# STUDIES ON THE GROWTH AND METABOLISM OF PLANT CELLS CULTURED ON FIXED-BED REACTORS

BY

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## DECLARATION

I hereby declare that, except where otherwise indicated, this thesis is the result of my own work.

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## ABBREVIATIONS

| | |
|---|---|
| g | gram(s) or unit of gravitational force |
| wt. | weight |
| fr. wt. | fresh weight |
| w/v | weight per volume (as percentage) |
| v/v | volume per volume (as percentage) |
| % | percentage |
| in | inch(es) |
| lb | pound(s) |
| m | metre(s), milli ($10^{-3}$) |
| μ | micro ($10^{-6}$) |
| n | nano ($10^{-9}$) |
| s | second(s) |
| M | molar |
| mol | mole |
| $^{o}C$ | degree(s) centigrade |
| ℓ | litre(s) |
| E | Einstein(s) |
| Ci | Curie(s) |
| Bq | Becquerel(s) |
| pH | negative log of the hydrogen ion concentration |
| $\bar{x}$ | mean |
| S.E. | standard error |
| 2, 4-D | 2, 4-dichlorophenoxyacetic acid |
| IAA | indolyl-3-acetic acid |
| NAA | naphthaleneacetic acid |
| PAL | phenylalanine ammonia-lyase |

| | |
|---|---|
| ATP | adenosine-5'-triphosphate |
| BUTYL PBD | 2-(4-tert-butyl phenol)-5-(4-biphenyl)-1,3,4-oxadiazone |
| TRITON-X-100 | octyl phenoxypolyethoxy ethanol |
| TLC | thin layer chromatography |
| GC | gas chromatography |

# CONTENTS

## ABSTRACT

The aim of this work was to investigate the growth of, and alkaloid production by, plant cells cultured on novel fixed-bed reactors.

Initially, the growth and alkaloid content of cultured cells of a large number of species was investigated; the cells were supplied with Murashige and Skoog medium, supplemented with $10^{-5}$M 2, 4-D and $10^{-5}$M kinetin. The alkaloid content of the cells was determined using a technique based on the cell-squash technique of Ogino et al. (1978). This method was quick and easy to use, and provided a semi-quantitative estimation of the levels of alkaloids accumulated by the cells.

From the various species grown in vitro, two were selected for further experimental purposes, namely Datura innoxia Mill. and Solanum nigrum L. Both species accumulated detectable levels of alkaloids and grew well on the nutrient medium used. The relationship between the growth, alkaloid content and chlorophyll content of the cells was investigated, in both callus and cell suspension cultures; evidence for an inverse relationship between growth and accumulation was obtained.

The cells were then grown in two fixed-bed reactor systems. In each, the cells were grown while physically stationary and in close association with each other, and liquid nutrient medium was moved across the cells. In the first, the 'flatbed' (devised by Yeoman et al., 1978), the cells were cultured while seated in the horizontal plane. In the second, cells were immobilized, using a novel technique developed in this thesis, in inert vertical columns. Both systems were characterised and found to facilitate the manipulation of the

environment of the cells (including precursor feeding and the levels of oxygen and light) and thereby allowed the accumulation of increased yields of alkaloids, compared with freely suspended cells. The ability of the cells (including cells of Capsicum frutescens Mill.) to release alkaloids into the nutrient medium was examined.

The results show that the column culture system in particular would be amenable to scaling-up for the industrial production of secondary metabolites.

# CHAPTER 1

## INTRODUCTION

# CHAPTER 1 : INTRODUCTION

A large number of chemicals used in the industries of pharmaceuticals, food flavourings and perfumes are of vegetable origin. These include a variety of compounds such as alkaloids, steroids, oils and pigments, many of which are still obtained from plant extracts. A number of useful flavours or perfumes, such as onion or rose oil, are complex mixtures, so in order to produce a commercially acceptable flavour or smell, the constituents must be extracted from a natural source or individually synthesised and then mixed in the correct proportions. Often chemical synthesis is difficult and expensive and may result in the production of a racemic mixture of compounds; some isomers may be physiologically inactive or inhibit the activity of useful isomers. However, compounds synthesised chemically are subjected to an intensive toxicological scrutiny, which is not so stringent for vegetable products. It is therefore advantageous if desired chemicals can be obtained from plant tissue.

Unfortunately, many of these products are not necessarily readily available from plant material. The majority of commercially useful substances originate from plants grown in tropical or sub-tropical regions, and the availability and cost of these materials is affected by economic and political factors in the countries of origin. Therefore, it would be advantageous if the compounds could be obtained locally.

For a number of years, plant cells and tissues cultured *in vitro* have been considered to be a potential source of specific secondary

metabolites. Unfortunately, however, the success of the fermentation industry in harnessing microorganisms to produce particular chemicals in abundance has not been repeated by plant cell culturists. With few exceptions, such as diosgenin production by Dioscorea cell cultures (Kaul and Staba, 1968), callus and suspended cell cultures tend to produce particular secondary metabolites in much lower quantities than are found in the whole plant, and the chemicals produced are often structurally different, or present in different proportions, to those of the whole plant (Boulanger et al., 1973; Forrest, 1969; Hiraoka and Tabata, 1974; Hirotani and Furuya, 1977; Ikuta et al., 1974; Jalal et al., 1979; Sejourne et al., 1981). The culturing of explants of specialised tissues or organs in which the desired compounds were thought to be synthesised in vivo, has not resulted in the production of 'specialised' (high-yielding) callus, for cultured cells merely de-differentiate, grow rapidly, and produce only low levels of secondary metabolites. A number of workers have concluded that the tissue of origin appears not to affect greatly the biosynthetic capacity of the callus (Dhoot and Henshaw, 1977; Furuya et al., 1972; Neuman and Mueller, 1974; Speake et al., 1964; Tabata et al., 1972; Tabata and Hiraoka, 1976).

The yield of secondary metabolites from cell cultures can be increased by a variety of techniques, and these are now described under three main headings, although it is likely that, in practice, at least two of the methods would be used in conjunction. The first approach involves the screening of a large number of 'wild type' cells to find one or more high-yielding strains. The second involves the production of mutants by the treatment of cells with

mutagens, followed by the selection of high-yielding, genetically novel, cell lines. The third broad approach involves the manipulation of culture conditions to maximise production.

The principal difficulty to be overcome with the first two techniques is the recognition of high-yielding cells or groups of cells. In cell cultures, there will be structural and biochemical heterogeneity in the cell population, as a result of genetic or epigenetic differences between cells (Blakely and Stewart, 1964; Davey et al., 1971; Meins and Binns, 1977; Ogino et al., 1978; Sunderland, 1977; Tabata and Hiraoka, 1976; Tabata et al, 1978; Zenk et al., 1977). Unless the desired product can be detected visually, i.e., is coloured or fluoresces, a distinction between high- and low-yielding cells or groups of cells cannot be readily made. This is true, whether the biochemical heterogeneity is the result of induced mutations (i.e., due to genetic factors) or is the result of natural variability in the cell population (i.e., due to epigenetic factors). Moreover, the use of mutagens to produce high-yielding cell lines is an empirical approach, and, similarly, the success of the selection of 'wild type' cells assumes that at least some cells in a culture do in fact accumulate relatively high levels of a specific metabolite. In this respect, the work of Ogino et al. (1978) in particular has demonstrated that, by the continued selection of cultured tobacco cell lines which accumulated relatively high levels of nicotine (compared with the rest of the cell population), callus cultures were obtained which accumulated 2.5% dry weight of nicotine. This was a large increase in the levels obtained - before selection, the callus had persistently shown a low nicotine content, corresponding to 0.004 to 0.05% dry weight.

However, once a high-yielding cell-line has been isolated, it is possible that its ability to accumulate particular secondary metabolites will vary through successive subcultures (Tabata et al., 1978); in order that selected cell lines can be used successfully in industry, it is important that stability of the biosynthetic capacity be established.

A different approach to that of cell line selection has been used in the work described in this thesis. Here, the aim was to increase the yields of secondary metabolites by manipulation of the environment of cultured cells. Alkaloids were the chosen metabolites, because they are easily detectable, their levels in cell cultures can be manipulated, their biosynthetic pathways have been well-studied, and they are commercially valuable compounds.

In the past, the manipulation of the environment of cultured cells has been shown to be a promising approach, but has often been an empirical one. Usually, cultures which had previously been shown to synthesise and accumulate to some extent a specific secondary compound were supplied with a range of mineral salts, different concentrations of growth-regulating substances (usually auxins and cytokinins) and primary carbon sources (usually sucrose), in the hope that a particular combination would induce the cells to accumulate high levels of the secondary compound(s). In this thesis, the manipulation of the environment of the cells to increase the yields of secondary compounds has been considered from three principal viewpoints. The first is the effects of manipulation on culture growth rate, the second is the effects of precursor supply to the cells, and the third is the induction of export of metabolites from the cells. The importance of all three

with respect to the regulation of the production of alkaloids and other secondary metabolites will now be considered.

## 1. Export of Metabolites

It has been suggested (e.g., Johnson and Waller, 1972) that the biosynthesis of the alkaloid ricinine in Ricinus communis may be under the control of a feedback regulation mechanism. If this is the case for other alkaloids, then to maximise production by cultured cells, the export of the metabolite(s) into the nutrient medium is essential. This would have the effect of lowering the endogenous concentration of the metabolite, and its synthesis would continue either until the external concentration increased to such an extent that the biosynthetic pathway was inhibited, or until the nutrients were depleted. For a continuous industrial process, the export of metabolites would be most advantageous. If a species should accumulate a desired product intracellularly, manipulation of the environment of the cells should be performed to increase membrane permeability to the compound(s), without damaging the cell population.

## 2. Precursor Supply

There is evidence in the literature that the levels of alkaloids produced by cultured cells can be increased by the addition of precursors to the nutrient medium (Chan and Staba, 1965; Dobberstein and Staba, 1968; Khanna and Jain, 1972; Konoshima et al., 1970; Ramawat and Arya, 1979; Sairam and Khanna, 1971; Tabata et al., 1972). This approach to increasing yields has been used in experiments described in this thesis. The mechanism or mechanisms

of the action are unresolved. It may be that the availability of precursors was limiting the rate of synthesis of the product, or that the precursor induced the de novo synthesis of the enzymes of the biosynthetic pathway. However, Phillips and Henshaw (1977) have shown that cells utilize precursors differentially according to the phase of the growth cycle, and this suggests that the physiological state of the cell is important in determining whether precursors will be incorporated into a specific metabolite, rather than the involvement of 'induction' mechansisms.

## 3. Growth Rate

In this thesis, manipulation of the growth rate of the cultures was considered to be particularly important for increasing yields, although it would be desirable to incorporate precursor addition and the induction of export into a programme designed to maximise the production of secondary metabolites. There is much information in the literature concerned with the relationship between the growth rate of cells, and their ability to produce secondary metabolites. Until about ten years ago, the concept that alkaloids tend to be synthesised mainly in young, actively-growing tissues, was prevalent. Solt (1957) determined that the amount of nicotine synthesised by cultured tobacco root tips is directly proportional to the mass of new tissues produced during steady-state growth. Dawson and Osdene (1972) state that such an observation may be interpreted to mean that a fixed number of nicotine molecules is produced, on average, for each new cell produced by the root apical meristem, and suggests that nicotine is produced principally in cells that are in active growth. Waller and Nowacki (1978) state, "To be emphasised is the

concept that the rate of their (alkaloids) biosynthesis is directly proportional to the synthetic activities of the tissue.".

However, there is a line of evidence now becoming established which suggests that the biosynthesis of alkaloids and other secondary metabolites occurs not in rapidly-growing cells, but rather in cells which are differentiating as opposed to dividing. Since the roots studied by Solt (1957) were differentiating as well as growing, and only a small proportion of cells in a root tip divide in any case (Clowes, 1976), her data can be interpreted as showing a correlation between nicotine accumulation and slowed growth and differentiation.

Evidence from work using plant cell and tissue cultures supports this hypothesis, namely that the accumulation of secondary metabolites is positively correlated with cell differentiation and inversely proportional to cell growth rate.

## The Relationship Between Accumulation and Differentiation

In 1969, Bhandary et al. examined the accumulation of atropine in callus of Atropa belladonna, and found that actively growing, undifferentiated callus did not produce detectable amounts of the alkaloid. In contrast, West and Mika (1957) have shown that A. belladonna callus, when cultured in conditions incompatible with cell proliferation and conducive to cell differentiation, accumulated small amounts of atropine. Thomas and Street (1970) also suggested that cellular organisation is necessary for alkaloid production in A. belladonna suspension cultures. Nikolaeva and Vollosovich (1972) similarly found that the production of tropane alkaloids by Datura spp.

and indole alkaloids by Rauwolfia serpentina tissue cultures was restricted to those conditions in which the level of auxin permitted organogenesis. Tabata et al. (1972) noted that roots regenerated from callus cultures of Scopelia parviflora accumulated the pattern of alkaloids characteristic of the intact plant, whereas the disorganised tissues did not; and if 'undifferentiated' callus (containing 0.009 to 0.01% dry weight alkaloids) was transferred to a medium conducive to root development, alkaloids accumulated to a level of 0.08% dry weight. Similarly, the production of the flavour components of onion (Allium cepa) were found to occur in tissue cultures to levels approaching those in fresh onion, but only in cultures in which roots had differentiated (Freeman et al., 1974). Tabata and Hiraoka (1976) found that the nicotine content of tobacco (Nicotiana tabacum) callus decreased to trace amounts in cultures which had lost the ability to regenerate roots. Light regimes inducing tissue differentiation and lignification in Citrus tissue cultures also stimulated flavonoid production (Brunet and Ibrahim, 1973).

A further indication that differentiation and accumulation are connected is given by a number of observations that the appearance of specialised cells in a variety of tissue cultures is correlated with the appearance of specific secondary compounds (Reinhard et al., 1968; Becker, 1970; Corduan and Reinhard, 1972; Neumann and Mueller, 1974).

## The Relationship Between Accumulation and Growth Rate

An inverse relationship between growth and accumulation and differentiation is suggested by the results of studies on cell and tissue cultures, following specific secondary metabolite production throughout a growth cycle. Thomas and Street (1970) found that

morphogenesis in cell suspension cultures of A. belladonna was favoured late in the growth cycle, and was increased by delaying subculture. In Glycine max cell cultures large changes in the activity of the enzymes phenylalanine ammonialyase (PAL) and a p-coumarate : CoA ligase occur just prior to the stationary phase, indicating the initiation of some facets of secondary metabolism (Hahlbrock et al., 1971). Speake et al. (1964) showed that tobacco suspension cultures accumulated maximal levels of alkaloids at the end of the growth cycle, a result identical in principle to that for caffeine production in tea (Camellia sinensis) callus cultures. A similar pattern of accumulation was reported by Townsley (1974), who followed the levels of a chocolate aromatic product in cell cultures of Theobroma cacao; accumulation occurred only in the mature-death stage of the growth curve. The production of the alkaloid trigonelline and the steroid diosgenin by tissue cultures of Trigonella foenum-graecum (Khanna and Jain, 1972, 1973) seems also to be inversely proportional to the growth index, and Takahashi and Yamada (1973) discovered that, in the presence of auxin (either IAA or 2, 4-D) there seemed to be an antithetical relationship between the growth rate of tobacco callus and nicotine production by that callus - it was suggested that phytohormones may control alkaloid production by affecting the growth rate of the cells. Similar results were obtained by Konoshima et al. (1970).

In 1973, Meyer-Teuter and Reinert altered the normal pattern of embryo formation in carrot (Daucus carota) tissue cultures on an inductive medium by effecting a retardation of growth, either by low temperature treatments or by transfer to a non-inductive medium. In both cases partial inhibition of growth resulted in a

prolonged period of competence of the cultures for embryo formation. Moreover, a close inverse relationship was found to exist between the rate of cell division and the percentage of cultures forming embryos.

There is therefore a general inverse relationship governing the production of alkaloids and at least some other types of secondary metabolites in cells, and it can be summarised by the simple formula

$$\text{secondary metabolite production and cell differentiation} \propto \frac{1}{\text{growth rate}}$$

It was the appreciation of this relationship between the growth rate of cell cultures and their ability to produce secondary metabolites which led to the development by Yeoman et al. (1978) of a cell culture system in which the growth rate of the cells could be manipulated with ease. The aim was that this would provide a means whereby secondary metabolite production could be increased.

## The Flatbed Cell Culture System

The principal feature of the flatbed cell culture system, which allows the simple manipulation of cell growth rate, is that the cells are kept physically stationary while the nutrient medium moves across them. Thus cells are grown as a callus-like mass while seated on a solid but permeable (fabric) substratum. Nutrient medium is supplied in liquid form, and drips from a reservoir into the cell culture vessel, where it moves across the fabric substratum by capillary action, so feeding the cells. It is then pumped back to the reservoir, and re-circulated (see Figure 1.1). By changing the nutrient medium in the reservoir, the chemical environment of the cells can be simply and

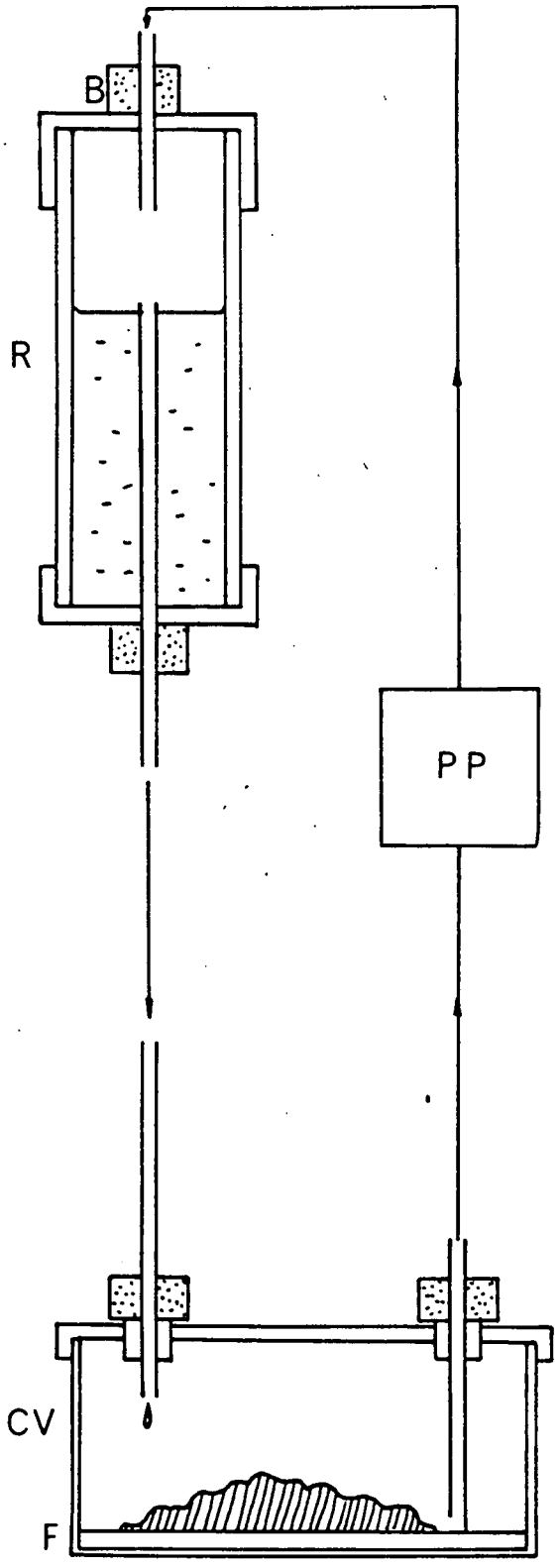



Figure. 1.1 The Flatbed Apparatus for the Culture of Plant Cells

R = nutrient medium reservoir

CV = culture vessel. containing cells

F = fabric substratum

PP = peristaltic pump

B = foam bung

effectively manipulated, and the growth rate of the cells can therefore be regulated. This method requires no physical manipulation of the cells; this is not the case with agar or liquid-suspension cultures. Further advantages of the flatbed culture system are related to the fact that (a) the cells grow like callus, i.e., in communication with each other and subject to physical and chemical gradients. This is advantageous, because secondary metabolites tend to accumulate to the highest levels in callus, as opposed to cell suspension cultures (see, e.g., Tabata et al., 1978); and (b) large quantities of (possibly toxic) precursors can be supplied to the cells at low concentrations.

In this thesis, the aim was to characterise cell growth on the flatbed system (which had been shown previously to allow secondary metabolite production, namely, capsaicin production by cells of Capsicum frutescens), to manipulate the environment of cells cultured on the flatbed, and to improve the culture system. This improvement comprised the development of a column culture system, involving the immobilisation of whole plant cells.

## Column Culture of Plant Cells

The aim of this work was to modify the design of the flatbed culture system so that cells would be immobilised in a biologically inert vertical column, rather than being seated horizontally in a flat culture vessel. Such a culture system was considered to be more amenable to scaling up to an industrial size than would be the flatbed.

The first reported instance of the immobilisation of whole higher plant cells on inert columns involved the use of calcium

alginate pellets in which cells of Morinda citrifolia, Catharanthus roseus and Digitalis lanata were embedded (Brodelius et al., 1979). Calcium alginate has been used also for the entrapment of animal cells (Nilsson and Mosbach, 1980), yeast cells (Cheetham et al., 1979; Kierstan and Bucke, 1977), bacterial spores (Dallyn et al., 1977), subcellular organelles and enzymes (Kierstan and Bucke, 1977), and the aluminium salt has similarly been used (Hackel et al., 1975) for yeast cell immobilisation. Results of investigations using this entrapment system indicate that cell metabolism is not adversely affected in a qualitative way, for those metabolic reactions which have been investigated so far, such as phenol oxidation (Hackel et al., 1975) and glucose-ethanol conversion (Kierstan and Bucke, 1977) by yeast cells, alkaloid biosynthesis (Brodelius et al., 1979; Brodelius and Nilsson, 1980) and respiration (Brodelius and Nilsson, 1980) in whole plant cells and the Hill reaction and ATP synthesis in immobilised chloroplasts and mitochondria respectively (Kierstan and Bucke, 1977) all take place within the environment of a calcium alginate cell. In most of these examples there is, however, an obvious diminuition of the rates of the reactions when compared to freely suspended cells, a noticeable exception being the increased efficiency of ajmalicine synthesis from tryptamine and secologanin in entrapped (compared with freely-suspended) cells of Catharanthus roseus (Brodelius and Nilsson, 1980).

Brodelius and Nilsson (1980) reported the immobilisation of whole plant cells in other carbohydrate matrices such as agarose, agar, carrageenan, gelatin, alginate plus gelatin, agarose plus gelatin and polyacrylamide.

The approach in this project may be briefly summarised as follows. Alkaloid-producing species were grown in vitro, and selected for their suitability for use on the flatbed culture system, on the basis of their ability to grow well and produce alkaloids on the nutrient medium available. The growth of the cells on the flatbed was characterised, and alkaloid production was determined before and after manipulation of the environment. The relationship between the growth and capacity for alkaloid production of cultured cells was investigated. The flatbed culture system was modified so as to be more suitable for scaling-up to an industrial size; this modification took the form of a column-culture system, in which cells were immobilised on biologically inert vertical columns.

# CHAPTER 2

## Experimental Materials And Methods

# CHAPTER 2 : EXPERIMENTAL MATERIALS AND METHODS

## Plant Material

Before any experimental work was started, a list of alkaloid-producing species was drawn up; as many as possible of these plants were obtained, grown up, and used for the initiation of callus cultures. The list is given in Table 2.1. Seeds were obtained from a wide variety of sources : Thompson and Morgan (Ipswich) Ltd., and a number of botanical gardens around the world. The Lupinus species, Solanum tuberosum and Solanum dulcamara were grown in the garden of the Botany Department, Edinburgh University. Seeds were germinated and seedlings grown up in damp Levington's compost.

The experimental material used in all but one of the flatbed or column experiments (in which Capsicum frutescens Mill. was used) was either Datura innoxia Mill. or Solanum nigrum L. The alkaloid metabolism of the former is the more well-known but it did not always grow well in the nutrient medium used; S. nigrum cells grew much better.

Table 2.1

Plants Considered for Use in the Production of Callus Cultures

ATROPA BELLADONNA

BERBERIS VULGARIS

COFFEA ARABICA

CEPHAELIS (URAGOGA) IPECACUANHA*

CHONDRODENDRON SPP.*

CINCHONA CUCUMBRA*

COLCHICUM AUTUMNALE

COLCHICUM LUTEUM

COPTIS JAPONICA*

DATURA CLORANTHA

DATURA INNOXIA

DATURA STRAMONIUM

DATURA SUAVEOLENS

EPHEDRA SPP.*

ERYTHROXYLUM COCA

HYOSCYAMUS MATICINS*

HYOSCYAMUS NIGER

LOBELIA INFLATA

LUPINUS ALBUS

LUPINUS ANGUSTIFOLIUS

LUPINUS HARTWEGI

LUPINUS LUTEUS

LUPINUS MUTABILIS

NERIUM OLEANDER

NICOTIANA TABACUM

PAPAVER ORIENTALE

PAPAVER SOMNIFERUM

PHYSOSTIGMA SPP.*

PILOCARPUS JABORANDI*

PILOCARPUS PENNATIFOLIUS*

RAUWOLFIA TETRAPHYLLA

RAUWOLFIA VOMITORIA

SCOPELIA ATROPOIDES*

SOLANUM CRISPUM

SOLANUM DULCAMARA

SOLANUM NIGRUM

SOLANUM TUBEROSUM

STRYCHNOS NUX-VOMICA*

THEA SINENSIS*

THEOBROMA CACAO*

TRIGONELLA BALANSAE

TRIGONELLA CORNICULATA

TRIGONELLA FOENUM-GRAECUM

TRIGONELLA MONSPELIACA

VERATRUM VIRIDE

VINCA ROSEA*

Those names marked with an asterisk were either unobtainable or the seeds failed to germinate.

# EXPERIMENTAL METHODS

## Section 1 Tissue and Cell Culture

### *(a) Nutrient Media*

All media (unless specifically stated otherwise) for the initiation and maintenance of callus, cell suspension, flatbed and column culture contained 4.71 g/l Murashige and Skoog medium (Murashige and Skoog, 1962), obtained from Flow Laboratories, Irvine, Scotland. The exact composition of the medium is given in Table 2.2. This was supplemented with 30 g/l sucrose, 2.2 mg/l ($10^{-5}$M) 2,4-dichlorophenoxyacetic acid (2,4-D), which is an auxin, and 2.1 mg/l ($10^{-5}$M) Kinetin, a cytokinin. The complete medium is hereafter referred to as full MS medium. For the experiment using cells of Capsicum frutescens (p.267 ) the callus was grown on 'pepper' nutrient medium. This medium was composed of 4.71 g/l Murashige and Skoog medium, 20 g/l sucrose, 2 µg/l ($10^{-8}$M) 2,4-D, 20 µg/l ($10^{-7}$M) Kinetin and 50 ml/l coconut milk. The coconut milk, obtained directly from coconuts, was prepared for use as follows : the milk was filtered through glass wool, autoclaved at 121°C (15 lb $in^{-2}$ steam pressure) for 15 minutes, and stored at -20°C. Immediately before use, it was centrifuged at 2500 g for 15 minutes to remove cell debris, and the supernatant was added to the medium prior to autoclaving.

Both full MS and pepper media were made up in glass-distilled water, and the pH was adjusted to 5.8 with 0.1 M KOH.

The medium for the initiation and maintenance of callus tissue also contained 10 g/l agar (Oxoid no. 3, Oxoid Ltd.). Callus cultures

Table 2.2

The composition of the Murashige and Skoog Medium (Flow Laboratories, Irvine, Ayrshire, Catalogue Number 26-100-20) is as follows :

| | | |
|---|---|---|
| $CaCl_2\ 2H_2O$ | 440.0 | mg/litre |
| $CoCl_2\ 6H_2O$ | 0.025 | mg/litre |
| $CuSO_4\ 5H_2O$ | 0.025 | mg/litre |
| FeNa EDTA | 36.70 | mg/litre |
| $H_3BO_3$ | 6.20 | mg/litre |
| $KH_2PO_4$ | 170.0 | mg/litre |
| KI | 0.83 | mg/litre |
| $KNO_3$ | 1900.0 | mg/litre |
| $MgSO_4\ 7H_2O$ | 370.0 | mg/litre |
| $MnSO_4\ 4H_2O$ | 22.30 | mg/litre |
| $Na_2MoO_4\ 2H_2O$ | 0.25 | mg/litre |
| $NH_4NO_3$ | 1650.0 | mg/litre |
| $ZnSO_4\ 7H_2O$ | 8.60 | mg/litre |
| myo-Inositol | 100.0 | mg/litre |
| Nicotinic acid | 0.50 | mg/litre |
| Thiamine HCl | 0.10 | mg/litre |
| Pyridoxine HCl | 0.50 | mg/litre |
| Glycine | 2.00 | mg/litre |

were kept on 10-15 ml of this solid medium in 9 cm diameter disposable pre-sterilised plastic Petri dishes (Sterilin, Teddington), and cell suspension cultures were maintained in 60 ml of liquid medium in 250 ml conical (Erlenmeyer) flasks.

*(b) Callus Culture*

Callus was derived from stem explants or by directly germinating seeds onto solid medium. Much difficulty was encountered in producing sterile cultures from root explants so these were not used.

Callus initiation

Sterilisation of explanted material was carried out as follows : seeds and stem segments (approximately 2-3 cm in length, the ends of which had been sealed with paraffin wax) were first washed for 10-15 seconds in absolute ethanol to pre-sterilise and de-wax the explant surfaces. They were then immersed, for 15 minutes, in 2% sodium hypochlorite solution (w/v) (20% v/v commercial solution), a sterilant, containing a few drops of detergent to act as a surfactant. The material was then rinsed three times in sterile distilled water, to remove all of the sterilant. The sterilised stem explants were then placed horizontally in pre-sterilised plastic Petri dishes, and with the use of sterile forceps and a scalpel, the ends of the explants (coated in paraffin wax) were cut off and discarded. Then the cylindrical explant was sliced into discs approximately 1-2 mm thick and transferred to Petri dishes containing the solid nutrient medium, upon the surface of which the explant discs were laid, cut surface down. The Petri dishes were sealed with Parafilm (American Can

Company) to prevent both contamination and water loss and the explanted tissues were allowed to produce callus under conditions of continuous fluorescent illumination provided by 'Warmwhite' fluorescent tubes, producing an illuminance of 200 μmol $m^{-2}s^{-1}$ (200 μE $m^{-2}s^{-1}$) at a temperature of 24 ± 1°C. The newly-formed callus was isolated from the explant after a period of approximately 6 weeks and was usually sub-cultured onto fresh medium every month; these intervals varied between species, however, according to the growth rate of the cells.

*(c) Cell Suspension Culture*

Culture initiation

Four or five small pieces of callus (approximately 100mg each) were placed in 50 or 60 ml of sterile liquid nutrient medium in 250 ml Erlenmeyer flasks which were then subjected to continuous rotary shaking (orbital diameter 1.5 cm, 96 r.p.m.) in continuous fluorescent illumination (200 μmol $m^2s^{-1}$) at 24 ± 1°C. The flasks were sealed with a double layer of (sterile) aluminium foil, secured with parafilm. Using this procedure, inidividual cells or small clumps of cells became separated from the larger pieces of callus, and continued to divide until growth was limited by environmental conditions.

Culture maintenance

The method of subculture depended upon the condition of the cell suspension, and was one of the following :

(i) If the culture was composed of small groups of cells, then 10 ml of the culture was pipetted (using a sterile 10 ml pipette, the tip of which had been cut off) into 50 ml fresh medium;

(ii) If the culture was composed mainly of large clumps of cells, smaller groups of cells were isolated by sieving (using a sieve of pore diameter 1 mm) into a sterile 100 ml measuring cylinder; the filtrate was allowed to settle for 10 minutes, after which time the supernatant was decanted off and 10 ml of the settled slurry of cells was pipetted into 50 ml fresh medium;

(iii) If the culture was composed of small cell groups but was very dense (i.e., too dense to pipette) then approximately 1 g fresh weight of cells was transferred, with the aid of a sterile perforated spoon, into 50 or 60 ml fresh medium.

The time interval between subcultures varied according to the growth rate of the cultures, but was usually 2 weeks.

*(d) Sterilisation of Equipment and Sterile Technique*

All glassware was cleaned in a solution of 2% (v/v) Decon 90 (Decon Laboratories, Brighton) in tap water for 2 days followed by thorough rinsing in tap and distilled water; it was dried in a hot air oven.

Glassware used in callus, cell suspension, flatbed and column culture, and subaseals, silicone and peristaltic rubber tubing, pan-scrubbers, scalpels and forceps, and nutrient media, were sterilised by autoclaving at 121$^{o}$C (15 lb in$^{-2}$ steam pressure) for 20 minutes.

All aseptic manipulations were carried out in a 'sterile room' which was reserved for such work; it was maintained in a sterile condition by irradiation with two ultra-violet lights (producing light of a wavelength of approximately 300 nm) when not in use, and was under a positive atmospheric pressure relative to its surroundings to prevent dust (and hence microbes) drifting in from adjacent rooms. Before sterile work commenced, and after it was finished, the bench was swabbed down with absolute ethanol; during, and in close proximity to, the manipulations, a Bunsen burner was kept alight in order to produce an up-draught of air, which acted to prevent any dust particles coming to rest on sterile material. Surgical gloves, sterilised by swabbing with absolute ethanol, were always worn during sterile work. During manipulations, scalpels and forceps were stored in absolute ethanol until required, when they were flamed by igniting the alcohol present on the surfaces of the instruments.

## Section 2 General Analytical Techniques

Unless otherwise stated, all chemicals obtained from British Drug Houses (BDH), Poole, Dorset, were Analar grade.

### *(a) Reagents for the Detection of Alkaloids*

A number of reagents, generally recognised for their specificity in reacting with alkaloids to give particular colours (but see the note concerning specificity below, p.26 ), were examined for their suitability for the screening work undertaken in this project.

#### (i) Dragendorff's Reagent

This was made up according to the Pharmacopoeia of Japan (1970) as follows :

1. 0.85 g of bismuth subnitrate (BDH), was dissolved in 10 ml glacial acetic acid (BDH); 40 ml distilled water was then added, and this comprised solution A.

2. Solution B comprised 8 g potassium iodide (BDH), dissolved in 20 ml distilled water.

3. x ml of 20% acetic acid were added to x/5 ml of a mixture of equal volumes of solutions A and B immediately before use.

#### (ii) Iodoplatinate Reagent

This was made up according to Clarke (1970) as follows :

10 ml 5% platinum chloride solution (made up as 0.5 g chloroplatinic acid (Fisons, Loughborough) dissolved in 5 ml M HCl plus 5 ml distilled water) and 5 ml conc. HCl (BDH) were added to 240 ml 2% potassium iodide (BDH) solution (w/v).

(iii) The Vitali-Morin Reagent and Test (described in Clarke, 1970)

A drop of the solution to be tested was placed on a glass slide or absorbed in filter paper and allowed to evaporate. Then a drop of fuming nitric acid was added, and evaporated to dryness over a boiling water bath. After cooling, the residue was moistened with a drop of 5% (w/v) solution of potassium hydroxide in ethanol (BDH). Colour reactions characteristic of different alkaloids are produced by this treatment.

(iv) The Marquis Reagent and Test (described in Clarke, 1970)

A drop of the solution to be tested was placed on a glass slide or absorbed on filter paper, allowed to dry, and moistened with the reagent (made up as 1 ml formalin solution, i.e., 40% v/v formaldehyde (BDH) in water) in 10 ml concentrated sulphuric acid (BDH). Colour reactions characteristic of different alkaloids are produced by this treatment.

A Note on the Specificity of Dragendorff's Reagent

Although the specificity of Dragendorff's reagent is such that it is considered a useful tool in the detection of plant alkaloids (by virtue of a precipitation reaction involving the formation of an insoluble heavy metal/alkaloid-nitrogen complex), the reagent does

give a false-positive precipitation with some non-nitrogenous compounds (which must possess conjugated carbonyl or lactone structural features) such as α-pyrones, chalcones, psoralens and a number of miscellaneous molecules containing the necessary reactive groups (including digitoxin and ouabain) (Farnsworth et al., 1962).

A similar criticism could be made of the iodoplatinate reagent which acts to precipitate alkaloids by a similar method to that of Dragendorff's reagent.

*(b) Tissue Analysis for Alkaloids*

(i) The Cell-Squash Analytical Technique

It was considered important to find a method for the estimation of the levels of alkaloids present in cultured cells which was quick, simple and fairly accurate; for these reasons the semi-quantitative 'cell-squash' technique of Ogino et al. (1978) was adopted and modified. The principle of the method is based on the fact that the intensity of the colour reaction produced as a result of reacting an alkaloid-detecting reagent with cell sap varies in approximately direct proportion to the concentration of alkaloids in the sap.

The method was performed as follows : pieces of callus, or clumps of cells from a suspension culture, of diameter approximately 5 mm, were placed between two pieces of filter paper (Whatman qualitative No. 1) and squashed between glass TLC plates (of dimension 20 x 20 x 0.3 cm), so that the cell contents were released and absorbed by the filter paper. The flattened cells were removed and discarded, and a drop of reagent (approximately 50 µl) was applied to the cell sap. The intensity of the resultant colour reaction was

given a value on an arbitrary scale (from 0 to 5), ranging from 'no visible reaction' (a colour intensity or I value of 0) to 'very strong visible reaction' (an I value of 5); the full scale is given in Table 2.3. The intensity of colours produced could be compared to those produced by a series of standard alkaloid solutions, and in this way, that is by reference, it was possible to quantify, to some extent, the method.

Different alkaloids often produced different colours with a particular reagent; the differences, however, were usually noticeable only at very high concentrations of the alkaloid (i.e., at concentrations much higher than would be found in plant material) or with pure samples.

In all 'cell squash' analyses, 12 estimations were made per tissue sample determination, and the mean I value was calculated.

The application of statistical methods to the I values is not feasible because of the skewed distribution of results it provides, and a precise conversion of I values to actual quantities of alkaloids (upon which statistical calculations could be performed) is not possible because of the very nature of the cell squash technique. Nevertheless, the difference in the levels of alkaloids represented by each I value is large (see Table 3.1.3) and by making a large number of determinations for each sample, a fairly accurate estimation of the alkaloid content of a piece of tissue can be obtained.

(ii) Qualitative Analysis of Alkaloids in Tissue

The cell squash analytical technique described above was most valuable for providing, rapidly, an estimation of the levels of alkaloids accumulated by cells. However, it gave only some idea of

Table 2.3

A Qualitative Description of 'I' Values

| Colour Intensity (I) Value | Description of Colour Reaction |
|---|---|
| 0 | no visible reaction |
| 1 | very faint reaction |
| 2 | faint reaction |
| 3 | light reaction |
| 4 | strong reaction |
| 5 | very strong reaction |

the total quantity, and not the composition, of the alkaloid complement present; for such qualitative analysis, it was necessary to subject an extract of tissue to thin layer chromatography.

## The Extraction of Alkaloids from Tissue

The method adopted was essentially that of Hultin and Torsell (1965) and was carried out as follows : a known weight of tissue (usually between 5 g and 7 g) was dried (at under 50°C) in a hot air oven and ground in 20 ml of methanol (BDH), with a little sand (approximately 1 g), in a pestle and mortar. A further 20 ml methanol was added, and the homogenate was allowed to stand overnight; thereafter the ground tissue plus methanol was warmed for 4 hours at 50°C, filtered, and the residue washed with 20 ml of methanol. The combined extracts (60 ml) were evaporated to dryness *in vacuo* at 40°C; the residue was suspended in 2 ml methanol, and 12 ml 1% w/v HCl (BDH) was added. The solution was shaken, filtered, and the residue washed with an additional 8 ml 1% HCl (w/v).

The filtrate was made basic with conc. $NH_4OH$ (.88 ammonia, BDH); an extract of this was obtained by shaking with 3 x 20 ml portions of chloroform (BDH) in a separating funnel-- this constituted FRACTION A. The aqueous solution was half-saturated with anhydrous $Na_2SO_4$ (BDH), and this was extracted with 3 x 20 ml portions of chloroform:ethanol (3:2 v/v) to produce FRACTION B, containing alkaloids not very soluble in chloroform such as solanine. Fractions A and B were filtered and evaporated to dryness *in vacuo* at 40°C separately. The respective residues were taken up in 1 ml of chloroform, and were then analysed chromatographically.

Thin Layer Chromatography of Alkaloid Extracts

Usually 10 or 25 µl of chloroform extracts of alkaloids were loaded onto commercially-prepared plastic TLC plates (20 x 20 cm) coated with a 0.2 mm layer of Kieselgel 60 (Merck). The alkaloids were separated in two solvent systems: either that described by Hultin (1966) composed of 100 ml of chloroform : glacial acetic acid : methanol (60:5:35 v/v) or that described by Clarke (1970), composed of 101.5 ml of methanol : .88 ammonia (100:1.5 v/v). The solvent tanks were allowed to become saturated with solvent vapour by leaving sealed for 1 hour before use. After 2 runs, new solvent was used.

Alkaloids were visualised by spraying, when dry, with Dragendorff's or iodoplatinate reagents, or by their ability to absorb or fluoresce under ultra-violet light.

$R_f$ values were calculated as the distance travelled by a compound from the origin, in a particular solvent system, divided by the distance travelled from the origin by the solvent front itself; different alkaloids have (usually) different $R_f$ values (which are determined by the molecular structure of the compound), and the same alkaloid may have different $R_f$ values in different solvent systems.

(c) *Analysis of the Nutrient Medium for Alkaloids*

A rapid indication of alkaloid presence/absence in the nutrient medium was obtained by reducing the volume of the medium to 1 or 2 ml by evaporation in vacuo at 40°C, and testing the residue with alkaloid reagents. If a positive reaction was obtained, and further

confirmation required, alkaloids could be extracted in acidified methanol and then chloroform, as described for tissue extraction, and chromatographed.

*(d) The Extraction of Capsaicin*

(i) From Cells

10 g of cells were dried at 50°C and ground in 30 ml of chloroform, with a little sand, in a pestle and mortar. The homogenate was then filtered and the residue was washed with a further 30 ml of chloroform. The combined filtrates (60 ml) were evaporated to dryness in vacuo at 40°C, and the residue was taken up in 1 ml of chloroform before being chromatographed.

(ii) From Nutrient Medium

x ml (usually 100 ml) of nutrient medium was vigorously shaken with 3 X x ml portions of chloroform, and the combined extracts (3x or 300 ml) were evaporated to dryness in vacuo at 40°C. The residue was taken up in 1 ml of chloroform before being chromatographed.

Thin Layer Chromatography of Capsaicin Extracts

At least 75 µl of the extract to be chromatographed was loaded onto commercially-prepared aluminium sheeting TLC plates (20 x 20 cm) coated with a 0.2 mm layer of polyamide 11 F254 (Merck). Two solvent systems were used for separation purposes: one was 100 ml of a mixture of chloroform : ethyl acetate (50 : 50 v/v), the second

was a 100 ml mixture of chloroform : methanol (98:2 v/v, Iwai et al., 1979). The solvent tanks were allowed to become saturated with solvent vapour by leaving sealed for 1 hour before use. After two runs, new solvent was used.

In order to visualise capsaicin, the plates were air-dried and then sprayed with a solution of 0.5 g of ferric chloride plus 0.25 g of potassium ferricyanide (BDH) in 50 ml of distilled water (Randerath,1968). Capsaicin appeared as a blue spot and was identified by co-chromatography with a 10 µl sample (containing 10 µg) of commercial capsaicin (Sigma London Chemical Co.), which had been dissolved in chloroform to make a stock solution of 1 mg capsaicin/ml chloroform.

*(e) Analysis of Some Essential Nutrients*

An indication of the growth rate of cells grown as flatbed and column cultures was obtained by examining the uptake kinetics of four essential nutrients (namely orthophosphate, ammonia, nitrate and sucrose) throughout the experimental period.

Each determination was by a spectrophotometric technique, and all optical densities were measured on a Pye Unicam spectrophotometer.

The methods employed were chosen because in each case there is minimal interference with the colour reactions resulting from the presence of other ions and nutrients. In any case, 'blank' solutions for 'zeroing' the spectrophotometer and for diluting nutrient samples were made up with full MS medium with the exclusion of the particular nutrient to be analysed.

(i) Orthophosphate Determination

The method of Allen (1940) was employed, as follows :

Amidol Reagent

A solution of 0.2 g of amidol (2,4-diaminophenol hydrochloride) plus 4.0 g of sodium metabisulphite (BDH) in 20 ml of distilled water, filtered before use.

The sample (usually 0.2 ml of medium plus 0.8 ml of distilled water) was decolourised by the addition of 0.44 ml of 60% perchloric acid (sp. gr. 1.54) (BDH); to this mixture was then added 4.0 ml of distilled water, 0.4 ml of amidol reagent and 0.2 ml of 8.3% (w/v) ammonium molybdate (BDH) in distilled water. After shaking to ensure thorough mixing, the solution was left to stand for 30 minutes to allow the colour to develop, and its optical density was measured at 620 nm. A calibration curve for orthophosphate is given (Figure 2.1).

(ii) Ammonium Determination

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

Reagents :

1. Salicylate reagent

34 g of sodium salicylate and 0.24 g of sodium nitroprusside (BDH) were dissolved in 500 ml of distilled water, and then made up to 1 litre with distilled water.

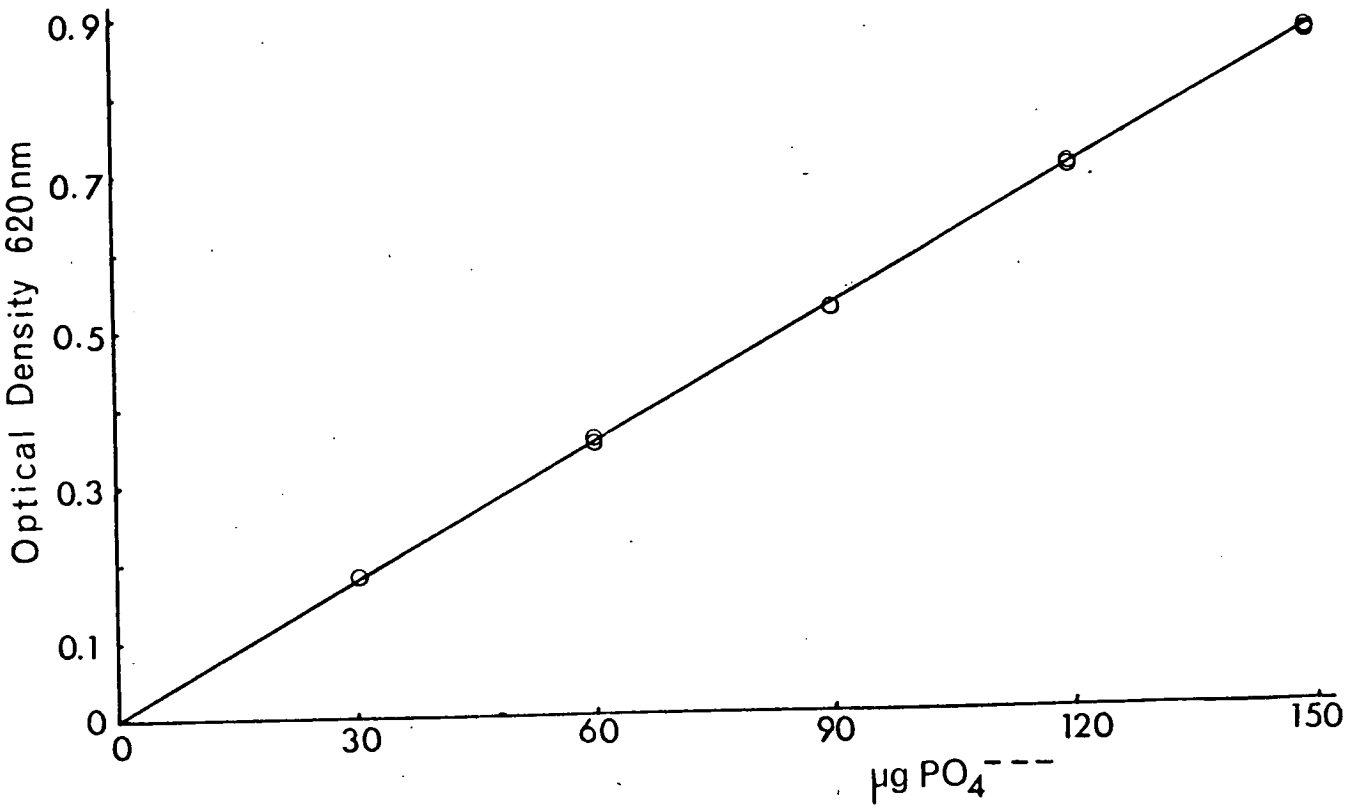



Figure 2.1 Orthophosphate Calibration Curve

2. Cyanurate reagent

0.25 g of sodium dichloroisocyanurate (BDH) was dissolved in 200 ml of distilled water, and to this was added 15 ml of a solution of NaOH (BDH) (400 g/l in distilled water). Distilled water was added to make this solution up to 1 litre.

In the sample (usually 50 µl of medium plus 0.95 ml of distilled water) was added 5 ml of salicylate reagent and 5 ml of cyanurate reagent, and after shaking the solution was allowed to stand for 30 minutes to allow the colour to develop. After this time optical density was measured at 660 nm. A calibration curve for ammonium is given (Figure 2.2).

(iii) Nitrate Determination

The method of Woolley et al. (1960) was employed :

Reagents :

1. 20% acetic acid (v/v in distilled water) containing 0.2 ppm Cu as $CuSo_4$ (BDH).

2. An intimate powder mixture of :

100 g $BaSO_4$

75 g citric acid

12 g $MnSO_4$ $4H_2O$

4 g sulphanilic acid

2 g powdered zinc

2 g 1-naphthylamine

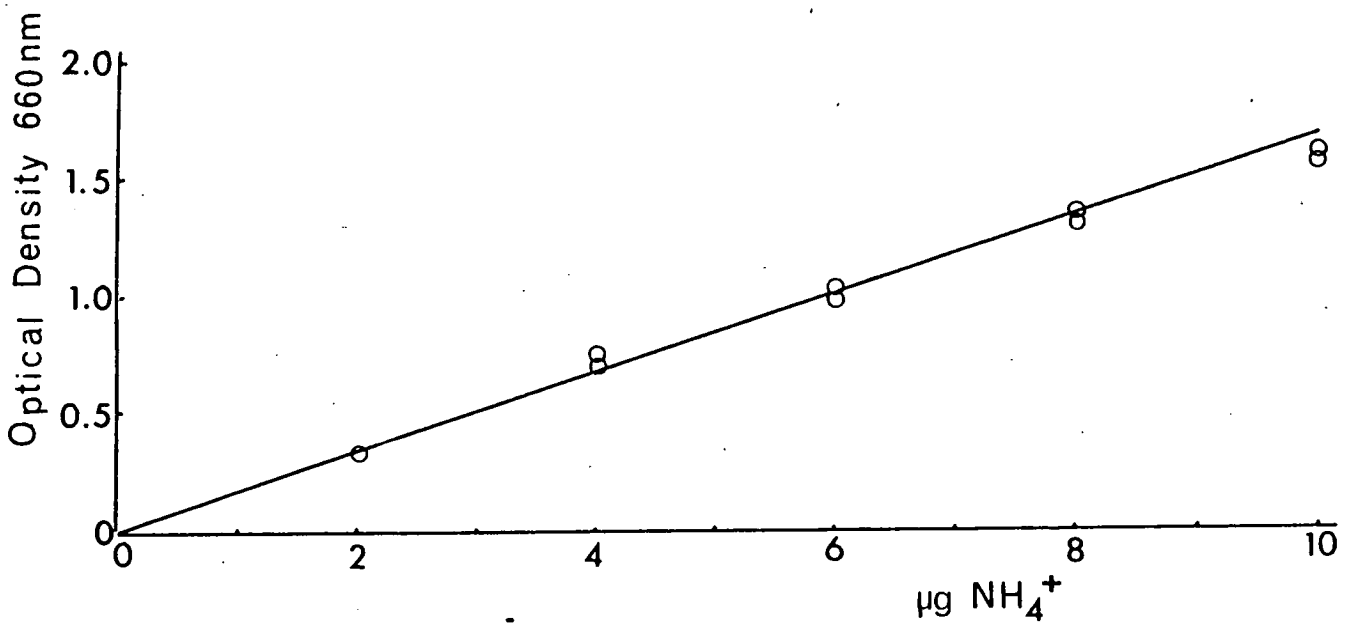



Figure 2.2 Ammonia Calibration Curve

all obtained from BDH, except 1-naphthylamine, from Sigma London Chemical Co. This solid mixture was prepared at least 15 days before use.

To the sample (usually 20 µl of medium plus 0.98 ml of distilled water) was added 9 ml of the acetic acid reagent. To this solution was added 0.8 g of the powder; the mixture was shaken for 15 seconds, left for 3 minutes, shaken for a further 15 seconds, and after standing for 3 more minutes was centrifuged at 1000 g for 3 minutes. The optical density of the clear supernatant was measured at 520 nm. A calibration curve for nitrate is given (Figure 2.3).

(iv) Sucrose Determination

Either of two methods was used :

A. THE ANTHRONE METHOD (described by Ashwell, 1957)

Anthrone reagent

2 g anthrone (Sigma London Chemical Co.) was dissolved in 1 litre of conc. $H_2SO_4$. The sample (1 µl of medium made up to 1 ml with distilled water) was thoroughly mixed with 2 ml of anthrone reagent at 10-15°C, and the mixture heated at 90°C for 10 minutes. After cooling, the optical density was measured at 620 nm. A calibration curve for sucrose is given (Figure 2.4).

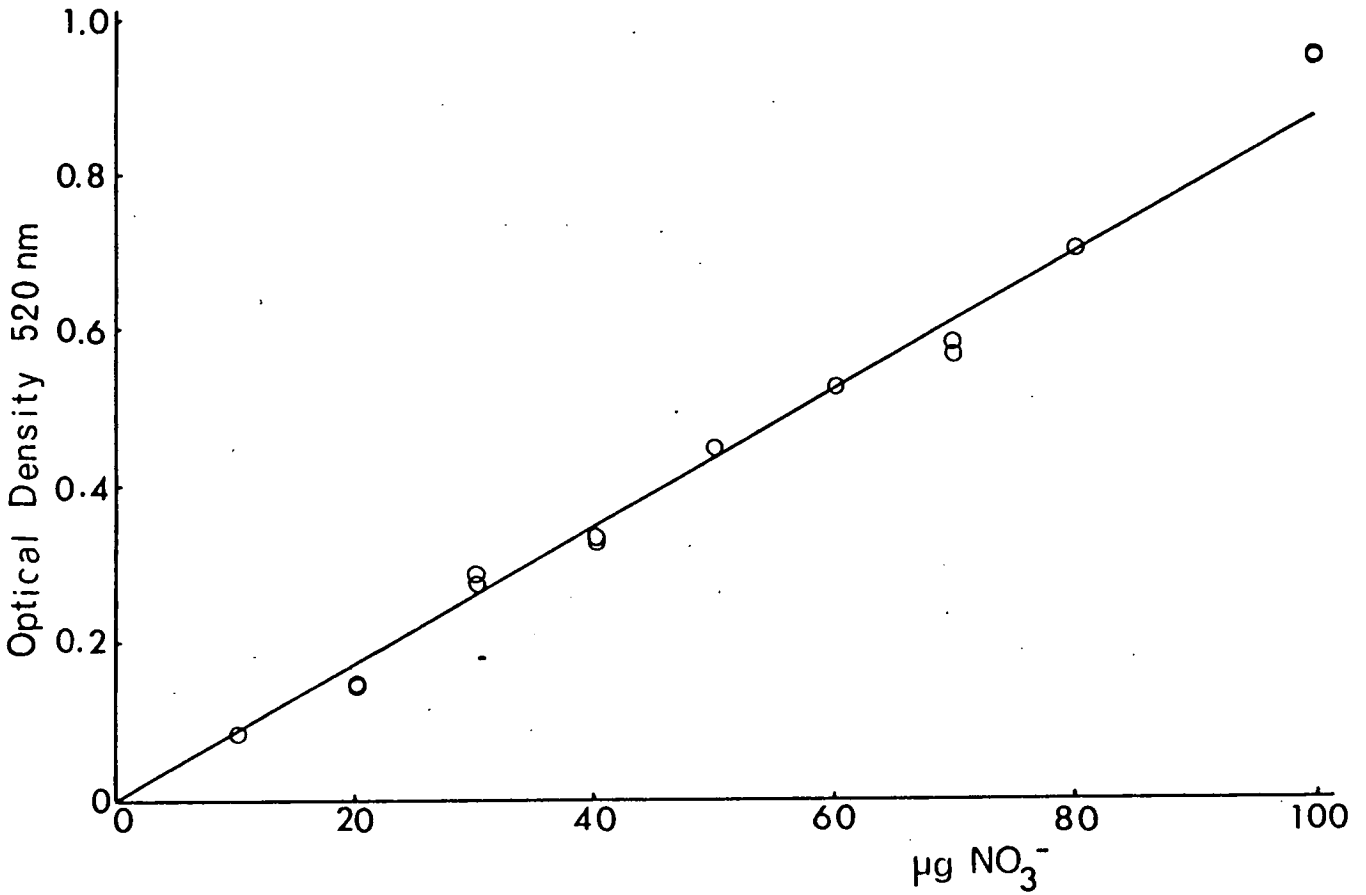



Figure 2.3 Nitrate Calibration Curve

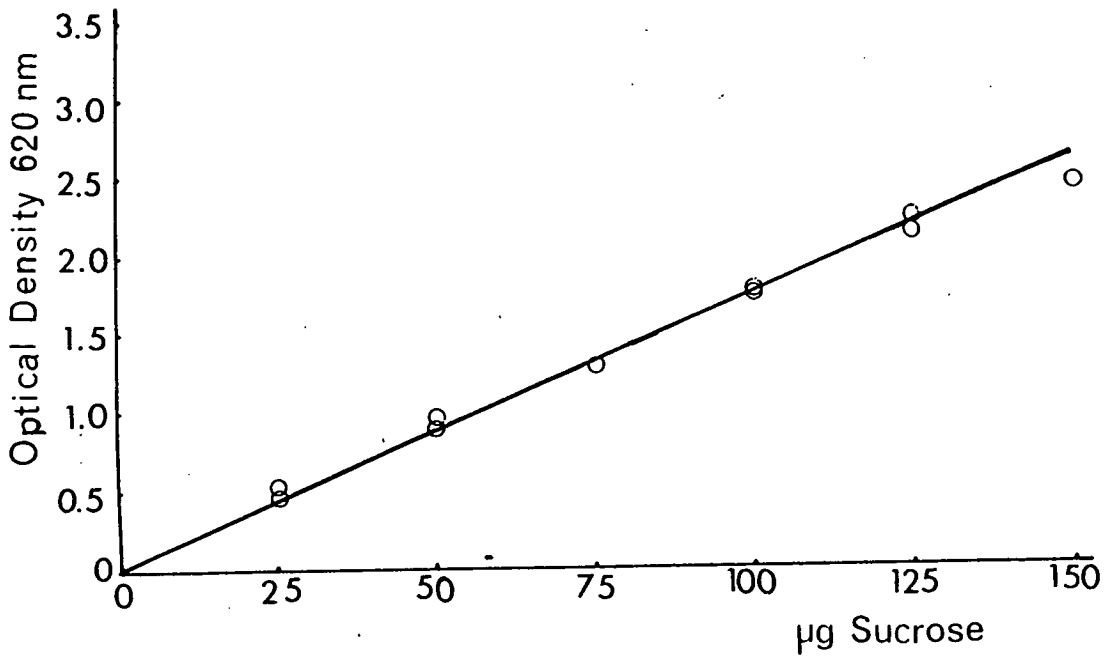



Figure 2.4 Sucrose Calibration Curve : The Anthrone Method

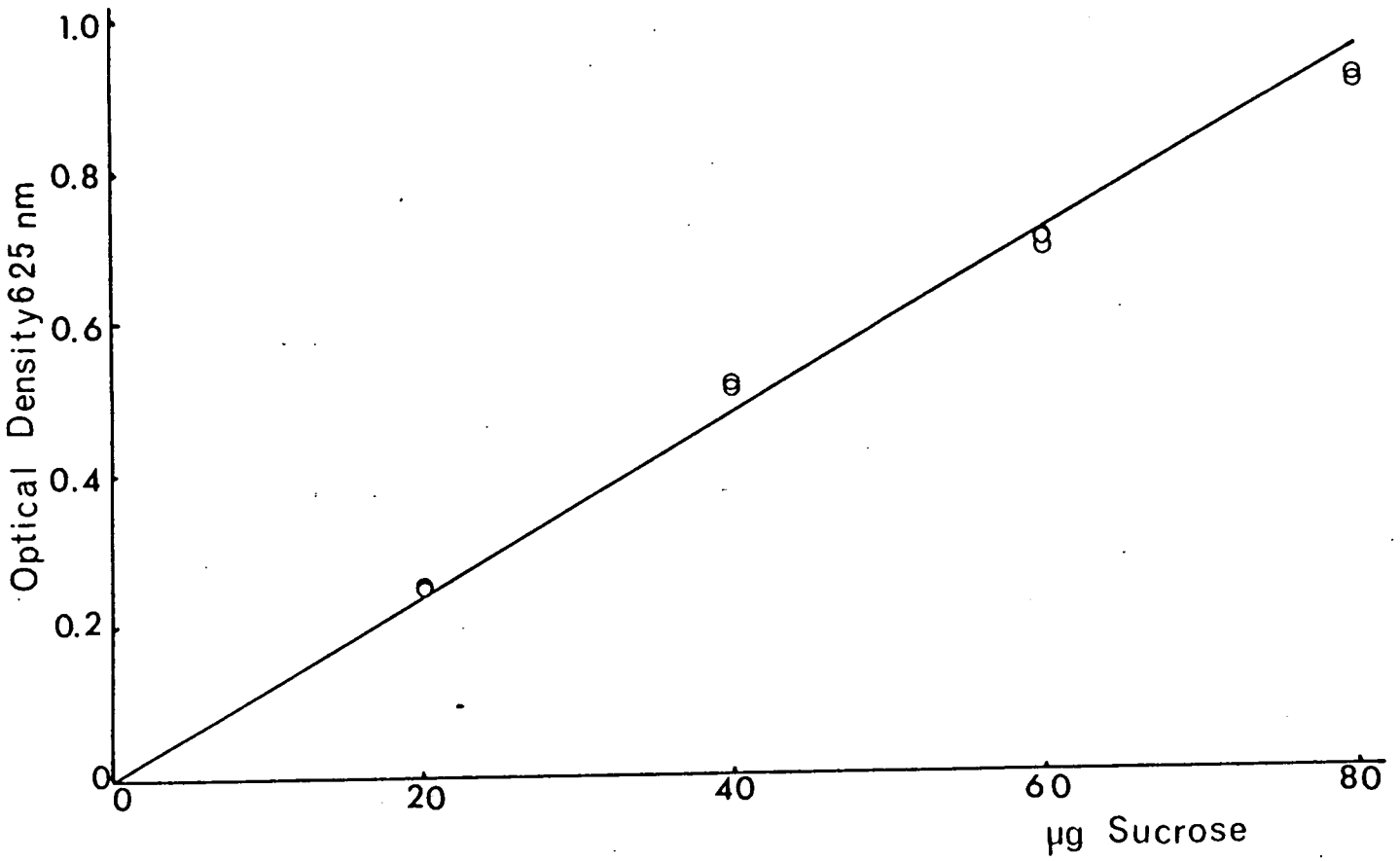



Figure 2.5 Sucrose Calibration Curve : The Somogyi-Nelson Method

B. THE SOMOGYI-NELSON METHOD (described in Moore, 1974)

Reagents :

1. Somogyi reagent

24 g of anhydrous $Na_2CO_3$ and 10 g of sodium potassium tartrate (BDH) were dissolved in 700 ml of distilled water. To this was slowly added, with stirring, 4 g of $CuSO_4$ $5H_2O$ (BDH) in 40 ml distilled water. Then was added 16 g anhydrous $NaHCO_3$ and 180 g anhydrous $Na_2SO_4$ (BDH). The solution was made up to 1 litre with distilled water, and stored in a warm place ($25^oC$).

2. Nelson's reagent

25 g of ammonium molybdate crystals (BDH) were dissolved in 450 ml of distilled water, and to this was added 21 ml of conc. $H_2SO_4$ and mixed. After addition of 3 g of $Na_2HA_5SO_4.7H_2O$ (BDH) dissolved in 25 ml of distilled water, the solution was mixed thoroughly and placed in an incubator ($37^oC$) for 24 hours. After this time the reagent was stored at $25^oC$, in the dark.

x ml of nutrient medium (usually 0.5 ml) was hydrolysed by boiling with x/5 ml 2M HCl for 20 minutes. After cooling the pH was adjusted to 6.0 with 2M NaOH.

1 µl of this solution was made up to 1 ml with distilled water, and to it was added 1 ml of Somogyi reagent, and mixed, followed by boiling for 10 minutes exactly. After cooling in cold water for 5 minutes, 1 ml of Nelson reagent was added, and the whole diluted with 7 ml of distilled water to make a final volume of 10 ml. The

optical density was measured at 625 nm. A calibration curve for sucrose is given (Figure 2.5).

*(f) Analysis of Callus Which Had Been Cultured for a Prolonged Period*

As well as alkaloid content, the chlorophyll content and karyotype of Datura innoxia callus was examined at intervals over a long period of culture (93 weeks), to discover something of the biosynthetic and genetic stability of cultured cells.

1. Chlorophyll Determination

The chlorophyll content of callus was determined by a method based on that described by Harborne (1973), and was performed as follows :

Tissue of known fresh weight (usually between 0.1 and 0.5 g) was ground in 10 ml 80% acetone (BDH), in a pestle and mortar, and the optical density was read immediately at 645 and at 663 nm. The chlorophyll content was calculated from the following formula :

$$\text{chlorophyll a (g/l)} = (0.0127 \times A663) - (0.00269 \times A645)$$

$$\text{chlorophyll b (g/l)} = (0.0227 \times A645) - (0.00468 \times A663)$$

where $A_{645}$ is optical density at 645 nm, and
$A_{663}$ is optical density at 663 nm.

Six determinations were made for each tissue sample, and the mean obtained.

## 2. Karotype Analysis

Callus was pre-treated by immersion for 4 hours in an aerated aqueous solution of colchicine (Sigma London Chemical Co.) (0.05% w/v), to ensure the arrest of mitosis at metaphase by elimination of the mitotic spindle. The cells were then fixed, usually overnight, in a solution of 3 parts absolute ethanol and 1 part glacial acetic acid.

After fixation the hydrolysis of the material (which also facilitates cell separation) was achieved by heating to 60°C in M HCl for 10-12 minutes in a hot-air oven. After the hydrolysing fluid was poured off, Feulgen (made up as described below) was added, and left to stain the nuclei for 1-2 hours.

The stained material was then placed on a clean slide and covered with a drop of 45% acetic acid. Then the material was tapped out with a blunt metal rod until reduced to a suspension of cells. For a temporary preparation, a clean cover slip was coated on one side with a thin layer of glycerol albumen; this was dried slightly in a spirit flame and placed, smeared side downwards, onto the cell suspension. The cells were squashed by gently pressing down on the cover slip through a layer of blotting paper and then examined microscopically.

Mean chromosome numbers were calculated as the average number from a random sample of thirty cells.

The Feulgen stain was prepared according to the method of Darlington and La Cour (1942) as follows :

1. 2 g of basic Fuchsin (BDH) was dissolved by pouring over it 200 ml of boiling distilled water.

2. The solution was shaken thoroughly and allowed to cool to 50°C.

3. The solution was filtered (Whatman qualitative No. 1 filter paper), and to the filtrate was added 30 ml of M HCl.

4. To this was added 3 g of potassium metabisulphite ($K_2S_2O_5$, BDH), and the solution was allowed to bleach for 24 hours in a tightly stoppered bottle, in the dark.

5. Then 1 g of activated charcoal (BDH) was added to decolourise the solution; this was shaken thoroughly for about a minute, and then rapidly filtered through coarse filter paper (Whatman qualitative No. 4).

6. When not in use, the solution was stored in a tightly stoppered bottle at 40°C in the dark.

*(g) The Determination of Cell Viability*

The method used involves the detection of live cells by virtue of their ability to cleave a diacetate moiety from an absorbed fluorescein diacetate molecule, leaving the fluorescein to fluoresce in ultra-violet light (Widholm, 1972).

For a rapid preparation of a solution giving a high degree of fluorescein accumulation, a stock solution of 2-5 mg/ml fluorescein diacetate (Sigma London Chemical Co.) in acetone was added dropwise to liquid full MS medium until the first persistent milkiness appeared; a solution thus prepared was used fresh (Heslop-Harrison and Heslop-Harrison, 1970).

Dyed cells were examined under a Vickers 41 Photoplan fluorescence microscope, which produced ultra-violet light of wavelength 250-480 nm. The emitted (fluoresced) light was yellow-green (500-550 nm) and was usually detectable after two or three minutes; the intensity of the fluorescence would develop for 10 to 15 minutes.

*(h) Determination of Oxygen Levels in Nutrient Medium*

Oxygen levels in nutrient medium were determined using an oxygen electrode (Rank Brothers, Botisham, Cambridge).

The electrode was calibrated as follows : oxygen was bubbled through approximately 100 ml of full MS liquid medium for 15 minutes, in order to saturate the medium with the gas. 1 ml of this solution was then placed into the sample chamber of the electrode, the chamber was sealed and a reading on the scale of a galvanometer so produced was the value representing 100% oxygen saturation of the medium (an actual value of 0.240 µmol $O_2$/ml medium). Then an excess quantity (approximately 20 µg) of sodium dithionite (BDH) was added to the liquid to remove all oxygen from it; on sealing the chamber, the galvanometer reading dropped to a value representing zero oxygen, and in this way a scale representing 0-100% oxygen saturation (i.e., 0-0.240 µmol oxygen/ml medium) was obtained, against which the actual levels in a real sample could be calibrated.

*(i) Scintillation Counting*

The radioactivity of the $^{14}C$ isotope incorporated into proteins and alkaloids, described in Results Part 2, was determined using an Intertechnique SL3000 scintillation counter. The scintillation

fluid employed was toluene-based, with butyl PBD as the scintillant and Triton-X-100 to emulsify any water produced by the reaction between KOH and HCl. The scintillation fluid was composed of 500 ml of Triton-X-100 (Hopkin and Williams) and 6.1 g of butyl PBD (Intertechnique) per litre of toluene (BDH). For the scintillation of extracted proteins, 0.5 ml of extract (i.e., protein dissolved in 0.1 M KOH) was neutralised with 0.5 ml of 0.1 M HCl (to reduce the effects of quenching (loss of counts) by the cloudiness of high-pH scintillation fluid) before being added to 4 ml of scintillation fluid. For the scintillation of alkaloids, the radioactive compounds were scraped from the TLC plates and added directly to 5 ml of scintillation fluid. Although the silica gel from the TLC plates would have caused a certain masking of counts, the degree of quenching was standardised in all samples by adding to the scintillation fluid an equal quantity of silica gel from the plates.

Each radioactive sample was subjected to scintillation counting for 10 minutes, and the results were expressed as counts per minute per gram fresh weight of cells.

## Cleaning of Radioactive Glassware

Radioactive glassware was cleaned by soaking for 2 days in a solution of 20 ml of Decon 90/litre of tap water (Decon Laboratories, Brighton), followed by brushing in a hot solution of teepol and thorough rinsing in tap water and distilled water. Glassware was dried in a hot air oven.

*(j) Statistical Analyses*

In certain experiments the significance of a difference between two sample means was tested; since the number of sample in each treatment was small (usually 3), and the sample variances were assumed to be equal, the Student t-test was performed on the data according to the method described in Parker (1973).

In those cases in which the data to be analysed was in the form of percentages, the values were subjected to arcsin transformation, and statistical manipulations were carried out on the transformed values, which were ultimately converted back to percentages. The rationale behind this procedure is as follows : since percentages must lie in the range 0% to 100%, they do not assume a normal distribution, in which the tails of the bell-shaped curve extend to infinity on either side of the mean. This means that, if a sample has a mean value of say 99%, its standard deviation will be smaller than a sample mean of about 50%, simply because the values are artificially lessened due to their being adjacent to the end of the scale. In other words, because percentage values do not conform to the normal distribution, statistical methods which assume that sample data are normally distributed about a mean cannot be employed in their analysis.

In order to eliminate this problem, percentage data can be transformed in such a way that they can be used in common statistical techniques, and in some experiments described in this thesis, data were so transformed using the arcsin transformation; tables for such a procedure are found in Fisher & Yates (1963).

Standard errors were calculated and Student t-tests were performed according to Parker (1973); where necessary, data was subjected to arcsin transformation before such calculations were performed.

## Section 3 The Flatbed Apparatus

A number of experiments were performed using a novel cell culture system devised by Yeoman et al. (1978), called the flatbed apparatus. The final construction of the apparatus is described here.

The principal feature of this culture system is the movement of liquid nutrient medium across physically stationary cells.

The medium drips under the influence of gravity from a cylindrical reservoir of dimensions 15 cm x 2.5 cm internal diameter and volume approximately 70 ml into a glass culture vessel (a circular dish plus lid of dimensions 9 cm diameter x 5 cm, volume approximately 350 ml) containing cells seated on a polypropylene fabric matting substratum* (Figure 1.1). The nutrient medium moves by capillary action across the fabric, thereby supplying the cells, and is thence pumped from the culture vessel (by means of a peritaltic pump) back into the reservoir and recirculated.

Subaseals (Gallenkamp) were used to seal the ends of the reservoir (subaseal no. 49) and the holes made in the lid (subaseal no. 23) to permit the addition and removal of medium. The subaseals were pierced by stainless steel needles (5 cm x 1 mm internal diameter) at the points of nutrient addition and removal in the apparatus, and were connected together by silicone rubber tubing (internal diameter 1-1.3 mm). Foam bungs were placed over the steel needles at openings in the seals, to reduce the chance of contamination. The whole apparatus was sealed, parafilm being used to seal the culture vessel.

* 'Fresh Mat' (Access Ltd., Crick, Northampton)

1. Preparation for Culture

The apparatus described was sterilised by autoclaving at 121°C (15 lb/in$^2$ steam pressure) for 20 minutes.

Cells, usually from stationary phase (4 weeks old) suspension cultures, were either sieved using a sieve of pore diameter 1 mm (to drain off nutrient medium) and placed (if the suspension cultures were of a low cell density) or poured directly (if the suspension cultures were of a high cell density) onto the fabric substratum, together with 10 ml sterile liquid nutrient medium (full MS); the dish was sealed with parafilm and the cells were left under standard callus culture conditions for 1 week without a further supply of nutrients - the point of this was to allow the cells to 'settle' together, and grow as a callus-like culture.

After 1 week, nutrient medium was supplied to the cells from the reservoir at a flow rate of 10 ml/minute.

During an experimental period, nutrient medium could be sampled for analysis by withdrawal from the top end of the reservoir with the aid of a hypodermic needle and syringe (samples were taken from the top end to reduce the chance of leakage).

The weight of cells from flatbed- or suspension-cultures was expressed in at least one of three ways :

1. The FRESH WEIGHT, which was determined by weighing cells after filtering off nutrient medium on a Buchner vacuum filter or on a Millipore vacuum filter; in both cases, the filter paper used was Whatman Qualitative No. 1.

2. The WET WEIGHT, which was determined by weighing after draining excess nutrient medium from the cells through a wire mesh (of pore diameter 1 mm).

3. The DRY WEIGHT, which was determined by weighing after drying the cells in an air oven at 90°C for 24 hours.

## 2. Peristaltic Pumps

Three peristaltic pumps were used in both flatbed and column experiments, namely the LKB Multiperpex 2115 (LKB, Croydon) and two Watson-Marlow models (the MHRE 200 and the 501 S50, Watson-Marlow Ltd., Falmouth). The LKB pump was found to be the most satisfactory; it caused little damage to the peristaltic tubing, even over a 15 day period of continuous usage, unlike the Watson-Marlow 501 S50.on which the peristaltic tubing began to split after 3 days. Moreover, it was capable of running six separate channels at once, unlike the Watson-Marlow MHRE 200, which had the facility for only two channels (although it had a less destructive effect on the peristaltic tubing than did the other Watson-Marlow pump).

# CHAPTER 3

## Experimental Results

# RESULTS PART 1

## The Production And Screening Of Callus And Cell Suspension Cultures

In this part, the preliminary work which provided the basis for the main studies on alkaloid production in cultured cells is described. It is divided into four sections. Section 1 comprises a study of the suitability of commonly-used reagents for alkaloid-detection and estimation of the cell-squash technique devised by Ogino et al., 1978. This is followed by an attempt to quantify the cell-squash technique, so that the actual alkaloid content of cells can be determined. In Section 2 are described the attempts to induce and maintain callus and cell suspension cultures from a large number of species from a variety of families. Once established, these cultures were subjected to the cell-squash method of alkaloid analysis. The objective of this 'screening programme' was to select the species which were suitable for culture on fixed-bed reactors. Two major criteria were used :

(a) the rate at which the callus tissues and suspended cells grew under the physical and chemical regimes used, and

(b) the level to which the detectable alkaloids were accumulated under these environmental conditions.

In Section 3, a study of the long-term stability of callus with regard to both its ability to retain the capacity to accumulate alkaloids and to maintain a normal genetic complement is described. The possible relationship between alkaloid accumulation and chlorophyll production is also examined. In Section 4 are described attempts made to identify the main alkaloids in callus of both S. nigrum and D. innoxia by TLC and to compare the alkaloid profiles of cultured tissues with those of tissues from whole plants.

# SECTION 1

## THE DEVELOPMENT OF A RAPID TECHNIQUE FOR THE SEMI-QUANTITATIVE ESTIMATION OF ALKALOIDS IN CULTURED CELLS

An essential preliminary to the selection of plants or cultures for the ability to accumulate a particular secondary metabolite is the development of a screening technique in which the desired secondary compound or compounds can be easily and quickly recognised. This is especially important if a large number of cultures (or plants) is to be examined. The advantages of working with cultures which produce coloured metabolites are obvious, and have been exploited by, for example, Alfermann et al. (1975) (anthocyanin production), Mizukami et al. (1978) (production of naphthoquinones), Nishi et al. (1974) (carotenoid synthesis) and Zenk et al. (1975) (anthraquinone production). Most alkaloids are colourless compounds, however, and so their detection requires the use of chemical reagents. Usually the qualitative and quantitative measurement of alkaloids in plant or animal tissues is performed using a chromatographic technique (see, for example, Clarke et al., 1970) or radioimmunoassay (see Zenk et al., 1977). Both techniques, however, require time-consuming preparative work before the sample is 'clean' enough for analysis. In this project it was considered important to establish at an early stage a rapid means of detecting and quantifying the alkaloids under investigation.

The method used was based upon the cell-squash technique of Ogino et al. (1978). In this method, cells are squashed onto filter paper; this absorbs the cell sap containing alkaloids which are then visualised by the application of a standard alkaloid-detecting reagent. The intensity of the colour which develops is related to the concentration of alkaloids in the cell sap. Ogino et al. (1978) determined that, for nicotine, the colour reaction was directly proportional to alkaloid concentration and

they used this method to select cell lines of tobacco (Nicotiana tabacum) callus which accumulated high levels of nicotine.

The results in this section firstly describe the suitability of reagents for the cell-squash technique, and then describe the quantification of this technique by calibrating the intensity of the colour reaction produced between reagents and cell sap (after squashing) against the intensity of colour reactions produced between reagents and solutions of known alkaloid concentrations.

## ALKALOID-DETECTING REAGENTS

A number of reagents have been used to detect alkaloids, and have been employed in both botanical and forensic contexts (e.g., Clarke, 1970). Four such reagents were examined for their suitability in the cell-squash technique of Ogino et al. (1978), namely Dragendorff's, iodoplatinate, Vitali-Morin and Marquis reagents.

Solutions of known concentrations of alkaloids were prepared, and a drop (about 30 µl) was spotted onto filter paper (Whatman, qualitative No. 1). Then a drop (about 30 µl) of a reagent was applied to the spot, and the reaction was noted. Because of the adverse effects of their constituent concentrated acids on the integrity of filter paper, the Vitali-Morin and Marquis reagents were found to be of little practical value; the iodoplatinate and (particularly) the Dragendorff's reagents were most useful and their reactions with seven alkaloids are described in Table 3.1.1.

Table 3.1.1

The minimum concentration of alkaloids for detection by Dragendorff's and iodoplatinate reagents

| | Alkaloid Concentration µg/ml | |
|---|---|---|
| ALKALOID | DRAGENDORFF'S | IODOPLATINATE |
| Atropine | 25 | 300 |
| Scopolamine | 250 | 250 |
| Hyoscyamine | 10 | 35 |
| α-Solanine | 1000 | >3000 |
| Nicotine | 50 | not determined |
| Sparteine | 50 | 20 |
| Trigonelline | 400 | >2000 |

The results show that in general, Dragendorff's reagent was the more sensitive, and was found to detect as little as approximately 0.5 µg of hyoscyamine and atropine, whereas the iodoplatinate could only detect a minimum of ten times that quantity of atropine. Moreover, Dragendorff's reagent could detect trigonelline at a concentration of 400 µg/ml, whereas iodoplatinate produced no visible reaction with a solution of 2 mg/ml.

## THE QUANTIFICATION OF THE CELL-SQUASH TECHNIQUE

Dragendorff's and iodoplatinate reagents were therefore employed in the cell-squash technique. It was found that, for the alkaloids tested, the intensity of the staining reaction produced with both reagents increased with an increase in alkaloid concentration (over the range of concentrations tested), as suggested by Ogino *et al*. (1978) who used the cell-squash method to determine *relative* levels of alkaloids in different cell lines. However, it was considered possible and useful to go one step further, that is, to designate broad categories of the intensity of the staining reaction (i.e., 'none', 'very faint', 'faint', 'light', 'strong' and 'very strong') and correlate these with standard solutions (i.e., of known concentrations) of particular alkaloids. This procedure would provide a means by which the concentration (and hence the amount) of alkaloids in a piece of plant tissue could be estimated. Each description of the reaction intensity was conveniently assigned an arbitrary 'I' (intensity) value, in a manner similar to that of Ogino *et al*. (1978), thus :

Table 3.1.2

| DESCRIPTION OF STAINING REACTION INTENSITY | I VALUE |
|---|---|
| none | 0 |
| very faint | 1 |
| faint | 2 |
| light | 3 |
| strong | 4 |
| very strong | 5 |

Intermediate I values, e.g., 1-2, 4-5, were recorded where considered appropriate.

In order to determine whether a particular I value could be equated with (i.e., whether the same intensity of staining reaction occurs with) the same concentration of different alkaloids, a number of standard solutions were prepared, one drop of each spotted onto filter paper (Whatman qualitative, No. 1) and I values were estimated for each (using a particular detection reagent). The results are expressed in Tables 3.1.3 (a) to (m).

Table 3.1.3

The relationship between I values and alkaloid concentration

(a) L-Hyoscyamine : Dragendorff's

| (L-Hyoscyamine) µg/ml | 0 | 10 | 20 | 40 | 80 | 160 |
|---|---|---|---|---|---|---|
| I value | 0 | 0-1 | 1 | 2 | 3-4 | 5 |

(b) Scopolamine (Hyoscine) : Dragendorff's

| (Scopolamine) µg/ml | 0 | 250 | 300 | 500 | 700 | 1000 |
|---|---|---|---|---|---|---|
| I value | 0 | 1 | 2 | 3 | 4 | 5 |

(c) Atropine : Dragendorff's

| (Atropine) µg/ml | 0 | 10 | 25 | 50 | 100 | 200 |
|---|---|---|---|---|---|---|
| I value | 0 | 0 | 1 | 2 | 3 | 5 |

(d) Nicotine : Dragendorff's

| (Nicotine) µg/ml | 0 | 10 | 25 | 50 | 100 | 200 |
|---|---|---|---|---|---|---|
| I value | 0 | 0 | 0 | 1-2 | 3-4 | 4-5 |

(e) Sparteine : Dragendorff's

| (Sparteine) µg/ml | 0 | 10 | 25 | 50 | 100 | 200 |
|---|---|---|---|---|---|---|
| I value | 0 | 0 | 0 | 1-2 | 3-4 | 5 |

(f) Trigonelline : Dragendorff's

| (Trigonelline) µg/ml | 0 → 300 | 400 | 500 | 700 | 1000 |
|---|---|---|---|---|---|
| I value | 0 | 0-1 | 2-3 | 4 | 5 |

(g) α-Solanine : Dragendorff's

| (α-Solanine) µg/ml | <1000 | 1000 | 1100-1300 | 1400-1600 | 1700-2000 | >2000 |
|---|---|---|---|---|---|---|
| I value | 0 | ~1 | 2 | 3 | 4 | 5 |

(h) L-Hyoscyamine : Iodoplatinate

| (L-Hyoscyamine) µg/ml | <30 | 35 | 75 | 100 | 150 | 250 |
|---|---|---|---|---|---|---|
| I value | 0 | 1 | 2-3 | 3 | 4 | 5 |

(i) Scopolamine (Hyoscine) : Iodoplatinate

| (Scopolamine) µg/ml | 0 | 250 | 300 | 500 | 700 | 1000 |
|---|---|---|---|---|---|---|
| I value | 0 | 1 | 2 | 2-3 | 4 | 4-5 |

(j) Atropine : Iodoplatinate

| (Atropine) µg/ml | <300 | 300 | 450 | 600 | 700-800 | >900 |
|---|---|---|---|---|---|---|
| I value | 0 | 1 | 2 | 3 | 4 | 5 |

(k) Sparteine : Iodoplatinate

| (Sparteine) µg/ml | 0 | 10 | 20 | 50 | 100 | 200 |
|---|---|---|---|---|---|---|
| I value | 0 | 0 | 1 | 2 | 3 | 5 |

(l) Trigonelline : Iodoplatinate

| (Trigonelline) µg/ml | 0 → 2000 |
|---|---|
| I value | 0 |

(m) α-Solanine : Iodoplatinate

| | |
|---|---|
| (α-Solanine) µg/ml | ≤3000 |
| I value | 0 |

It is apparent from the date in Tables 3.1.3 (a) to (m) that different groups of alkaloids are differentially precipitated and detected by each reagent, and, moreover, the two reagents may be differentially sensitive to a particular alkaloid, as indicated above.

Thus this information gives a semi-quantitative basis to the cell-squash technique; that is, the I value produced by applying a drop of reagent to the spot of cell exudate after squashing gives some indication of the concentration of alkaloids in the sap, albeit only a rough indication. The approximate amount of alkaloid per unit weight of tissue can be estimated from an I value if the proportion of tissue dry weight which can be attributed to alkaloids at a particular I value is known. The approximate % dry weight of alkaloids in callus was estimated as follows : callus (or cell clumps from a suspension culture) of a diameter of about 5 mm were found to have a dry weight of the order of 3-4 mg, and to contain 30-40 µl of fluid (gauged by comparing the size of the spot of cell-exudate after squashing onto filter paper with the size of spot produced by spotting known volumes of water onto identical filter paper). If the I value recorded by the cell sap indicates an alkaloid concentration of, say 100 µg/ml, then the callus piece contains 0.03 → 0.04 x 100 = 3-4 µg of alkaloid. The % dry weight is then

$$\frac{\text{weight of alkaloid}}{\text{weight of tissue}} \times 100$$

$$= \frac{3 \rightarrow 4}{3000 \rightarrow 4000} \times 100$$

$$= \underline{\sim 0.1\% \text{ dry weight}}$$

So, if an I value of 4 represents an alkaloid concentration of, say 100 µg alkaloid/ml cell sap, and 100 µg/ml represents about 0.1% dry weight of alkaloid in the cell, then an I value of 4 represents a level of alkaloid production of about 1 mg alkaloid/g dry weight of callus.

Table 3.1.4 gives the concentrations of alkaloids in the cell sap and the amount of alkaloids per g dry weight of cells for different groups of alkaloids, as detected by Dragendorff's reagent.

With this information available it was concluded that the approximate levels of alkaloids accumulated by cultured cells could be estimated with an adequate degree of accuracy for the purposes of screening a large number of plant species.

Table 3.1.4 (a)

The concentration and amount of 5 different groups of alkaloids in cells which correspond, approximately, with observed I values (after staining with Dragendorff's reagent)

| | 'TROPANE' | | 'PYRIDINE' | |
|---|---|---|---|---|
| I Value | µg alk $ml^{-1}$ cell sap | mg alk $g^{-1}$ dry weight cells | µg alk $ml^{-1}$ cell sap | mg alk $g^{-1}$ dry weight cells |
| 0 | 0 - 10 | 0 - 0.10 | 0 - 50 | 0 - 0.50 |
| 1 | 10 - 25 | 0.10 - 0.25 | 50 - 75 | 0.50 - 0.75 |
| 2 | 25 - 50 | 0.25 - 0.50 | 75 - 100 | 0.75 - 1.00 |
| 3 | 50 - 100 | 0.50 - 1.00 | 100 - 150 | 1.00 - 1.50 |
| 4 | 100 - 150 | 1.00 - 1.50 | 150 - 200 | 1.50 - 2.00 |
| 5 | >~150 | >~1.50 | >~200 | >~2.00 |

Table 3.1.4 (b)

| | 'PIPERDINE' | | 'QUINOLIZIDINE' | | 'SOLANUM' | |
|---|---|---|---|---|---|---|
| I Value | µg alk $ml^{-1}$ cell sap | mg alk $g^{-1}$ dry weight cells | µg alk $ml^{-1}$ cell sap | mg alk $g^{-1}$ dry weight cells | µg alk $ml^{-1}$ cell sap | mg alk $g^{-1}$ dry weight cells |
| 0 | 0 - 400 | 0 - 4.0 | 0 - 50 | 0 - 0.50 | <~1000 | <~10.0 |
| 1 | 400 - 50 | 4.0 - 5.0 | ~50 | ~0.50 | ~1000 | ~10.0 |
| 2 | 500 - 600 | 5.0 - 6.0 | 50 - 75 | 0.50 - 0.75 | ~1200 | ~12.0 |
| 3 | 600 - 700 | 6.0 - 7.0 | 75 - 100 | 0.75 - 1.00 | ~1500 | ~15.0 |
| 4 | 700 - 1000 | 7.0 - 10.0 | ~150 | ~1.50 | ~1700 - 2000 | ~17.0 - 20.0 |
| 5 | >~1000 | >~10.0 | >~200 | >~2.00 | ~3000 | ~30.0 |

# SECTION 2

## THE ESTABLISHMENT AND SCREENING OF CELL CULTURES FOR ALKALOIDS

Once the rapid semi-quantitative technique for the estimation of alkaloids had been established, the next stage of the project was undertaken, namely the induction of callus from a large number of plant species and the determination of the levels of alkaloids accumulated by the cultured tissues.

Thirty-two species known to accumulate alkaloids in the whole plant (listed in 'Materials', chapter 2, table 2.1) were grown from seed and tissue explants were placed on agar plates containing full MS medium. Although it is probable that other nutrient media would have had different effects on cell growth rate and alkaloid production, no attempt was made to find the optimal combination of nutrients and growth-regulatory substances for each individual species - that in itself would have been a massive operation. The performance of the explants is presented in Table 3.1.5, and it can be seen that the nutrient medium employed was conducive to the growth of about one third of the original number of explants. Those individuals which failed to proliferate were discarded, while the actively-growing cells were separated from the original explants and subcultured onto fresh medium. Two months after callus initiation, the appearance and alkaloid content of each species in culture was recorded, and the results are presented in Table 3.1.5.

It was observed empirically from these results that fast-growing callus cultures were often friable, i.e., the cell density was low. Microscopical examinations of cell macerates (Table 3.1.6) indicated that friable callus cells of a number of species (e.g., Atropa belladonna, Datura innoxia, Solanum nigrum) may have been actively dividing (the cells were small, rounded, highly cytoplasmic) whereas the cells from more compact callus, e.g., of

Table 3.1.5

Alkaloid contents of callus cultures

| SPECIES | DESCRIPTION | MEAN I VALUE | APPROXIMATE ALKALOID CONTENT mg alk/g dry weight callus |
|---|---|---|---|
| Datura innoxia | friable, pale, actively-growing | 0-1 | <0.1 |
| | compact, green, slower-growing | 2 | 0.3 |
| D. innoxia (haploid) | green, slow-growing | 4 | 1.3 |
| D. stramonium | pale green | 2-3 | 0.5 |
| | green/brown, slow-growing | 3-4 | 1.0 |
| D. clorantha | dark brown, undifferentiated, not growing | 1 | 0.1 |
| | compact, green, slow-growing | 4 | 1.3 |
| Hyoscyamus niger | friable, pale green | 2 | 0.3 |
| Atropa belladonna | friable, pale green | 2-3 | 0.5 |
| | friable, white | 1 | 0.1 |
| Solanum dulcamara | brown, very slow-growing | 2 | 12 |
| | green, highly differentiated | 3-4 | 15-20 |
| S. nigrum | friable, pale | 0-1 | <10 |
| | compact, some differentiation | 1-2 | 12 |
| | green | 1-2 | 12 |
| Nicotiana tabacum | mixed appearance (green/greyish/ white), fairly compact | 2 | 0.5 |
| Lupinus luteus | brown, very slow-growing | 0 | <0.5 |
| L. mutabilis | brown, very slow-growing | 0-1 | 0.5 |

(continued)

Table 3.1.5 (continued)

| | | | |
|---|---|---|---|
| L. angustifolius | brown, very slow-growing | 2 | 0.7 |
| | faster-growing, green, clumped | 4 | 1.5 |
| Trigonella balansae | friable, pale | 0 | ? |
| T. corniculata | friable, pale | 2 | ? |
| | compact, green, slower-growing | 4 | ? |
| T. foenum-graecum | friable, pale, actively-growing | 0 | ? |

Solanum dulcamara, Nicotiana tabacum) were not (the cells mostly were parenchymatous, i.e., larger and more vacuolated than the more friable cells).

It may be seen from the results in Table 3.1.5 that most of the cultures produced detectable levels of alkaloids, and, in general, actively-growing cultures were less green and accumulated the lowest levels of alkaloids. The more compact, slower-growing cultures, on the other hand, were greener and accumulated the highest levels of alkaloids. The most striking example of this phenomenon is given by the callus of S. dulcamara. This grew very slowly indeed, requiring subculture only every 3 months, yet was bright green in colour and was structurally highly differentiated, producing an abundance of roots, shoots and leaves, and relatively high levels of alkaloids (I values of 4-5, representing an alkaloid content of 20-30 mg/g dry weight callus). Occasionally, regions of colourless faster-growing callus developed which accumulated much lower levels of alkaloids (I values of 0-2, i.e., less than about 12 mg/g dry weight callus). However, some of this colourless callus gave I values of 3-4 (about 15-20 mg alkaloids/g dry weight callus) - this was relatively compact tissue; it was the more friable colourless callus which contained the lowest levels of alkaloids.

Callus cultures of a number of species exhibited a notable heterogeneity of appearance, both between tissue pieces on the same or different agar plates, and between areas in a single mass of callus. This variability, most notable as differences in the colour of the callus, was especially prominent in tissue of S. nigrum,

Table 3.1.6

'Cell division' in friable and compact callus

Cells were macerated with a needle on a glass slide, and examined and counted without staining.

| | ATROPA BELLADONNA FRIABLE CALLUS | | | SOLANUM DULCAMARA COMPACT CALLUS | |
|---|---|---|---|---|---|
| Replicate | * Number of 'Dividing' Cells | † Number of 'Non-dividing' cells | Replicate | * Number of 'Dividing' Cells | † Number of 'Non-dividing' cells |
| 1 | 249 | 51 | 1 | 3 | 297 |
| 2 | 287 | 13 | 2 | 31 | 269 |
| 3 | 283 | 17 | 3 | 28 | 272 |
| $\bar{x}$ | 273 | 27 | | 30.6 | 279.3 |

* = small, round, highly cytoplasmic cells

† = large, vacuolated cells

Datura stramonium, and N. tabacum (Table 3.1.5). Callus of S. nigrum for example, was found to vary in colour from white to green to very dark brown or grey across a single piece of tissue, and the levels of alkaloids accumulated within a particular callus were found also to vary; levels were almost invariably highest in the greenest parts (Table 3.1.7).

Some species, such as A. belladonna, produced callus which was more homogeneous in appearance, although the extent of greening of the whole culture was found to change throughout a passage period (i.e., the period between successive subcultures). This phenomenon is described below, and in Results Part 2, for other species.

## THE INITIATION OF CELL SUSPENSION CULTURES

An attempt was made to initiate cell suspension cultures from the most friable callus cultures, in order that large quantities of cells could be grown up rapidly for use in the flatbed- and column-culture systems. Under the conditions used (i.e., on full MS liquid medium) the only cells to grow satisfactorily were those of A. belladonna, S. nigrum and D. innoxia; the best of these was S. nigrum, which in four weeks grew to a density representing a packed cell volume of 80-85% (i.e., the ratio of the volume of cells to the volume of cells plus nutrient medium after spinning 10 ml of a cell suspension at 1000 g for 10 minutes).

There was found to be some heterogeneity in the cell suspension cultures, although to a smaller extent than in callus cultures. In the cultures of S. nigrum, the majority of the cells were organised in the form of pale clumps of 3 or 4 cells, but there were also

Table 3.1.7

Heterogeneity in *S. nigrum* callus

| CALLUS APPEARANCE | MEAN I VALUE | APPROXIMATE ALKALOID LEVELS mg/g dry weight callus |
|---|---|---|
| white, friable | 0.5 | <10 |
| grey, friable | 0.9 | 10 |
| grey, compact | 1.7 | 12 |
| brown, compact | 1.5 | 11 |
| green, compact | 2.3 | 13 |

present larger clumps which were either a pale creamy colour or occasionally green; these green clumps accumulated the highest levels of alkaloids (see Table 3.1.8). Towards the end of the growthcycle (after 3 - 4 weeks into the passage period), the incidence of green clumps usually increased. However, this was not invariably the case, for some cultures merely turned brown and died after 4 weeks, with no greening at all. In these brown cultures there was not the dramatic increase in the levels of accumulated alkaloids observed in those which had turned green(Table 3.1.8).

In (most notably) A. belladonna suspension cultures there was found (again occasionally, not invariably) a distinct structural differentiation of the suspended cells. This involved the formation of unusual shoot-like organs and embryoids in the stationary phase (from after 25-30 days into the passage period), accompanied by an obvious increase in greening, particularly of the embryoids. Such structures contained relatively high levels of alkaloids, of I values 4-5, representing at least 1 mg tropane alkaloids/g dry weight cells (Table 3.1.8). Similar observations have been made by Thomas and Street (1970).

The main conclusion from the observations of alkaloid accumulation in the callus and suspended cells is that there appears to be a positive correlation between the state of greening and structural organisation (a term used here to describe compactness and embryoid and root and shoot formation) and the levels to which alkaloids are accumulated. Moreover, it would appear that there is an inverse relationship between the growth rate of the culture and the extent of its greening, structural organisation and alkaloid

Table 3.1.8

The alkaloid contents of suspended cells

| SPECIES | APPEARANCE | MEAN I VALUE | APPROXIMATE ALKALOID CONTENT mg/g dry weight cells |
|---|---|---|---|
| Atropa belladonna | pale undifferentiated clumps | 1 | 0.1 |
| | green clumps, ± embryoid | 4-5 | 1.5 |
| Solanum nigrum | pale undifferentiated clumps | 1 | 10 |
| | green/brown clumps | 3-4 | 16 |
| | green clumps | 4 | 18 |
| | brown clumps | 1-2 | 12 |
| Datura innoxia | pale undifferentiated clumps | 1-2 | 0.3 |
| | green clumps | 4 | 1.3 |

content. It was only in the green or differentiated cultures that cells accumulated alkaloids to levels approaching those in the whole plant (stem tissue squashes of D. innoxia and D. stramonium onto filter paper gave I values of 5, but no upper limit of the amount of alkaloids present could be set by this cell-squash technique); the majority of the cultures produced much lower levels than did whole plants, and suspended cells tended to produce lower levels than callus (when compared at periods of fastest growth).

With regard to the selection of species for their suitability for use on the flatbed and column culture systems, Datura innoxia and Solanum nigrum were considered the most appropriate: they both were found capable of accumulating alkaloids in both callus and cell suspension culture, but perhaps more importantly they grew rapidly and to a high cell density in liquid culture, and this characteristic was most desirable. The fact that only relatively low levels of alkaloids seemed to be produced in the fast-growing suspensions was thought not to be a serious drawback, for it was hoped that manipulation of the growth conditions would induce a transition from the 'cell suspension habit' (i.e., fast growth, dispersed cells, low alkaloid production) to the 'callus habit' (i.e., slower growth, denser cultures, relatively high alkaloid production). The cell-squash technique was useful for the semi-quantitative analysis of alkaloids, but it was considered important, at this stage, to analyse callus qualitatively.

# SECTION 3

## AN ATTEMPT TO IDENTIFY THE MAIN ALKALOIDS IN CALLUS AND WHOLE PLANT TISSUE OF *SOLANUM NIGRUM* L. AND *DATURA INNOXIA* MILL.

The results from the previous section show that the cell squash method of alkaloid determination may be used to estimate the level of total alkaloids in a tissue sample, but does not give an indication of the qualitative composition of or the ratios of different alkaloids in the sample. The aim of this section was to identify as many as possible of the main alkaloids in extracts of whole plant tissue and callus of both S. nigrum and D. innoxia. Two methods were used : (1) a comparison of the $R_f$ values of spots (visualised by alkaloid-specific reagents) produced after chromatography of extracts with those of commercially-obtained samples of known alkaloids; and (2) by comparing the $R_f$ values of spots from extracts with those values for known alkaloids that are listed in the literature (see Clarke, 1970).

## TO DETERMINE THE EFFICIENCY OF THE EXTRACTION OF ALKALOIDS ACCORDING TO THE METHOD OF HULTIN AND TORSELL (1965)

The aim of this preliminary experiment was to determine the efficiency of the alkaloid extraction procedure used for the TLC analyses described in this section. Since most alkaloids are basic, methods for their extraction employ the use of a weakly acidic alcoholic solvent, from which they are precipitated with a concentrated solution of ammonia.

Hultin and Torsell (1965) have described a relatively simple procedure for the extraction of alkaloids which was originally designed for use in the screening of a large number of plants in the field. Before employing this technique (described in 'Methods', p.30 ), the approximate efficiency of the extraction method was

estimated by determining how much of a known weight of a pure sample of alkaloid could be recovered from a mixture of alkaloid and sand (which was used in the tissue-grinding procedure in an actual analysis of tissue) after going through the whole extraction process.

Thus, 0.1 g of scopolamine hydrochloride (Sigma London Chemical Co.) was ground up with approximately 1 g of sand in 20 ml of methanol in a pestle and mortar; a further 20 ml of methanol was added and the mixture was left to stand overnight. The procedure was then continued as described for the extraction of alkaloids from tissue, involving the completion of the extraction in acidified methanol, the precipitation of the alkaloid with 0.88 ammonia and extraction in chloroform. When this stage was reached, the aqueous and chloroform fractions were evaporated to dryness in vacuo at 40°C.

It was found that no scopolamine was present in the evaporation flask after the drying down of the aqueous extract (as demonstrated by the addition to the flask of approximately 0.5 ml of Dragendorff's reagent). However, a large proportion (0.0873 g) of the original quantity of scopolamine hydrochloride was recovered by scraping it from the inside of the flask (and was shown to be scopolamine hydrochloride by virtue of its colour reaction (pink-orange) with Dragendorff's reagent and co-chromatography of 10 µg of the extracted material with 10 µg of the original compound of chloroform : acetic acid : methanol (60 : 5 : 35 v/v) on TLC plates of Kieselgel 60 (as described in 'Methods') at an $R_f$ value of 0.15 (visualised by spraying with Dragendorff's reagent). The addition of approximately

0.5 ml of Dragendorff's reagent to the inside of the flask in which the chloroform extract was dried revealed that, though invisible, a readily detectable quantity of scopolamine lined the glass, and precipitated as pink-orange clots.

It was therefore concluded that this extraction procedure was satisfactory for the purposes required in this project.

Once the extraction procedure was established, an attempt was made to identify the main alkaloids present in callus and whole plant tissue of S. nigrum and D. innoxia.

## THE EXTRACTION AND IDENTIFICATION OF SOLANUM NIGRUM ALKALOIDS

### 1. In Whole Plant Tissue

The tissue from which alkaloids were extracted was a 5 g mixture of stem, leaf and mature fruit (in an approximate ratio of 1 : 1 : 1 by weight) derived from mature plants (i.e., at the mature fruit stage) which had been grown up from seed in the greenhouse of the Botany Department, Edinburgh, in the manner described in the 'Materials and Methods' chapter. Alkaloids were extracted from the dried tissue according to the method of Hultin and Torsel (1965) giving two fractions of alkaloids, designated fraction A and fraction B respectively (the latter containing, if present, solanine, which is more soluble in ethanol than in chloroform). Samples (10 µl and 25 µl) of the extracts (fractions A and B) were loaded onto commercially-prepared TLC plates (coated with 0.2 mm Kieselgel 60, Merck) and separated in either of two solvent systems : one was a 100 ml mixture of chloroform : acetic acid : methanol

(60 : 5 : 35 v/v) (Hultin, 1966), and the plate sprayed, when dry, with Dragendorff's or iodoplatinate reagents; the other was a 101.5 ml mixture of methanol : 0.88 ammonia (100 : 1.5 v/v) (Clarke, 1970) and the plate sprayed, when dry, with Dragendorff's or iodoplatinate reagents. The use of two solvent systems and two detection reagents provided a means of double-checking the number of separate alkaloids in a particular extract; the spots separated in the methanol : ammonia system and sprayed with the iodoplatinate reagent were checked against the list of $R_f$ values (for that solvent system and spray given in Clarke (1970)). 5 µg of samples of the only two Solanum steroidal glycoalkaloids readily obtainable, namely α-solanine (composed of a glucose, a galactose and a rhamnose residue attached to the steroidal alkamine moiety solanidine) and α-chaconine (composed of two rhamnose and one galactose residue attached to solanidine) (Sigma London Chemical Co.) were dissolved in methanol and run alongside samples of the tissue extracts for identification purposes.

The results of subjecting the tissue extracts to TLC are given in Figures 3.1.1(a) and (b).

## A. THE CHLOROFORM : ACETIC ACID : METHANOL SYSTEM

### FRACTION A

In this fraction, four separate alkaloids were distinguished, the $R_f$ values of which were 0.86, 0.81, 0.10 and 0.08 (detectable in only the 25 µl sample).

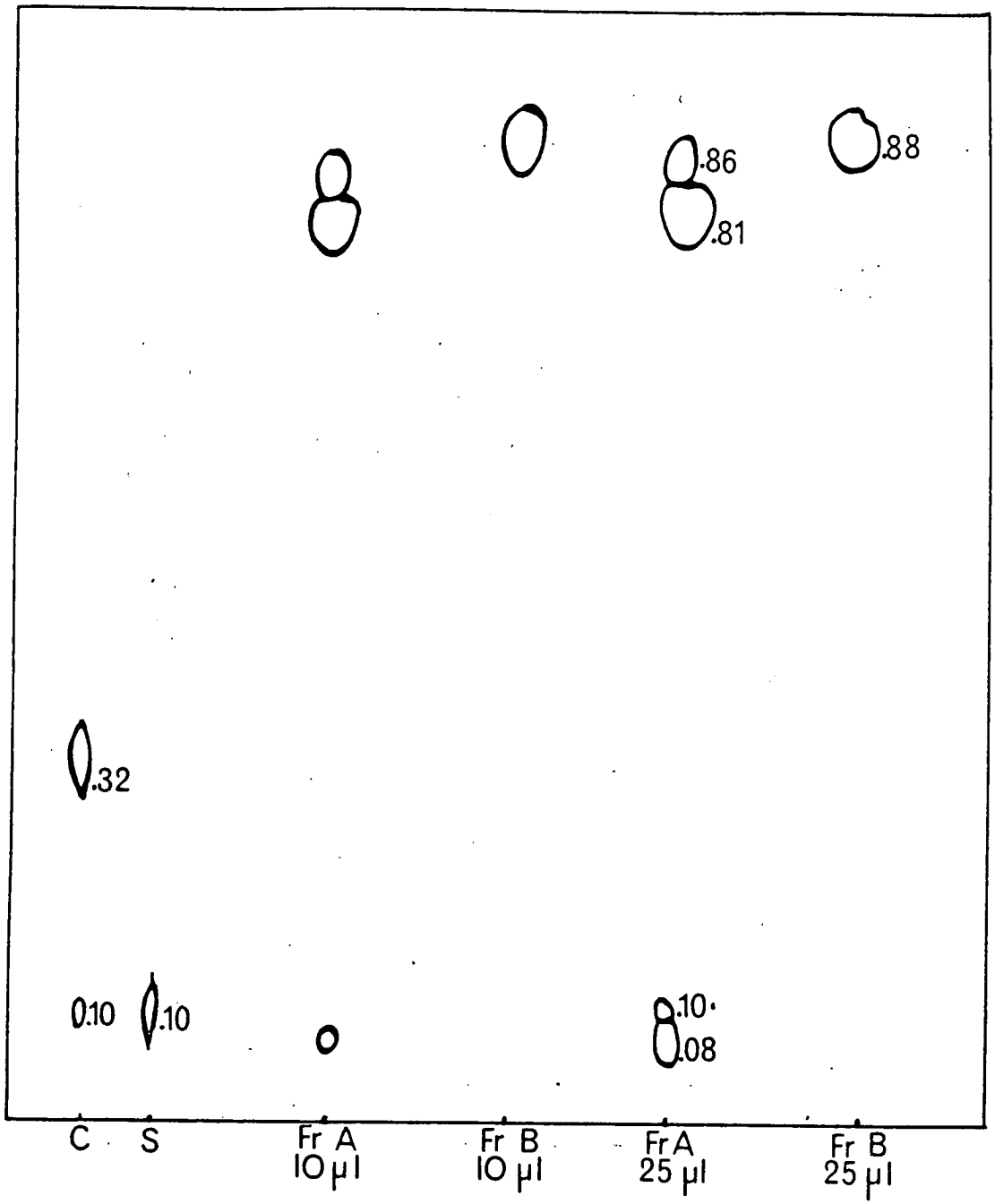
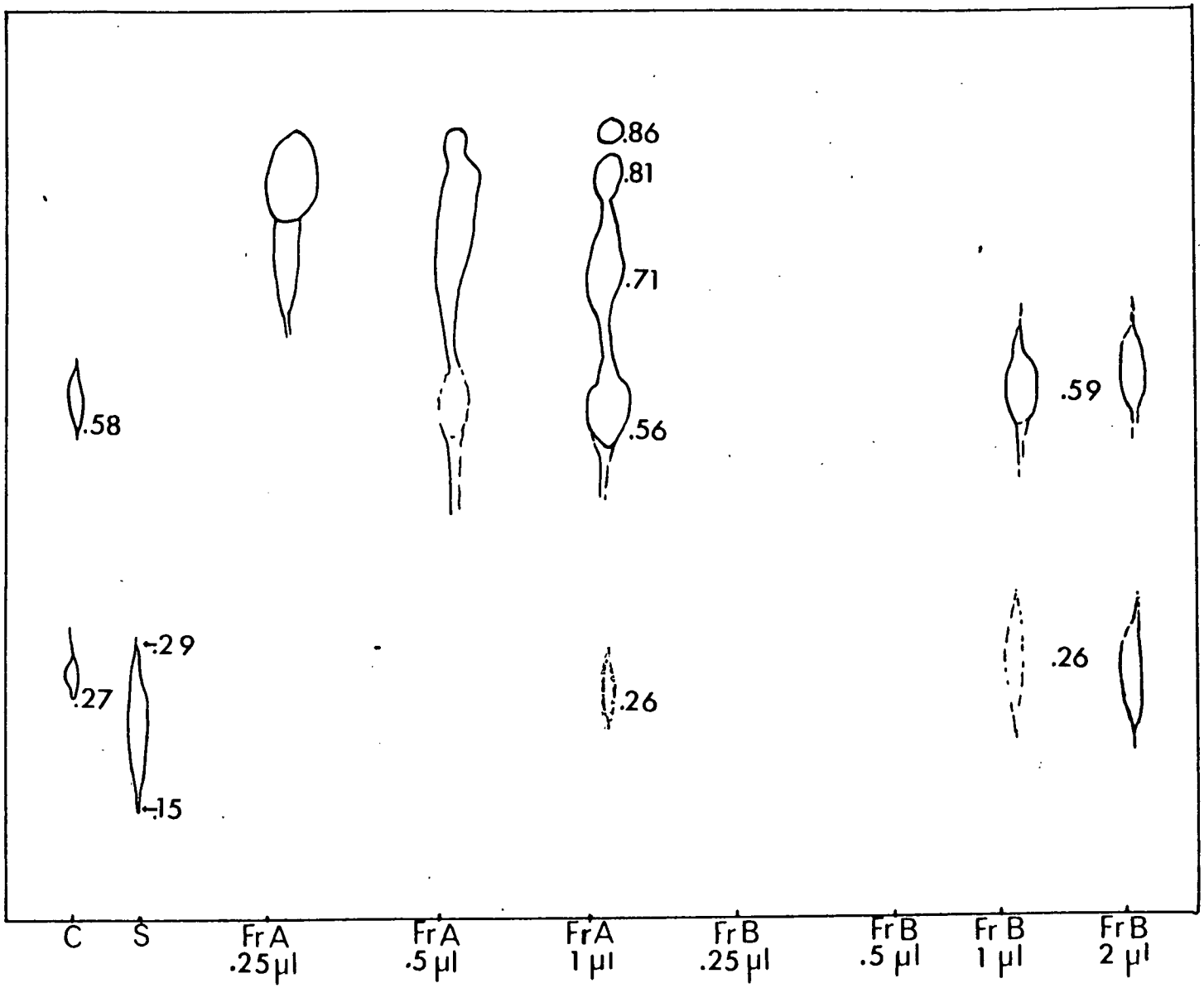



Figure 3.1.1 (a) TLC of Extracts of S. nigrum Whole Plant Tissue in the Chloroform : Acetic Acid : Methanol System, Sprayed with Dragendorff's Reagent

C = α-chaconine

S = α-solanine




C = α-chaconine

S = α-solanine

Figure 3.1.1 (b) TLC of Extracts of S. nigrum Whole Plant Tissue in the Methanol : Ammonia System Sprayed with Iodoplatinate Reagent

FRACTION B

In this fraction, one alkaloid was present, of $R_f$ value 0.88, detectable in both the 10 µl and 25 µl samples.

The standard samples had $R_f$ values of 0.10 (α-solanine) and 0.34 (α-chaconine); the latter sample appeared to contain a small amount of impurity, detectable at $R_f$ 0.10, which was probably α-solanine.

## B. THE METHANOL : AMMONIA SYSTEM

FRACTION A

10 µl and 25 µl proved to be too high loading volumes to be of any real analytical value - streaking of the alkaloids was such that individual spots were obscured. Therefore, sample volumes of 0.25 µl, 0.5 µl and 1 µl were run. It was then found that in the 1 µl sample four or five alkaloids were separated, of $R_f$ values 0.86, 0.81, 0.71, 0.56, and possibly 0.26: the spot was very faint; fewer alkaloids were visualised in the samples of smaller volume.

FRACTION B

As was the case for Fraction A, the 10 µl and 25 µl samples streaked, so smaller volumes (0.25 µl, 0.5 µl, 1.0 µl and 2.0 µl) were loaded and separated. One alkaloid was present in the 1 µl and 2 µl samples, of $R_f$ value 0.59; another was present, but was extremely faint, at the position representing an $R_f$ value of 0.26.

In this solvent system, α-solanine had an $R_f$ value of between 0.29 and 0.13 (appearing as a streak) and α-chaconine an $R_f$ value

of 0.58 (with a smaller, additional spot of $R_f$ 0.27, which was probably $\alpha$-solanine as an impurity in the sample).

A number of tropane alkaloids were chromatographed in the two solvent systems, to determine whether the tissue extracts contained any compounds which separated with comparable $R_f$ values, which are given in Table 3.1.9.

Both Solanum alkaloids and tropane alkaloids, when sprayed with Dragendorff's reagent after separation in either of the solvent systems, were visualized as pink-orange spots on a yellow background. When sprayed with the iodoplatinate reagent, the two groups of alkaloids appeared either grey on a pink-red background (if separated in the chloroform : acetic acid : methanol solvent system) or a creamy pink-brown on an off-white background (if separated in the methanol : ammonia system).

The results suggest that the extract of S. nigrum whole plant tissue contained five or possibly six distinct alkaloids detectable by the techniques employed, four in Fraction A and one or perhaps two in Fraction B. It is possible that other alkaloids, present in smaller quantities than those detected, exist in whole plant tissue, but the problem of streaking dictated that it was not possible to load higher concentrations of extract sample onto the plastes in order to try and visualise these 'minor' compounds.

Difficulties arise when attempting to positively identify the alkaloids. Firstly, unless one knows what is likely to be present in an extract, one can only give a very tentative identification to a particular compound based on a comparison of the observed rate

Table 3.1.9

$R_f$ values of tropane alkaloids

(a) In chloroform : acetic acid : methanol (60 : 5 : 35 v/v)

(detectable with either Dragendorff's or iodoplatinate reagents)

| | Tropane Alkaloid | $R_f$ Value |
|---|---|---|
| 1.6 µg | Hyoscyamine | 0.49 |
| 3.3 µg | Scopolamine | 0.15 |
| 5.8 µg | Atropine | 0.32 |
| 1.4 µg | Tropine | 0.14 |

(b) In methanol : ammonia (100 : 1.5)

(detectable with either Dragendorff's or iodoplatinate reagents)

| | Tropane Alkaloid | $R_f$ Value |
|---|---|---|
| 1.6 µg | Hyoscyamine | 0.19 |
| 3.3 µg | Scopolamine | 0.64 |
| 5.8 µg | Atropine | 0.21 |
| 1.4 µg | Tropine | not detected |
| 2.8 µg | Tropine | 0.07 |

of migration in a particular solvent system with the $R_f$ values given in the literature for a wide range of alkaloids, from a variety of taxa (such as given in Clarke, 1970). Moreover, it was found that in some cases (such as those of α-solanine and scopolamine) there was a significant discrepancy between the listed and observed $R_f$ values of a particular (pure sample of) alkaloid, possibly as a result of using different plate coatings - Clarke (1970) does not name the type of TLC plate or plates he used. Thus, co-chromatography was considered to be the surest method of identification, but, of course, is only possible if a large range of standards is available, and as indicated above, if the candidates likely to be present are known beforehand.

With these limitations in mind, it was possible to gain a tentative identification of some compounds, and to eliminate the possibility of others. The alkaloid present in Fraction A, separated in the chloroform : acetic acid : methanol system, of $R_f$ value 0.10 (and possibly also that of $R_f$ 0.08) was probably α-solanine. It is known to be present in fruits of S. nigrum and was found to co-chromatograph with the α-solanine standard. Indeed, the spot size from the extract was increased at an $R_f$ value of 0.10 when 5 μg of α-solanine was added to the loaded extract sample as an internal standard. Nevertheless, some doubt remains as to whether this Fraction A compound really was α-solanine, because Fraction A alkaloids were extracted in chloroform, and α-solanine is only sparingly soluble in this solvent. However, the Fraction B alkaloid (extracted in a chloroform : ethanol mixture) of $R_f$ 0.26 in the methanol : ammonia system was probably α-solanine (as suggested by co-chromatography with internal and external standards).

The spot of $R_f$ value 0.56 in the methanol-ammonia system was found to co-chromatograph with both internal and external (5 µg) standards of α-chaconine, and may be tentatively identified as such.

The fraction A compound (in the methanol : ammonia system) of an $R_f$ value of 0.71 was possibly the steroidal aglycone solanidine, by comparison with the data given by Clarke (1970), which gives an $R_f$ value for this molecule of 0.68 - certainly it is readily soluble in chloroform, unlike solanine.

There appear to have been no tropane alkaloids present in detectable quantities in the extracts examined. The other alkaloids present could not be identified even tentatively.

## 2. Alkaloids in S. nigrum Callus

The callus used in the extractions was originally derived from stem tissue of mature plants, and had been maintained and sub-cultured (at monthly intervals for 6 months) on full MS medium on agar plates. The extraction procedure employed was the same as that described for the whole plant tissue; 5 g of callus was dried, ground and subjected to extraction. As was the case for the whole plant extracts, chromatographic separation was carried out in two solvent systems, the chloroform : acetic acid : methanol and the methanol : ammonia systems. The results are presented in Figures 3.1.2 (a) and (b).

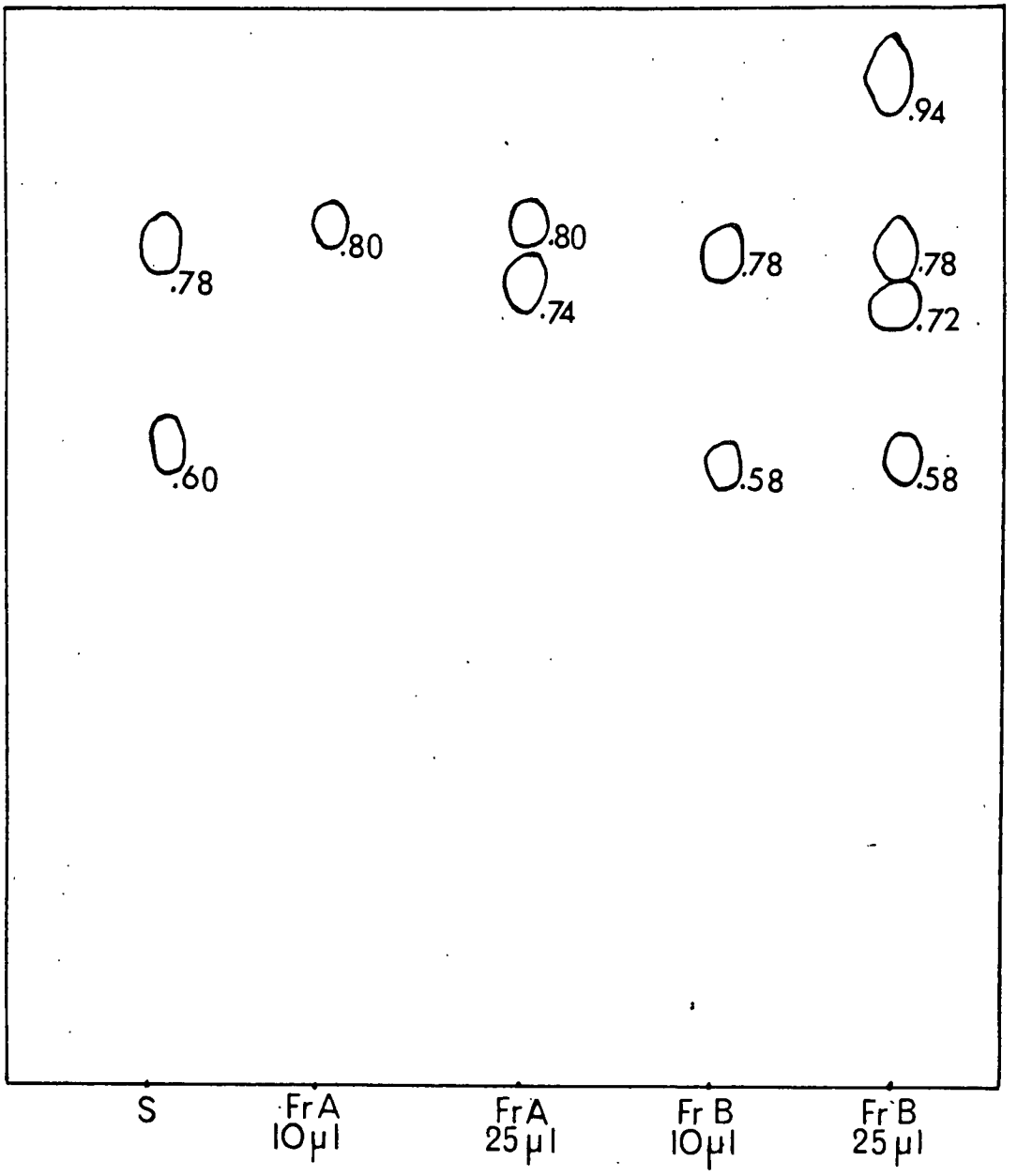



Figure 3.1.2 (a) TLC of Extracts of S. nigrum Callus in the Methanol : Ammonia System, Viewed Under U.V. Light

S = α-solanine

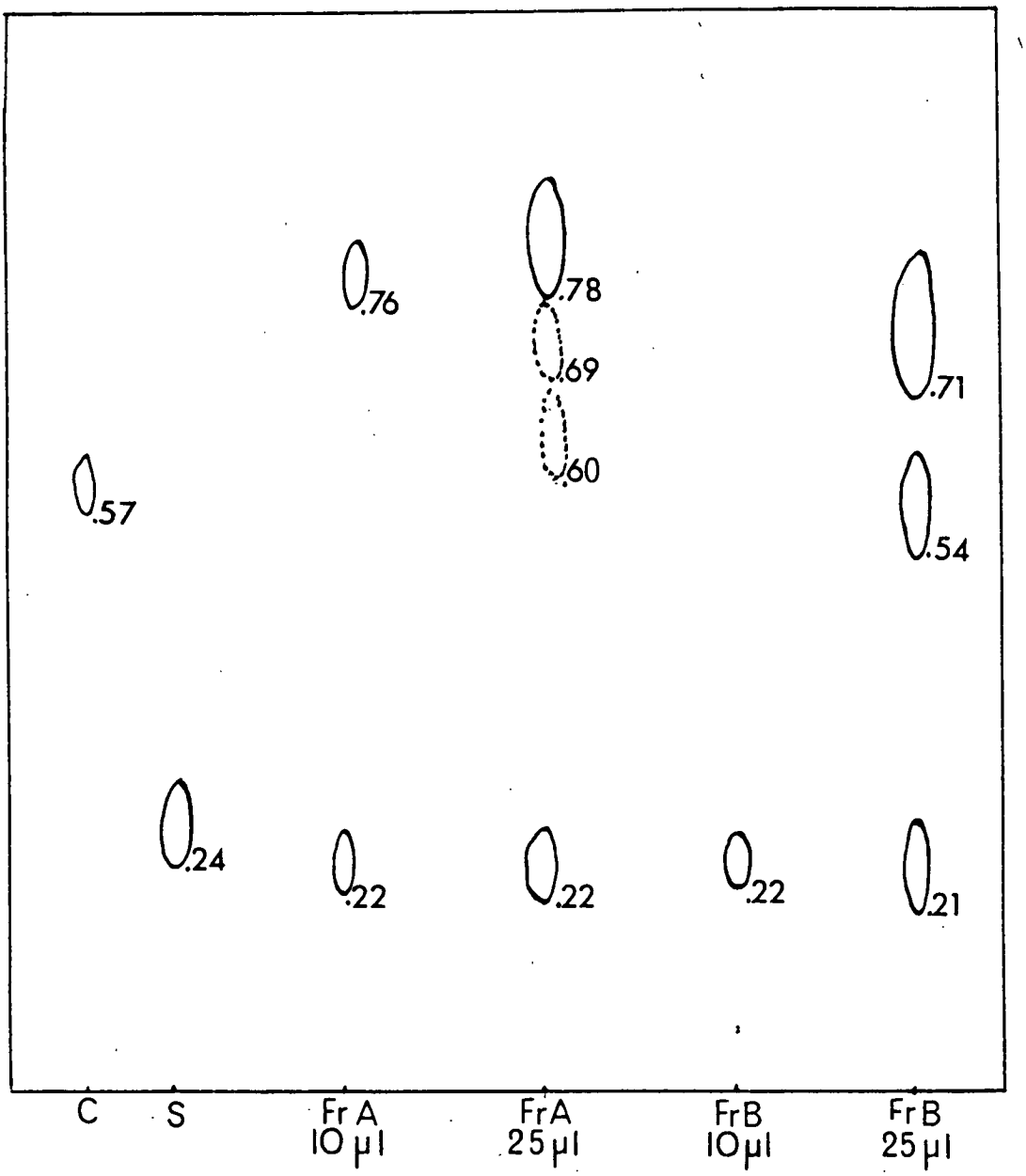



Figure 3.1.2 (b) TLC of Extracts of S. nigrum Callus in the Methanol : Ammonia System, Sprayed with Iodoplatinate Reagent

C = α-chaconine

S = α-solanine

## A. THE CHLOROFORM : ACETIC ACID : METHANOL SYSTEM

10 and 25 µl of extract samples were loaded onto Kieselgel 60 plates, together with 5 µg of the two standards, α-solanine and α-chaconine. The plate was sprayed with Dragendorff's reagent.

### FRACTION A

In this fraction, one alkaloid was visualised (in only the 25 µl sample), of $R_f$ value 0.83.

### FRACTION B

In this fraction, one alkaloid was visualised (in both the 25 µl and the 10 µl sample, of $R_f$ value 0.83.

## B. THE METHANOL : AMMONIA SYSTEM

### (a) Viewed under ultra-violet light

Some alkaloids are capable of absorbing or fluorescing when exposed to ultra-violet light and the reactions of a number of compounds with u.v. are listed in Clarke (1970). In order, therefore, to gain more information on the number and composition of alkaloids in the extracts, a plate was viewed under ultra-violet light (Figure 3.1.2 (a)).

The α-solanine standard was not detectable under u.v., but two other spots had been separated and seen to faintly absorb the light; they had $R_f$ values of 0.78 and 0.60 respectively.

FRACTION A

Two alkaloids were distinguishable by virtue of their u.v.-absorbing properties, of $R_f$ values 0.80 and 0.74.

FRACTION B

Four alkaloids were found to absorb u.v. in this fraction, and separated at $R_f$ values 0.94, 0.78, 0.72 and 0.58.

(b) Sprayed with iodoplatinate reagent

The α-solanine standard appeared to be composed of only one compound ($R_f$ 0.24) when sprayed with the reagent. The α-chaconine standard chromatographed at $R_f$ 0.57.

FRACTION A

Four alkaloids were visualised, of $R_f$ values 0.78, 0.69, 0.60 and 0.22 - the latter three compounds were very faint and only detectable in the 25 µl sample.

FRACTION B

Three alkaloids were visualised, of $R_f$ values 0.71, 0.54 and 0.21; the first two were very faint and only apparent in the 25 µl sample.

See Figure 3.1.2(b).

The data derived from exposure of the plate to ultra-violet light indicates that the α-solanine standard contained at least two impurities, possibly solanidine ($R_f$ 0.78) and α-chaconine ($R_f$ 0.60). Both α-solanine (the spots at $R_f$ 0.22 and 0.21) and α-chaconine ($R_f$ 0.60) seem to have been present in the callus, as visualised with iodoplatinate in the methanol : ammonia solvent system; solanidine (one of the spots at $R_f$ 0.80, 0.78 or 0.72?) may also have been present. A number of other alkaloids were noted, but could not be identified.

## A COMPARISON OF S. NIGRUM CALLUS AND WHOLE PLANT TISSUE

The Fraction A of both callus and whole plant tissue appeared to contain a roughly similar complement of alkaloids, although the compounds of higher $R_f$ values were more abundant, both in number and in weight, in whole plant extracts.

There may have been an additional Fraction B alkaloid in the callus extract (compared with the whole plant tissue extract) of $R_f$ 0.71.

## THE EXTRACTION AND IDENTIFICATION OF DATURA INNOXIA ALKALOIDS

### 1. In Whole Plant Tissue

The tissue from which the alkaloids were extracted was a 5 g mixture of stem and leaf tissue (in a ratio of 1 : 1 by weight) derived from mature plants (at the fruiting stage) which had been grown up from seed in the greenhouse of the Botany Department, Edinburgh, in the manner described in the 'Materials and Methods' chapter. Alkaloids were extracted from the dried tissue according to the method of Hultin and Torsell (1965). Samples of extracts were separated on commercially prepared TLC plates (coated with 0.2 mm Kieselgel 60, Merck) and separated in the two solvent systems, and sprayed, in the manner described above for S. nigrum tissue.

Four standards of tropane alkaloids (dissolved in methanol) were chromatographed, for identification purposes, alongside extracts; these were hyoscyamine, scopolamine (hyoscine), atropine and tropine (Sigma London Chemical Co.).

The results of subjecting the tissue extracts to TLC are given in Figures 3.1.3 (a) and (b).

#### A. THE CHLOROFORM : ACETIC ACID : METHANOL SYSTEM

The following quantities of standard tropane alkaloids were loaded onto the plates, and their $R_f$ values in this solvent system are given : 1.63 µg hyoscyamine, $R_f$ value 0.49; 3.3 µg scopolamine (hyoscine), $R_f$ value 0.15; 5.8 µg atropine, $R_f$ value 0.32; and 1.4 µg tropine, $R_f$ value 0.14.

H = hyoscyamine

S = scopolamine

A = atropine

T = tropine

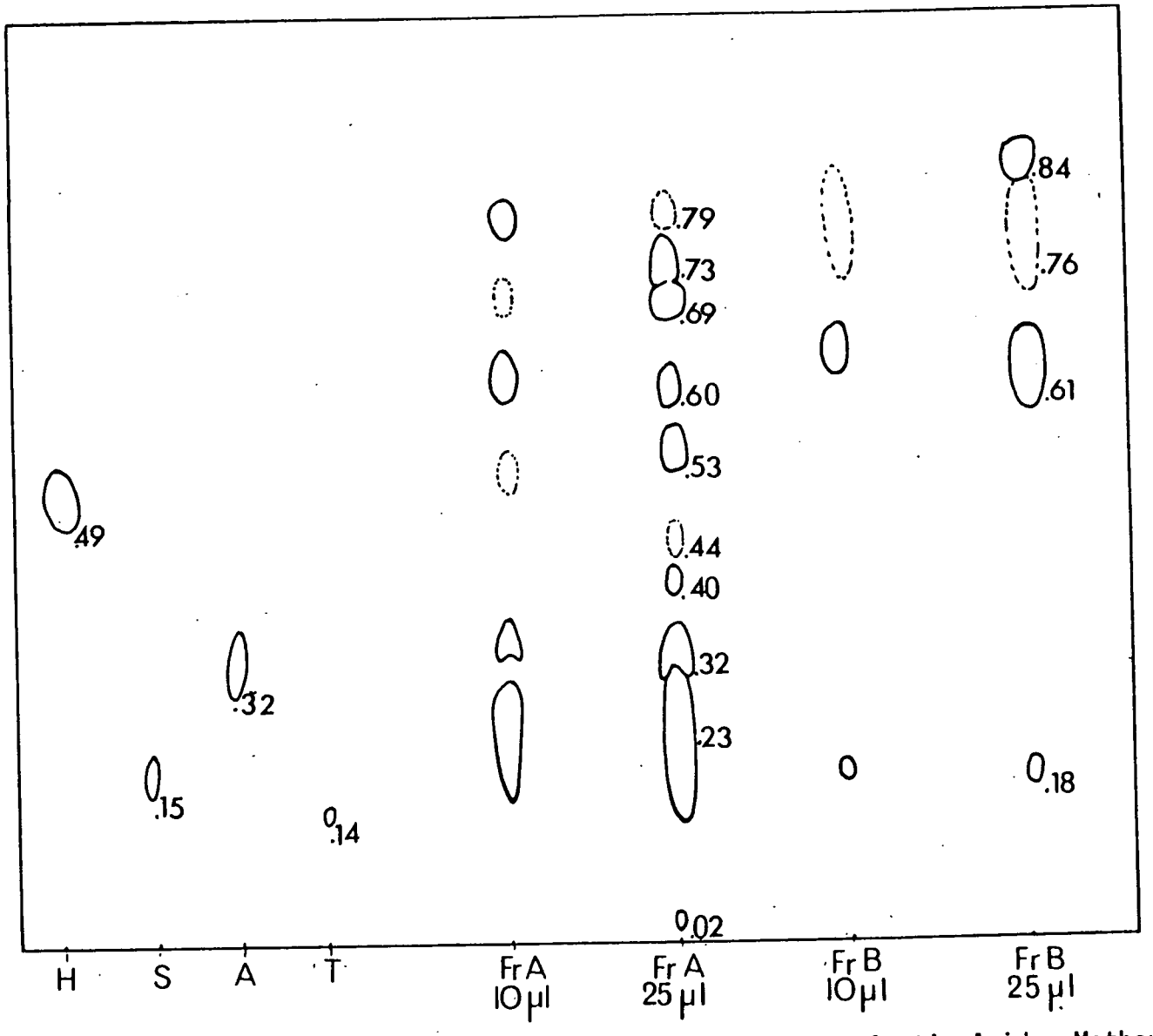
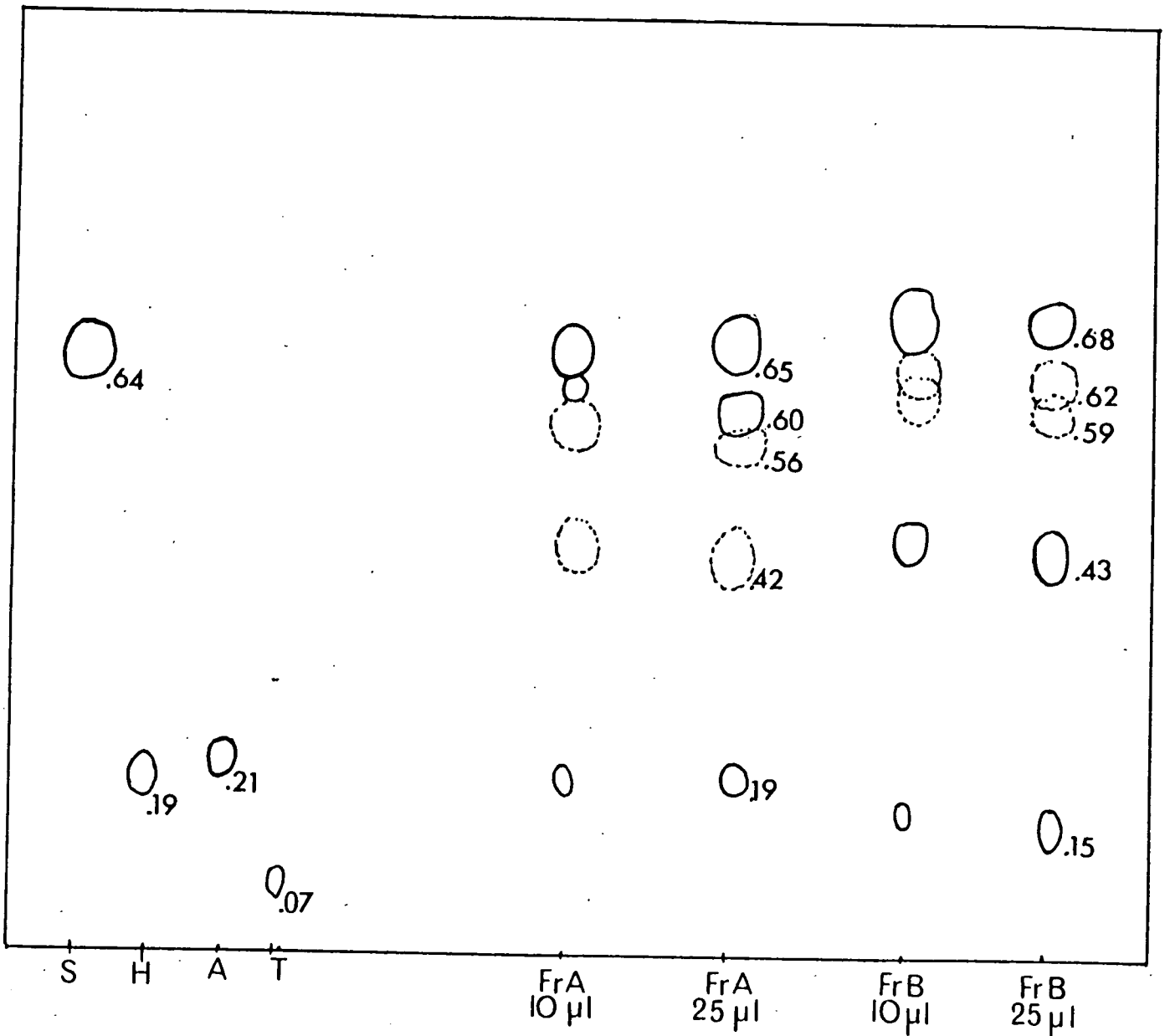



Figure 3.1.3 (a) TLC of Extracts of D. innoxia Whole Plant Tissue, in the Chloroform : Acetic Acid : Methanol System, Sprayed with Dragendorff's Reagent




S = scopolamine

H = hyoscyamine

A = atropine

T = tropine

Figure 3.1.3 (b) TLC of Extracts of D. innoxia Whole Plant Tissue in the Methanol : Ammonia System, Sprayed with Iodoplatinate Reagent

FRACTION A

Ten separate alkaloids were distinguished after spraying with Dragendorff's reagent (which was more sensitive in the detection of tropane alkaloids than iodopolatinate, as described in Results Part 1, Section 1); two of these were, however, only faintly visible. The $R_f$ values of the compounds were : 0.79 (faintly visible), 0.73, 0.69, 0.60, 0.53, 0.44 (faintly visible), 0.40, 0.32, 0.23 and 0.02. The $R_f$ value of the penultimate alkaloid (0.23) was measured from the middle of the streaky spot (which ranged from $R_f$ 0.30 - 0.13).

FRACTION B

Four separate alkaloids were distinguished, of $R_f$ values 0.84, 0.76, 0.61, and 0.18. The compound of $R_f$ 0.76 was a long spot, ranging from $R_f$ 0.80 - 0.67.

B. THE METHANOL : AMMONIA SYSTEM

The TLC plates were sprayed with iodoplatinate reagent, as recommended by Clarke (1970).

FRACTION A

Five separate alkaloids were noted, of $R_f$ values 0.65, 0.60, 0.56 (faintly visible), 0.42 (faintly visible) and 0.19.

FRACTION B

Five separate alkaloids were distinguished, of $R_f$ values 0.68, 0.62 (faintly visible), 0.59 (faintly visible), 0.43 and 0.15.

From the data presented above it seems likely that at least three of the alkaloids used as standards, i.e., hyoscyamine, scopolamine and atropine, were present in the whole plant tissue extract, scopolamine being the most abundant; their presence was confirmed by co-chromatography of the extract samples with internal standards. Tropine may have been masked by other alkaloids after chromatography in the chloroform : acetic acid : methanol solvent system; the methanol : ammonia data, however, suggest it was not present, but it may have been in the extract at a level below that necessary for detection.

Since there was some discrepancy between the observed $R_f$ values of standards and those reported by Clarke (1970), it was not possible to identify any of the other alkaloids present. It seems likely that most of the alkaloids (most notably scopolamine) in the Fraction B were simply there because they had not been taken up completely in the Fraction A, due to their relatively high concentrations in the extract samples, rather than because of any chemical peculiarities; the use of three internal standards (hyoscyamine, scopolamine and atropine) supported this view by virtue of their co-chromatography with some of the Fraction B alkaloids. Nevertheless, there seemed to be present alkaloids which were only found in Fraction B, at $R_f$ values 0.84 (in the chloroform : acetic acid : methanol systems) and at 0.68 (in the methanol : ammonia system).

## 2. Alkaloids in D. innoxia Callus

The callus used in the extractions was originally derived from stem tissue of mature (fruiting) plants, and had been maintained and subcultured (at monthly intervals) on full MS medium on agar plates for 5 months. The extraction procedure which was employed was the same as that described for the whole plant tissue above. 7 g of callus was dried before extraction. The extracts obtained (i.e., fractions A and B) were separated in the chloroform : acetic acid : methanol solvent system and sprayed with Dragendorff's reagent; the results of the D. innoxia whole plant extraction described above suggested that this was the more useful separation and detection procedure. Two types of commercially prepared TLC plates were used - the usual Kieselgel 60, and polyamide F254 - coated aluminium sheeting (Merck); it was hoped that a second type of TLC plate would aid identification by separating the alkaloids in a different way to the Kieselgel plates. Tropane alkaloid standards were co-chromatographed alongside the extracts, as described for the whole plant TLC procedure described above.

The results are presented in Figure 3.1.4.

### 1. The Polyamide Plates

These plates were found to be quite useless for use in the analytical technique employed, for on spraying with Dragendorff's reagent after the separation procedure, the whole plate was uniformly stained orange, and no spots were detectable whatsoever; the reagent spray appeared to react in some way with the polyamide coating of the plate.

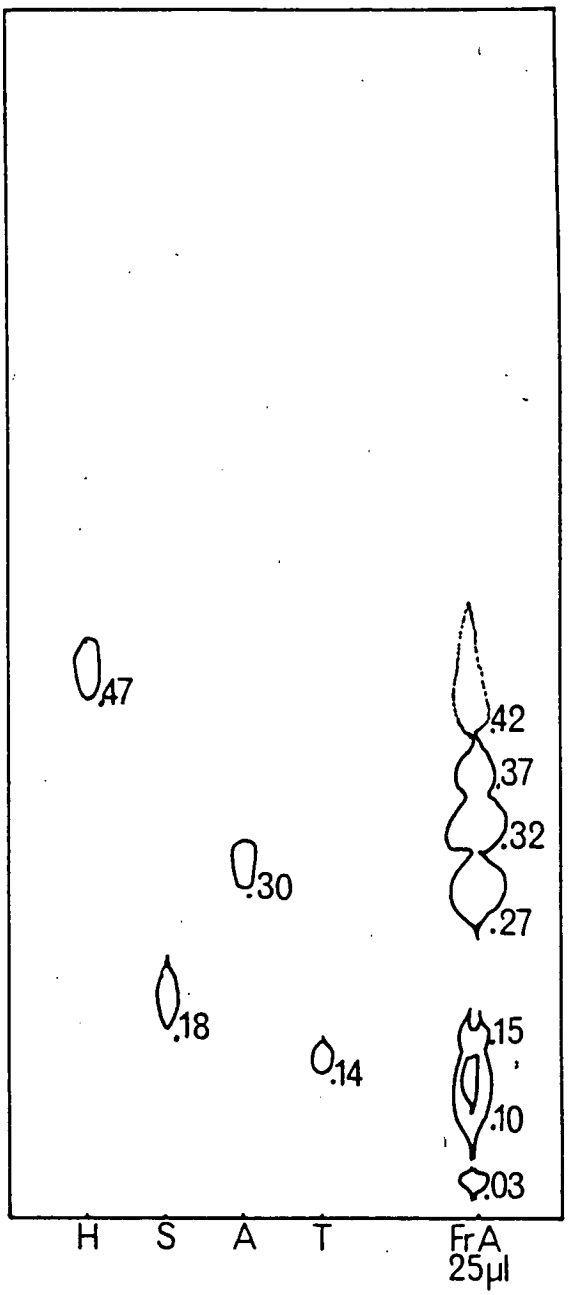



Figure 3.1.4 TLC of Extracts of D. innoxia Callus in the Chloroform : Acetic Acid : Methanol System, Sprayed with Dragendorff's Reagent

H = hyoscyamine

S = scopolamine

A = atropine

T = tropine

## 2. The Kieselgel Plates

The $R_f$ values of the four alkaloid standards used were in close agreement with the values previously determined; i.e., hyoscyamine = 0.47, atropine = 0.30, scopolamine (hyoscine) = 0.18 and tropine = 0.14.

### FRACTION A

10 µl and 25 µl samples were loaded onto the plates. Seven spots were visualised, of $R_f$ values 0.42, 0.37, 0.32, 0.27, 0.15, 0.10 and 0.03. Those of $R_f$ values 0.42, 0.27 and 0.03 were the faintest.

### FRACTION B

No alkaloids were detectable, even in 50 µl samples.

The use of internal standards indicated that the Fraction A alkaloids of $R_f$ values 0.42, 0.32, 0.15 and 0.10 were probably hyoscyamine, atropine, scopolamine and tropine respectively, although there was a slight doubt as to whether the compound of $R_f$ value 0.10 was definitely tropine.

## A COMPARISON OF D. INNOXIA CALLUS AND WHOLE PLANT TISSUE

There is little doubt that there was a larger number and amount (unit weight of alkaloids per unit weight of tissue) of alkaloids in D. innoxia whole plant tissue than in the callus. There were distinct similarities in composition between the two types of tissue, and it appears that most or perhaps all of the compounds present in the callus were also present in the whole plant tissue. Hyoscyamine, scopolamine and atropine were almost certainly present in both callus and whole plant tissue, as well as other alkaloids, of $R_f$ values approximately 0.40, 0.02 in the chloroform : acetic acid : methanol solvent. The long, streaked alkaloid of the whole plant Fraction A of $R_f$ 0.30 - 0.13 (in this solvent system) may in fact have been composed of at least three separate compounds, a possibility suggested by the presence in the callus of three individual alkaloids over this approximate range of $R_f$ values. The additional alkaloids detectable in the whole plant tissue extract were confined to the higher range of $R_f$ values, above about 0.50; it was not possible to identify these latter compounds.

The results of Section 3 demonstrate that callus cultures of D. innoxia and S. nigrum contain both fewer and lower levels of alkaloids than does the respective whole plant tissue. Similar observations have been made by Boulanger et al. (1973, for eduline and furoquinoline alkaloids of Ruta graveolens), Forrest (1969, for polyphenols of Camellia sinensis), Hiraoka and Tabata (1974, for alkaloids in suspension cultures of D. innoxia), Hirotani and Furuya (1977, for cardenolides of Digitalis purpurea), Ikuta et al. (1974, for alkaloids of the papaveraceae), Jalal et al. (1979, for the formation of flavones in Andrographis paniculata) and Sejourne et al. (1981, for alkaloids of Choisya ternata).

The studies of these first three sections of results were performed on relatively new callus and suspension cultures, none more than 6 months old and most 2-3 months old. It was thought that the biosynthetic capacity of older callus might be lower than that observed in these data, and a long-term study of the biosynthetic stability of D. innoxia callus was performed to determine whether it would decline. This is now described.

# SECTION 4

## THE STABILITY OF CALLUS DURING A PROLONGED PERIOD OF CULTURE

It is possible that callus, maintained in culture for long periods of time, will exhibit some gradual decline in ability to synthesise or accumulate alkaloids or other secondary products, or in morphogenetic capacity (Thomas and Street, 1970; Meyer-Teuter and Reinert, 1973; Tabata and Hiraoka, 1976). The precise reasons for this are unclear. It may be that chromosomal abnormalities, developed during rapid and unorganised cell proliferation (Bayliss, 1973; Sunderland, 1977) provide a genetic basis for the observed demise of the biosynthetic potential. It is also possible that the metabolic character of cultured cells can alter as a result of epigenetic changes, as in the case of, for example, cytokinin habituation (Meins and Binns, 1977).

In order to determine whether alkaloid accumulation by D. innoxia callus was stable over a prolonged period of culture, the levels of alkaloids were determined (by the cell-squash technique) at intervals over a period of 93 weeks. The chlorophyll content of the tissue was also determined, to investigate whether there was any correlation between the levels of alkaloids and chlorophyll. At weeks 4, 30, 62 and 89, determinations were made of mean chromosome number, to discover if any variations in alkaloid and chlorophyll levels were associated with the incidence of chromosomal aberrations. Cells were subcultured at approximately monthly intervals as usual.

The mean I values and chlorophyll contents of the callus at known intervals are given in Table 3.1.10, and the mean chromosome number for sampled cells are given in Table 3.1.11.

The results presented demonstrate that, even after a period of 93 weeks in culture, the cells still retained the capacity for alkaloid and chlorophyll accumulation. There was no great divergence

Table 3.1.10

The alkaloid and chlorophyll contents of callus of *D. innoxia* throughout a long period of culture

| Duration of Culture (week) | Days after Subculture | Mean I Value | Approximate Amounts of Alkaloids mg/g dry weight | Mean Chlorophyll Content µg/g fresh weight |
|---|---|---|---|---|
| 0 | 3 | 3.7 | 0.75 - 1 | 49.5 ± 1.5 |
| 2 | 17 | 3.5 | 0.75 - 1 | 62.0 ± 4.7 |
| 4 | 31 | 3.7 | 0.75 - 1 | 49.3 ± 2.1 |
| 6 | 14 | 3.5 | 0.75 - 1 | 40.8 ± 5.4 |
| 20 | 31 | 3.3 | 0.5 | 33.7 ± 3.3 |
| 23 | 20 | 2.7 | 0.5 | 37.0 ± 6.4 |
| 27 | 24 | 2.8 | 0.5 | 47.9 ± 7.6 |
| 30 | 10 | 2.8 | 0.5 | 40.1 ± 3.3 |
| 33 | 31 | 2.7 | 0.5 | 37.2 ± 1.9 |
| 42 | 22 | 3.8 | 1 | 41.5 ± 6.2 |
| 47 | 3 | 3.6 | 0.75 - 1 | 48.7 ± 2.9 |
| 51 | 1 | 3.1 | 0.5 | 42.9 ± 3.4 |
| 67 | 4 | 3.5 | 0.75 - 1 | 43.8 ± 5.5 |
| 87 | 0 | 3.4 | 0.75 | 49.3 ± 6.1 |
| 93 | 3 | 3.4 | 0.75 | 39.2 ± 2.6 |

in the relative amounts of alkaloids and chlorophyll over the culture period. The callus was always of a fairly compact nature, and as can be seen from Table 3.1.10, was also invariably green, even when growing at its fastest (at about 10-20 days after subculture). Moreover, the levels of accumulated alkaloids were not strongly correlated with the time of analysis (number of days after subculture, and this presumably reflects differences in growth rate). This stability of the callus was apparent despite the fact that, as the culture period progressed to 30 weeks, the ploidy level of the callus cells increased to between septaploid and octoploid by week 89. This result suggests that the amount of genetic material was not crucial in determining the levels of alkaloid and chlorophyll accumulation.

## A BRIEF SUMMARY OF RESULTS PART 1

Results from 'Part 1', and particularly from Section 2, have suggested in general and rather empirically, that cultures accumulate alkaloids to the highest levels when they are of a green and compact nature, and to the lowest levels when they are pale and friable. It was felt that, since the greening and greatest degree of contact between cultured cells occurs towards the end of the growth cycle, and that the production of secondary metabolites has been demonstrated, in many cases, to be greatest at this stage, it would be important to investigate further the relationship between alkaloid accumulation and phase of growth cycle (or growth rate) of cultured cells. The results of such an investigation are now described.

# RESULTS PART 2

## The Relationship Between The Phase Of The Cell Growth Cycle And Alkaloid Content

'Results Part 2' is divided into three sections. In Section 1 are described studies to determine whether there are detectable changes in the levels of alkaloids produced during the cell growth cycle, and whether any such changes were positively correlated with changes in the chlorophyll levels of cells. In Section 2, radioactive ornithine was supplied to suspended cells to determine whether the accumulation of labelled alkaloids and proteins occurred differentially in fast- and slow-growing cells. In the final section is described an experiment in which the growth rate of cultured cells was altered using a growth-regulating substance (2,4-D), to determine whether this would affect the levels of alkaloids and chlorophyll accumulated.

# SECTION 1

## THE LEVELS OF ALKALOIDS PRODUCED IN CULTURES OF *S. NIGRUM* CELLS DURING GROWTH CYCLES

## CHANGES IN THE LEVELS OF ALKALOIDS AND CHLOROPHYLL DURING THE GROWTH CYCLE OF *SOLANUM NIGRUM* CALLUS

The aim of this experiment was to determine the changes in the levels of both alkaloids and chlorophyll during a single growth cycle of *S. nigrum* stem callus, in order to discover if there is a correlation between callus growth rate, chlorophyll content and alkaloid content.

5 g of callus (which had been subbed for 4 months at monthly intervals before use) was placed onto each of 27 agar plates containing full MS medium, and maintained under the standard callus culture conditions described above. At intervals of 5 days over a period of 40 days, 3 plates were sampled and determinations were made of callus fresh weight, chlorophyll content and alkaloid content (by the cell-squash technique). The value for the chlorophyll content obtained for each sample was the mean of 6 determinations.

From the results presented in Table 3.2.1, and Figure 3.2.1, it can be seen that there was a lag phase of 10-15 days before the fresh weight began to increase. Subsequently, the growth rate reached a peak between days 15 and 30, and slowed down thereafter as the cells reached stationary phase.

It can be seen from the results in Table 3.2.1 that the chlorophyll and alkaloid contents of the cells varied in a similar manner throughout the growth cycle. A major decrease occurred between days 10 and 20, when the culture growth rate was greatest, i.e., in the exponential/linear growth phase. Thus, over the first 25 days, the alkaloid content fell from I value 3 to 2 (15→10 mg alkaloids/g dry weight

Table 3.2.1

Changes in fresh weight, alkaloid content and chlorophyll content of S. nigrum callus during a growth cycle

| Day After Subculture | Fresh Weight (g) | Average I Value | Mean Chlorophyll Content µg chl/g fresh weight callus |
|---|---|---|---|
| 0 | 5.0 ± 0.0 | 3.4 | 32.5 ± 3.4 |
| 5 | 5.0 ± 0.0 | 3.1 | 38.4 ± 5.2 |
| 10 | 5.4 ± 0.1 | 2.9 | 32.3 ± 1.3 |
| 15 | 5.9 ± 0.0 | 2.0 | 24.3 ± 2.6 |
| 20 | 7.6 ± 0.0 | 2.1 | 20.8 ± 2.2 |
| 25 | 8.7 ± 0.1 | 2.2 | 18.3 ± 4.8 |
| 30 | 9.1 ± 0.1 | 3.1 | 29.5 ± 5.4 |
| 35 | 9.3 ± 0.1 | 3.7 | 28.9 ± 2.1 |
| 40 | 9.3 ± 0.1 | 3.7 | 36.8 ± 4.6 |

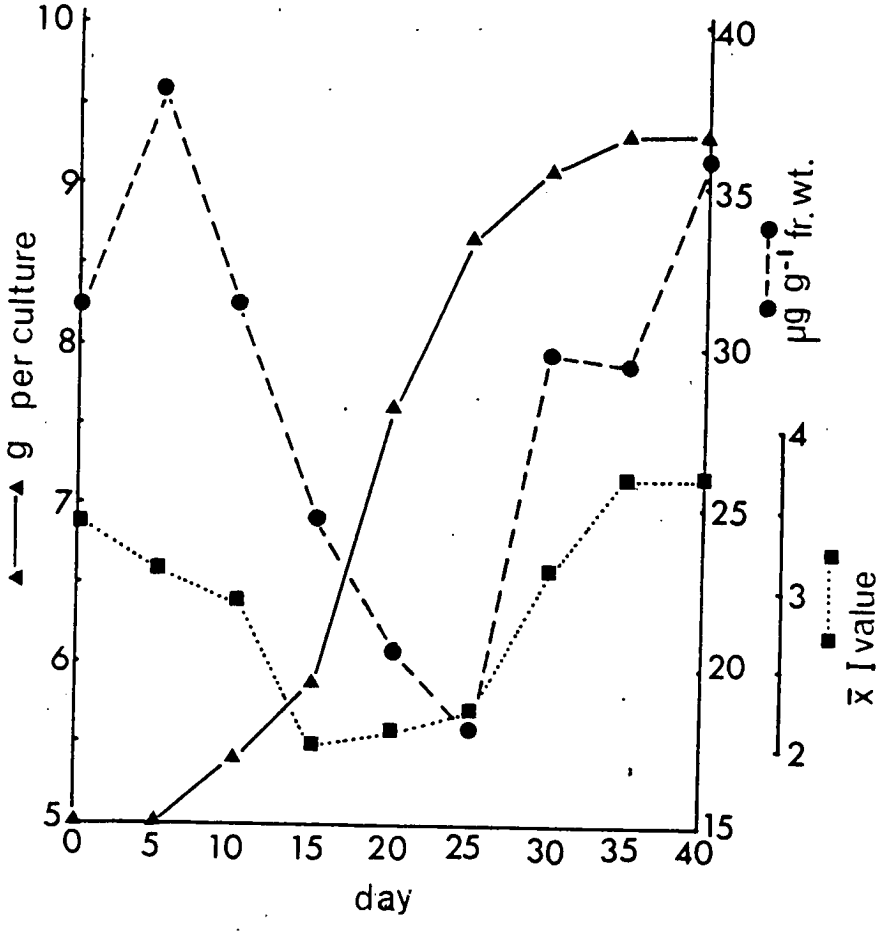



Figure 3.2.1 Changes in Fresh Weight (Triangles), Chlorophyll Content (Circles) and Alkaloid Content (Squares) of S. nigrum Callus over 40 Days

callus). As growth slowed down and the stationary phase was approached (after about day 30) the alkaloid and chlorophyll contents of the callus increased to levels similar to those at the beginning of the growth cycle. Thus at day 40 the cells registered a mean I value of 3.7 (16-17 mg alkaloids/g dry weight cells) and mean chlorophyll levels of 36.8 µg/g fresh weight cells. From these results it would appear that S. nigrum callus exhibits a temporal, as well as structural, heterogeneity with regard to both alkaloid and chlorophyll accumulation. The apparent positive correlation between the two is consistent with the general concepts gleaned from the screening work, namely that alkaloid and chlorophyll accumulation are inversely proportional to culture growth rate.

## CHANGES IN THE LEVELS OF ALKALOIDS PRODUCED BY SUSPENSION CULTURES OF SOLANUM NIGRUM DURING A GROWTH CYCLE

The results of the previous experiment suggested that the alkaloid content of callus of S. nigrum varied according to the stage of the growth cycle (i.e., time after subculture). The aim of this experiment was to determine whether similar changes would occur in cell suspension cultures.

Cell suspension cultures of S. nigrum were initiated by spooning approximately 1 g fresh weight of cells (derived from a mixture of four-week-old, stationary phase, suspension cultures) into 250 ml Erlenmeyer flasks containing 50 ml of liquid full MS medium. The initiating suspensions had been originally produced from 6-month-old stem callus, and had been suspended and subcultured for 2 months before use in this experiment.

Three flasks were sampled every two days for the duration of the experiment (24 days), and determinations were made of cell fresh weight, dry weight and alkaloid content (by the cell-squash technique). The nutrient medium was also examined for the presence of alkaloids, in order to determine whether these compounds had been released.

From the results presented in Table 3.2.2, and Figure 3.2.2, it may be seen that there was a lag phase of 4 days before there was any increase in the fresh and dry weight of the cultures. The fresh and dry weight then increased sharply over the next 10 days. At day 14, there was a temporary drop in the rate of increase of both fresh and especially dry weight, followed 4 days later by a resumption of growth for another 2 days. After this time, growth slowed down again.

The alkaloid accumulation data in Table 3.2.2 and Figure 3.2.2 show there was an increase in the levels of alkaloids accumulated in the lag phase, from a mean I value of 2.7 (approximately 14 mg alkaloids/g dry weight cells) to a value of 4.0 (approximately 20 mg alkaloids/g dry weight cells), but between days 6 and 16 there was a gradual decrease in the levels in the rapidly-growing cells, from mean I values of approximately 4, representing about 20 mg alkaloids/g dry weight cells, to mean I values of approximately 2.5 (representing about 12 mg alkaloids/g dry weight cells). A sharp increase in alkaloid content was observed at day 18 from a mean I value of 2.5 to a mean I value of 3.2 (representing an increase of approximately 2-3 mg alkaloids/g dry weight cells). This was when the growth of the cultures temporarily slowed, and as growth resumed the levels of alkaloids dropped slightly (back to a mean I value of 2.5 by day 22)

Table 3.2.2

Changes in fresh weight, dry weight and alkaloid content of S. nigrum suspended cells during a growth cycle

| Day of Culture | Mean Fresh Weight (g) per Flask | Mean Dry Weight (g) per Flask | Mean I Value |
|---|---|---|---|
| 0 | 0.93 ± 0.07 | 0.04 ± 0.00 | 2.7 |
| 2 | 0.96 ± 0.08 | 0.04 ± 0.01 | 3.9 |
| 4 | 0.89 ± 0.12 | 0.04 ± 0.01 | 4.0 |
| 6 | 1.73 ± 0.18 | 0.08 ± 0.01 | 3.5 |
| 8 | 2.63 ± 0.25 | 0.12 ± 0.00 | 3.6 |
| 10 | 3.24 ± 0.07 | 0.17 ± 0.01 | 3.3 |
| 12 | 6.36 ± 0.53 | 0.35 ± 0.02 | 2.8 |
| 14 | 8.85 ± 0.82 | 0.44 ± 0.03 | 2.8 |
| 16 | 9.35 ± 0.70 | 0.45 ± 0.04 | 2.5 |
| 18 | 9.76 ± 1.04 | 0.35 ± 0.01 | 3.2 |
| 20 | 13.71 ± 0.22 | 0.61 ± 0.02 | 2.8 |
| 22 | 14.06 ± 0.33 | 0.66 ± 0.02 | 2.5 |
| 24 | 14.78 ± 0.18 | 0.71 ± 0.01 | 3.4 |

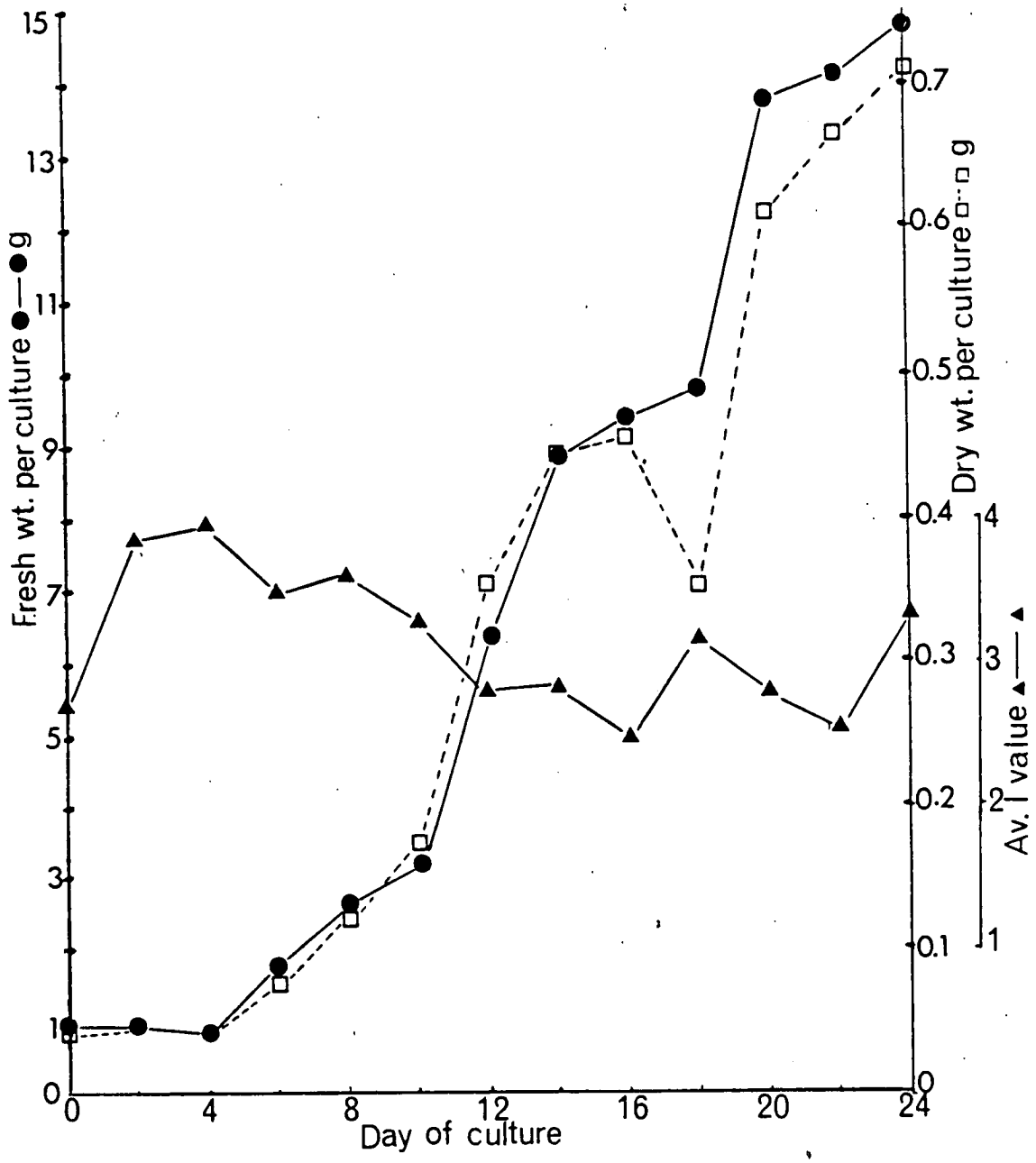



Figure 3.2.2 Changes in Fresh Weight (Circles), Dry Weight (Squares) and Alkaloid Content (Triangles) of Suspended S. nigrum Cells over 24 Days

until growth slowed down once more. Thus, at day 24, the mean I value rose to 3.4, representing approximately 15 mg alkaloids/g dry weight cells. No alkaloids were detected in the nutrient medium during any phase of the growth cycle.

It was found in this experiment that there was distinct biochemical and morphological heterogeneity within a single suspension culture. Differentiated, that is, green and compact, cell clumps were found throughout the growth cycle, and contained relatively high levels of alkaloids (mean I values of approximately 4, representing about 20 mg alkaloids/g dry weight cells). However, between days 6 and 24 there was found relatively more colourless and friable material (in fact, comprising the bulk of the tissue). This tissue contained relatively low levels of alkaloids of mean I values of between 2 and 3, representing approximately 15 mg alkaloids/g dry weight cells.

The results of this experiment suggest a relationship between the phase of the growth cycle and the levels of alkaloids accumulated by suspended cells. This may be correlated with an increased proportion of pale, friable cells during the middle part of the growth cycle when the cultures were growing rapidly.

The results of the two experiments in this section suggest an inverse relationship between alkaloid content (and, in the former experiment, chlorophyll content) and culture growth rate. This relationship was more marked in callus than in suspension cultures.

The aim of the next section was to further investigate the relationship between alkaloid accumulation and phase of the growth cycle by examining the incorporation of radio-actively-labelled ornithine into the alkaloids and proteins of fast- and slow-growing cells.

# SECTION 2

## THE INCORPORATION OF RADIOACTIVELY-LABELLED ORNITHINE INTO FAST- AND SLOW-GROWING CELLS

## THE INCORPORATION OF RADIOACTIVELY-LABELLED ORNITHINE INTO THE PROTEINS AND ALKALOIDS OF FAST- AND SLOW-GROWING CELLS OF DATURA INNOXIA

The aim of this experiment was to determine whether there is a differential incorporation of ornithine into the proteins and alkaloids of fast- and slow-growing cells. Ornithine is a precursor of both Datura alkaloids and proteins (via arginine). The results of the previous section indicate that alkaloids are accumulated to the greatest extent in stationary phase cells, and in this experiment this simple hypothesis was tested. Phillips and Henshaw (1977) have shown that radioactively-labelled phenylalanine is differentially incorporated into the phenolics and proteins of fast- and slow-growing cells of Acer pseudoplatanus, and the method of this experiment is based on their approach.

The radioactive precursor used was an aqueous solution of DL-(1- $^{14}C$) ornithine - HCl containing 2% ethanol (v/v), with a specific activity of 40-60 mCi/m mol (1.5 - 2.2 G Bq/m mol) (the Radiochemical Centre, Amersham). It was stored at -5$^{o}C$.

From a 4 week-old, stationary phase, suspension culture of D. innoxia (produced from stem callus and subcultured for 4 months) six suspension cultures were initiated in 250 ml Erlenmeyer flasks. At linear phase (day 12) three of the cultures were mixed together, and from this mixture was pipetted 10 ml of (fast-growing) suspended cells into each of four 50 ml Erlenmeyer flasks. In this way, cells were randomised; and the ratio of the volume to surface area of the culture medium was maintained. This procedure was repeated at day 30, i.e., for slow-growing, stationary phase, cells.

To each 10 ml suspension culture was added 0.5 μ Ci (0.0185 M Bq) of labelled ornithine (in 10 μl). This was incubated for 24 hours, after which time proteins and alkaloids were extracted. An incubation time of 24 hours was chosen for two reasons. Firstly, previous work in this laboratory suggests that the uptake of $^{14}$C-ornithine by suspended cells is linear up to at least 5 hours, but a longer period was required to obtain sufficient incorporation for counting. Secondly, a longer incubation period might result in the cells leaving the desired phase of the growth cycle.

## ALKALOID EXTRACTION AND ANALYSIS

After the incubation period, the cells of each culture were filtered on a vacuum filter using Whatman qualitative No. 1 filter paper, weighed, and washed with 3 x 10 ml of boiling 80% (v/v in distilled water) methanol respectively, to extract the alkaloids. The extracts were evaporated to dryness *in vacuo* at 40$^{o}$C and the residues were taken up in 1 ml of methanol; 50 μl samples were chromatographed on 0.2 mm Kieselgel 60 TLC plates (Merck) in the chloroform : acetic acid : methanol solvent system as described previously (p.31 ). The TLC plates were either sprayed with Dragendorff's reagent, to visualise the alkaloids, or were autoradiographed by exposure to Dupont Cronex X-ray film at room temperature for 4 weeks. The Cronex film was developed in Polycon (May and Baker) developer for 4 minutes, stopped in a bath of 4.5% (v/v in tap water) acetic acid for 30 seconds, and fixed in Perfix (May and Baker) for 5 minutes.

In order to further check that radioactive spots corresponded

to alkaloids, TLC plates were sprayed and autoradiographed, and a comparison of the two was made. Moreover, standards of 1 µg DL-ornithine (Sigma London Chemical Co.) and 1 µg DL-arginine (Sigma London Chemical Co.) were chromatographed in the chloroform : acetic acid : methanol solvent system and visualised with ninhydrin-acetic acid reagent, as described in Randerath (1968); their $R_f$ values were calculated.

Radioactive spots were scraped from the TLC plates and assayed using a scintillation counter. The alkaloid contents of the cells were estimated using the cell-squash technique.

PROTEIN EXTRACTION AND ANALYSIS

Once the cells had been treated with methanol, the excess label was removed: the cells were washed with 3 x 10 ml of 5% (w/v in distilled water) trichloracetic acid (TCA), which also precipitated the protein, and then spun in a centrifuge (at 2500 g for 15 minutes). The supernatant, containing excess label, was discarded. The cells were then left to stand overnight in 10 ml of 0.1 M KOH, to dissolve the protein. The cell wall material was then spun down (at 2500 g for 15 minutes) and to the supernatant (containing the protein) was added 10 ml of 5% TCA. This was left on ice for 1 hour, to allow the protein to precipitate completely. The protein was spun down (at 2500 g for 15 minutes), and was once more washed with 10 ml of 5% TCA to ensure the removal of any remaining excess label. The protein was spun down as before, the supernatant was discarded, and the protein was dissolved in 10 ml of 0.1 M KOH, ready for scintillation.

The radioactivity of the extracts was expressed as number of counts/g fresh weight of cells/minute.

## QUALITATIVE ALKALOID ANALYSIS

The results of the qualitative analysis of the cell extracts for alkaloids are given in Table 3.2.3 and Figures 3.2.3 (a) and (b) and 3.2.4 (a) and (b). In the table are given the $R_f$ values detected (a) after spraying with Dragendorff's reagent and (b) by autoradiography. Commercial standards of hyoscyamine, scopolamine, atropine and tropine, were chromatographed alongside the extracts to aid identification, and the $R_f$ values are given in Table 3.2.3. 0.1 μCi of $^{14}$C-ornithine (in 2 μl) was also chromatographed, but was found to remain at the origin in the solvent system used.

From the results presented, it is apparent that radioactivity was incorporated into a number of compounds, which were not necessarily the same in each replicate extract. It is also apparent that not necessarily the same compounds were visualised by spraying and autoradiography. Since $^{14}$C-labelled ornithine and arginine were immobile in the solvent system, it can be concluded that none of the radioactive spots beyond the origin was ornithine. By comparing the $R_f$ values of the radioactive spots (a) with those of the alkaloid standards,(b) with those of chromatographed extracts which were sprayed, and (c) with those of compounds previously found in D. innoxia whole plant tissue and callus (Results Part 1, Section 3), it seems certain that all the radioactive spots represent labelled alkaloids.

Table 3.2.3

TLC of alkaloids extracted from fast- and slow-growing cells

| Extract Sample | $R_f$ VALUES | | | |
|---|---|---|---|---|
| | 'Fast' Cells | | 'Slow' Cells | |
| | Sprayed | Autoradiographed | Sprayed | Autoradiographed |
| 1 | 0.07 | 0.17 | 0.22 | 0.31 |
| | | 0.03 | 0.11 | 0.12 |
| | | | 0.06 | 0.04 |
| 2 | 0.74 | 0.04 | 0.22 | 0.13 |
| | 0.08 | | 0.06 | 0.05 |
| 3 | 0.54 | 0.06 | 0.22 | 0.12 |
| | 0.09 | | 0.06 | 0.05 |
| 4 | 0.74 | 0.19 | 0.22 | 0.19 |
| | 0.17 | 0.03 | 0.06 | 0.11 |
| | | | | 0.04 |

$R_f$ values of 'standards' with 'fast' cells :

scopolamine = 0.18

atropine = 0.28

hyoscyamine = 0.43

tropine = 0.12

$R_f$ values of 'standards' with 'slow' cells :

scopolamine = 0.19

atropine = 0.29

hyoscyamine = 0.42

tropine = 0.13

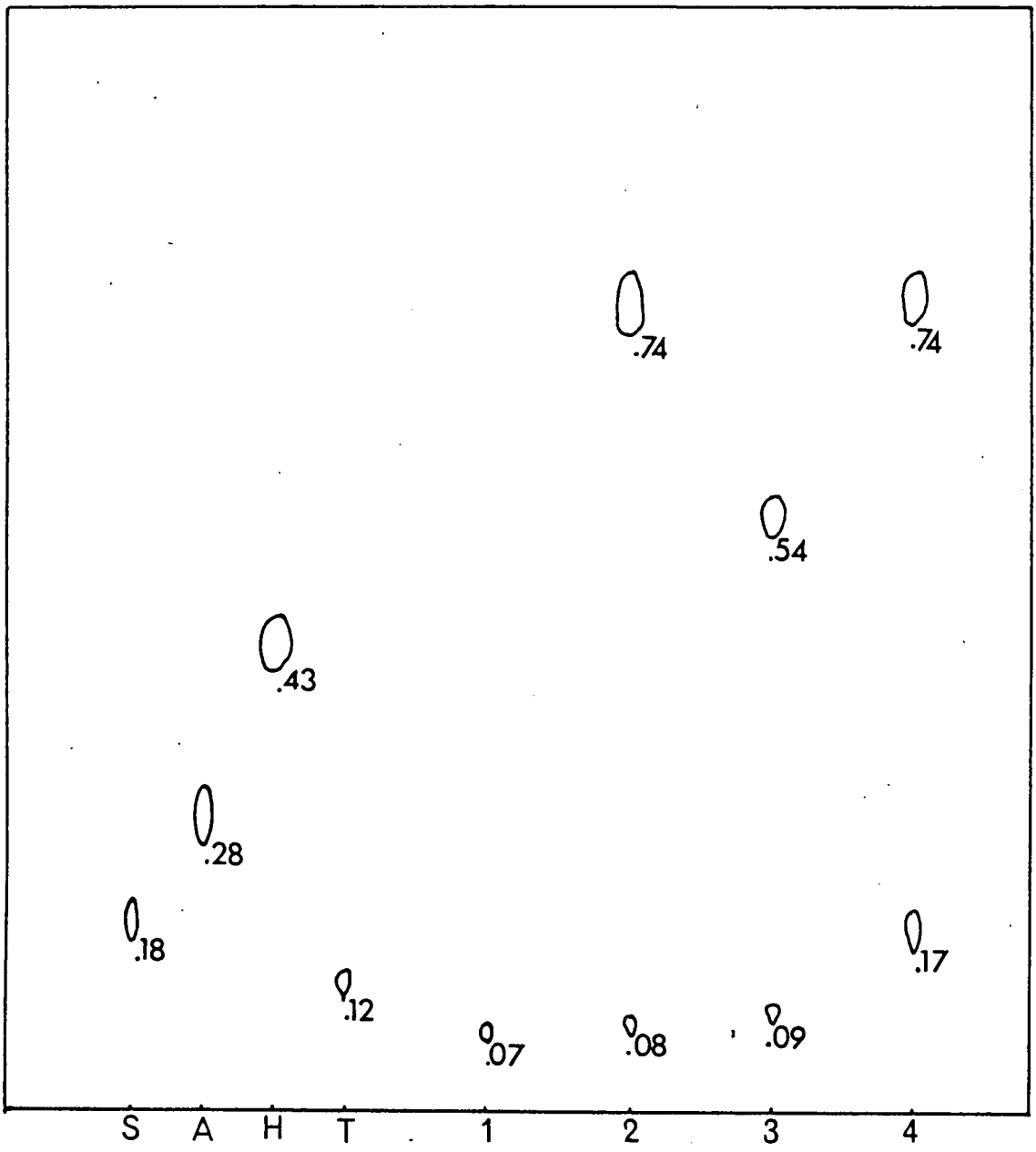



Figure 3.2.3 (a) TLC of Extracts of Fast-Growing Cells of D. innoxia after $^{14}$C-Ornithine Incorporation, Sprayed with Dragendorff's Reagent

S = scopolamine

A = atropine

H = hyoscyamine

T = tropine

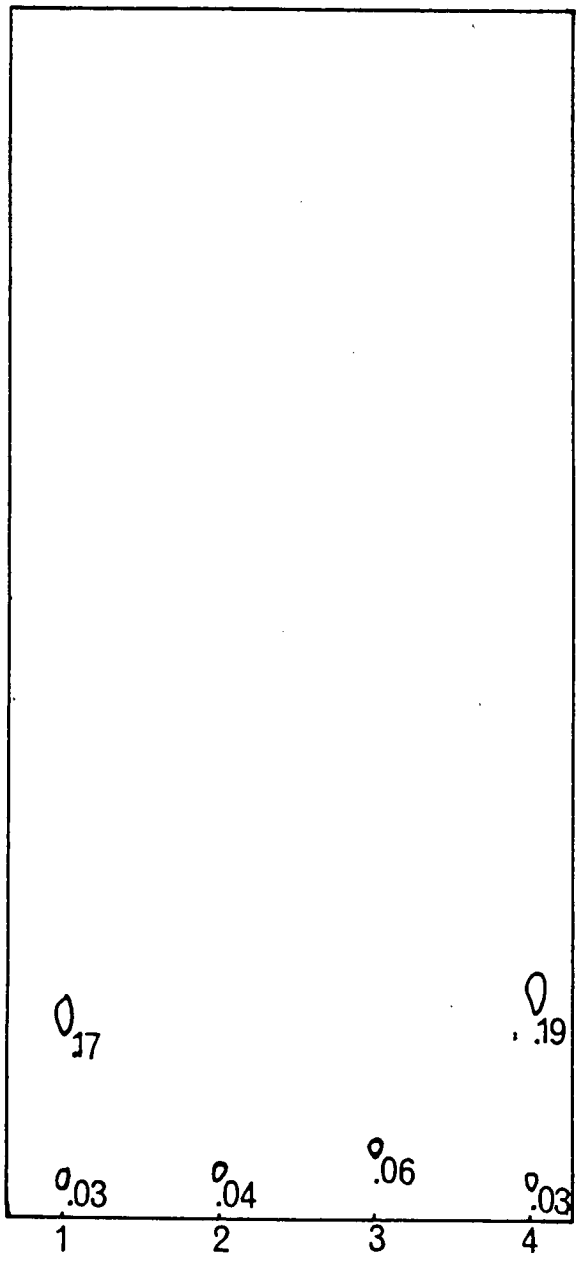



Figure 3.2.3 (b) Autoradiograph of Extracts of Fast-Growing Cells of D. innoxia after $^{14}C$-Incorporation and TLC

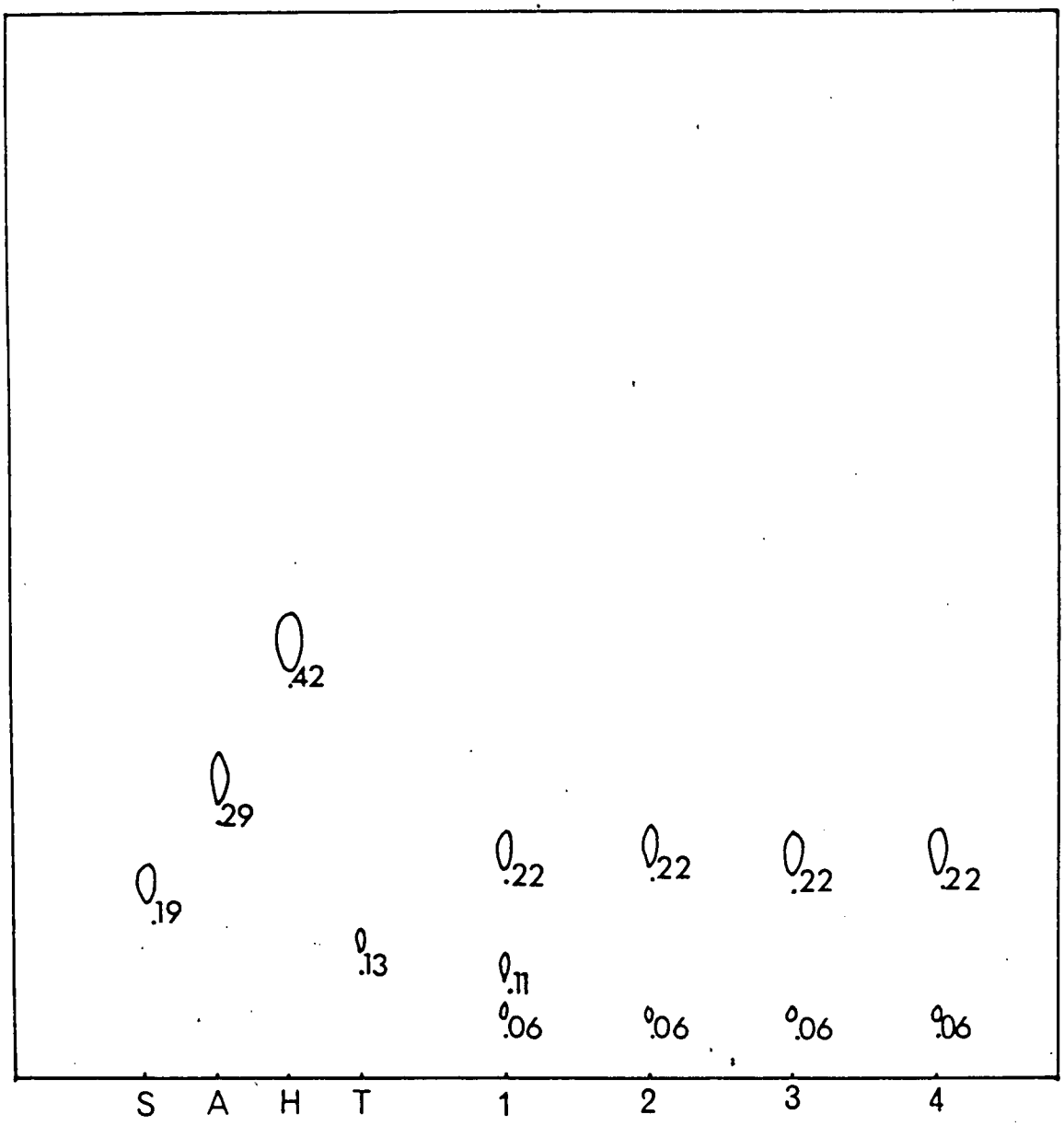



Figure 3.2.4 (a) TLC of Extracts of Slow-Growing Cells of D. innoxia After $^{14}$C-Ornithine Incorporation, Sprayed with Dragendorff's Reagent

S = scopolamine

A = atropine

H = hyoscyamine

T = tropine

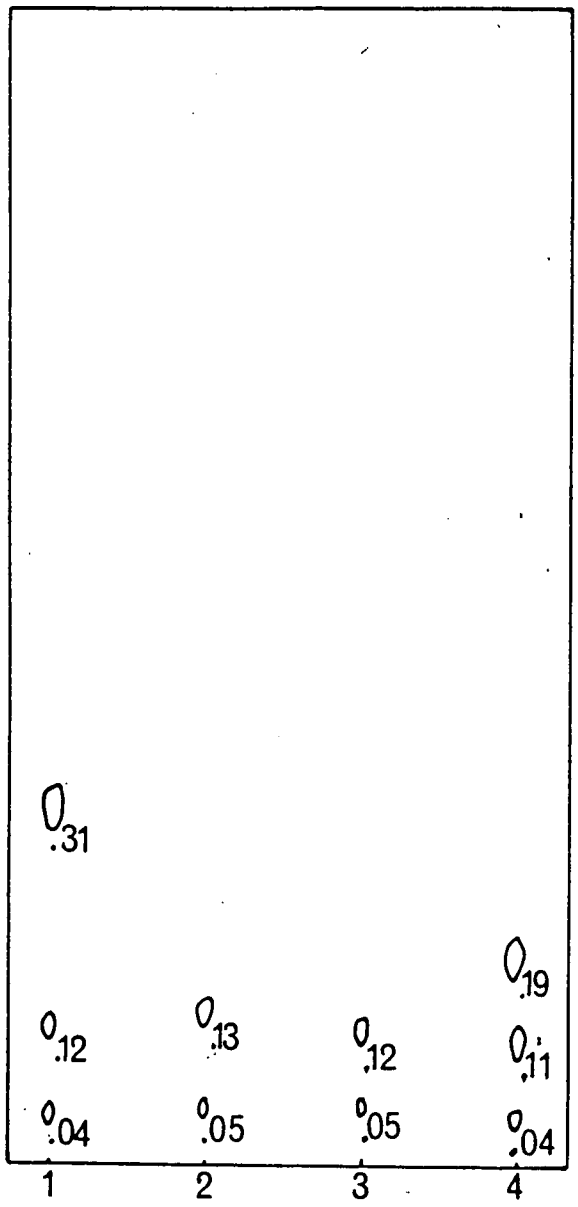



Figure 3.2.4 (b) Autoradiograph of Extracts of Slow-Growing Cells of D. innoxia after $^{14}$C-Ornithine Incorporation and TLC

The alkaloid profiles of (i) the fast-growing cells and (ii) the slow-growing cells are now described.

(i) The Fast-Growing Cells

It is apparent from the results in Table 3.2.3 and Figures 3.2.3 (a) and (b) that there were qualitative and quantitative differences between replicate extracts, and in some replicates different alkaloids were detected by spraying compared with autoradiography. The spots on the sprayed plates were mainly unidentified alkaloids, including those of $R_f$ values 0.07, 0.08 and 0.09 (probably a single compound) in replicates 1, 2 and 3; the compound of $R_f$ 0.74 in samples 2 and 4; and the alkaloid of $R_f$ 0.54 in sample 3. The compound of $R_f$ 0.17 in sample 4 was probably scopolamine.

The autoradiographed samples all contained alkaloids of $R_f$ values between 0.03 and 0.06, and these were probably the same, unidentified compound; samples 1 and 4 contained alkaloids of $R_f$ values 0.17 and 0.19 respectively; these were both probably scopolamine. No radioactivity was detected at $R_f$ values of approximately 0.08 or 0.54 or 0.17 (scopolamine), although these alkaloids were detectable on sprayed plates. Thus radioactive label was not incorporated into all the alkaloids present, but autoradiography did show up compounds not detectable by spraying (i.e., scopolamine in sample 1 and the unidentified alkaloids of $R_f$ values between 0.03 and 0.06). From their $R_f$ values, it seems likely that these unidentified compounds were alkaloids which, because they were present at low concentrations, were only detectable by autoradiography.

(ii) The Slow-Growing Cells

From the results in Table 3.2.3 and Figures 3.2.4 (a) and (b), it can be seen that on all the sprayed plates were visualised an unknown alkaloid of $R_f$ value 0.06. This was probably the same compound as was detected in the sprayed extracts of the fast-growing cells (samples 1 and 2) and the autoradiographed fast-growing cell extracts (in all four samples). The sprayed plates of extracts of the slow-growing cells also all showed the presence of an alkaloid of $R_f$ value 0.22, which was probably scopolamine. Sample 1 contained an alkaloid of $R_f$ 0.11, which was probably tropine.

Autoradiography of the chromatographed samples produced a different pattern of alkaloids to the sprayed plates. Sample 1 contained alkaloids of $R_f$ values 0.12 and 0.04 (similar to the sprayed plate) but also contained an alkaloid of $R_f$ 0.31, which was possibly atropine. The scopolamine, visualised by the spraying of chromatographed samples 2 and 3, was not visible on autoradiographed plates, but alkaloids of $R_f$ values 0.13 and 0.12 respectively were present (probably tropine), also detectable in sample 4. Only in sample 4 was scopolamine detectable in both sprayed and autoradiographed plates.

A COMPARISON OF FAST- AND SLOW-GROWING CELLS

There is little similarity between the TLC profiles of the extracts of fast- and slow-growing cells. The former contained no tropine or atropine, although they did contain alkaloids of $R_f$ values 0.74 and 0.54. The fast-growing cells contained alkaloids of $R_f$ values between 0.07 and 0.09, which were probably different compounds

(or perhaps a different single compound) to that of $R_f$ value 0.06 of the slow-growing cells. The only 'over-lap' between the fast- and slow-growing cells was the presence of scopolamine and the unknown compounds of $R_f$ values of approximately 0.05.

## QUANTITATIVE ANALYSIS

The result of the cell-squash analysis of the fast- and slow-growing cell cultures are given in Table 3.2.5. It can be seen that the stationary phase cultures contained higher levels of alkaloids than did the linear phase cells, the former having a mean I value of 2.3 (representing approximately 0.5 mg alkaloids/g dry weight cells) compared with a value of 1.2 for the latter (representing approximately 0.25 mg alkaloids/g dry weight cells).

The results of the $^{14}C$-ornithine incorporation experiment presented in Table 3.2.6 show that more radioactivity was incorporated into the alkaloids of the slow-growing cells than of the fast-growing cells. In the slow-growing cells, 4.8% of the total incorporated radioactivity was present in the alkaloid fraction (with 95.2% in the protein fraction), whereas in the fast-growing cells, only 0.01% of the total incorporated radioactivity was present in the alkaloid fraction (with 99.9% in the protein fraction).

Incorporation of radioactivity was much greater in the fast-growing cells compared with the slow-growing cells, with totals of 112,264 and 2193 cpms/g fresh weight respectively (Tables 3.2.4 and 3.2.6). These values represent percentages uptake of approximately 2½% and 0.4% respectively of the total number of counts added to the cultures. These values were calculated as the total number of counts

Table 3.2.4

The incorporation of $^{14}$C-ornithine-HCl into proteins

| Replicate | Counts per Minute per Gram Fresh Weight Cells: Fast-Growing | Slow-Growing |
|---|---|---|
| 1 | 78,230 | 2,440 |
| 2 | 134,382 | 2,594 |
| 3 | 89,460 | 1,688 |
| 4 | 146,908 | 1,628 |
| $\bar{x} \pm$ S.E. | 112,245 $\pm$ 16,752 | 2,087 $\pm$ 250 |

Table 3.2.5

| Alkaloid Content (Mean I Value) | |
|---|---|
| Fast-Growing | Slow-Growing |
| 1.2 | 2.3 |

Table 3.2.6

The incorporation of $^{14}$C-ornithine-HCl into alkaloids

| Replicate | Counts per Minute per Gram Fresh Weight Cells: Fast-Growing | Slow-Growing |
|---|---|---|
| 1 | 18.9 | 114.9 |
| 2 | 24.0 | 113.4 |
| 3 | 16.7 | 90.3 |
| 4 | 15.5 | 106.0 |
| $\bar{x} \pm$ S.E. | 18.8 $\pm$ 1.9 | 102.6 $\pm$ 5.6 |

from extracts of approximately 0.1 g fresh weight of cells as a percentage of the number of counts given by 0.5 μCi of $^{14}$C-ornithine-HCl. Although total incorporation was therefore much greater in the fast-growing cells, five times as many counts were present in the alkaloid fraction of the slow-growing cells than of the fast-growing.

Although the number of counts recorded will give a useful indication of the degree of incorporation of ornithine into proteins and alkaloids, it is essential to recognise that the turnover of molecules, that is, the balance of the differences in their rates of synthesis and degradation, will determine the final number of radioactive counts. The results presented in Tables 3.2.4 and 3.2.6 do demonstrate that fast-growing cells incorporate considerably more ornithine into proteins than do slow-growing cells, and that relatively more alkaloids are labelled in slow-growing cells than in fast-growing. It is tempting to suggest, from this information, that fast-growing cells synthesise relatively more proteins whereas slow-growing cells synthesise relatively more alkaloids, but such an interpretation must be approached with caution; this is not only for the reasons of 'turnover' given above, but also because it is possible that radioactively-labelled ornithine is utilised in the cell in a different way to endogenous ornithine.

Nevertheless, these results are consistent with the view that alkaloids are accumulated to the highest levels in slow-growing, stationary phase cells, while proteins are produced to the greatest extent in fast-growing cells.

The main points to come out of this experiment are as follows :

1. The fast- and slow-growing cells had different TLC profiles for alkaloids.

2. The slow-growing cells contained a greater number of radioactively-labelled alkaloids, and a greater total number of radioactive counts in alkaloids than the faster-growing cells.

3. The extracted protein of the faster-growing cells contained a greater number of radioactive counts than did that of the slower-growing cells.

The aim of the next section of results is to describe an experiment which investigated the effects of artificially changing cell growth rate on the accumulation of alkaloids and chlorophyll and on the friability of callus.

# SECTION 3

# THE EFFECTS OF 2,4-D ON GROWTH, FRIABILITY AND ALKALOID AND CHLOROPHYLL CONTENTS

There is evidence in the results described so far that the levels of alkaloids accumulated in callus and cell cultures are related to both the phase of the growth cycle (including such possibilities as the growth rate of the cells and their chemical environment, which must be continually changing) and the extent to which they are green and compact. In order to investigate these relationships further, an attempt was made to increase the friability and growth rate of D. innoxia callus by a sample change in the auxin concentration in the nutrient medium supplied to the cells, and to determine whether there were corresponding changes in alkaloid accumulation and chlorophyll production after a 4-week period.

Thus stem callus (which was 4 months old and had been sub-cultured at monthly intervals, and was normally maintained on full MS nutrient medium, i.e., containing $10^{-5}$M 2,4-D) was divided into small pieces (of approximately 0.5 g fresh weight). These pieces were randomised by mixing together, and 5 g of tissue was placed onto each of either nine agar plates containing full MS medium or nine plates containing full MS medium supplemented with $10^{-6}$M 2,4-D instead of $10^{-5}$M 2,4-D; this was the only alteration in the composition of the medium. At time 0 and at weeks 2 and 4 after plating, three plates from each treatment were sampled and determinations were made of callus fresh weight, alkaloid content (by the cell-squash technique) and chlorophyll content.

At week 4, callus from the three unopened plates from each treatment was subcultured onto six agar plates containing full MS medium (i.e., containing the higher concentration of auxin), and 4 weeks later the tissue was sampled and determinations were made of callus fresh weight, alkaloid content (by the cell-squash technique) and chlorophyll content.

The results presented in Table 3.2.7 describe the effects of transferring callus from full MS medium containing $10^{-5}$M 2,4-D to the reduced MS medium containing $10^{-6}$M 2,4-D. It can be seen that the callus on the reduced medium showed a greater percentage increase in fresh weight, after both 2 and 4 weeks of culture. By 4 weeks, the fresh weight of the callus on the reduced MS medium had increased by 221.4%, whereas the fresh weight of the callus on the full MS medium had increased by 96.1%. The friability of the callus on the reduced MS medium was observed to be dramatically greater on the reduced auxin medium than on the full medium.

The results in Table 3.2.7 also demonstrate that after 2 and 4 weeks of culture, the callus grown on the reduced MS medium contained lower levels of alkaloids and chlorophyll than did the callus grown on full MS medium. By week 4, the mean I value of the callus grown on reduced medium was 0.75 (representing approximately 0.1 mg alkaloids/g dry weight cells) compared with 2.7 (approximately 0.5 mg alkaloids/g dry weight cells) for the callus on full MS medium. The mean chlorophyll content for the callus grown on reduced medium was 26.2 µg chlorophyll/g fresh weight cells compared with 37.2 µg chlorophyll/g fresh weight cells for the callus on full MS medium.

The results in Table 3.2.7 show that when the 'reduced medium' callus was returned to full MS medium and grown thereon for a 4-week period, the percentage increase in fresh weight, friability, alkaloids content and chlorophyll content of the callus were comparable to those of the control callus which had been grown continuously on full MS medium.

| Week of Culture | % Increase Fresh Weight | | Alkaloid Content Mean I Value | | Chlorophyll Content µg chl./g.f.wt.callus | |
|---|---|---|---|---|---|---|
| | $10^{-5}$M 2,4-D | $10^{-6}$M 2,4-D | $10^{-5}$M 2,4-D | $10^{-6}$M 2,4-D | $10^{-5}$M 2,4-D | $10^{-6}$M 2,4-D |
| 0 | - | - | 2.8 | 2.8 | 47.9 ± 7.6 | 47.6 ± 7.6 |
| 2 | 20.3 ± 5.5 | 62.4 ± 7.2 | 2.8 | 1.8 | 40.1 ± 3.3 | 27.3 ± 6.9 |
| 4 | 96.1 ± 8.3 | 221.4 ± 10.9 | 2.7 | 0.75 | 37.2 ± 1.9 | 26.2 ± 3.1 |
| subculture | $10^{-5}$M 2,4-D | $10^{-5}$M 2,4-D | $10^{-5}$M 2,4-D | $10^{-5}$M 2,4-D | $10^{-5}$M 2,4-D | $10^{-5}$M 2,4-D |
| 8 | 83.4 ± 4.7 | 70.2 ± 10.4 | 2.7 | 2.8 | 42.4 ± 3.2 | 41.5 ± 6.2 |

Table 3.2.7

The effects of $10^{-5}$M and $10^{-6}$M 2,4-D on growth, alkaloid content and chlorophyll content of D. innoxia callus

The results of this experiment therefore suggest that the growth rate of cells affects the friability, alkaloid content and chlorophyll content.

## A BRIEF SUMMARY OF RESULTS PART 2

The results presented in all three sections are consistent with the concept suggested in Results Part 1, namely that there seems to be an inverse relationship between the growth of a culture and its alkaloid content, chlorophyll content and compactness. The growth curve experiments in Section 1 suggested that the inverse relationship may be more pronounced in callus cultures than in cell suspensions. The radioactive-labelling experiment indicated that proportionally more ornithine is incorporated into alkaloids (than proteins) in slow-growing, compared with fast-growing cells. The results of the third section have shown that the levels of alkaloids and chlorophyll and callus friability can be altered by manipulating the growth rate of the cells.

In the next part of the results are described experiments performed on the flatbed apparatus, which was designed to facilitate manipulation of the chemical environment of the cells. Particular attention was paid to the characterisation of culture growth in relation to alkaloid accumulation.

# RESULTS PART 3

## Characterisation Of The Flatbed Culture System

It is clear from the results described in Parts 1 and 2 that cultures of cells and tissues accumulate alkaloids to the greatest extent when they are compact and/or green and growing relatively slowly. These characteristics are best achieved when the cells are cultured on an agar medium, but manipulations of the environment, particularly sequential chemical treatments, are impossible without disturbing the cells. Therefore it was important to develop a culture system in which this could be facilitated.

A means of producing slow-growing, 'partially differentiated' cells in culture has already been described by Yeoman et al., (1978), namely the 'flatbed' apparatus, which is described in Methods, Chapter 2. Using this system of cultures, cells (derived from fast-growing suspension cultures) tend to grow relatively slowly and in close association with one another, in a similar way to callus cultures.

In this part are described attempts to define some of the environmental conditions to which the flatbed-cultured cells were subjected, and the effects of manipulating the environment on the growth and metabolism of the cultures.

There are three sections of results. In Section 1 are presented the results of some preliminary experiments in which the growth of the cultures was investigated. In Section 2, the effects of altering the environment of the cells on their ability to grow and accumulate alkaloids was investigated. In Section 3, the effects of the dripping of nutrient medium directly onto the cells was examined.

# SECTION 1

# PRELIMINARY EXPERIMENTS ON THE CHARACTERIZATION OF THE FLATBED SYSTEM

In this section a study has been made of the growth of cells in the flatbed culture system. The first experiment was designed to show whether the fabric substratum was inhibitory to the growth of the cells and their ability to synthesise alkaloids. The second set of experiments examines the nutritional environment of the cells and correlates the uptake of four essential nutrients with cell growth rate. The third set of experiments examines the levels of oxygen available to the cells in the system.

## TO DETERMINE WHETHER THE FABRIC SUBSTRATUM HAS CYTOTOXIC PROPERTIES

The cells cultured in the flatbed apparatus were seated on a substratum of polypropylene matting. Since the cells were in direct contact with the fabric substratum and also received some of their nutrients by an upward diffusion through the mesh, it was considered important to determine whether the fabric had any detectable intrinsic cytotoxic properties which would limit the growth and viability of the cultures.

Accordingly, the effects of the fabric on the increase in fresh weight of and production of alkaloids by suspension cultures of S. nigrum was studied. Six cell suspension cultures were prepared by adding 10 ml of a mixture of two 10 day-old (i.e., rapidly-growing) suspension cultures (which had been maintained for 4 months) to 50 ml of liquid full MS medium in 250 ml Erlenmeyer flasks. Fabric matting was washed thoroughly in distilled water, dried and autoclaved (as it was before use in flatbed cell-culture); to each of three 'test' flasks was added 1 g of the sterile matting, and then these and the three 'control' culture flasks (simply cell suspensions without the

inclusion of fabric matting) were allowed to grow for 3 weeks under normal suspension culture conditions, and at the end of this period determinations were made of cell fresh weight (after draining on a Buchner funnel) and alkaloid content (by the cell-squash technique). The results are given in Table 3.3.1.

It can be seen that there was no significant retardation of the growth rate of the suspended cells by the fabric matting, the mean fresh weight of the 'treated' cultures, in fact, being not significantly different from that of the controls (12.73 g compared with 12.56 g); alkaloid accumulation was similarly unaffected. It was therefore concluded that the matting had no observable toxic properties and could be safely used for the production of cultures.

The growth of the cells on the flatbed system is now considered.

## NUTRIENT UPTAKE ON THE FLATBED

It is axiomatic that, in order to grow, cultured cells must be supplied with nutrients, and therefore some measure of growth can be obtained by following the rate of disappearance of nutrients from the medium. Accordingly, the levels of four essential nutrients, i.e., orthophosphate, nitrate, ammonia and sucrose, were followed over a time course.

In this series of experiments, each treatment of cultured cells was replicated three times, i.e., three flatbeds were each supplied with their own reservoir. In analyses from nutrient uptake experiments, it is important that the samples removed from each reservoir are of small volumes, so that the total amount of nutrients

Table 3.3.1

The effect of fabric substratum on the growth of and alkaloid production by S. nigrum suspension cultures

| Replicate | Fresh Weight (g) | | | Alkaloid Content Mean I Value | | |
|---|---|---|---|---|---|---|
| | Time 0 | Week 3 | | Time 0 | Week 3 | |
| | | Control | Fabric | | Control | Fabric |
| 1 | 0.84 | 12.72 | 13.40 | 2.1 | 2.9 | 2.8 |
| 2 | 0.62 | 12.32 | 12.38 | 2.3 | 2.7 | 2.9 |
| 3 | 0.79 | 12.90 | 12.39 | 2.3 | 2.8 | 2.8 |
| MEAN ± S.E. = | 0.75 ± 0.07 | 12.65 ± 0.17 | 12.72 ± 0.34 | 2.2 | 2.8 | 2.8 |

in the system (i.e., reservoir, culture vessel and cells) remains as near constant as possible. For this reason, the minimum amount of medium required for each analysis was calculated, divided by three, and that volume sampled from each of the three reservoirs of a particular treatment and then combined. This procedure ensured that (a) the total level of nutrients in the apparatus was reduced minimally and (b) an overall estimate of nutrient uptake was obtained for each treatment.

## UPTAKE OF NUTRIENTS BY *DATURA INNOXIA* CELLS CULTURED ON THE FLATBED APPARATUS

Approximately 45 g wet weight of *D. innoxia* cells, which had been maintained in suspension culture for 3 months, were set up in flatbeds as described in Methods, Chapter 2. In the first of two experiments, the nutrient medium from three flatbed reservoirs was sampled, mixed and analysed (as described previously) at the end of a 7-day culture period. In the second experiment, medium from three reservoirs was sampled, mixed and analysed every 2 days over a 10-day culture period. The results of each experiment are given in Tables 3.3.2 and 3.3.3 respectively, and in Figures 3.3.1 (a) - (d) for the second experiment.

The results in Table 3.3.2 show that, by the end of the 7-day period there was removed from the medium 87.7% of the initial amount of orthophosphate, 60.0% of the initial sucrose, and 17.3% of the initial ammonium ions. A more detailed picture of the uptake kinetics, and includes information on nitrate ion uptake, is presented in Table 3.3.3. It may be seen that there was a rapid uptake of

Table 3.3.2

Nutrient uptake by Datura innoxia cells after a 7-day culture period

| | % Nutrient Uptake | | |
|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | Sucrose |
| Day 7 | 87.8 | 17.3 | 60.0 |

Table 3.3.3

Nutrient uptake by D. innoxia cells during a 10-day culture period

| | % Nutrient Uptake | | | |
|---|---|---|---|---|
| Day | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 2 | 93.6 | 22.5 | 30.7 | 50.7 |
| 4 | 92.7 | 21.4 | 71.6 | 47.2 |
| 6 | 88.9 | 26.1 | 73.3 | 56.9 |
| 8 | 90.4 | 14.2 | 59.5 | 72.1 |
| 10 | 66.9 | 27.3 | 41.7 | 37.5 |

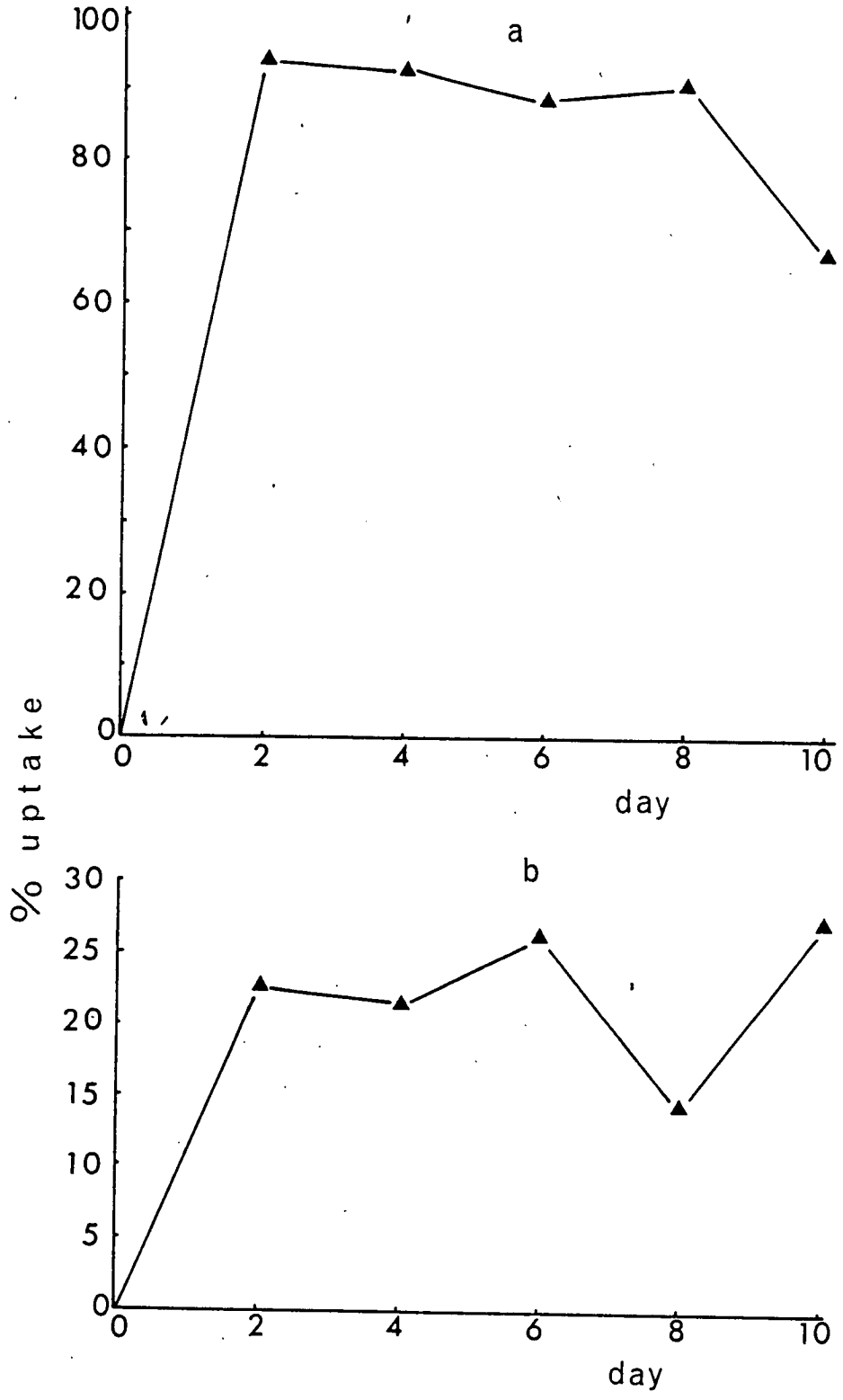



Figure 3.3.1 Nutrient Uptake by D. innoxia Cells on the Flatbed

(a) Orthophosphate

(b) Ammonia

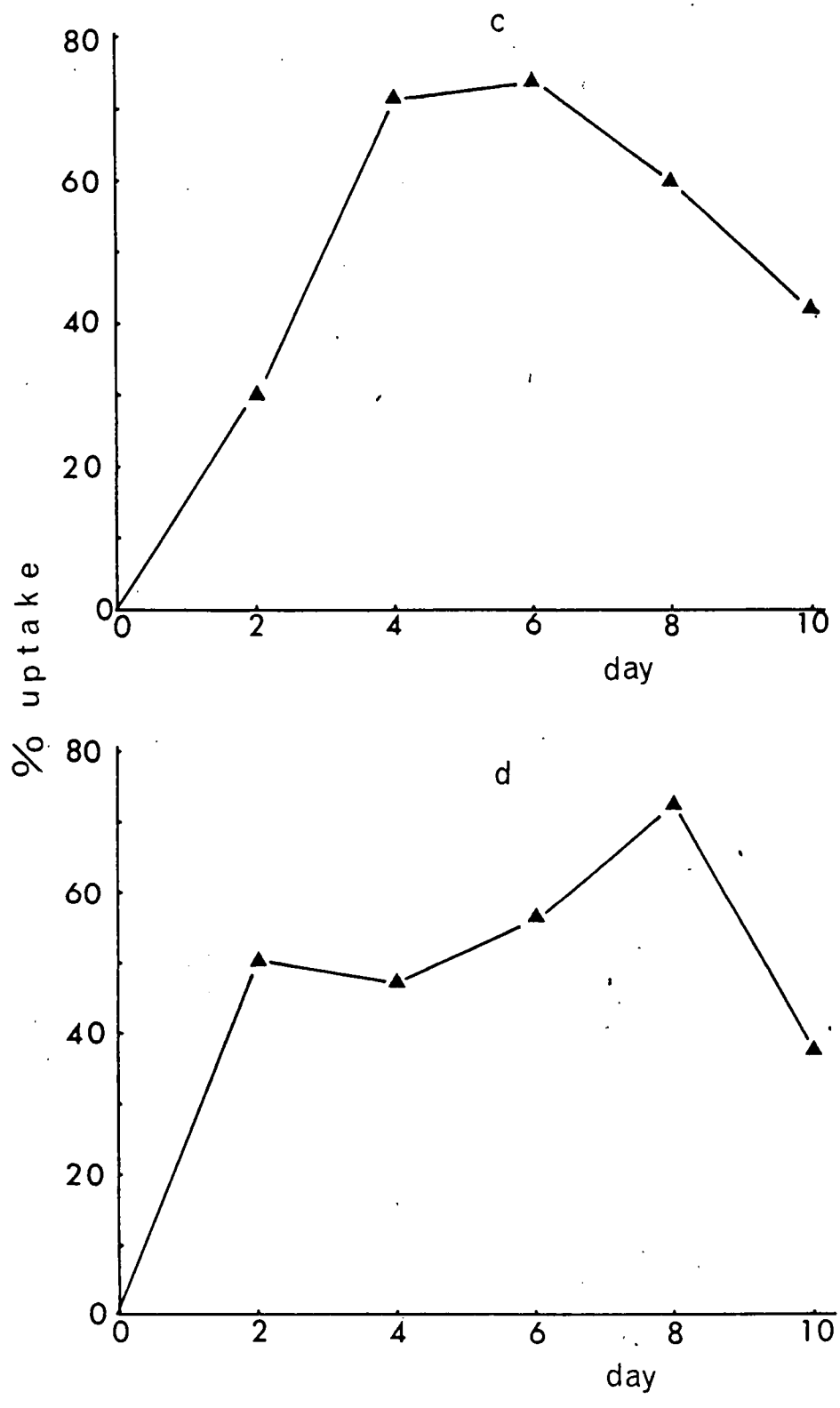



Figure 3.3.1 (continued)

(c) Nitrate

(d) Sucrose

orthophosphate during the first two days (93.6%), which levelled off thereafter. Between days 8 and 10 there was an increase in the detectable levels in the medium of nearly 25% of the initial amount of orthophosphate, possibly due to cell lysis. Similarly, there was a fairly rapid uptake of sucrose during the initial two days of culture, in which time nearly 60% of the available sucrose was removed from the medium, and after day 8 cell lysis was again indicated as the levels in the medium increased. A relatively small quantity of ammonium ions was absorbed over 10 days: just over 20% of that present at day 0 was removed during the first two days, after which time the rate of uptake slowed considerably. Between days 6 and 8 there was an increase in the concentration of ammonia in the medium, but uptake continued after this period. Nitrate was taken out of the medium in a more or less linear manner over the first four days of culture, until about 70% of the available ion was removed from the medium. Between days 6 and 10 some nitrate (equivalent to approximately 30% of that available) was released from the cells.

In order to determine whether the observed nutrient uptake from the medium was due solely to the cells, or whether the flatbed apparatus itself contributed to the removal, medium was passed through the apparatus in the usual way, but in the absence of cells. The medium was sampled at intervals, and the results are given in Table 3.3.4.

It may be seen that the flatbed apparatus appeared to adsorb the four nutrients to some extent, but not irreversibly or even very strongly so. Thus in a rapidly-growing flatbed culture of

Table 3.3.4

Nutrient uptake by the flatbed apparatus itself

| | % Nutrient Uptake | | | |
|---|---|---|---|---|
| Time (hr) | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 8 | 22.0 | 19.1 | 10.3 | 7.9 |
| 24 | 19.5 | 14.6 | 21.4 | 5.3 |
| 32 | 19.5 | 11.5 | 19.0 | 5.5 |
| 48 | 17.1 | 11.5 | 9.6 | 5.9 |
| 96 | 4.9 | 5.8 | 3.0 | 0.2 |

D. innoxia the cells would be expected to account for the uptake of about 95% of the total removed orthophosphate, about 70% of the total removed ammonia, about 95% of the total removed nitrate and about 99% of the total removed sugar. The remainder is due to the apparatus itself, but no clear explanation for this phenomenon can be given - presumably the glass or the fabric matting adsorbs the nutrients.

## THE RELATIONSHIP BETWEEN GROWTH RATE AND RATE OF NUTRIENT UPTAKE IN FLATBED CULTURES

The flatbed cultures demonstrably remove nutrients from the liquid medium, and it might be expected that the rate of uptake would be positively correlated with the growth rate of the cells. If such a relationship was found to exist, a ready indication of culture growth rate could be obtained by monitoring the levels of the nutrients in the medium. In order to test this hypothesis, known weights (45 - 50 g wet weight) of cells of S. nigrum derived from stationary phase fast- and slow-growing suspended cell lines (see Table 3.3.7) (which had apparently arisen spontaneously from different pieces of initiating stem callus and had been maintained and subcultured separately for approximately 4 months) were loaded onto the flatbed apparatus so that there were three beds of fast-growing and three beds of slow-growing cells. The cells of each 'treatment' were randomised before loading; this was effected by mixing together three stationary-phase suspension cultures of each cell line respectively, and putting one third of each mixture onto a flatbed. Nutrient medium was sampled from each reservoir and mixed (for each group of three flatbeds) at daily intervals and analysed for

orthophosphate, ammonia, nitrate and sucrose. At the end of the 7-day culture period, determinations were made of cell fresh weight, viability and alkaloid content (by the cell-squash technique) and a comparison was made with suspended cells. The results are expressed in Tables 3.3.5, 3.3.6 and 3.3.7, and Figures 3.3.2 (a) - (d). The fresh weight data demonstrate that the cell lines, which grew quickly and slowly respectively in suspension culture, grew in a similar manner when cultured on the flatbed (but more slowly). There is a noticeable difference in the extent to which the two cell lines removed all four nutrients from the medium when cultured on the flat-bed. The nutrient uptake data of the faster-growing cells were noticeably up on the slower ones even after 24 hours growth, and this trend of a greater rate of uptake by the 'fast' cell line continued throughout the duration of the experiment; by day 7, the 'fast' cells had taken up over 20% more orthophosphate than the 'slow' cells, 40% more ammonia, 20% more nitrate and nearly 20% more sucrose. It may therefore be concluded that, as was expected, the rate of nutrient uptake could be used as a guide to the rate of growth of the cells.

There was also a small difference in alkaloid content between the two cell lines, the fast-growing cultures having a mean I value of 1.4 (about 10 mg alkaloid/g dry weight cells) and the slow-growing cultures a mean I value of 2.0 (about 12 mg alkaloid/g dry weight cells); this may have been related to the growth rate of the cells, but could equally likely have been because the 'slow' line might have been a high yielder of alkaloids irrespective of its growth rate. Some workers have demonstrated that, by simple selection procedures, high- and low-yielding strains can be isolated from a single piece of callus (Tabata and Hiraoka, 1976; Ogino *et al.*,

Table 3.3.5

Nutrient uptake by fast- and slow-growing *S. nigrum* cell lines on the flatbed

| | % NUTRIENT UPTAKE | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FAST | | | | SLOW | | | |
| Day of Culture | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 1 | 76.1 | 39.7 | 59.1 | 54.4 | 52.4 | 30.9 | 51.8 | 25.3 |
| 2 | 82.1 | 46.1 | 59.2 | 53.5 | 60.5 | 32.3 | 53.1 | 25.9 |
| 3 | 89.3 | 49.7 | 62.4 | 56.3 | 62.1 | 30.7 | 55.9 | 30.4 |
| 4 | 99.7 | 56.0 | 76.0 | 69.2 | 57.3 | 28.3 | 60.3 | 53.5 |
| 5 | 97.4 | 58.9 | 70.5 | 69.1 | 56.1 | 36.3 | 60.1 | 54.1 |
| 6 | 98.9 | 86.8 | 82.9 | 71.5 | 63.5 | 41.6 | 62.4 | 49.3 |
| 7 | 99.0 | 87.0 | 89.5 | 77.4 | 76.9 | 46.9 | 66.1 | 60.2 |

Table 3.3.6

Growth viability and alkaloid content of two cell lines on the flatbed, after a culture period of 7 days

| | FAST CELL LINE | | | SLOW CELL LINE | | |
|---|---|---|---|---|---|---|
| Replicate | % Increase Fresh Weight | % Cell Viability | Mean I Value | % Increase Fresh Weight | % Cell Viability | Mean I Value |
| 1 | 21.3 | 69.3 | 1.8 | 11.3 | 68.2 | 2.0 |
| 2 | 17.2 | 77.8 | 1.0 | 5.8 | 72.2 | 2.1 |
| 3 | 29.7 | 70.4 | 1.3 | 3.9 | 74.6 | 1.8 |
| $\bar{x} \pm$ S.E. = | 22.6 ± 0.2 | 72.6 ± 0.1 | 1.4 | 6.7 ± 0.2 | 73.3 ± 0.1 | 2.0 |

Table 3.3.7

Growth, viability and alkaloid content of two cell lines in suspension culture, after a culture period of 18 days

| | FAST CELL LINE | | | SLOW CELL LINE | | |
|---|---|---|---|---|---|---|
| Replicate | % Increase Fresh Weight * | % Cell Viability | Mean I Value | % Increase Fresh Weight * | % Cell Viability | Mean I Value |
| 1 | 1227 | 78.2 | 1.4 | 844 | 72.4 | 1.8 |
| 2 | 1473 | 73.0 | 1.3 | 929 | 78.3 | 1.6 |
| 3 | 1590 | 69.4 | 1.3 | 826 | 71.1 | 1.2 |
| $\bar{x} \pm$ S.E. = | 1430 ± 106 | 73.6 ± 0.1 | 1.3 | 866 ± 32 | 74.0 ± 0.1 | 1.5 |

* Initial Fresh Weight = approximately 1 g

(a) Orthophosphate

(b) Nitrate

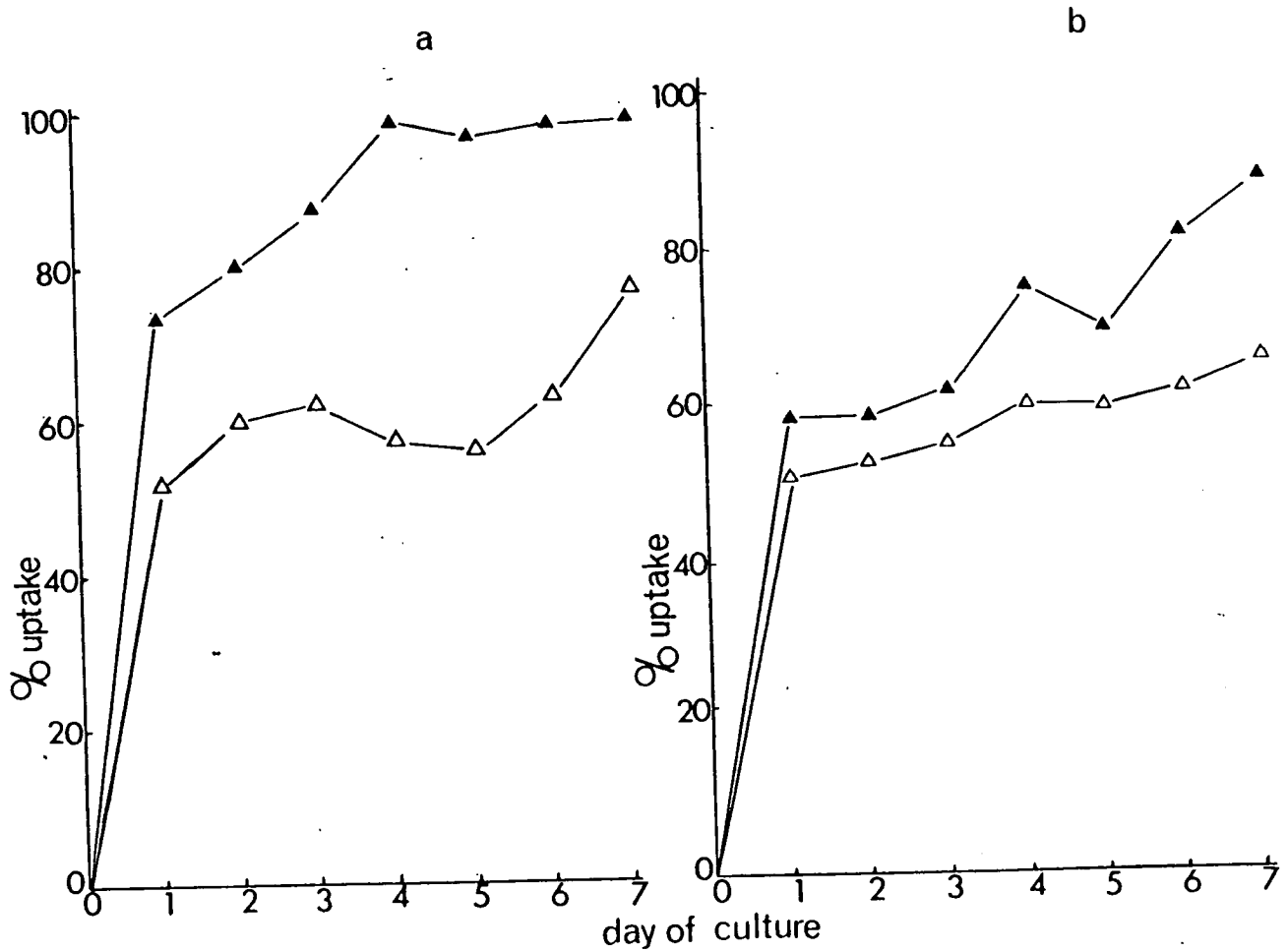



Figure 3.3.2 Nutrient Uptake by Fast- (Solid Triangles) and Slow- (Open Triangles) Growing Cell Lines of S. nigrum on the Flatbed

(c) Ammonia

(d) Sucrose

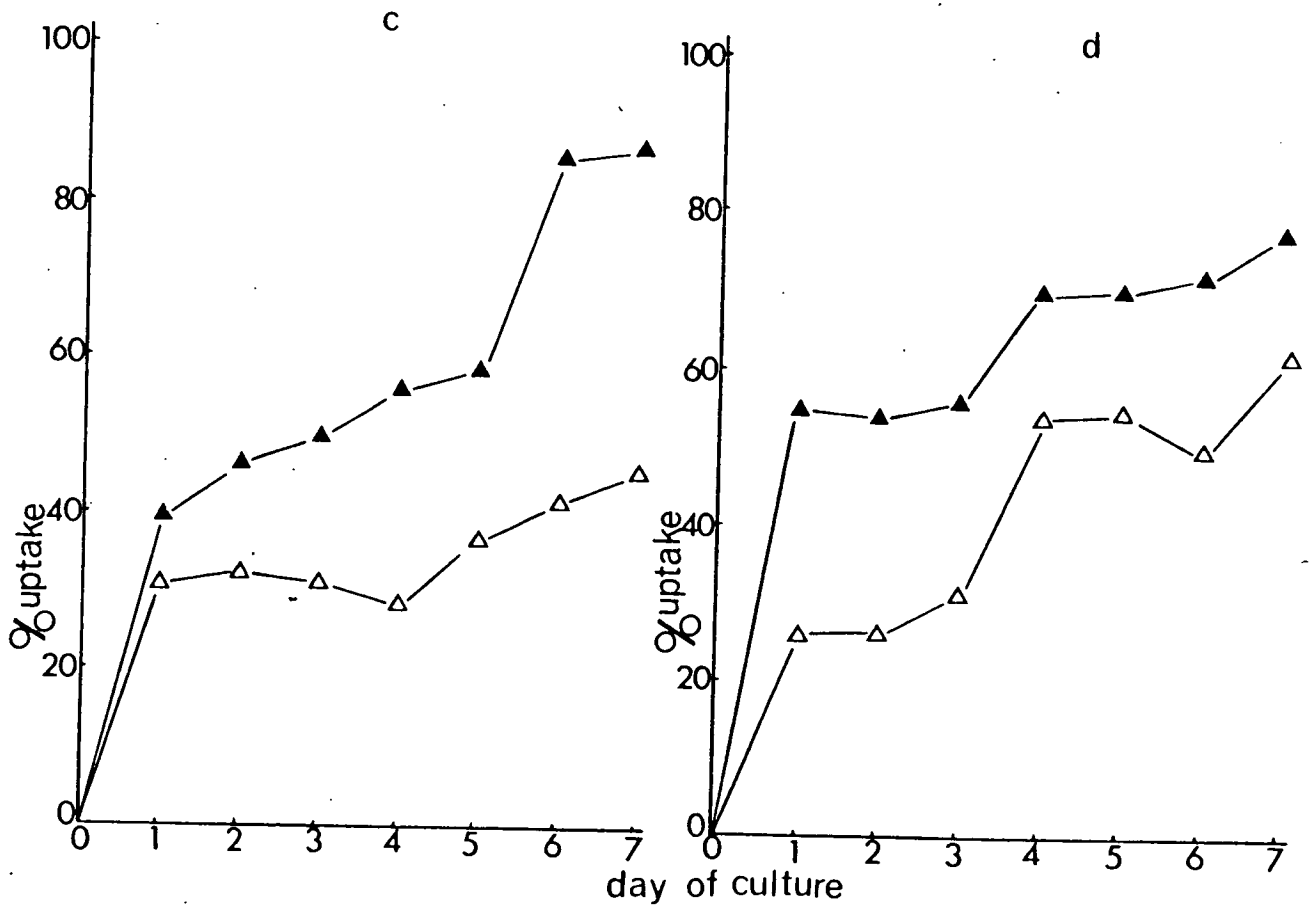



Figure 3.3.2 (continued)

1978). It may also be seen from the results that both cell lines cultured on the flatbed grew more slowly than when cultured as liquid suspensions. Furthermore, the percentage viability of the cells was between 70 and 75%, whether they were grown on the flatbed or as suspended cells. The alkaloid content of the 'fast' cells was approximately the same in both culture systems (I value of 1.3 - 1.4), but the flatbed-grown 'slow' cells accumulated higher levels of alkaloids (approximately 12 mg/g dry weight cells) than did the suspended 'slow' cells (approximately 10-11 mg/g dry weight cells).

The growth characteristics of cells cultured on the flatbed are now investigated.

## OXYGEN LEVELS IN THE CIRCULATING MEDIUM SUPPLYING CELLS OF *SOLANUM NIGRUM* ON THE FLATBED

The aim of this experiment was to determine whether cells of *S. nigrum* could obtain oxygen from the nutrient medium when cultured on the flatbed apparatus. Since this apparatus is sealed during operation, cells are supplied only with oxygen (a) present in the air of the culture vessel at the time of sealing, (b) dissolved in the nutrient medium (and obtained from the air in the medium reservoir) and (c) from any diffusion of air into the apparatus through seals, joints and rubber tubing.

Six flatbeds were therefore set up in the usual way. Three contained known weights of between 45 - 50 g of cells (wet weight) and three contained no cells. By analysis of the medium supplied to the flatbeds without cells and and comparison of the results with the other flatbeds, it was possible to estimate the extent to which

the cells themselves removed oxygen from the medium. Medium was sampled from each of the six flatbeds at daily intervals for a period of 7 days, and the dissolved oxygen levels were determined using an oxygen electrode. The percentage increase in wet weight was calculated for each flatbed. The oxygen uptake results are expressed in Table 3.3.8.

The medium in the flatbeds without cells contained, throughout the 7 day culture period, levels of oxygen of about 0.049 μmol $O_2$ $ml^{-1}$ medium which represented 97-98% air saturation. The presence of cells resulted in a significant uptake of oxygen from the medium, such that by day 7 about 30% of the oxygen originally present in the medium was removed (Figure 3.3.3); the mean wet weight increased by approximately 30% over this period. Thus, the growing cells apparently made use of the oxygen dissolved in the medium, but would also have obtained the gas from the air in the culture vessel. It cannot be determined precisely from the data presented here whether the cultures were under oxygen stress, but a rough estimation can be made. According to the species, the extinction point (i.e., that concentration of oxygen below which anaerobic metabolism will proceed) is of the order of 5% $O_2$ (v/v) in the atmosphere. Assuming that the gases of air each have approximately the same solubility in liquid medium, then 5% available oxygen is represented by 0.0124 μmol $O_2$ per ml medium. The results in Table 3.3.8 show that this low value was not reached. It therefore seems likely that even if the free space in the culture vessel had become anaerobic, there would be sufficient oxygen dissolved in the medium after 7 days to allow further growth; this would, however, be limited by the slow rate of diffusion of oxygen in water.

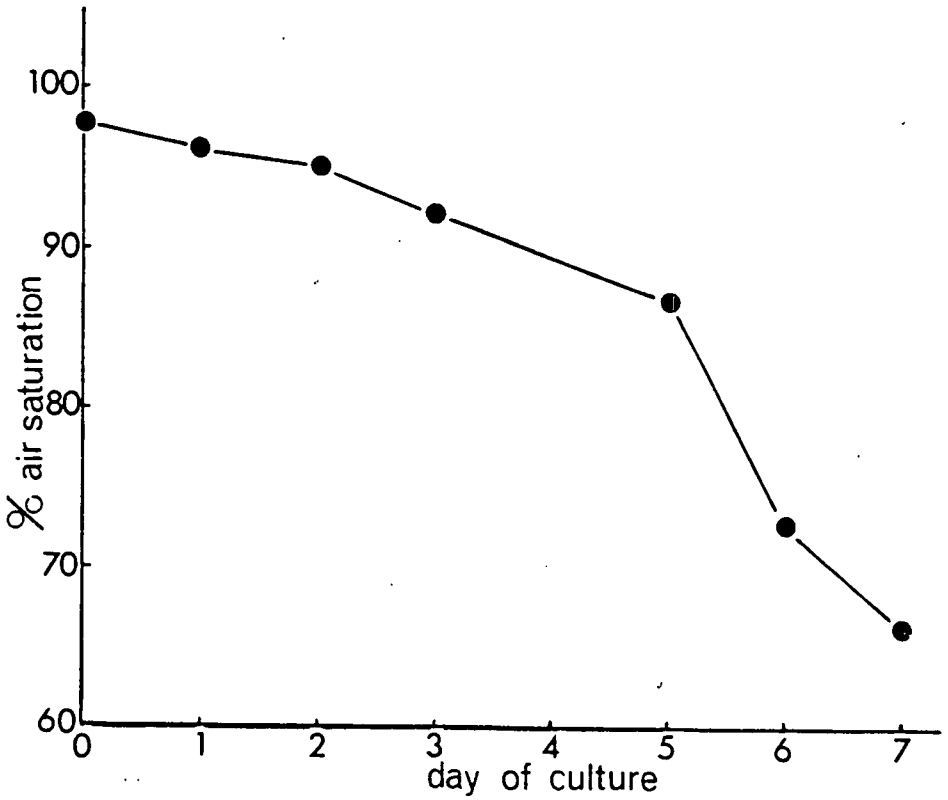



Figure 3.3.3 Oxygen Uptake by Cells of S. nigrum on the Flatbed

100% Air Saturation ≡ 0.05 μmol $O_2$/ml medium

Table 3.3.8

Oxygen levels in the nutrient medium

| Day of Culture | PLUS CELLS | | MINUS CELLS | |
|---|---|---|---|---|
| | Mean % Air Saturation | Mean µmol $O_2$ $ml^{-1}$ Medium | Mean % Air Saturation | Mean µmol $O_2$ $ml^{-1}$ Medium |
| 0 | 97.6 ± 0.1 | 0.049 ± 0.000 | 97.7 ± 0.1 | 0.049 ± 0.000 |
| 1 | 96.0 ± 0.1 | 0.048 ± 0.000 | 97.6 ± 0.1 | 0.049 ± 0.000 |
| 2 | 95.1 ± 0.1 | 0.047 ± 0.000 | 97.6 ± 0.1 | 0.049 ± 0.000 |
| 3 | 92.1 ± 0.2 | 0.046 ± 0.000 | 97.7 ± 0.1 | 0.049 ± 0.000 |
| 4 | 89.2 ± 0.2 | 0.045 ± 0.000 | 97.2 ± 0.1 | 0.049 ± 0.000 |
| 5 | 86.8 ± 0.4 | 0.043 ± 0.000 | 97.2 ± 0.1 | 0.049 ± 0.000 |
| 6 | 72.8 ± 0.1 | 0.036 ± 0.000 | 97.3 ± 0.1 | 0.049 ± 0.000 |
| 7 | 66.4 ± 0.1 | 0.033 ± 0.000 | 97.2 ± 0.1 | 0.049 ± 0.000 |

The results of this section have shown that cells of *D. innoxia* and *S. nigrum* were capable of growth (as indicated by the percentage nutrient uptake data) and alkaloid production when cultured on the flatbed apparatus, and also utilized oxygen dissolved in the nutrient medium. Growth on the flatbed was slower than in suspension culture. Although the growth of the cells did not appear to be oxygen-limited, most of the orthophosphate had been taken up within 2 days. The fabric substratum did not inhibit growth or alkaloid production by *S. nigrum* cells.

In the next section of results are described experiments in which the environment of the cells was deliberately manipulated.

# SECTION 2

## THE EFFECTS OF ENVIRONMENTAL CHANGES ON THE GROWTH AND METABOLISM OF CELLS CULTURED ON THE FLATBED

This second section of flatbed results describes some physiological and metabolic studies performed on cells cultured in the flatbed apparatus. The experiments described in Section 1 attempted to define some of the features of the growth of flatbed-grown cells; the next set of experiments involved the manipulation of the physical and chemical environment of the cells, in order to determine the effects on cell growth and alkaloid accumulation.

The first experiment examines the effects of relatively high and low oxygen levels. In the second, ornithine was supplied to the cells in an attempt to increase alkaloid accumulation. The third set of experiments investigate the effects on alkaloid accumulation of the dripping of nutrient medium directly onto the cells.

## THE EFFECTS OF HIGH AND LOW OXYGEN LEVELS (IN THE NUTRIENT MEDIUM) ON CELLS OF *SOLANUM NIGRUM* CULTURED ON THE FLATBED

The aim of this experiment was to discover whether the level of oxygen in the nutrient medium affected the growth and metabolism of *S. nigrum* cells cultured on the flatbed. In this experiment, the medium supplied to the cells was either saturated with air to provide an oxygen level of approximately 0.050 μmol $ml^{-1}$ medium or with a nitrogen/air mixture to provide a dissolved oxygen level of 0.020 μmol $ml^{-1}$ medium. In the latter case, the aim was not to completely starve the cells of oxygen, but to subject them to some degree of oxygen stress; that is, subjecting them to oxygen levels lower than those experienced under normal flatbed conditions, in which the only oxygen available to the cells was that present in the culture

vessel and nutrient medium at the time of setting up the apparatus or may have leaked in through seals and joints.

Accordingly, six flatbeds were set up as described in Methods, Chapter 2, each with 45-50 g wet weight of cells from suspensions which had been maintained for 3 months. After 2 days, during which time the medium was not aerated, the sterile gas mixtures were bubbled through the reservoirs via an 'input needle' inserted in the bottom of each reservoir and after sterilisation by passing through an air filter (Microflow Ltd., Fleet, Hampshire). Three flatbeds were subjected to each treatment.

## The Treatments

### (a) The $N_2$/Air Mixture

The nutrient medium was saturated with a mixture of approximately 99% $N_2$ and 1% air (containing 20% $O_2$ v/v) v/v, at an arbitrary flow rate of 520 ml of gas/50 ml medium/minute for 8 hours, to reduce the oxygen level in the medium to about 0.020 µmol $ml^{-1}$; after this initial 8 hour period, the flow rate was greatly reduced (to 60 ml of gas/50 ml of medium/minute) in order to reduce the rate of evaporation of the medium. The residual gas in the culture vessel was removed by blowing the sterile gas mixture through the nutrient medium entrance port for 2 minutes and allowing it to come out through a hollow needle in the medium exit port.

### (b) Air

The nutrient medium was aerated at an initial flow rate of 520 ml of air/50 ml of medium/minute for 8 hours, as described in

the $N_2$/air mixture. After the 8 hour period, the flow rate was reduced to 60 ml of air/50 ml of medium/minute, to limit evaporation of the medium and yet maintain high oxygen levels. The culture vessels were purged with air, in the manner described above. The reservoirs were gassed for 8 days, making a total experimental period of 10 days (from the time nutrient medium was first supplied from the reservoirs).

At the same time, three flatbeds were set up without any cells; each contained 50 ml of medium which was gassed with the $N_2$/air mixture as described above. This was so that a comparison could be made of oxygen uptake by $N_2$/air-treated flatbeds with and without cells and the uptake due to the cells alone could be estimated. In these 'control' flatbeds the reservoirs were gassed for 9 days.

Gas flow rates were determined and controlled using gas flow meters (Meterate, Hemel Hempstead).

## Nutrient and Oxygen Levels

For the three 'control' flatbeds (i.e., without cells) the nutrient medium was sampled immediately before gassing (time 0) and then on days 1, 2, 5, 7 and 9, for oxygen level determinations. The nutrient medium of the six 'test' flatbeds (i.e., with cells) was sampled at the beginning of the experiment (time 0) and at days 2, 3, 4, 6, 8 and 10, and measurements were made of the levels of dissolved oxygen and nutrients (orthophosphate, ammonia, nitrate and sucrose).

The results presented in Table 3.3.9 show that, in the absence of cells, the $N_2$/air mixture succeeded in producing much-reduced oxygen levels in the medium, being maintained at about 40% air saturation (i.e., 0.02 µmol $O_2$ $ml^{-1}$ medium) over the time period examined. The results in Table 3.3.10 show the effects of both gas treatments on the levels of oxygen in the medium supplied to cells, and it can be seen that the air-treated medium maintained a high level of oxygen (about 0.050 µmol $O_2$ $ml^{-1}$, equivalent to air-saturation), while the $N_2$/air treatment produced the reduced oxygen levels recorded in the 'control' flatbeds. By day 8 the oxygen levels were lower than this (0.013 µmol $ml^{-1}$), presumably due to uptake by the cells.

APPEARANCE OF THE CULTURES AFTER TREATMENT

In both air- and $N_2$/air-treated cultures, regions became distinguished which were darkly coloured. These were seemingly formed as a result of the dripping of nutrient medium onto the cells, and were apparent by day 3. These areas were designated 'drip areas'. In the air-treated cultures there was still a distinction observed between the 'drip areas' and the rest of the cells by day 10, but by this time the whole of the $N_2$/air-treated cultures were of a more or less uniformly dark appearance.

FRESH WEIGHT

The fresh weight increases of the cells are given in Table 3.3.11. The air-treated cultures showed a mean increase of 15.0% by the end of the experimental period, while the $N_2$/air-treated cultures showed a mean increase of 6.7%. In order to determine whether this difference was statistically significant, a Student t-test was performed on the arcsin-transformed data. It was found that, in the reduced oxygen regime, the growth rate of the cultures was significantly slower (at $p = 0.02 - 0.01$, $t = 3.804$, 4 degrees of freedom) than in the flatbeds of higher oxygen levels.

NUTRIENT UPTAKE

The uptake of all four nutrients examined (see Table 3.3.12 and Figures 3.3.4 (a) - (d)) proceeded with an initially rapid rate of uptake over the first four or so days, and was followed by a more gradual but steady uptake, in the case of both treatments. It is apparent, however, that the percentage uptake was lower for all four

Table 3.3.9

Oxygen levels in the $N_2$/air-treated medium, in the absence of cells

| Day | % Air Saturation | µmol $O_2$ $ml^{-1}$ Medium |
|---|---|---|
| 0 | 98 | 0.047 |
| 1 | 42 | 0.020 |
| 2 | 40 | 0.019 |
| 5 | 39 | 0.019 |
| 7 | 43 | 0.021 |
| 9 | 41 | 0.020 |

Table 3.3.10

Oxygen levels in air- and $N_2$/air-treated media, in the presence of cells

| Day of Culture | % Air Saturation | | µmol $O_2$ $ml^{-1}$ medium | |
|---|---|---|---|---|
| 0 | 98 | | 0.047 | |
| 2 | 97 | | 0.047 | |
| | $N_2$/Air | Air | $N_2$/Air | Air |
| 3 | 40 | 100 | 0.019 | 0.050 |
| 4 | 45 | 100 | 0.022 | 0.050 |
| 6 | 41 | 100 | 0.020 | 0.050 |
| 8 | 29 | 100 | 0.013 | 0.050 |
| 10 | 37 | 100 | 0.018 | 0.050 |

Table 3.3.11

Growth and alkaloid content of the cultures after gas treatment

| Treatment | Replicate | % Increase Fresh Weight | % Cell Viability | Mean I Values |
|---|---|---|---|---|
| | 1 | 8.2 | 81.7 | 1.3 |
| AIR | 2 | 21.3 | 75.2 | 1.5 |
| | 3 | 17.0 | 75.9 | 1.4 |
| | $\bar{x} \pm$ S.E. = | 15.0 ± 0.3 | 77.7 ± 0.1 | 1.4 |
| | 1 | 10.3 | 40.7 | 2.3 |
| $N_2$/AIR | 2 | 3.2 | 38.7 | 2.5 |
| | 2 | 7.7 | 59.9 | 2.6 |
| | $\bar{x} \pm$ S.E. = | 6.7 ± 0.2 | 46.4 ± 0.4 | 2.5 |

no alkaloids were detected in the medium of either treatment

Table 3.3.12

Nutrient uptake during gas treatment

% NUTRIENT UPTAKE

| Day of Culture | $PO_4^{---}$ | | $NH_4^+$ | | $NO_3^-$ | | SUCROSE | |
|---|---|---|---|---|---|---|---|---|
| | Air | $N_2$/Air | Air | $N_2$/Air | Air | $N_2$/Air | Air | $N_2$/Air |
| 1 | 70.4 | 73.2 | 31.3 | 38.8 | 55.2 | 50.1 | 48.3 | 45.2 |
| 2 | 82.1 | 79.3 | 35.1 | 39.1 | 60.7 | 58.3 | 47.9 | 46.1 |
| 4 | 96.3 | 90.4 | 35.1 | 40.7 | 71.3 | 62.5 | 60.7 | 52.2 |
| 6 | 92.4 | 91.1 | 59.6 | 55.7 | 70.4 | 70.7 | 72.5 | 71.8 |
| 8 | 98.3 | 97.4 | 67.4 | 61.3 | 83.1 | 75.7 | 89.3 | 70.6 |
| 10 | 99.1 | 89.7 | 66.1 | 60.5 | 83.9 | 75.5 | 87.4 | 71.4 |

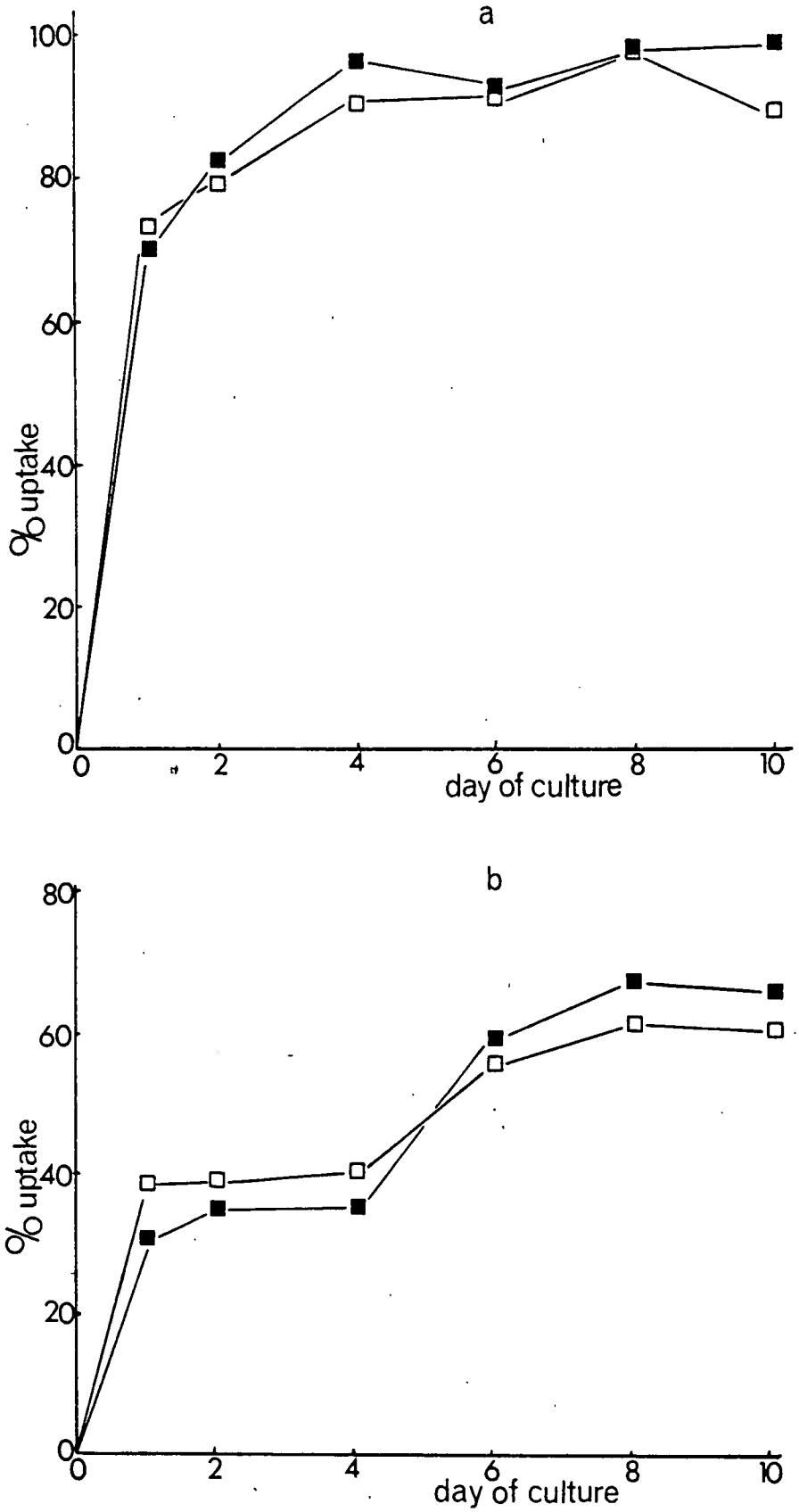



Figure 3.3.4 Nutrient Uptake by S. nigrum Cells in the Presence of Air (Solid Squares) and $N_2$/Air (Open Squares) on the Flatbed

(a) Orthophosphate

(b) Ammonia

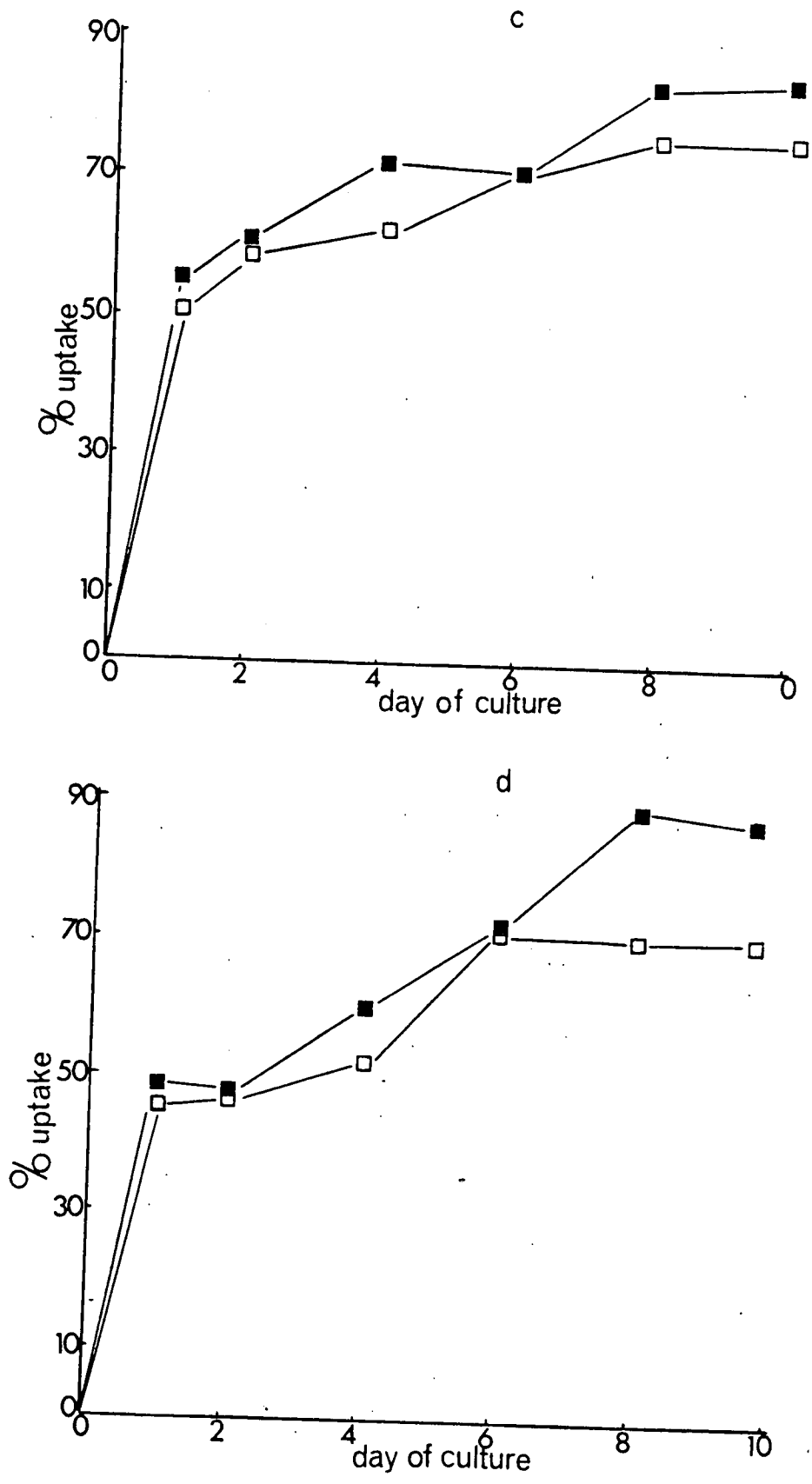



Figure 3.3.4 (continued)

(c) Nitrate

(d) Sucrose

nutrients in the cultures supplied with the lower oxygen level; at day 10, 99.1% of the orthophosphate had disappeared from the aerated medium compared with 89.7% in the $N_2$/air-treated cultures, and this pattern was repeated for ammonia (66.1% (air) against 60.5% ($N_2$/air)), nitrate (83.9% (air) against 75.5% ($N_2$/air)) and sucrose (87.4% (air) against 71.4% ($N_2$/air)).

## CELL VIABILITY

The results of cell viability estimations of the cells are given in Table 3.3.11. The air-treated cultures showed a mean percentage cell viability of 77.7% by the end of the experimental period, compared with a mean percentage cell viability of the $N_2$/air-treated cultures of 46.6%. In order to determine whether this difference was statistically significant, a Student t-test was performed on the arcsin-transformed data, and it was found that there was a significantly higher percentage viability in the aerated cultures (between $p = 0.02 - 0.01$, $t = 4.529$, 4 degrees of freedom).

## ALKALOID CONTENT

The mean I values obtained for each flatbed culture are given in Table 3.3.11 and the results show there is a significant difference in the levels accumulated in cells subjected to the different treatments. The mean I values for cells derived from those parts of the cultures not showing any 'drip' effects (for the alkaloid levels of the drip areas are considered separately, Results, Part 3, Section 3), were 1.4 for the air-treated cultures (i.e., about 10-11 mg alkaloids/g dry weight cells) and 2.5 for the $N_2$/air-treated cultures (about 13-14 mg alkaloids/g dry weight cells), based on calibration with standard

solutions of α-solanine.

Three main conclusions can be drawn from these results.

1. The lower levels of oxygen in the nutrient medium were associated with an increased accumulation of alkaloids in the cells (as detected by the cell-squash technique).

2. The growth rate of the cells (described by the fresh weight data and the nutrient uptake data) was lower in the $N_2$/air-treated flatbeds.

3. The percentage cell viability of the $N_2$/air-treated cells was lower than in the air-treated cultures.

This experiment can be considered to be one in which a regime of nutrient stress was imposed. In the next, the effects of precursor feeding are described.

## THE EFFECTS OF ORNITHINE ON THE GROWTH AND METABOLISM OF CULTURED SOLANUM NIGRUM CELLS

The aim of this experiment was to determine whether ornithine, when added to the nutrient medium, would cause an increase in alkaloid accumulation by cells of S. nigrum when cultured on agar and on the flatbed. The effects of ornithine on cell viability and growth were also investigated.

Hyoscyamine has been shown to accumulate in callus of S. nigrum which was supplied with ornithine (Fernandez-Perez, unpublished data). If the synthesis of the alkaloid was limited by the endogenous availability of the precursor, then it might be expected that its exogenous application would cause an increase in the accumulation of the product, assuming that the rate of degradation did not exceed the rate of synthesis. Indeed, Yeoman et al.(1980) have reported that by supplementing the nutrient medium of flat-bed cultured cells of Capsicum frutescens with precursors of capsaicin the level of production of this compound was greatly raised over the level of production in the absence of precursors.

The experiment was performed in two sequential parts. In the first, ornithine was added to full MS medium in 1% agar to produce final concentrations of 1, 5, 10, 50 and 100 mM. Cells of S. nigrum (approximately 5 g wet weight, from 4 week-old, stationary phase, suspension cultures which had been maintained for 2 months) were weighed, plated out and cultured for 4 weeks. After this period, investigations were made of culture appearance, alkaloid content (by the cell-squash technique), percentage increase in wet weight and percentage cell viability. From the results of this part of the

experiment, the optimal ornithine concentration, which promoted the greatest alkaloid accumulation with least loss in cell viability, was determined.

In the second part of the experiment, ornithine at the optimal concentration was supplied to S. nigrum cells cultured on the flatbed. After a culture period of 7 days, investigations were made of culture appearance, percentage nutrient uptake, percentage increase in wet weight, alkaloid content, and percentage cell viability.

PART 1 : ON AGAR PLATES

Three replicate agar plates were produced for each ornithine concentration, making a total of 18 plates; these included 3 control plates which contained no ornithine.

Results : The Appearance of the Cultures

There appeared to be a variability in the extent of cell growth, within a particular treatment, presumably due to clonal differences within the original cell suspension cultures.

The Growth and Alkaloid Content of the Cultures

Some of the cultures contained areas of growing cells while others did not; the alkaloid content of each region was examined, and the results are presented, together with the data for percentage increase wet weight and percentage cell viability, in Tables 3.3.13 and 3.3.14.

Table 3.3.13

The effects of ornithine on alkaloid content, growth and viability of S. nigrum cells on agar plates. The means and standard errors of these results are presented in Table 3.3.14.

MEAN I VALUE

| mM Ornithine | Rep. | Growing Cells | Non-Growing Cells | % Increase Fresh Weight | % Cell Viability |
|---|---|---|---|---|---|
| | 1 | 0.2 | - | 130.6 | 68.3 |
| 0 | 2 | 0.1 | - | 94.3 | 76.5 |
| | 3 | 0.1 | - | 128.4 | 53.4 |
| | 1 | 3.3 | - | 35.1 | 63.8 |
| 1 | 2 | 0.3 | - | 89.3 | 53.1 |
| | 3 | 4.0 | - | 30.4 | 59.5 |
| | 1 | 1.9 | - | 32.7 | 64.3 |
| 5 | 2 | 2.5 | 3.2 | 31.5 | 64.3 |
| | 3 | - | 1.3 | 5.3 | 61.4 |
| | 1 | 2.1 | 1.5 | 15.3 | 60.5 |
| 10 | 2 | 2.2 | 1.9 | 11.7 | 67.5 |
| | 3 | 2.2 | 0.9 | 5.1 | 59.2 |
| | 1 | 2.8 | 2.6 | 30.3 | 71.6 |
| 50 | 2 | - | 1.2 | 4.7 | 62.3 |
| | 3 | - | 0.3 | 9.8 | 60.7 |
| | 1 | - | 0.3 | 0.0 | 13.3 |
| 100 | 2 | - | 0.6 | 0.0 | 11.0 |
| | 3 | - | 0.3 | 0.0 | 10.7 |

Table 3.3.14

The means per treatment of the data from Table 3.3.13

| mM Ornithine | Mean I Value: Growing Cells | Mean I Value: Non-Growing Cells | Mean % Increase Fresh Weight | Mean % Cell Viability |
|---|---|---|---|---|
| 0 | 0.1 | - | 117.8 ± 11.7 | 66.4 ± 0.5 |
| 1 | 2.6 | - | 53.3 ± 4.3 | 58.8 ± 0.1 |
| 5 | 2.2 | 2.3 | 21.2 ± 1.5 | 67.5 ± 0.2 |
| 10 | 2.2 | 1.4 | 10.3 ± 0.3 | 62.4 ± 0.1 |
| 50 | 2.8* | 1.4 | 13.3 ± 1.2 | 64.9 ± 0.1 |
| 100 | - | 0.4 | 0 | 11.6 ± 0.0 |

* 1 reading only

The results show that cells treated with ornithine at concentrations up to 50 mM accumulated significantly higher levels of alkaloids (mean I values ranging from 2.2 to 2.8, representing 12-15 mg alkaloids/g dry weight cells) than did untreated cells (of mean I values of approximately 0.1, representing less than 10 mg alkaloids/g dry weight cells), without severely reducing the percentage cell viability (64.9 $\pm$ 0.1% for cells supplied with 50 mM ornithine compared with 66.4 $\pm$ 0.5% for the cells without ornithine). The higher concentration of ornithine (100 mM) reduced the mean percentage viability to 11.6%, and there was no dramatic increase in the levels of alkaloids in these cells.

Ornithine had an inhibitory effect on the growth of the cells at all concentrations investigated. There was a lower percentage increase in wet weight of cells grown on higher concentrations of ornithine.

The results in Tables 3.3.13 and 3.3.14 show that, although growing cells may accumulate higher levels of alkaloids than non-growing cells, those cells which were growing most rapidly (see, for example, the 1 mM ornithine plate, replicate 2) tended to accumulate relatively low levels compared with cells growing more slowly.

PART 2 : ON THE FLATBED APPARATUS

It was decided, on the basis of the data obtained from the 'agar plate' experiment, that the nutrient medium used in the flatbed experiment should contain ornithine at a concentration of 5 mM, for the following reasons :

1. 5 mM ornithine promoted increased alkaloid accumulation in *S. nigrum* cells;

2. this concentration would not allow rapid cell growth (as 1 mM might have done), and should therefore increase the potential for greater alkaloid accumulation;

3. 5 mM ornithine did not cause a significant reduction in the percentage cell viability.

40-50 g wet weight of cells of *S. nigrum* (which had been maintained for 3 months) were weighed and set up on flatbeds as described in Methods, Chapter 2. 'Treated' cells were supplied with 5 mM ornithine which was added at day 3 of culture, while 'control' cultures were grown simply on full MS medium. Flatbed cultures were sampled at days 0 and 3 (i.e., before the addition of ornithine) and at days 5 and 7 (± ornithine). An investigation was made of the appearance of the cultures, and determinations were made of alkaloid content (by the cell-squash technique), percentage increase wet weight, dry weight and percentage cell viability. The percentage nutrient uptake was also measured, at daily intervals, throughout the experiment.

## Results : The Appearance of the Cultures

The most striking observation was that, by day 5 of culture, a variously dark brown-/rusty brown-coloured 'drip area' had formed in both ornithine-treated and control cultures, and the cells in the drip areas were compacted or clumped.

The Growth and Alkaloid Content of the Cultures

The results are presented in Tables 3.3.15 and 3.3.16. It can be seen that 5 mM ornithine had little effect in increasing the alkaloid content of the cells over the 7 day culture period, and analysis of the nutrient medium revealed that alkaloids were not released by the cells in detectable quantities. At day 5 there was no difference in the alkaloid content of ornithine-treated and untreated cells (with mean I values of 1.5 and 1.6 respectively); by day 7 there was a trend towards higher levels of alkaloids in the treated cells than in the controls (with mean I values of 2.1 and 1.6 respectively, representing a difference of about 1-2 mg alkaloids/g dry weight cells). It is evident that in day 5 cultures (i.e., two days after the addition of ornithine to the nutrient medium) there was no increase in alkaloid levels (in either the treated or the control cultures) over the levels in day 0 cultures, but the treated cultures exhibited an increase by day 7, having a mean I value of 2.1 (approximately 12 mg alkaloids/g dry weight cells) compared with a value of 1.3 (approximately 10 mg alkaloids/g dry weight cells)for the day 0 cultures and 1.6 (approximately 11 mg alkaloid/g dry weight cells) for the day 7 control cultures.

Statistical analysis of the percentage increase wet weight data in Table 3.3.16 shows that, at days 5 and 7, there is no significant effect of 5 mM ornithine on growth ($t = 0.769$ at $p = 0.1$, with 4 degrees of freedom, at day 7). Similarly, there was no significant difference in the percentage cell viability of treated and untreated cultures at day 5 ($t = 0.783$ at $p = 0.1$, 4 degrees of freedom) or at day 7 ($t = 0.332$ at $p = 0.1$, 4 degrees of freedom).

Table 3.3.15

The effects of 5 mM ornithine on alkaloid content, growth and viability of S. nigrum cells cultured on the flatbed apparatus

The result of statistical treatment of this data is given in Table 3.3.16.

| Day of Culture | | Repl. | Initial Wet Weight (g) | Final Wet Weight (g) | % Increase Wet Weight | Dry Weight (g) | % Cell Viability | Mean I Value |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 43.0 | 43.0 | 0 | 0.611 | 67.9 | 1.2 |
| 0 | | 2 | 45.0 | 45.0 | 0 | 0.702 | 73.2 | 1.3 |
| | | 3 | 46.0 | 46.0 | 0 | 0.881 | 77.4 | 1.4 |
| | | 1 | 46.0 | 47.0 | 2.2 | 0.849 | 75.2 | 1.2 |
| 3 | | 2 | 43.0 | 43.5 | 1.2 | 0.652 | 58.7 | 1.2 |
| | | 3 | 45.0 | 47.0 | 4.4 | 0.729 | 77.5 | 1.7 |
| | | 1 | 47.0 | 54.0 | 14.9 | 1.168 | 60.8 | 1.3 |
| | +ORN | 2 | 46.0 | 47.5 | 3.2 | 1.189 | 73.2 | 1.7 |
| 5 | | 3 | 36.5 | 44.5 | 21.9 | 1.128 | 65.8 | 1.6 |
| | | 1 | 47.0 | 47.5 | 1.1 | 0.956 | 65.8 | 1.7 |
| | -ORN | 2 | 52.5 | 52.5 | 0.0 | 1.247 | 78.2 | 1.3 |
| | | 3 | 54.0 | 54.0 | 0.0 | 1.091 | 68.1 | 1.9 |

continued ...

Table 3.3.15 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 7 | +ORN | 1 | 42.0 | 44.0 | 4.8 | 0.803 | 81.8 | 1.9 |
| | | 2 | 43.0 | 45.0 | 4.6 | 0.788 | 71.4 | 2.2 |
| | | 3 | 52.5 | 55.5 | 5.7 | 1.290 | 79.3 | 2.2 |
| | -ORN | 1 | 52.0 | 54.0 | 3.8 | 0.899 | 76.1 | 1.8 |
| | | 2 | 52.0 | 55.0 | 5.8 | 1.047 | 68.9 | 1.4 |
| | | 3 | 49.0 | 53.5 | 9.2 | 1.184 | 82.5 | 1.6 |

Table 3.3.16

The means and standard errors per treatment of the data from Table 3.3.15

| Day of Culture | Mean Initial Fresh Weight (g) | Mean Final Fresh Weight (g) | Mean % Increase Fresh Weight | Mean Dry Weight (g) | Mean % Cell Viability | Mean I Value |
|---|---|---|---|---|---|---|
| 0 | 44.7 ± 0.9 | 44.7 ± 0.9 | - | 0.731 ± 0.08 | 72.8 ± 0.3 | 1.3 |
| 3 | 45.5 ± 0.5 | 45.8 ± 1.2 | 2.5 ± 0.1 | 0.743 ± 0.06 | 70.8 ± 0.4 | 1.4 |
| 5 + ORN | 43.2 ± 3.3 | 48.7 ± 2.8 | 6.0 ± 0.4 | 1.162 ± 0.02 | 66.1 ± 0.2 | 1.5 |
| 5 - ORN | 51.2 ± 2.1 | 51.3 ± 2.0 | 0.1 ± 0.1 | 1.098 ± 0.06 | 70.9 ± 0.2 | 1.6 |
| 7 + ORN | 45.8 ± 3.3 | 48.2 ± 3.7 | 5.0 ± 0.0 | 0.960 ± 0.16 | 77.7 ± 0.1 | 2.1 |
| 7 - ORN | 51.0 ± 1.0 | 54.2 ± 0.4 | 6.1 ± 0.1 | 1.043 ± 0.08 | 68.9 ± 0.4 | 1.6 |

Nutrient Uptake

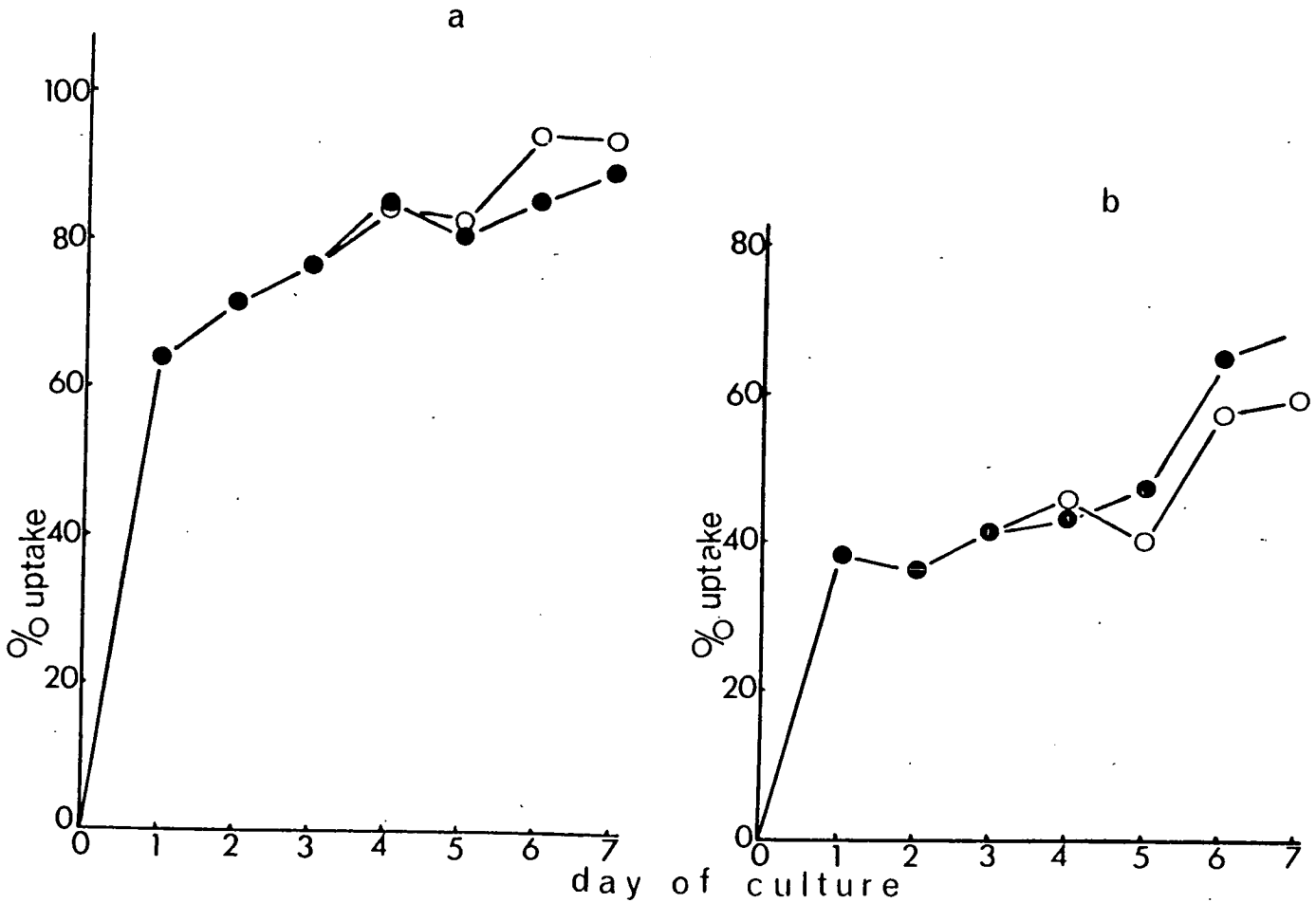
The results of the daily nutrient medium analysis are given in Table 3.3.17 and Figures 3.35. (a) - (d). The data show that both treated and untreated cultures removed nutrients from the medium at a fairly rapid rate, particularly orthophosphate, 90% of which was removed at day 7. In all cases there was a steady rate of uptake after an initially sharp removal of the nutrients, and the relatively high final percentage uptake figures for treated and untreated cells are characteristic of growing cultures. The only possibly significant indication of effects of ornithine on retarding nutrient uptake was reflected in the data for nitrate and sucrose. In these respective cases there was, at day 7, a 15% and 20% difference in uptake between treated and untreated cultures.

From this experiment, it may be concluded that 5 mM ornithine, supplied for a period of up to 4 days, had a small effect in raising the levels of alkaloids in S. nigrum cells cultured on the flatbed. Alkaloids were not detected in the medium at the end of the experimental period. The nutrient uptake and percentage increase wet weight data suggest that the growth of the flatbed-grown cells was slightly inhibited by the presence of ornithine (compared to control cultures) but the inhibitory effect was greater in agar-grown cells. The levels of alkaloids accumulated in cells on agar and on the flatbed were comparable.

Table 3.3.17

Nutrient uptake by flatbed-cultured cells of *S. nigrum* in the presence and absence of 5 mM ornithine

| Duration of Culture (hr) | % UPTAKE | | | |
|---|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 24 | 63.8 | 38.3 | 55.6 | 42.0 |
| 48 | 71.6 | 36.7 | 56.9 | 37.3 |
| 72 | 76.4 | 41.2 | 58.9 | 41.4 |
| 96 | 85.0 | 43.6 | 69.3 | 68.9 |
| 96 + ORN | 84.2 | 45.9 | 64.9 | 43.5 |
| 120 | 80.3 | 47.8 | 67.5 | 56.4 |
| 120 + ORN | 82.5 | 40.7 | 59.6 | 37.5 |
| 144 | 85.0 | 65.2 | 74.7 | 71.4 |
| 144 + ORN | 94.2 | 57.8 | 66.2 | 50.8 |
| 168 | 89.3 | 68.7 | 80.3 | 79.4 |
| 168 + ORN | 93.9 | 59.5 | 66.3 | 59.5 |




(a) Orthophosphate

(b) Ammonia

Figure 3.3.5 Nutrient Uptake by Cells of S. nigrum on the Flatbed in the Presence (Open Circles) and Absence (Solid Circles) of 5 mM Ornithine

(c) Nitrate

(d) Sucrose

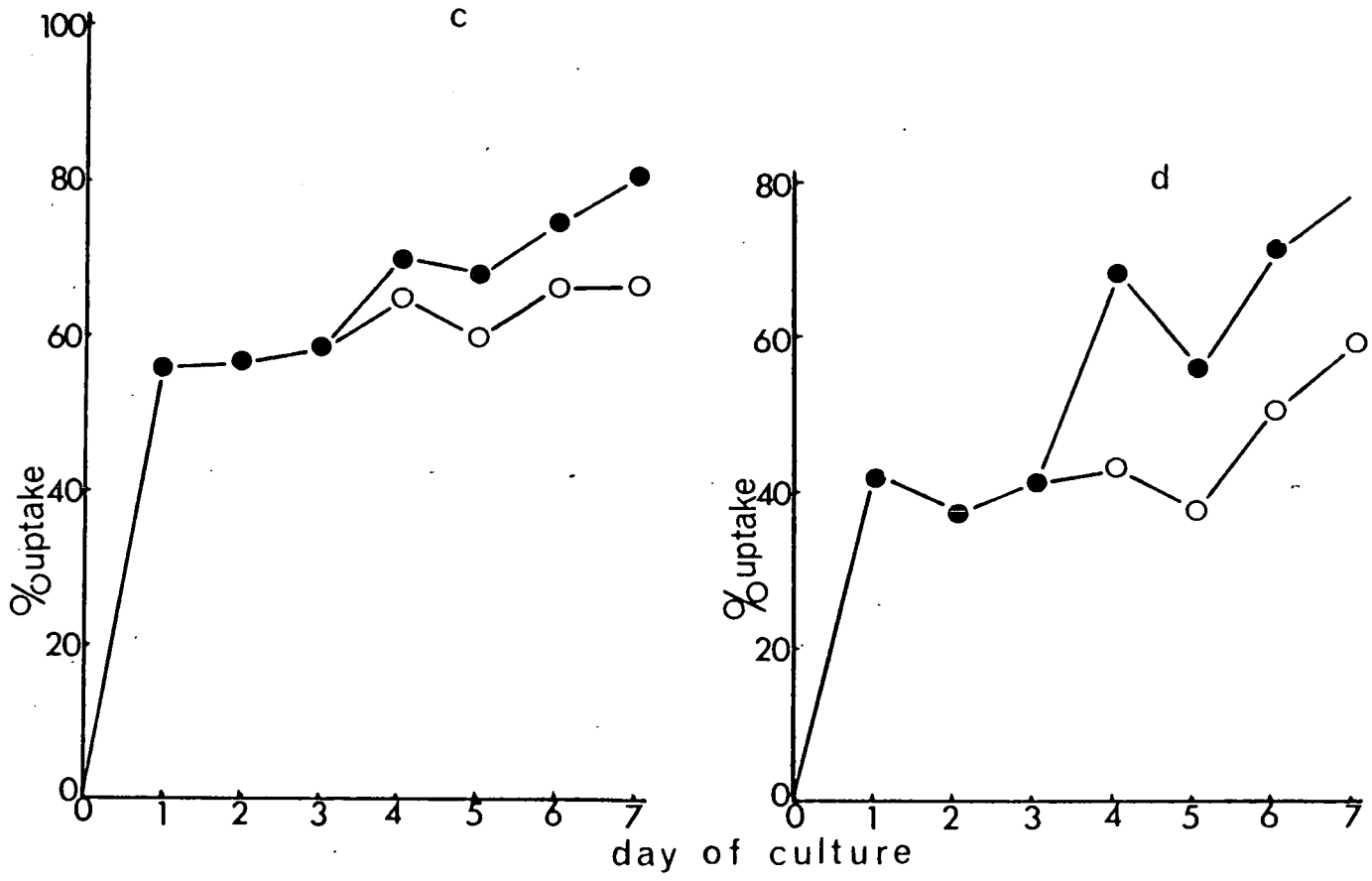



Figure 3.3.5 (continued)

From the results presented in this section, it is apparent that the flatbed apparatus was amenable to investigations involving the manipulation of the environment of cultured cells. Cells subjected to relatively low oxygen levels, or supplied with ornithine, accumulated increased levels of alkaloids. In the former case, at least, this effect was associated with reduced growth of the cultures.

When flatbed-grown cells were directly dripped upon by the nutrient medium, they underwent a number of striking changes. These are now described, and the results of experiments to investigate further the phenomenon are presented.

# SECTION 3

## AN INVESTIGATION OF THE 'DRIP-AREA' PHENOMENON

In this section is described and investigated the 'drip-area' phenomenon, which was briefly described in the flatbed experiments which examined the effects of high and low oxygen levels and the presence of ornithine.

## THE PRODUCTION OF NUTRIENT MEDIUM 'DRIP-AREAS'

In both the gas-treated and the ornithine-treated flatbed cultures, it was noticed that, after 3 or 4 days, a change occurred in the appearance of the region of the cultures on to which the nutrient medium dripped as it entered the culture vessel. In these so-called 'drip-areas', the tissue invariably turned dark- or rusty-brown and was thereby distinct from the rest of the culture, which was usually of a pale creamy colour. Moreover, the cells of the drip-areas were often more compacted or clumped than the cells of the bulk of the culture.

The apparent induction of differentiation in the cells of the drip-areas promoted an investigation into the levels of alkaloids produced by them.

## DRIP-AREAS IN THE AIR/$N_2$-AIR-TREATED FLATBEDS

In Table 3.3.18 are presented the results of a comparison of alkaloid production by cells of the drip-areas and the remainder of cells of the cultures (designated as cells of the 'bulk areas') for both air-treated and $N_2$/air-treated flatbeds. Alkaloid levels were determined by the cell-squash technique.

Table 3.3.18

Alkaloid levels in the 'drip' and 'bulk' areas of air- and $N_2$/air-treated flatbed cultures of Solanum nigrum

| | AIR TREATMENT : | | $N_2$/AIR TREATMENT : | |
|---|---|---|---|---|
| | Bulk Area | Drip Area | Bulk Area | Drip Area |
| Mean I Value | 1.4 | 2.6 | 2.5 | 2.8 |

Table 3.3.19

Alkaloid levels in the 'drip' and 'bulk' areas of 5 mM ornithine-treated (+ ORN) and untreated (- ORN) flatbed cultures of S. nigrum

| | Mean I Value | |
|---|---|---|
| Day of Culture | Bulk Area | Drip Area |
| 5 - ORN | 1.6 | 3.0 |
| 5 + ORN | 1.5 | 2.5 |
| 7 - ORN | 1.6 | 3.3* |
| 7 + ORN | 2.1 | 3.2 |

* in only 1 flatbed did medium drip directly onto cells

In the air-treated cultures, there was a significant difference in the levels of alkaloids accumulated by the cells of the drip-areas (mean I value = 2.6) and the bulk areas (mean I value = 1.4) respectively. The difference between these mean I values represents a difference in the quantity of alkaloids produced by the cells of the order of approximately 5 mg alkaloids/g dry weight of callus.

By the end of the experimental period, the drip-areas in the $N_2$/air-treated flatbeds had become indistinguishable from the bulk, due to a general darkening of the cultures. This visual homogeneity was reflected in the alkaloid levels of the cells of the former drip-areas and the bulk areas (with mean I values of 2.8 and 2.5 respectively); the levels in each part of these cultures were relatively high, and comparable with those of the drip-area cells of the air-treated flatbeds.

## DRIP-AREAS IN THE FLATBEDS OF THE ORNITHINE EXPERIMENT

The I values for the drip and bulk areas in the flatbed cultures which were untreated or treated with 5 mM ornithine are given in Table 3.3.19.

It can be seen that at day 5 of the experimental period (i.e., 2 days after the addition of ornithine) and at day 7, both the treated and untreated cultures accumulated higher levels of alkaloid in the drip-area cells than in the bulk area cells. The presence of ornithine in the medium appears to have had little effect on either increasing or decreasing the disparity between the two areas of cells, for at day 7, the mean I values of the drip-areas of treated and untreated cultures were 3.2 and 3.3 respectively.

## VIABILITY OF THE DRIP-AREA CELLS

The percentage cell viability of the drip-area cells in the ornithine feeding experiment was determined, both for ornithine-treated and non-treated cultures; the results are given in Table 3.3.20.

It can be seen that after 5 and 7 days of continuous dripping of the nutrient medium onto the cells, there was a slightly lower percentage viability of the drip-area cells compared to the cells of the bulk of the culture. The presence or absence of ornithine in the medium appeared not to influence the viability of drip-area cells. On average, the viability of the drip-area cells was approximately 10% lower than for cells of the rest of the culture, with values of about 60 and 70% respectively.

There is little doubt that the dripping of nutrient medium directly onto S. nigrum cells resulted in the increased accumulation of alkaloids (detectable by the cell-squash technique) by those cells. It is conceivable that this result is due to a flooding effect, causing the cells to suffer from, for example, low oxygen levels (suggested by the apparent 'spreading' of the phenomenon to the whole of the culture in $N_2$/air-treated flatbeds), high carbon dioxide levels, cell damage and ethylene production, direct pressure of dripping liquid, leaching effects, or locally high levels of nutrients.

Experiments to gain further information on the nature of this effect are now described.

Table 3.3.20

The mean percentage viability in the 'drip' and 'bulk' areas of 5 mM ornithine-treated (+ ORN) and untreated (- ORN) flatbed cultures of S. nigrum

| | Mean % Cell Viability | |
|---|---|---|
| Day of Culture | Bulk Area | Drip Area |
| 5 - ORN | 70.9 ± 0.2 | 62.2 ± 0.3 |
| 5 + ORN | 66.1 ± 0.2 | 58.0 ± 0.7 |
| 7 - ORN | 68.9 ± 0.4 | 61.8* |
| 7 + ORN | 77.7 ± 0.1 | 60.5 ± 1.1 |

* in only 1 flatbed did medium drip directly onto cells

## THE EFFECTS OF DRIPPING DISTILLED WATER AND 20% STRENGTH NUTRIENT MEDIUM ONTO FLATBED-CULTURED CELLS OF SOLANUM NIGRUM

In this experiment sterile distilled water and 20% strength MS nutrient medium were allowed to drip directly onto S. nigrum cells cultured on the flatbed. The rationale behind such an experiment was that, if the production of drip-areas was the result of some dripping effect associated with mechanical cell damage, or oxygen depletion due to 'drowning' of the cells, then such a phenomenon should occur, in principle, as a result of the dripping of dilute nutrient medium or water containing no nutrients.

Known weights (approximately 45-50 g wet weight) of cells of S. nigrum (which had been maintained for approximately 5 months) were set up on flatbeds as described in Methods, Chapter 2. Three culture vessels, the controls, were supplied with 50 ml of full MS liquid nutrient medium, and three 'test' flatbeds were supplied with 50 ml of distilled water only. An obvious flaw in this method is that the 'test' cells received no nutrient supply whatsoever, and a comparison of results with the control cultures must take this into account. Therefore, in a second experiment, cells were subjected to the effects of the dripping of much diluted nutrient medium, with the aim of establishing a regime which would permit some cell growth, but would eliminate any effect on drip-area production caused by the presence of nutrients in the medium. In this second experiment, therefore, three 'test' flatbeds (set up as in the first experiment) were supplied with one-fifth strength full MS medium. In each experiment, culture appearance (drip-area production) was investigated and determinations were made of percentage increase in wet weight,

percentage cell viability and alkaloid content (by the cell-squash method) after the 17-day and 10-day culture periods respectively. In the second experiment, the percentage nutrient uptake from the medium was determined at the end of the (in this case) 10-day culture period. Results are given in Tables 3.3.21, 3.3.22 and 3.3.23.

In both experiments, the nutrient medium control cultures characteristically developed dark brown drip areas containing clumped cells. However, in neither the distilled water-treated nor the dilute medium-treated cultures was there any visual differentiation. The percentage increase wet weight of the nutrient-free cultures was predictably lower than in the controls, but this was not the case in the cultures grown on 20% medium (Tables 3.3.20 and 3.3.21). The percentage cell viability was lower in the distilled water cultures (64.1 $\pm$ 0.1%) than in the controls (77.9 $\pm$ 0.1%); in the controls (in both experiments), the viability of the drip-area cells was of the order of 10-15% lower than in the cells of the bulk of the cultures. The viability of the dilute medium cells was slightly lower than the bulk area cells in the control cultures, but it was not as reduced as that of the distilled water-treated cells. The nutrient uptake data for the second experiment (Table 3.3.23) demonstrate that not all the nutrients examined were completely utilized in either the full medium or the 20% medium cultures, although in the latter case approximately 95% of the orthophosphate and 85% of the sucrose was removed from the medium.

The alkaloid content data are in agreement with the visual data, that is, no recognisable drip-areas were formed in any cultures other

Table 3.3.21

The effects of dripping distilled water on :

(a) % Increase Wet Weight of the Cultures

| Replicate Culture | % Increase Fresh Weight | |
|---|---|---|
| | Nutrient Medium | Dist. $H_2O$ |
| 1 | 33.5 | 35.6 |
| 2 | 38.3 | 25.7 |
| 3 | 39.1 | 29.3 |
| $\bar{x} \pm$ S.E. = | 37.6 ± 0.0 | 30.1 ± 0.1 |

(b) Cell Viability of the Cultures

| Replicate Culture | Mean % Cell Viability | | | |
|---|---|---|---|---|
| | Nutrient Medium | | Dist. $H_2O$ | |
| | Drip Area | Bulk Area | Drip Area | Bulk Area |
| 1 | 59.9 | 78.8 | 44.3 | 43.9 |
| 2 | 67.3 | 75.2 | 48.7 | 40.9 |
| 3 | 65.1 | 79.6 | 39.2 | 40.3 |
| $\bar{x} \pm$ S.E. = | 64.1 ± 0.1 | 77.9 ± 0.1 | 43.7 ± 0.1 | 41.7 ± 0.0 |

(c) Alkaloid Content of the Cultures

| Replicate Culture | Mean I Value | | | |
|---|---|---|---|---|
| | Nutrient Medium | | Dist. $H_2O$ | |
| | Drip Area | Bulk Area | Drip Area | Bulk Area |
| 1 | 3.2 | 1.6 | 0.6 | 0.5 |
| 2 | 3.4 | 1.9 | 0.6 | 0.6 |
| 3 | 3.5 | 1.4 | 0.6 | 0.5 |
| $\bar{x} \pm$ S.E. = | 3.4 | 1.6 | 0.6 | 0.5 |

These data were obtained at the end of a 17-day experimental period.

Table 3.3.22

The effects of dripping 20% nutrient medium on :

(a) % Increase Wet Weight of the Cultures

| Replicate Culture | % Increase Fresh Weight | |
|---|---|---|
| | Full Medium | 20% Medium |
| 1 | 21.3 | 20.1 |
| 2 | 19.4 | 22.3 |
| 3 | 19.2 | 18.3 |
| $\bar{x} \pm$ S.E. = | 19.9 ± 0.0 | 20.2 ± 0.0 |

(b) Cell Viability of the Cultures

| Replicate Culture | Mean % Cell Viability | | | |
|---|---|---|---|---|
| | Full Medium | | 20% Medium | |
| | Drip Area | Bulk Area | Drip Area | Bulk Area |
| 1 | 62.4 | 81.7 | 60.1 | 62.3 |
| 2 | 66.6 | 72.4 | 52.1 | 56.2 |
| 3 | 60.7 | 77.1 | 61.1 | 59.9 |
| $\bar{x} \pm$ S.E. = | 63.3 ± 0.1 | 77.2 ± 0.1 | 57.8 ± 0.1 | 59.5 ± 0.1 |

(c) Alkaloid Content of the Cultures

| Replicate Culture | Mean I Value | | | |
|---|---|---|---|---|
| | Full Medium | | 20% Medium | |
| | Drip Area | Bulk Area | Drip Area | Bulk Area |
| 1 | 3.9 | 2.2 | 1.8 | 1.4 |
| 2 | 3.9 | 1.4 | 2.2 | 2.1 |
| 3 | 3.3 | 2.2 | 1.8 | 1.8 |
| $\bar{x}$ = | 3.7 | 1.9 | 1.9 | 1.8 |

These data were obtained at the end of a 10-day experimental period.

Table 3.3.23

Percentage nutrient uptake in 20% medium and control cultures after a 10-day culture period

| Nutrient | Replicate Culture | % Nutrient Uptake | |
|---|---|---|---|
| | | Full Medium | 20% Medium * |
| Phosphate | 1 | 84.3 | 92.3 |
| | 2 | 80.1 | 94.4 |
| | 3 | 87.7 | 97.8 |
| Ammonia | 1 | 52.4 | 44.5 |
| | 2 | 59.1 | 56.7 |
| | 3 | 59.1 | 59.3 |
| Nitrate | 1 | 62.3 | 43.2 |
| | 2 | 60.2 | 33.3 |
| | 3 | 59.1 | 34.3 |
| Sucrose | 1 | 66.1 | 82.1 |
| | 2 | 68.2 | 89.4 |
| | 3 | 62.4 | 89.9 |

* % uptake from the initial 20%

than those which had been supplied with full nutrient medium. In the 20% medium experiment, the mean I values of the control bulk area and the reduced medium bulk and drip areas were all of the order of 1.9 (about 11-13 mg alkaloids/g dry weight cells), whereas the control drip-area cells registered a mean I value of 3.7 (nearer 20 mg alkaloids/g dry weight cells), significantly higher. A similar general pattern was observed in the distilled water experiment, but here the distilled water-treated cultures accumulated particularly low levels of alkaloids (mean I values of approximately 0.5) (about 10 mg alkaloids/g dry weight cells).

The results of this experiment show that there was a significant difference in the effects of dripping dilute nutrient medium or distilled water as opposed to full nutrient medium onto cells. Apart from the expected differences in effect on cell growth and viability, there was an increased accumulation of alkaloids and an induction of pigment production (giving the drip-areas a dark colouration) associated with the presence in the medium of a nutrient or nutrients (including growth-regulatory substances) at relatively high concentrations. The possibility that the production of drip-areas was the result of a physical effect of dripping liquid can be eliminated by the results of this experiment.

The stability of the alkaloid-accumulating capacity of the drip-area cells was investigated in the following experiment.

## THE STABILITY OF THE CAPACITY OF DRIP-AREA CELLS TO ACCUMULATE RELATIVELY HIGH LEVELS OF ALKALOIDS (IN LIQUID AND ON SOLID NUTRIENT MEDIA)

The aim of this experiment was to investigate whether the accumulation of relatively high levels of alkaloids by drip-area cells occurred when the cells were isolated from flatbed cultures and grown in liquid suspension culture or on agar.

45-50 g of S. nigrum cells, which had been maintained for 4 months, were set up on each of three flatbeds as described previously. Full MS liquid medium was allowed to drip onto part of the cultures to ensure the production of drip-areas. At day 10, approximately 15 g wet weight of drip-area cells were isolated from each of the three flatbeds, and the alkaloid content for each isolate was determined by the cell-squash technique. Cells at this stage were designated 'time 0' cells. From each isolate, 2 g wet weight of cells was cultured (a) in each of three 250 ml Erlenmeyer flasks containing 50 ml of liquid nutrient medium and (b) on each of three nutrient agar plates. The suspension cultures were grown up for 15 days and the agar plate cultures for 21 days; this was to allow determinations of the alkaloid contents of relatively fast-growing cells, and so provide information on the stability of the alkaloid-accumulating capacity of the drip-area cells under such conditions. At the end of the culture period, the three suspensions were returned (after weighing and sampling) to flatbed cultures, to determine whether drip areas could again be produced.

The growth data of the cells in suspension and agar plate culture are given in Table 3.3.24, and the alkaloid content data for the original flatbed cultures and the suspension and agar plate cultures are in Table 3.3.25.

The cultured drip-area cells grew fairly rapidly in both liquid and agar plate culture, and when sampled at the end of the respective growth periods exhibited a significant drop in the levels of detectable alkaloids, from an initial mean of 3.6 (about 15-17 mg/g dry weight) (i.e., on the flatbed) to a mean of 1.2 (about 10 mg/g dry weight cells) in suspension culture and 2.1 (about 11-13 mg/g dry weight cells) on agar plates.

The suspension cultures, on return to flatbed culture and subjection to dripping for 10 days, developed drip-areas with relatively high alkaloid levels (Table 3.3.26); moreover, culture darkening and clumping, which had disappeared in suspension culture (in which the cells became friable and pale in colour) returned to the 'second generation' drip-area cells.

The results in this section demonstrate that the levels of alkaloids produced by flatbed-cultured cells can be manipulated by effecting changes in the cells' environment : low oxygen levels, the presence of ornithine in the nutrient medium, and the dripping of nutrient medium directly onto the cells all served to increase the levels of alkaloids accumulated. At least some of these effects were associated with cell growth rate changes.

Table 3.3.24

Growth of drip-area cells of S. nigrum in liquid and solid culture

| | % Increase Wet Weight | |
|---|---|---|
| Flatbed Isolate | Suspension Culture* | Agar Plate Culture† |
| 1 | 946 | 205 |
| 2 | 832 | 202 |
| 3 | 881 | 183 |
| $\bar{x} \pm$ S.E. = | 886 ± 33 | 197 ± 7 |

* at the end of a 15-day period

† at the end of a 21-day period

Table 3.3.25

Alkaloid contents of drip-area cells of S. nigrum before and after liquid and solid culture

| | Mean I Values | | |
|---|---|---|---|
| | Time 0 | After Culture | |
| Flatbed Isolate | Drip-Area | Suspension* | Agar Plate† |
| 1 | 3.7 | 1.0 | 1.9 |
| 2 | 3.6 | 1.4 | 2.1 |
| 3 | 3.6 | 1.2 | 2.2 |
| $\bar{x}$ = | 3.6 | 1.2 | 2.1 |

* at the end of a 15-day period

† at the end of a 21-day period

Table 3.3.26

The alkaloid contents of 'second generation' flatbed cultures

| | Mean I Values | |
|---|---|---|
| Replicate Culture | Drip-Area | Bulk Area |
| 1 | 3.5 | 1.8 |
| 2 | 3.7 | 1.8 |
| 3 | 3.8 | 2.2 |

## A BRIEF SUMMARY OF 'RESULTS, PART 3'

There are three or four important points to come out of the results presented in 'Part 3'. It would appear that the principle of cell culture employed in the flatbed system, namely the movement of a solution of nutrients across a callus-like mass of physically stationary cells, does allow cell growth and alkaloid production to proceed. It was established that the cells were able to take up nutrients (including oxygen) from the medium, and in fact may have suffered from phosphate limitation. The relatively slow growth of the cells (compared to their growth rate in suspension culture, from which they were derived) was probably due in part at least to the phosphate limitation, and together with the callus-like habit of the cells, with more cell-cell contact than in cell suspension culture, this may have contributed to the generally higher levels of alkaloids produced in the flatbed cells than in suspension cultures. The addition of 5 mM ornithine to the nutrient medium resulted in an increased accumulation of alkaloids. It is also apparent that what would appear to be a simple change in the cells' environment, such as the dripping of nutrient medium onto the cells, can cause dramatic changes in the levels of alkaloids produced. It is significant that alkaloids were accumulated intracellularly and not released into the nutrient medium.

The next part of the Results chapter describes the development of a column culture technique, more suitable than the flatbed for industrial 'scaling-up'. The manipulation of the environment of cells grown while immobilised is described.

# RESULTS PART 4

## THE IMMOBILISATION OF CELLS

## THE DEVELOPMENT OF A CELL CULTURE TECHNIQUE INVOLVING THE IMMOBILISATION OF CELLS IN VERTICAL COLUMNS

It has been shown that cells cultured in the flatbed system can accumulate alkaloids to higher levels than cells in suspension culture. However, despite this fact and the obvious advantages of a culture system in which the cell population can be manipulated successfully, the flatbed has one considerable disadvantage when scaling-up for industrial use is contemplated. This is that cell culture apparatus in a horizontal mode is awkward to design and operate and would require a considerable floor area. In order to resolve this problem it was therefore decided to design and develop a column system of culture, in which cells would be attached to a biologically inert substratum inside a vertical column, and which would preserve the basic advantages of the flatbed system. In addition to the important advantage of 'scaling-up' to an industrial level of operation, column culture also offers certain advantages to the laboratory worker, such as better control of nutrient flow rate over the cells, more even illumination of the cells, and saved space. It also ensures that a greater proportion of cells (than in a flatbed culture vessel) would be dripped on by the medium, thus creating an enlarged 'drip-area' of cells, in which it has already been shown that alkaloid accumulation was enhanced.

Part 4 of the results describes the development and characterisation of such a culture system. The development of the system was in effect a simple step from the flatbed; the culture vessel was transformed from an essentially horizontal format to a vertical one. As in the flatbed, liquid nutrient medium was allowed to run over the cells,

under the influence of gravity, from a reservoir. On reaching the bottom of the column containing the immobilised cells, the medium was collected, pumped back into the reservoir, and recycled.

The major problem to be overcome was how the cells should be physically supported in the column. Clearly, some support was essential, because the effect of dripping liquid nutrient medium would be to pack the cells into a dense and largely anaerobic agglomeration of tissue at the bottom of the column. It was therefore decided that a structural substratum should be employed, to which the cells could be attached. This would ensure that the cells were supported firmly along the length of the column.

As may be seen in Table 3.4.1, a variety of techniques have been used to immobilise cells, mainly in relation to animal and microbial cell culture (reviewed by Durand and Navarro, 1978; Jack and Zajic, 1977). The basic requirements for such a technique are stability and lack of cytotoxicity of the substratum, and the method of immobilisation must be such that the cells can easily take up and release nutrients and metabolites.

Because of the inability of plant cells to adhere to glass to any extent, the use of glass beads as a substratum was considered inappropriate. The coating of the beads with a 'sticky' substance, to which the cells would attach, such as agar or using, for example, plastic beads coated with lectins, was thought to be too complicated - what was required was a quick, simple and effective immobilisation procedure which would permit easy 'scaling-up'.

Table 3.4.1

Examples of the immobilisation of whole cells

(a) Immobilisation in an inert substratum

| Oganism | Immobilisation Substratum | Reference |
|---|---|---|
| MORINDA CITRIFOLIA<br>CATHARANTHUS ROSEUS<br>DIGITALIS LANATUS | alginate beads | Brodelius et al., 1979 |
| CATHARANTHUS ROSEUS | alginate, agarose, agar, carrageenan, alginate and gelatin, agarose and gelatin, gelatin, polyacrylamide | Brodelius & Nilsson, 1980 |
| SACCHAROMYCES UVARUM | alginate | Cheetham et al., 1979 |
| BACILLUS STEAROTHERMOPHILUS SPORES | alginate | Dallyn et al., 1977 |
| CANDIDA TROPICALIS | alginate, polyacrylamide, polystyrene | Hackel et al., 1975 |
| SACCHAROMYCES CEREVISIAE<br>KLUYVEROMYCES MARXIANUS<br>CHLOROPLASTS, MITOCHONDRIA | alginate | Kierstan & Bucke, 1977 |
| HeLa, K562 (ERYTHROLEUKAEMIC)<br>HUMAN SKIN FIBROBLASTS<br>HUMAN KIDNEY CARCINOMA<br>RAT COLON CARCINOMA | alginate, agarose, chitosan, | Nilsson & Mosbach, 1980 |
| ARTHROBACTER SIMPLEX | alginate | Ohlson et al., 1979 |
| ESCHERICHIA COLI | polyacrylamide | Chibata et al., 1974 |

Table 3.4.1 (continued)

| | | |
|---|---|---|
| CORYNEBACTERIUM SIMPLEX | polyacrylamide | Larsson et al., 1976 |
| CURVULARIA LUNATA | polyacrylamide | Mosbach & Larsson, 1970 |
| UMBILICARIA PUSTULATA | polyacrylamide | Mosbach & Mosbach, 1966 |
| LACTOBACILLUS BULGARICUS | polyacrylamide | Ohmiya et al., 1977 |
| ESCHERICHIA COLI | | |
| SACCHAROMYCES LACTIS | | |
| CORYNEBACTERIUM SIMPLEX | collagen | Constantinides, 1980 |
| STREPTOMYCES VENEZUELAE | collagen | Saini & Vieth, 1975 |
| ASPERGILLUS & PENICILLIUM SPORES | ecteola-cellulose | Johnson & Ciegler, 1969 |
| HUMAN DIPLOID CELLS | deae-gels | Van Wezel, 1976 |
| ESCHERICHIA COLI | metal hydroxide precipitates | Kennedy et al., 1976 |
| SACCHAROMYCES CEREVISIAE | | |
| SERRATIA MARCESCENS | | |
| ACETOBACTER sp. | | |
| SACCHAROMYCES PASTORIANUS | agar | Toda & Schoda, 1975 |
| MICROCOCCUS DENITRIFICANS | liquid-surfactant membranes | Mohan & Li, 1975 |

Table 3.4.1 (continued)

| | | |
|---|---|---|
| ASPERGILLUS FOETIDUS | steel wool nets | Atkinson et al., 1979 |
| BACILLUS SUBTILIS | | |
| SACCHAROMYCES CEREVISIAE | | |
| (b) Adsorption to an inert substratum : | | |
| FOETAL CALF BUCCAL CELLS | dextran microbeads | Beaudry et al., 1979 |
| CHICK EMBRYO FIBROBLASTS | | |
| $HE_p$-2 and L CELLS | | |
| CHICK EMBRYO FIBROBLASTS | dextran microbeads | Levine et al., 1977 |
| HUMAN FIBROBLASTS | | |
| BHK CELLS | glass beads | Spier & Whiteside, 1976(a) |
| BHK CELLS | deae-sephadex microbeads | Spier & Whiteside, 1976(b) |
| EMBRYONIC RABBIT SKIN CELLS | deae-sephadex microbeads | Van Wezel, 1967 |
| HUMAN EMBRYONIC LUNG CELLS | | |
| FILAMENTOUS FUNGI | rotating metal disc | Blain et al., 1979 |
| (c) Adsorption to inert substratum via biological macromolecules : | | |
| VARIOUS HUMAN CELL LINES | protein-coated agarose | Carlsson et al., 1979 |
| SHEEP ERYTHROCYTES | | |
| (d) Covalent bonding to otherwise inert substratum : | | |
| MICROCOCCUS LUTEUS | carboxymethyl cellulose | Jack & Zajic, 1976 |

The use of metal netting and gel matrices both were considered to have advantages. An immobilisation procedure involving the attachment of cells to an open net would allow the formation of air spaces and provide a large surface area for cell entrapment, while the use of gels would allow a stronger, more stable immobilisation. Therefore, an immobilisation technique was developed which incorporated elements of both these methods.

There are three sections of results. In the first is described the development of the immobilisation procedure and the final construction of the column apparatus. In the second section are described a number of basic studies, similar to those performed on the flatbed apparatus, designed to investigate the growth, viability and ability to accumulate alkaloids of immobilised cells. In the final section are described metabolic studies on entrapped cells, which not only provide additional physiological data but also indicate the usefulness and potential of the column culture system, in both the laboratory and in industry.

# SECTION 1

## THE IMMOBILISATION PROCEDURE

The aim of the immobilisation procedure was to entrap cells in 'baskets' of nylon netting with the aid of a gel. These 'baskets' acted as the basic unit of the column, which was packed with them. Air spaces were formed between the baskets, the netting was strengthened by the gel, and the cells were in contact with each other and capable of growth through the baskets.

It was decided that nylon panscrubbers (Spong, Basildon, Essex) or pieces of them, were a suitable form of netting in which the cells could be embedded. This material was easily cut up and was able to withstand autoclaving. Two gels were investigated for their suitability as cell entrapment substrata, namely agar and calcium alginate. The immobilisation procedures which were carried out are now described.

(a) CELL IMMOBILISATION USING AGAR

The first substratum used to immobilise cells was agar. It was an obvious first choice, having been used in tissue culture for many years as a 'seat' for cells; it is also freely permeable to nutrients. It therefore fulfilled three basic requirements : mechanical strength, lack of toxicity and permeability. It has been used independently for plant cell immobilisation by Brodelius and Nilsson (1980) and for yeast cell immobilisation by Toda and Shoda (1975).

A 2% solution (w/v) of agar (Oxoid No. 3) was prepared in distilled water, autoclaved at 121$^{o}$C (15 lb in$^{-2}$ steam pressure) for 20 minutes, and allowed to cool to 35-40$^{o}$C in a water bath. Cells from 4 week-old, stationary phase, suspension cultures were sieved (using a sieve of pore size 1 mm) and mixed 1:1 (v/v) with the molten

agar at 35-40°C. Sterile (autoclaved at 121°C for 20 minutes) pieces of panscrubber netting, of approximately cylindrical shape and of dimensions approximately 2-3 cm x 1 cm x 1 cm, were dipped into the cell-agar mixture using sterile forceps, and the resultant panscrubber/cell-agar units were inserted into glass columns (of dimensions 15 cm x 2.5 cm internal diameter) as the agar was beginning to solidify. The result was a column containing a number (usually about 10)of nylon 'baskets' each containing a mass of agar-embedded cells. Each column contained approximately 5 g wet weight of cells.

(b) CELL IMMOBILISATION USING CALCIUM ALGINATE

The second immobilisation substrate used was calcium alginate gel, which had been previously used for the immobilisation of yeast cells, bacterial spores, subcellular organelles and enzymes (see Table 3.4.1). It has also been independently used for the immobilisation of plant cells by Brodelius et al. (1979).

The method of cell immobilisation described here is based on the method of Kierstan and Bucke (1977), used by them for the entrapment of yeast cells, enzymes and subcellular organelles.

A 2% solution (w/v) of sodium alginate (Sigma London Chemical Company, practical grade type IV) was prepared in either distilled water or full MS liquid medium, autoclaved at 121°C (15 lb $in^{-1}$ steam pressure) for 20 minutes, and allowed to cool to room temperature. This solution was then mixed 1:1 (v/v) with cells sieved (as for the agar immobilisation procedure) from a 4 week-old

suspension culture, and sterile (autoclaved) panscrubber netting was dipped into the mixture, using sterile forceps. The panscrubber pieces containing the still liquid mixture of sodium alginate and cells were then quickly transferred into a sterile solution of 0.05 M calcium chloride (BDH) in distilled water, and left there for 10 minutes to allow the calcium alginate gel to solidify within the nylon netting, so entrapping the cells. The calcium alginate is made stable by the cross-linking of alginate molecules by the divalent calcium cations (see Cheetham et al., 1979). When solid, the panscrubber/cell-alginate units were washed three times in sterile distilled water and inserted into glass columns.

Each column contained approximately 5 g wet weight of cells.

## THE COLUMN APPARATUS

The column apparatus (Figure 3.4.1), as used in experiments, was a simple modification of the flatbed apparatus, the culture vessel of the latter system being replaced by a glass column containing immobilised cells.

The nutrient medium (50 ml) was contained in a reservoir (identical to that described for the flatbed system) and dripped, under the influence of gravity, into the vertical column of entrapped cells (the column being a glass tube of identical type to that used as a medium reservoir). Medium was removed from the bottom of the column, pumped back to the reservoir and recycled. As for the flatbed, subaseals (Gallenkamp, No. 49) were used to seal the glass tubes, and these were pierced by stainless steel needles (5 cm x 1 mm internal diameter) at the points of nutrient addition/

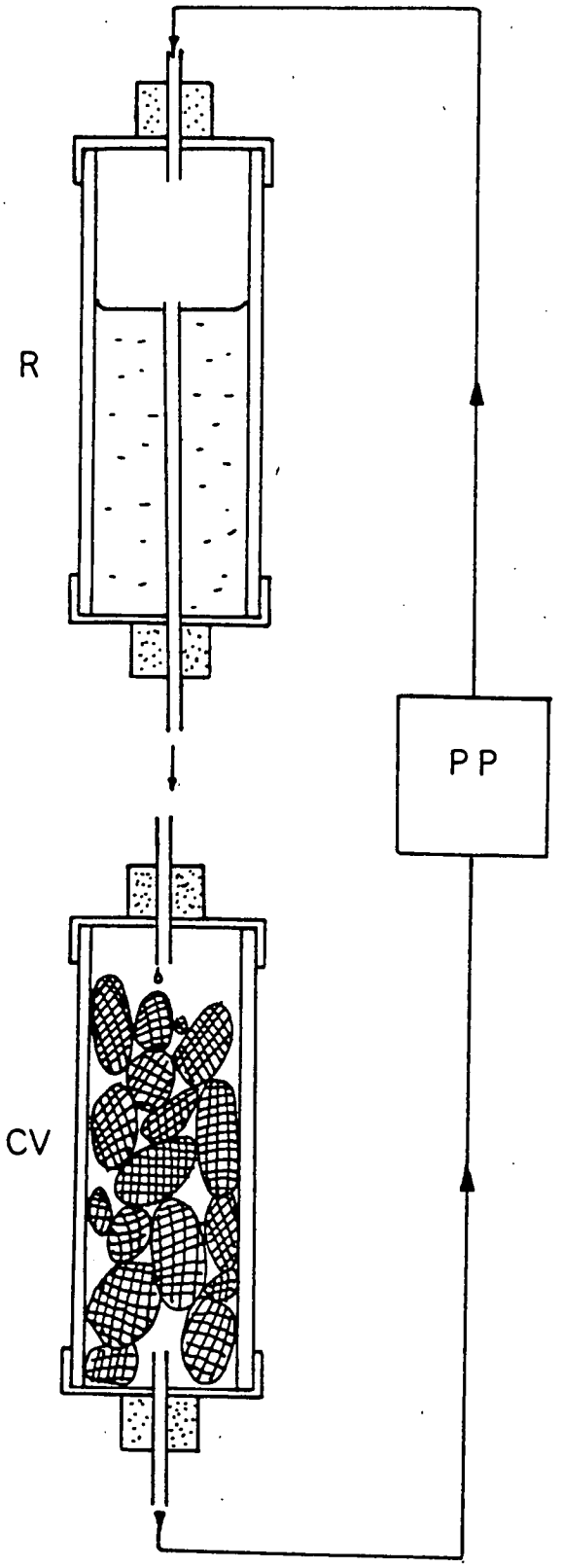



Figure 3.4.1 The Column Apparatus for the Culture of Immobilised Cells

R = nutrient medium reservoir

CV = cell culture vessel

PP = peristaltic pump

removal, and were connected together by silicone rubber tubing (1-1.3 mm internal diameter); foam bungs were slipped on over the needles at the openings in the seals to reduce the chance of contamination. Medium was circulated at a rate of 10 ml/minute.

Before use, the glass tubes, subaseals, needles and tubing were autoclaved in the usual way. Sterile nutrient medium and (obviously) cells were added after the autoclaving of the apparatus.

During an experimental period, nutrient medium was removed from the top of the reservoir, as in the flatbed experiments.

The results of Section 1 have therefore shown that two substrates, i.e., agar and calcium alginate gels, were chosen for the immobilisation of plant cells, and a method of producing 'baskets' of cells entrapped and supported in nylon mesh/gel units was described. These units were inserted into glass columns.

The next section of results describes experiments performed to characterise aspects of the column culture system.

# SECTION 2

## BASIC EXPERIMENTS TO CHARACTERISE THE COLUMN CULTURE SYSTEM

In this section are described basic investigations into the growth, viability and ability to accumulate alkaloids of cells of S. nigrum immobilised in columns of both agar and calcium alginate. The first experiment is concerned with determining whether the pan-scrubbers (with which the cells were in very close contact) had cytotoxic properties, the result of which would affect the design of the column apparatus. Nutrient uptake by cells entrapped in both agar and alginate columns was followed as an indication of the capacity for cell growth, and oxygen uptake was also examined, to discover whether there was an oxygen limitation on cell growth. The effects of illuminating the cells compared with growing them in the dark was investigated, a factor which would be an important consideration when designing a large-scale (industrial) column culture system.

## TO DETERMINE WHETHER THE NYLON PANSCRUBBERS HAVE CYTOTOXIC PROPERTIES

The cells cultured on the columns were obviously in close proximity to the nylon meshwork of the panscrubber baskets, and it was important to determine whether this supportive material was cytotoxic, adversely affecting the viability of the cells.

The method of investigating such possible effects was the same as that used for the fabric substratum of the flatbed apparatus; that is, by determining whether the material inhibited alkaloid accumulation and fresh weight production by S. nigrum suspension cultures.

Panscrubber netting was thoroughly rinsed in distilled water (as it was before the cell immobilisation procedure), dried, autoclaved and added to newly subcultured suspension cultures; the control flasks, which contained no panscrubbers, were those also used as controls for the investigation of the toxicity of the fabric substratum (both investigations had been carried out simultaneously). After 3 weeks growth the fresh weight and alkaloid content (by the cell-squash technique) of the flask cultures were determined, and the results are given in Table 3.4.2.

There was no significant retardation of the growth rate of the suspended cells by the panscrubbers, and alkaloid accumulation was similarly unaffected; it was therefore concluded that they had no detrimental effects and could be used safely for the production of cell cultures.

Table 3.4.2

The effect of panscrubber netting on the growth of and alkaloid production by S. nigrum suspension cultures

| | Fresh Weight (g) | | | Mean I Value | | |
|---|---|---|---|---|---|---|
| | | Week 3 | | | Week 3 | |
| Replicate | Time 0 | Control | Panscrubbers | Time 0 | Control | Panscrubbers |
| 1 | 0.84 | 12.72 | 12.31 | 2.1 | 2.9 | 2.7 |
| 2 | 0.62 | 12.32 | 12.94 | 2.3 | 2.7 | 2.8 |
| 3 | 0.79 | 12.90 | 12.90 | 2.3 | 2.8 | 2.8 |
| $\bar{x} \pm$ S.E. = | 0.75 ± 0.07 | 12.65 ± 0.17 | 12.72 ± 0.20 | 2.2 | 2.8 | 2.8 |

## NUTRIENT UPTAKE ON THE AGAR COLUMN

The cells immobilised in agar (or any other supportive substrate) are in a physical state unlike that of any other method of culture, that is, buried inside a solid matrix. In order to find out whether the cells were capable of growth, or even of staying alive under such conditions, determinations were made of percentage nutrient uptake by the cells, their percentage viability and alkaloid content.

## NUTRIENT UPTAKE BY AGAR COLUMNS IN THE ABSENCE OF CELLS

A preliminary experiment was performed to discover whether nutrients were removed from the medium by the agar column itself. Three columns were set up as described previously, but without any cells. Samples of the nutrient medium were taken from each column at intervals, mixed, and analysed for orthophosphate, ammonia, nitrate, and sucrose, and the results are given in Table 3.4.3.

It can be seen that there was a rapid removal of phosphate, ammonia and nitrate from the medium, reaching a maximum level (removed) by the second day of culture (when the percentage uptake was between about 20% and 30%). After this period, some of each of these three nutrients was released back into the medium, having been in some way ad- or absorbed by the agar. The data for the sucrose (measured as reducing sugar by the anthrone method) shows an increase in reducing sugar in the medium at the beginning of the culture period, suggesting that some of the agar was washed from the column. There seems to have been some uptake of sucrose after this time (approximately 17%). Thus, the agar columns removed nutrients from the medium to some extent, but not irreversibly so.

Table 3.4.3

Nutrient uptake by agar columns in the absence of cells

| Duration of Culture (hr) | % Nutrient Uptake | | | |
|---|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 0 | 0 | 0 | 0 | 0 |
| 3 | 17.7 | 22.6 | 14.8 | -8.2 |
| 19 | 20.6 | 17.5 | 15.2 | 1.6 |
| 27 | 21.3 | 21.7 | 27.8 | 5.6 |
| 43 | 22.7 | 24.6 | 34.9 | 8.9 |
| 99 | 22.0 | 19.1 | 17.1 | 4.9 |
| 148 | 21.3 | 20.4 | 23.6 | 4.2 |

## NUTRIENT UPTAKE BY, AND THE VIABILITY AND ALKALOID CONTENT OF, SOLANUM NIGRUM CELLS IMMOBILISED IN 2% AGAR

The aim of this investigation was to determine the growth (by following nutrient uptake), viability and ability to accumulate alkaloids of S. nigrum cells immobilised in a 2% agar gel.

Cells of S. nigrum (which had been maintained for approximately 3 months) were immobilised in 2% agar in the usual manner, and inserted into four columns. Nutrient medium (full MS) was supplied to the cells for a period of 334 hours, and was sampled at time intervals. The medium sampled from each column was mixed before analysis for the nutrients orthophosphate, ammonia, nitrate and sucrose. At the end of the experimental period the alkaloid content and the viability of the cells were determined, as was the pH and alkaloid content of the medium.

The nutrient uptake results are given in Table 3.4.4 and Figures 3.4.2 (a) - (d). There was, after the first day, a gradual uptake of both phosphate and ammonia (in the latter case until about 250 hours) up to values of 25.5% and 28.8% uptake respectively. The uptake of nitrate fluctuated much more than did that of the previous two nutrients, but there was an overall removal of the nutrient from the medium. The pattern of sucrose uptake was initially masked by an increase in the amount of reducing sugar in the medium (due, presumably, to the washing off of agar and cells from the column) but there was a distinct uptake of sucrose after the first day of culture.

Table 3.4.4

Percentage nutrient uptake by S. nigrum cells immobilised in agar

| Duration of Culture (hr) | % Nutrient Uptake | | | |
|---|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 0 | 0 | 0 | 0 | 0 |
| 17 | 19.3 | 12.6 | 23.8 | -33.5 |
| 22 | 20.7 | 18.0 | 18.8 | -21.7 |
| 39 | 20.7 | 15.5 | 25.7 | - 7.6 |
| 46 | 22.8 | 17.2 | 24.2 | 6.3 |
| 117 | 21.4 | 21.4 | 15.1 | - 9.7 |
| 166 | 22.8 | 26.6 | 27.7 | 1.1 |
| 253 | 24.1 | 28.8 | 24.8 | 12.5 |
| 360 | 25.5 | 23.9 | 25.9 | 12.5 |

(a) Orthophosphate

(b) Ammonia

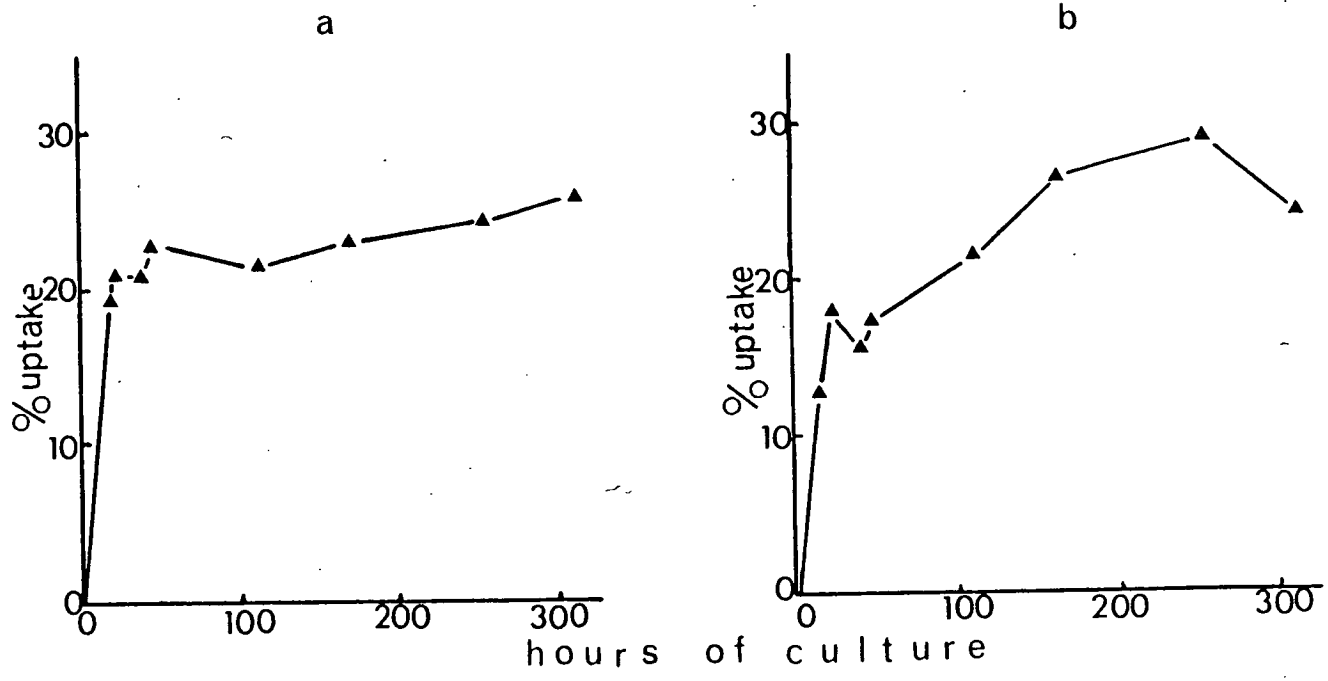



Figure 3.4.2 Nutrient Uptake by Agar-Immobilised Cells of S. nigrum

(c) Nitrate

(d) Sucrose

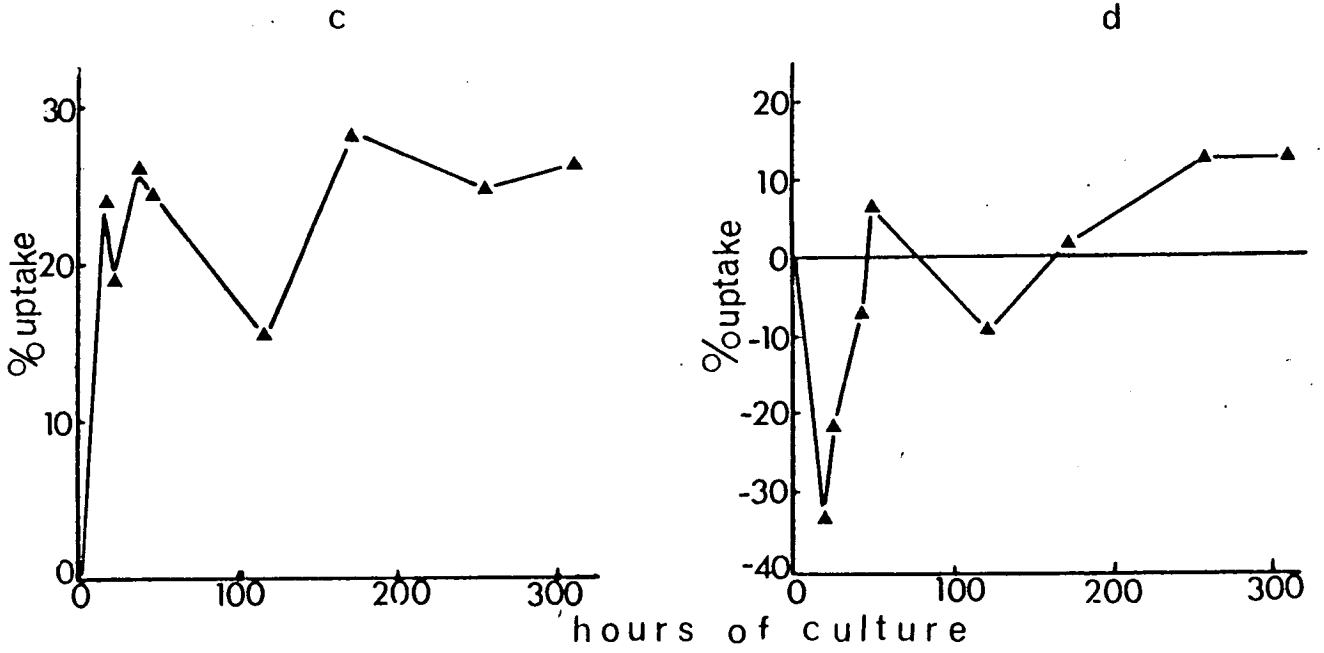



Figure 3.4.2 (continued)

The percentage viability and alkaloid content of the cells are given in Table 3.4.5. It is evident that the percentage viability was not severely reduced by immobilisation and culturing for the experimental period of 334 hours, with a mean for the four columns of 62%, a figure comparable to drip-area cells of the flatbed. The level of alkaloids accumulated by the column cells was, however, not as spectacularly high as it was for drip-area cells, the mean I value for the four columns being 2.2 (representing about 11-13 mg alkaloids/g dry weight cells), a result similar to that found for cells from the main (bulk) part of a flatbed cell culture.

Analysis of the nutrient medium, combined from the 4 columns and reduced in volume to approximately 1 ml and tested with Dragendorff's reagent, revealed that the cells did not release alkaloids to a detectable level. The pH of the medium after 334 hours was 5.1, i.e., it had become slightly acidified by 0.7 units.

It can be concluded from this initial column experiment that the immobilisation of cells of S. nigrum in warm agar seemed to have no seriously deleterious effect on their viability or ability to accumulate alkaloids (i.e., when compared to a 'typical' suspension culture, with a viability of about 70-80% and a mean I value of approximately 2, i.e., about 11-13 mg alkaloids/g dry weight cells). However, the observed rates of nutrient uptake do suggest that the cells are fairly slow-growing; any uptake over the first few hours of culture due to the cells would have been masked by the adsorptive properties of the agar itself, but there was, nevertheless, a definite and steady removal of nutrients by the cells, although by 300 hours the quantities removed were only

Table 3.4.5

The cell viability and alkaloid content of cells of *S. nigrum* immobilised in agar columns

| Replicate Column | % Cell Viability | Mean I Value |
|---|---|---|
| 1 | 62.5 | 2.0 |
| 2 | 56.2 | 2.3 |
| 3 | 59.4 | 2.3 |
| 4 | 69.8 | 2.1 |
| $\bar{x} \pm$ S.E. = | 62.0 $\pm$ 0.1 | 2.2 |

of the order of between 5% and 30% of that taken up by the flatbed-grown cells (according to the particular nutrient).

The next experiments describe the use of calcium alginate as a substratum for cell immobilisation.

## THE IMMOBILISATION OF SOLANUM NIGRUM CELLS IN CALCIUM ALGINATE

A second substratum employed in the attempt to immobilise cells was a gel of 2% (w/v) calcium alginate. A similar preliminary experiment to that involving the use of agar described above was performed to investigate the growth (i.e., nutrient uptake), viability and capacity for alkaloid production of entrapped cells of S. nigrum.

## NUTRIENT UPTAKE BY CALCIUM ALGINATE COLUMNS IN THE ABSENCE OF CELLS

To ascertain whether the alginate column itself was capable of removing nutrients from the medium, three columns were set up as described previously, but without any cells. Samples of the nutrient medium were taken from each column at intervals, mixed and analysed for phosphate, ammonia, nitrate and sucrose; the results are given in Table 3.4.6. It can be seen that there was an uptake from the medium of all four nutrients, which increased, in the cases of phosphate and ammonia, to 17.6% and 18.5% respectively by day 5. After this time there was some release from the alginate back into the medium, so that by day 9 approximately 13% of each had been taken up. Nitrate uptake fluctuated; there was an uptake of nearly 25% at the end of the first day, but there was after this period a general release of nitrate from the column so that by day 9 almost

Table 3.4.6

Nutrient uptake by calcium alginate columns in the absence of cells

| Day of Culture | % Nutrient Uptake | | | |
|---|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 12.2 | 10.7 | 24.8 | 3.2 |
| 3 | 16.9 | 16.2 | 19.0 | 19.0 |
| 5 | 17.6 | 18.5 | 20.3 | 1.3 |
| 7 | 12.8 | 15.5 | 11.6 | 26.5 |
| 9 | 12.8 | 13.4 | 0.6 | 4.6 |

all was present in the medium. The pattern of sucrose uptake fluctuated dramatically throughout the culture period, varying from 1.3% to as much as 26.5% uptake, eventually being recorded at 4.6% at day 9. The calcium alginate did not interfere significantly with the sucrose assay; moreover, it did not appear to dissolve, despite the presence of phosphate in the medium (see Ohlson et al., 1979).

## NUTRIENT UPTAKE BY, AND THE VIABILITY AND ALKALOID CONTENT OF, SOLANUM NIGRUM CELLS IMMOBILISED IN 2% CALCIUM ALGINATE

The aim of this experiment was to examine the growth (nutrient uptake), viability and ability to accumulate alkaloids of cells entrapped in a 2% calcium alginate gel.

Cells of S. nigrum, which had been maintained for 5 months, were immobilised in a 2% calcium alginate gel as described previously and inserted into 3 columns. Nutrient medium (full MS) was supplied to the cells for a period of 8 days, and was sampled at daily intervals. The medium sampled from the columns was mixed before analysis, and was analysed for orthophosphate, ammonia, nitrate and sucrose, and for oxygen. At the end of the experimental period, the alkaloid content and the percentage viability of the cells were determined, as was the pH and the alkaloid content of the medium.

The nutrient uptake data are given in Table 3.4.7 and Figures 3.4.3 (a) - (d). All four nutrients were taken up at more or less steady rates. Phosphate was removed from the medium to the greatest extent, such that only 30% of the initial amount remained after 8 days; the rate of uptake was approximately linear. The uptake of the other three nutrients was most steady after

Table 3.4.7

Percentage nutrient uptake by S. nigrum cells immobilised in calcium alginate

| Day of Culture | % Nutrient Uptake | | | |
|---|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 13.6 | 21.4 | 16.6 | 12.9 |
| 2 | 14.3 | 17.2 | 15.6 | 15.1 |
| 3 | 25.8 | 16.4 | 27.5 | -20.0 |
| 4 | 34.0 | 24.7 | 26.4 | -2.2 |
| 5 | 49.7 | 30.7 | 29.5 | 20.7 |
| 6 | 57.2 | 33.9 | 33.9 | 29.6 |
| 7 | 60.3 | 36.1 | 39.8 | 31.7 |
| 8 | 70.0 | 39.0 | 40.2 | 34.6 |

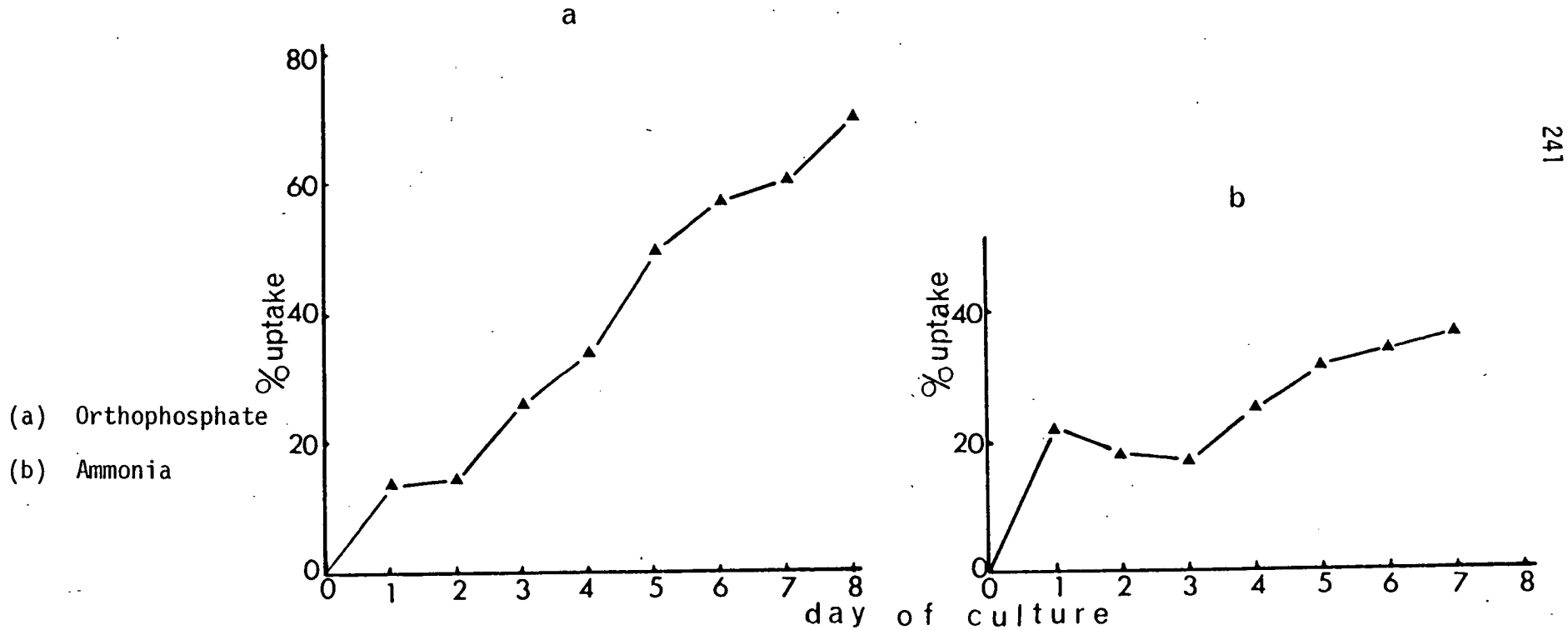



Figure 3.4.3 Nutrient Uptake by Alginate-Immobilised Cells of S. nigrum

(c) Nitrate

(d) Sucrose

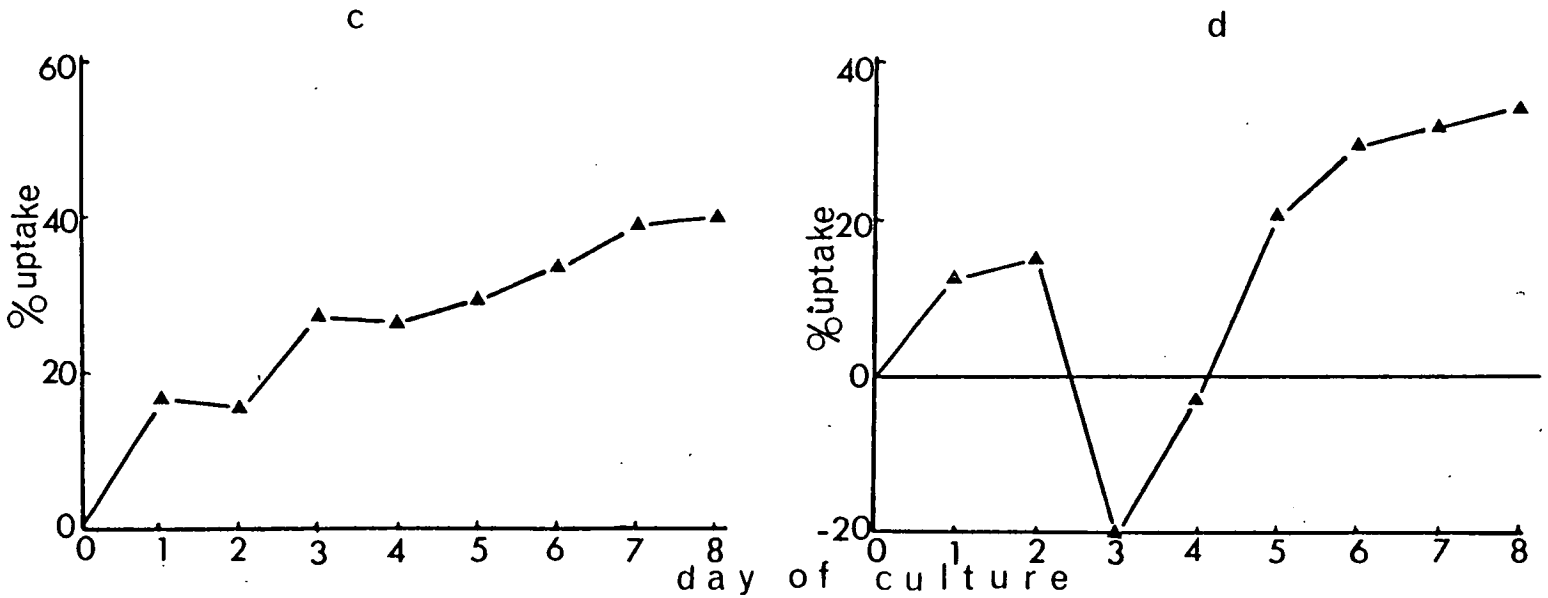



Figure 3.4.3 (continued)

days 3 or 4; by day 8, about 40% of the ammonia and nitrate, and about 35% of the sucrose, had been taken up. At day 3 there was an increase in the quantity of reducing sugar in the medium, presumably due to a washing off from the column of cells or cell wall materials.

The percentage viability and alkaloid content of the immobilised cells are given in Table 3.4.8, and it is apparent that, at the end of the 8 day culture period, the percentage viability of the cells was not severely reduced (with a mean value of 62.7% for the three columns) and was closely comparable with that for agar-entrapped cells. The alkaloid content of the cells was also similar, with a mean I value of about 2.4 (about 12 or 13 mg/g dry weight cells) across the three columns.

Analysis of the nutrient medium from the 3 columns at day 8 revealed that alkaloids were not released by the cells. The pH of the medium was, at this time, 5.4, i.e., was 0.4 units more acidic than it had been initially.

From these results, it can be concluded that cells embedded in a 2% calcium alginate gel were capable of alkaloid production and growth (in the latter case, it would appear, better than when entrapped in 2% agar) without a dramatic loss in viability (i.e., when compared to a suspension culture).

Oxygen uptake by cells immobilised in alginate is now described.

Table 3.4.8

Cell viability and alkaloid content of alginate-immobilised *S. nigrum* cells (after 8 days)

| Replicate | % Cell Viability | Mean I Value |
|---|---|---|
| 1 | 62.0 | 2.3 |
| 2 | 57.7 | 2.4 |
| 3 | 68.3 | 2.4 |
| $\bar{x} \pm$ S.E. = | $62.7 \pm 0.1$ | $\bar{x} = 2.4$ |

## OXYGEN CONSUMPTION BY CELLS OF *SOLANUM NIGRUM* IMMOBILISED IN 2% CALCIUM ALGINATE

In order to gain more information concerned with the activity of entrapped cells, and to discover something of the physical conditions associated with the column culture technique, the level of oxygen in the nutrient medium throughout a culture period was followed.

## OXYGEN LEVELS IN THE NUTRIENT MEDIUM OF CALCIUM ALGINATE COLUMNS, IN THE ABSENCE OF CELLS

A preliminary investigation was carried out to ascertain any changes in the oxygen levels in the medium which occur over a 9 day experimental period, due to uptake by the column apparatus itself. Three columns were set up as described previously, but without cells, and nutrient medium was sampled at intervals, mixed and analysed for oxygen in an oxygen electrode, using the method described in 'Materials and Methods'. The results of this experiment are given in Table 3.4.9; oxygen levels remained between about 0.044 and 0.048 µmols oxygen per ml medium, representing between 88 and 97% air saturation.

## OXYGEN LEVELS IN THE NUTRIENT MEDIUM SUPPLYING COLUMNS OF *SOLANUM NIGRUM* CELLS IMMOBILISED IN 2% CALCIUM ALGINATE

Oxygen uptake by cells immobilised in a 2% calcium alginate gel was used as an indication of cell growth and viability. Three columns were set up as described previously and measurements were made of the oxygen level in the nutrient medium of the columns over the 8 day culture period, and the results are presented in Table 3.4.10

Table 3.4.9

Oxygen levels in the nutrient medium supplied to calcium alginate columns, in the absence of cells

| Day of Culture | % Air Saturation | µmol Oxygen $ml^{-1}$ Medium |
|---|---|---|
| 0 | 96.0 | 0.048 |
| 1 | 97.0 | 0.048 |
| 2 | 88.0 | 0.044 |
| 3 | 89.5 | 0.045 |
| 5 | 87.5 | 0.044 |
| 7 | 95.0 | 0.047 |
| 9 | 94.0 | 0.047 |

and illustrated in Figure 3.4.4.

It can be seen that there was a rapid drop in the level of oxygen in the medium after the first day of culture, and the rate of uptake began to level off at day 4, at a value of approximately 30% air saturation (0.015 µmol $O_2$ $ml^{-1}$ medium). There was a much slower decline in the oxygen level over the next 4 days, to 28% air saturation. The increase in the rate of oxygen uptake corresponded approximately to the initiation of steady nutrient uptake, suggesting that the cells were metabolically active after the first couple of days of culture, followed after days 5 or 6 by a period of reduced activity. Since the oxygen level representing 5% available oxygen (the extinction point) is approximately 0.012 µmol $O_2$ $ml^{-1}$ medium (as described previously, p.164 ), it seems likely from the results in Table 3.4.10, that the immobilised cells were suffering from oxygen limitation by between days 4 and 8.

The effects of another environmental factor, namely light, on the immobilised cells are now described.

## THE EFFECTS OF ILLUMINATION AND DARKNESS ON THE GROWTH AND METABOLISM OF SOLANUM NIGRUM CELLS IMMOBILISED IN CALCIUM ALGINATE

The aim of this experiment was to determine whether there was any significant difference in the growth and metabolism of light- and dark-grown immobilised cells. Having discovered that alginate-entrapped cells of S. nigrum grew to some extent under the experimental conditions used, it was decided to determine the importance of light. In all previous experiments, the cultures were illuminated with 200 µmol $m^{-2}$ $sec^{-1}$ fluorescent white light,

Table 3.4.10

Oxygen levels in the nutrient medium supplying columns of *S. nigrum* cells immobilised in calcium alginate

| Day of Culture | % Air Saturation | $\mu$ mol Oxygen $ml^{-1}$ Medium |
|---|---|---|
| 0 | 92.4 | 0.046 |
| 1 | 91.0 | 0.045 |
| 2 | 65.0 | 0.032 |
| 3 | 48.4 | 0.024 |
| 4 | 30.9 | 0.015 |
| 5 | 29.6 | 0.015 |
| 6 | 29.6 | 0.015 |
| 7 | 29.0 | 0.014 |
| 8 | 28.0 | 0.014 |

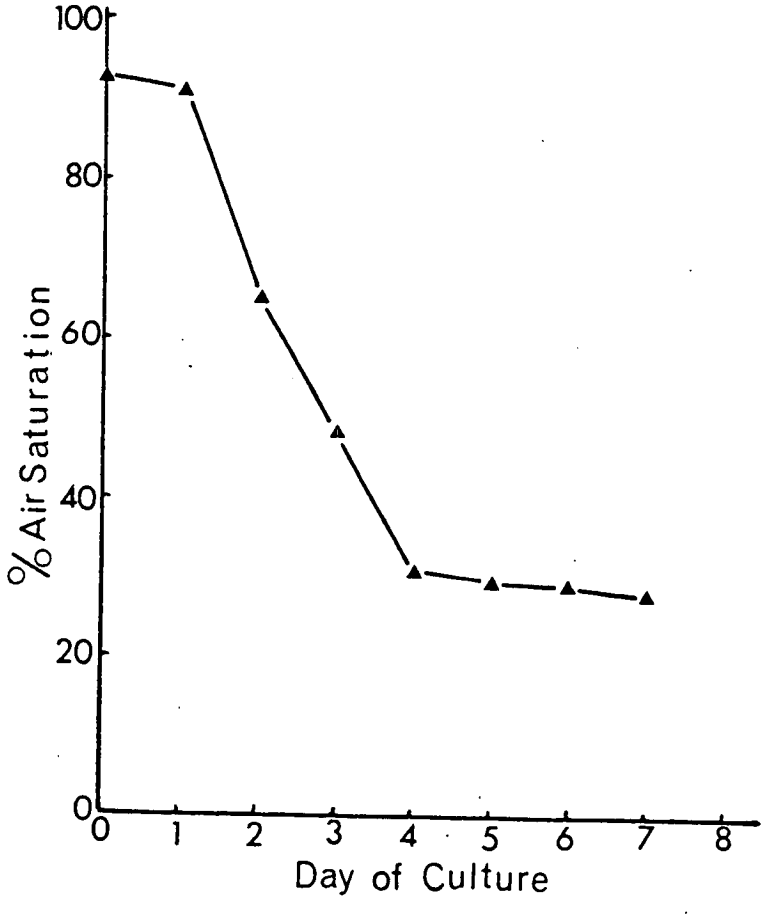



Figure 3.4.4 Oxygen Uptake by Alginate-Immobilised Cells of S. nigrum

100% Air Saturation ≡ 0.05 µmol $O_2$/ml medium

but it was not known whether this was essential.

To investigate this, six alginate columns were constructed with immobilised cells of S. nigrum, as described previously, although the sodium alginate solution was prepared with full MS liquid medium so that the cell cultures might become more readily established on the columns. Three columns were wrapped in aluminium foil, to exclude all light from the cells, and three were illuminated, each by a single 8 watt 'warm white' fluorescent tube (Thorn), providing an illuminance of 150 $\mu$mol $m^{-2}$ $sec^{-1}$ (150 $\mu E m^{-2}$ $sec^{-1}$).

The cells were supplied with 50 ml of full MS liquid nutrient medium. After a culture period of 8 days, determinations were made of the alkaloid content of the cells (by the cell-squash technique) and of the nutrient medium, and percentage cell viability and culture appearance were recorded. The nutrient medium was sampled every 24 hours, and the percentage uptake of orthophosphate, ammonia, nitrate and sucrose was determined and used as an indication of cell growth rate.

The nutrient uptake data are given in Table 3.4.11 and Figures 3.4.5 (a) - (d). The most notable point is that the illuminated cultures removed nutrients from the medium more quickly and to a greater extent than did the cultures kept in the dark. The uptake of phosphate by the illuminated columns was approximately linear over the first 5 days of culture (reaching a value of nearly 75% uptake), followed by a sharp decline in the rate of uptake and some release of phosphate from presumably lysing cells. The dark-grown cultures, however, took up only about 50% of the available phosphate over the initial 6 days, after which time there was some

Table 3.4.11

Percentage nutrient uptake by light- and dark-grown immobilised *S. nigrum* cells

| Day of Culture | % Nutrient Uptake | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $PO_4^{---}$ | | $NH_4^+$ | | $NO_3^-$ | | Sucrose | |
| | Light | Dark | Light | Dark | Light | Dark | Light | Dark |
| 1 | 17.2 | 18.2 | 20.3 | 19.4 | 16.4 | 16.7 | 22.1 | 17.8 |
| 2 | 30.5 | 28.4 | 22.5 | 21.1 | 20.3 | 21.1 | 26.5 | 25.1 |
| 3 | 49.8 | 31.6 | 27.9 | 28.3 | 30.4 | 24.2 | 7.3 | 8.2 |
| 4 | 58.2 | 40.0 | 30.5 | 29.1 | 29.9 | 25.3 | 14.1 | 10.0 |
| 5 | 72.5 | 49.3 | 38.7 | 32.3 | 34.1 | 27.4 | 24.7 | 21.3 |
| 6 | 74.1 | 51.3 | 40.2 | 31.8 | 43.3 | 29.5 | 30.4 | 28.7 |
| 7 | 70.4 | 48.7 | 36.3 | 28.5 | 49.4 | 30.2 | 32.3 | 33.4 |
| 8 | 68.5 | 41.2 | 35.4 | 26.9 | 44.5 | 26.8 | 30.2 | 29.1 |

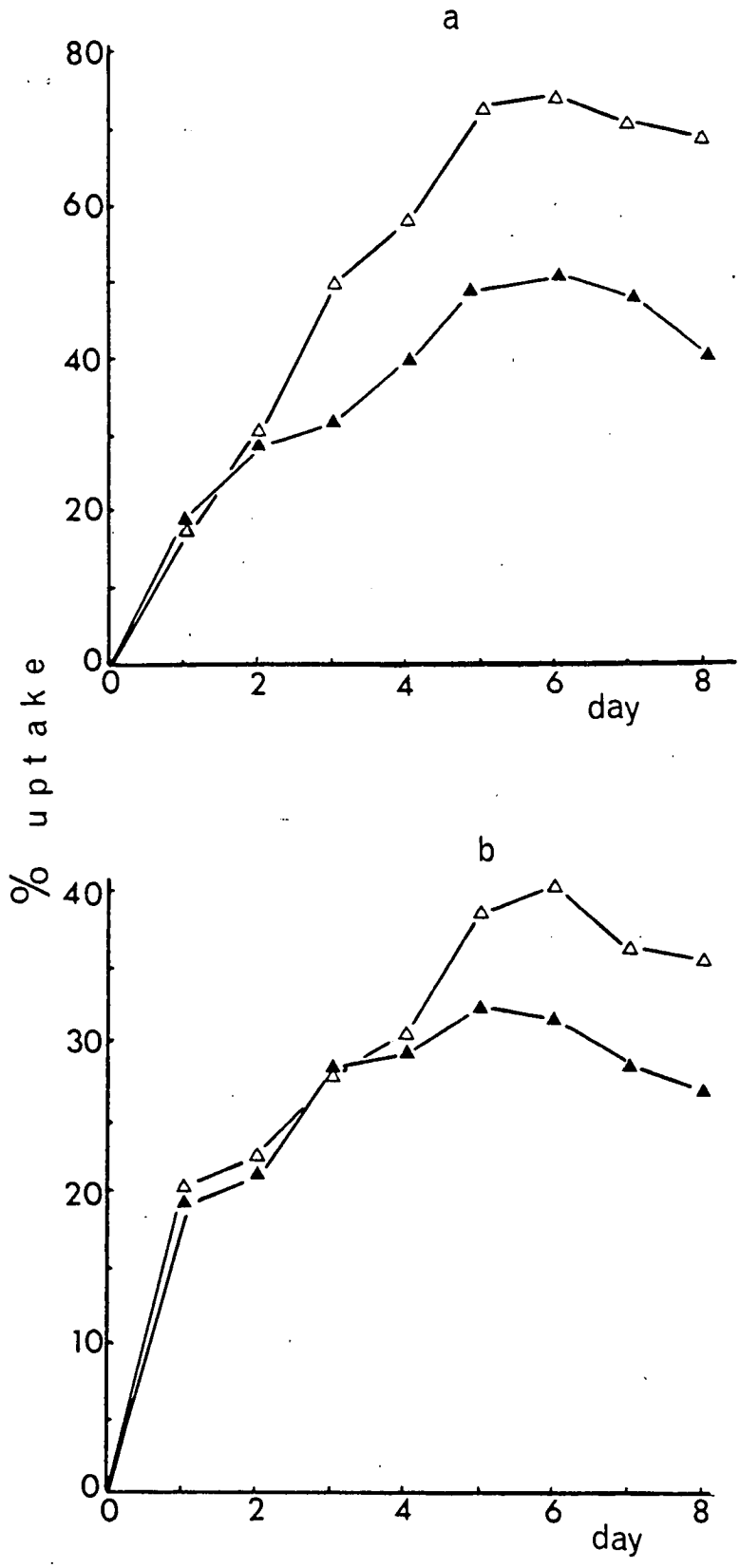



Figure 3.4.5 Nutrient Uptake by Alginate-Immobilised Cells of S. nigrum, Grown in the Light (Open Triangles) and in the Dark (Solid Triangles)

(a) Orthophosphate

(b) Ammonia

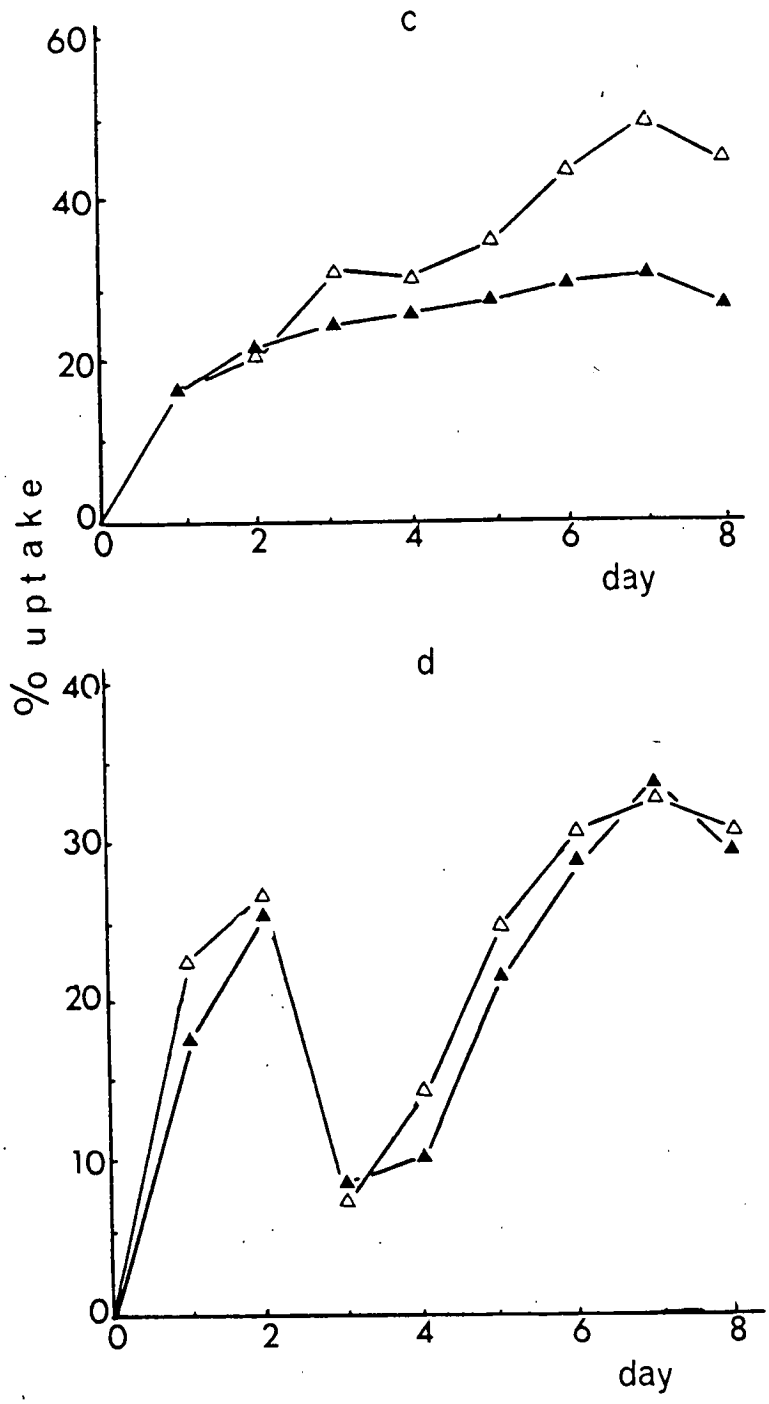



Figure 3.4.5 (continued)

(c) Nitrate

(d) Sucrose

release of phosphate back into the medium. Phosphate was taken up to a greater extent than any of the other nutrients measured, but the pattern of uptake was similar for ammonia and nitrate. The illuminated cultures removed about 40% of the ammonia and 50% of the nitrate and the dark cultures removed 32% of the ammonia and 30% of the nitrate from the medium. The pattern of sucrose uptake was masked somewhat by what was assumed to be interference of the analytical method by cells and cell wall material washed from the columns, but there was nevertheless a distinct removal of sucrose. The disparity in percentage uptake between the treatments exhibited by the other three nutrients was not apparent with sucrose. The pattern of uptake by the dark-grown columns closely followed that of the illuminated columns, and was at only a slightly lower level : at the end of the 8 day culture period, the illuminated columns had taken up 30.2% and the dark-grown 29.1% of the sucrose.

## APPEARANCE OF THE CULTURES

By the end of the experimental period the dark-grown cultures had become darker in appearance than the illuminated cultures; no greening was obvious in the illuminated cultures.

## VIABILITY OF THE CULTURES

The percentage cell viability data for the two sets of columns are given in Table 3.4.12. The illuminated cultures had a greater proportion of live cells than did the darkened cultures, the mean percentage viability for the two treatments being 68.5% and 56.2% respectively.

Table 3.4.12

Percentage cell viability of illuminated and dark-grown column cultures of *Solanum nigrum*

| Replicate Column | % Cell Viability | | |
|---|---|---|---|
| | Light | Dark | Day 0 Cells |
| | | | % Viability = 69.7% |
| 1 | 71.3 | 52.5 | |
| 2 | 64.5 | 61.3 | |
| 3 | 69.7 | 54.7 | |
| mean $\overline{+}$ S.E. = | 68.5 $\pm$ 0.1 | 56.2 $\pm$ 0.1 | |

Table 3.4.13

Alkaloid content of illuminated and dark-grown column cultures of *Solanum nigrum*

| Day 8 : Replicate Column | Light | Dark | Day 0 Cells |
|---|---|---|---|
| | | | Mean I Value = 2.2 |
| 1 | 2.4 | 0.9 | |
| 2 | 2.4 | 0.7 | |
| 3 | 2.3 | 0.9 | |
| mean = | 2.4 | 0.8 | |

ALKALOID CONTENT OF THE CULTURES AND MEDIUM

On reducing (*in vacuo* at 40°C) the volume of the nutrient medium from the columns of the respective treatments, followed by spotting out and testing with Dragendorff's reagent, it was found that alkaloids had not been released by either light- or dark-grown cells.

The alkaloid contents of the cells, both before and after culture, are given in Table 3.4.13. It can be seen that the alkaloid levels in the illuminated cultures were approximately the same at the end of the culture period as at the beginning (the cells containing of the order of 12-13 mg alkaloids/g dry weight); the dark-grown cultures, on the other hand, contained much reduced levels (less than 10 mg alkaloids/g dry weight cells).

It would seem that cell growth was relatively better in the illuminated columns than in the darkened columns, as indicated by the nutrient uptake data. The cell viability results also suggest that light was important in producing viable cultures, a point, moreover, indicated simply by the general appearance of the cultures, although this is a subjective parameter. Although the darkened (in both senses) cultures appeared to be growing the more slowly, they contained lower levels of alkaloids than the illuminated cells, and it therefore seems that slow growth *in itself* does not automatically determine that alkaloids should be produced : light is apparently a contributory factor. Since greening was not observed in the illuminated cultures, other effects of light must be of importance in maintaining 'normal' cell metabolism.

From the results of the preliminary experiments, the following points emerge. The panscrubbers had no detectable cytotoxic properties, and the cells were capable of taking up nutrients from the medium while entrapped in either agar or calcium alginate (although the cells apparently grew better in the latter matrix , and appeared to be limited in their growth by phosphate availability). The percentage viability of the cultures was of the order of 60-70%, a figure which suggests that the apparent oxygen limitation had no severely detrimental effects. The illumination of the cultures seems to be essential to maintain this high level of cell viability and to allow the cells to grow and produce alkaloids.

The next section of results describes the effects of supplying precursors to immobilised cells.

# SECTION 3

# THE EFFECTS OF PRECURSORS ON THE VIABILITY OF AND SECONDARY METABOLITE PRODUCTION BY IMMOBILISED CELLS

This third section of results describes experiments which involved the 'feeding' of cells with precursors of specific secondary compounds. In the first experiment, cells of D. innoxia were treated with ornithine, to determine the effects of this precursor on the qualitative and quantitative composition of alkaloids accumulated in the cells. In the second experiment, cells of Capsicum frutescens were treated with a precursor of capsaicin, iso-capric acid, to determine (1) whether capsaicin could be produced by immobilised cells (and this was compared with production by cells grown in the flatbed system) and (2) whether the immobilisation procedure inhibited in any way the release of capsaicin from the cells (as occurs in flatbed-cultured cells).

## THE EFFECTS OF 5 mM ORNITHINE ON THE GROWTH, VIABILITY AND ALKALOID CONTENT OF IMMOBILISED CELLS OF D. INNOXIA

The work of Forche and Yeoman (unpublished data) has demonstrated that the addition of ornithine to the nutrient medium supplying cell suspension cultures of D. innoxia led to an increase in the amounts of tropane alkaloids accumulated in the cells. In this experiment, cells of the same species were immobilised on calcium alginate columns and supplied with 5 mM ornithine, to determine whether an increase in alkaloid content could be achieved.

Cells of D. innoxia, which had been maintained for 2 months, were immobilised, as described previously, in columns of calcium alginate. Three 'control' columns were supplied with full MS medium, whereas the three 'test' columns were supplied with full MS medium containing 5 mM ornithine. At the end of a 9 day culture period, determinations were made of the alkaloid content of the nutrient medium and the cells (by both the cell-squash technique and by TLC), and percentage cell viability. Throughout the experiment, the nutrient medium was sampled at intervals (medium was taken from each of the three columns of a particular treatment and mixed) and analysed for orthophosphate, ammonia, nitrate and sucrose, to provide an indication of culture growth rate. Thin layer chromatography of cell extracts was carried out (according to the method of Hultin and Torsell, 1965, described in Methods, Chapter 2) to determine whether the ornithine treatment affected the number or ratio of alkaloids produced.

Table 3.4.14 contains the results obtained for the relative alkaloid contents and percentage cell viability of the treated and

Table 3.4.14

Alkaloid content and viability of ornithine-treated and untreated cells of *D. innoxia* after a 9-day immobilisation period in alginate columns

| | Replicate Column | Mean I Value | % Cell Viability |
|---|---|---|---|
| CONTROLS (-ORN) | 1 | 3.2 | 78.3 |
| | 2 | 2.9 | 64.1 |
| | 3 | 2.9 | 71.9 |
| | | $\bar{x}$ = 3.0 | $\bar{x}$ = 71.9 ± 0.2 |
| PLUS ORN | 1 | 3.1 | 62.1 |
| | 2 | 3.7 | 66.2 |
| | 3 | 3.6 | 61.7 |
| | | $\bar{x}$ = 3.5 | $\bar{x}$ = 63.3 ± 0.1 |

untreated cultures at the end of the culture period. The mean I value for the treated cells was higher (3.5) than for the controls (3.0), representing a difference of only about 0.5 mg tropane alkaloid/g dry weight of cells. No alkaloids were detectable in the nutrient medium after 9 days. The TLC results revealed little qualitative difference in the alkaloid content of the treated and untreated cells (Figure 3.4.6). 100 µl samples of fractions A and B, and standards of hyoscyamine, scopolamine, atropine and tropic acid were loaded onto Kieselgel 60 TLC plates as described previously and separated in the chloroform : acetic acid : methanol solvent system, and the plates, when dry, were sprayed with Dragendorff's reagent. In both ornithine-treated and untreated cells, three spots only were visible in fraction A, of $R_f$ values 0.36, 0.32 and 0.16. No alkaloids were visualised in the fraction B. The spots at 0.32 and 0.16 co-chromatographed with both internal and external standards of atropine and scopolamine respectively, but the other alkaloid was not identified. The area of the alkaloid spot at $R_f$ 0.16 (probably scopolamine) was slightly larger in area in the ornithine-treated cells compared with the controls; the other two spots were of approximately the same size in treated and untreated cells.

The nutrient uptake data (Table 3.4.15 and Figures 3.4.7 (a) - (d)) indicate that, in general, the ornithine-treated cells grew more slowly than the untreated cells : although there was no real difference in phosphate uptake between the two sets of cultures, there was a differential amount of uptake of the other nutrients, especially towards the end of the culture period (after about day 5); by day 9, the percentage uptake of ammonia, nitrate and sucrose was of the order of 10% lower in the ornithine-treated compared to the

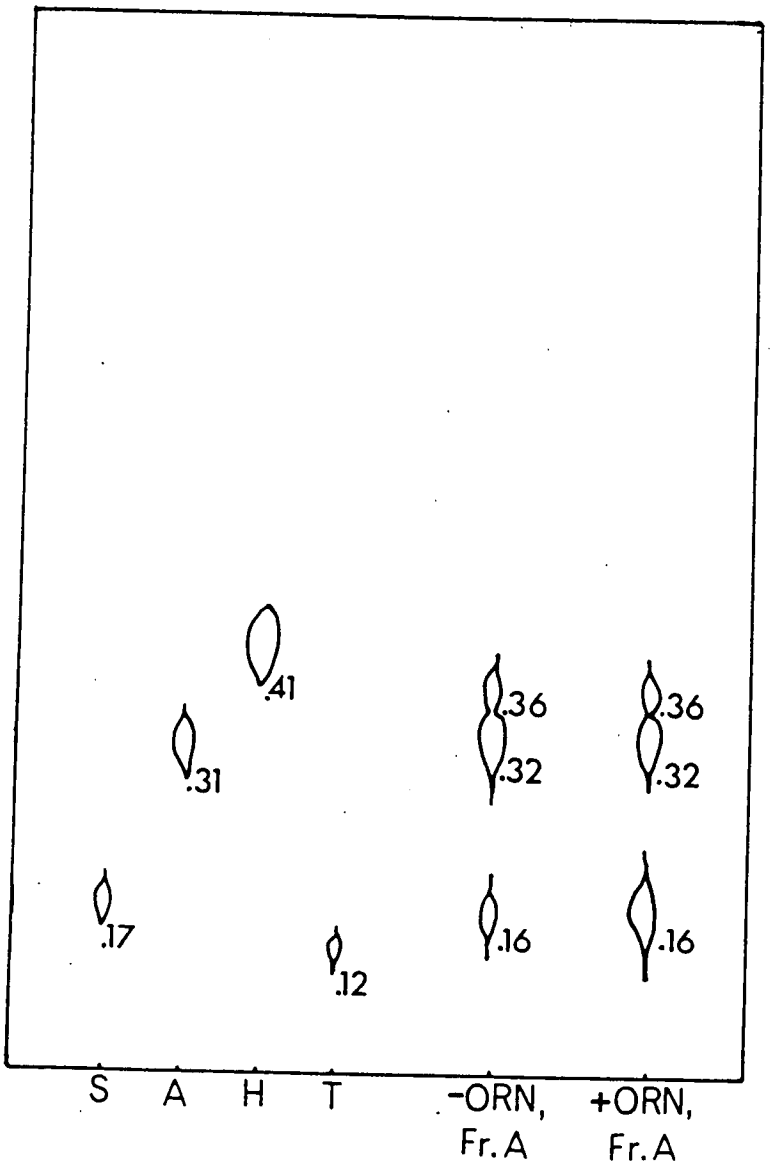



Figure 3.4.6 TLC of Extracts of Immobilised Cells of D. innoxia ± 5mM Ornithine

S = scopolamine

H = hyoscyamine

A = atropine

T = tropine

Table 3.4.15

Nutrient uptake by immobilised D. innoxia cells ± ornithine

| Day of Culture | % Nutrient Uptake | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $PO_4^{---}$ | | $NH_4^+$ | | $NO_3^-$ | | Sucrose | |
| | +ORN | -ORN | +ORN | -ORN | +ORN | -ORN | +ORN | -ORN |
| 1 | 81.8 | 84.2 | 21.0 | 31.2 | 30.3 | 42.0 | 26.6 | 33.7 |
| 3 | 93.7 | 96.4 | 17.8 | 24.9 | 32.3 | 35.9 | 21.0 | 30.9 |
| 5 | 96.8 | 97.2 | 26.3 | 25.5 | 36.4 | 36.2 | 44.9 | 35.4 |
| 7 | 95.7 | 97.2 | 29.3 | 34.9 | 38.5 | 44.2 | 48.4 | 51.7 |
| 9 | 96.8 | 96.5 | 38.7 | 47.2 | 36.1 | 44.2 | 40.8 | 50.3 |

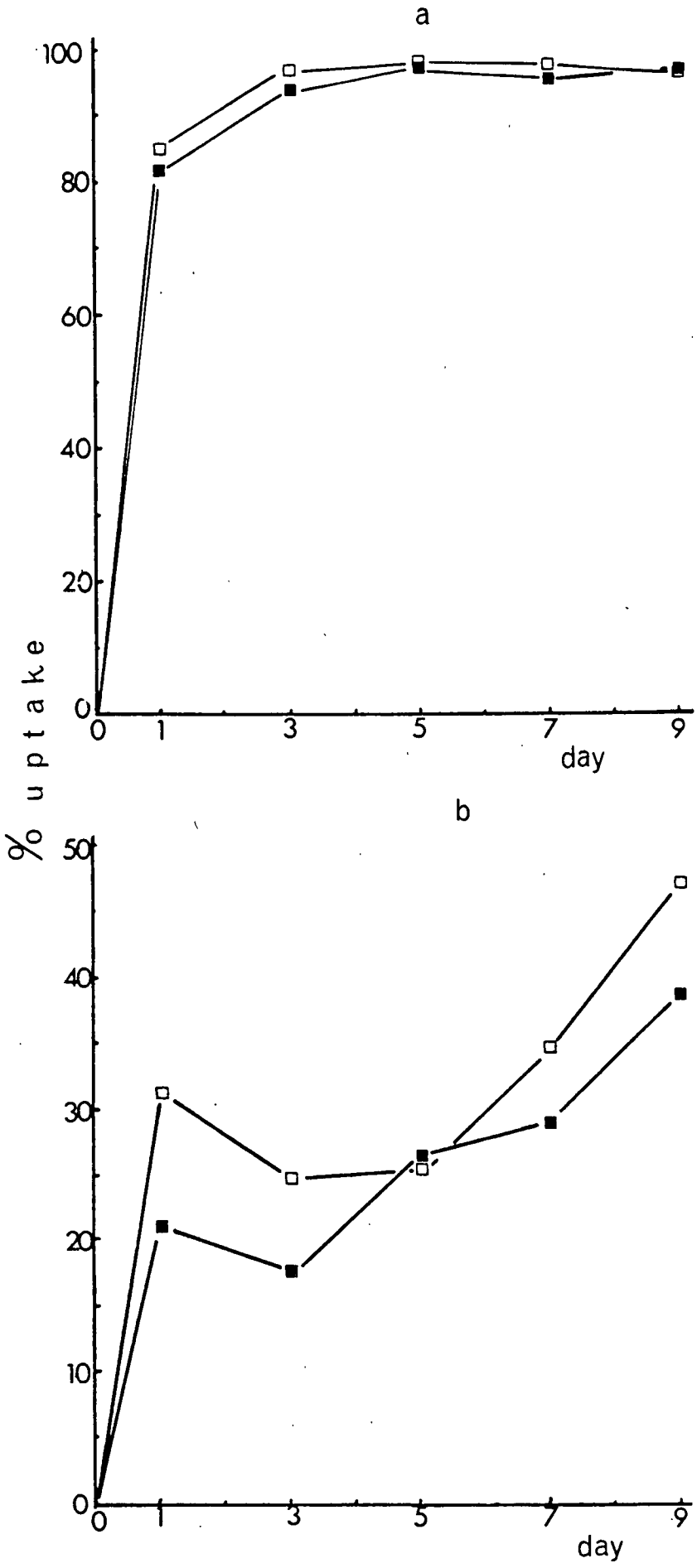



Figure 3.4.7 Nutrient Uptake by Immobilised Cells of D. innoxia in the Presence (Solid Squares) and Absence (Open Squares) of 5mM Ornithine

(a) Orthophosphate (b) Ammonia

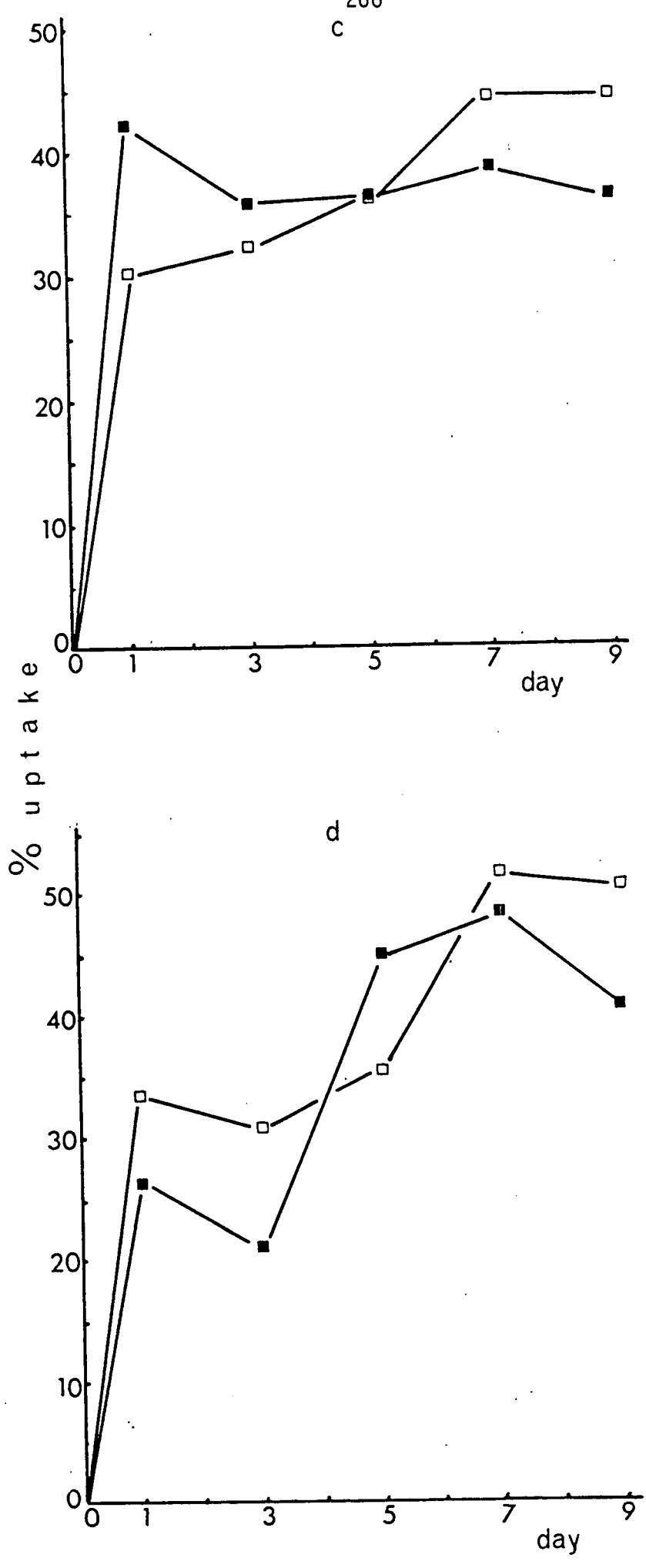



Figure 3.4.7 (continued)

(c) Nitrate (d) Sucrose

untreated cultures. This apparent adverse effect of ornithine on cell growth seemed to be reflected in a lower percentage cell viability of the treated cells ( a mean of 63.3% compared with 71.9%).

It may therefore be concluded that 5 mM ornithine caused a reduction in the growth and viability of cells of D. innoxia and had a small effect in increasing the yield of tropane alkaloids produced by the immobilised cells; this was mainly an increase in the amount of scopolamine. Since the cells accumulated and retained the alkaloids, it was decided to determine whether the immobilisation of the cells prevented the release of the compounds into the nutrient medium.

## CAPSAICIN PRODUCTION BY, AND GROWTH OF, CELLS OF CAPSICUM FRUTESCENS MILL. CULTURED ON CALCIUM ALGINATE COLUMNS AND FLATBEDS

The aim of this experiment was to determine whether cells of C. frutescens, immobilised in calcium alginate gel, were capable of growth and of the production and release of capsaicin. In the latter case, a comparison was made with cells cultured on the flatbed. The experiments described so far have shown that, at least in the cases of the species employed, immobilised cells did not release alkaloids into the medium, but accumulated them intracellularly. In order to discover whether the alginate gel itself was inhibitory to the release of specific secondary compounds, cells of C. frutescens were immobilised; Yeoman et al.(1980) have shown that, when cultured on the flatbed apparatus, cells of this species release capsaicin into the medium rather than accumulate it. A comparison was

therefore made of capsaicin production by flatbed cultures and column cultures.

(a) Column Culture

Approximately 10 g of 2 week-old friable callus of Capsicum frutescens (derived from stem tissue, and maintained and subcultured at monthly intervals on agar plates containing 'pepper' medium for a period of 4 months) was immobilised on each of three alginate columns. The cells were supplied with 50 ml of full MS liquid nutrient medium containing a final concentration of 5 mM iso-capric acid (8-methyl nonenoic acid), a precursor of capsaicin. During the 10 day culture period, nutrient medium was sampled from the three columns and mixed before analysis for orthophosphate, ammonia, nitrate and sucrose (to provide an indication of cell growth rate). Capsaicin was extracted in chloroform from both nutrient medium (100 ml) and cells (10 g), and analysed by TLC (according to the procedure in 'Methods', p.32 ) and by GC.

(b) Flatbed Culture

Three flatbeds were set up, each containing approximately 50 g of 2 week-old friable stem callus of Capsicum frutescens (as was used in the column cultures described above). Each flatbed culture was supplied with 50 ml of full MS medium containing iso-capric acid at a final concentration of 5 mM, and at the end of a 10 day culture period, the cells and nutrient medium were analysed for capsaicin by TLC and GC.

## RESULTS OF COLUMN CULTURE

### (a) Nutrient Uptake

The nutrient uptake data are given in Table 3.4.16 and Figures 3.4.8 (a) - (d). It is apparent that the cells were capable of growth under the conditions of entrapment, all four nutrients being taken up to relatively high levels (69.7%, 47.5%, 81.6% and 78.8% by day 7 for $PO_4^{---}$, $NH_4^+$, $NO_3^-$ and sucrose respectively); there seems to have been a significant amount of cell lysis between days 7 and 9, indicated by a large release of nutrients (most notably $NH_4^+$, $NO_3^-$ and sucrose) back into the medium - in fact, approximately half of the amount which had been taken up before lysis.

### (b) TLC

100 µl extracts from cells and nutrient medium were loaded onto polyamide TLC plates, together with 10 µg of a capsaicin external standard and 8.6 µg of an iso-capric acid external standard (i.e., 10 µl of 5 mM iso-capric acid). The samples were separated in 100 ml of a chloroform : ethylacetate (50:50 v/v) solvent system, and when dry the plate was sprayed with the $KFeCN^-/FeCl_3$ reagent.

The capsaicin standard was visualised as a blue spot (on a white background) of $R_f$ value 0.84, and the iso-capric acid standard as a pale creamy spot (on a white background) of $R_f$ value 0.69.

Table 3.4.16

Nutrient uptake by cells of *C. frutescens* immobilised in alginate columns

| Day of Culture | % Nutrient Uptake | | | |
|---|---|---|---|---|
| | $PO_4^{---}$ | $NH_4^+$ | $NO_3^-$ | Sucrose |
| 3 | 31.0 | 36.6 | 42.3 | 40.6 |
| 5 | 41.4 | 35.9 | 27.9 | 51.5 |
| 7 | 69.7 | 47.5 | 81.6 | 78.8 |
| 9 | 61.7 | 18.2 | 46.7 | 42.9 |

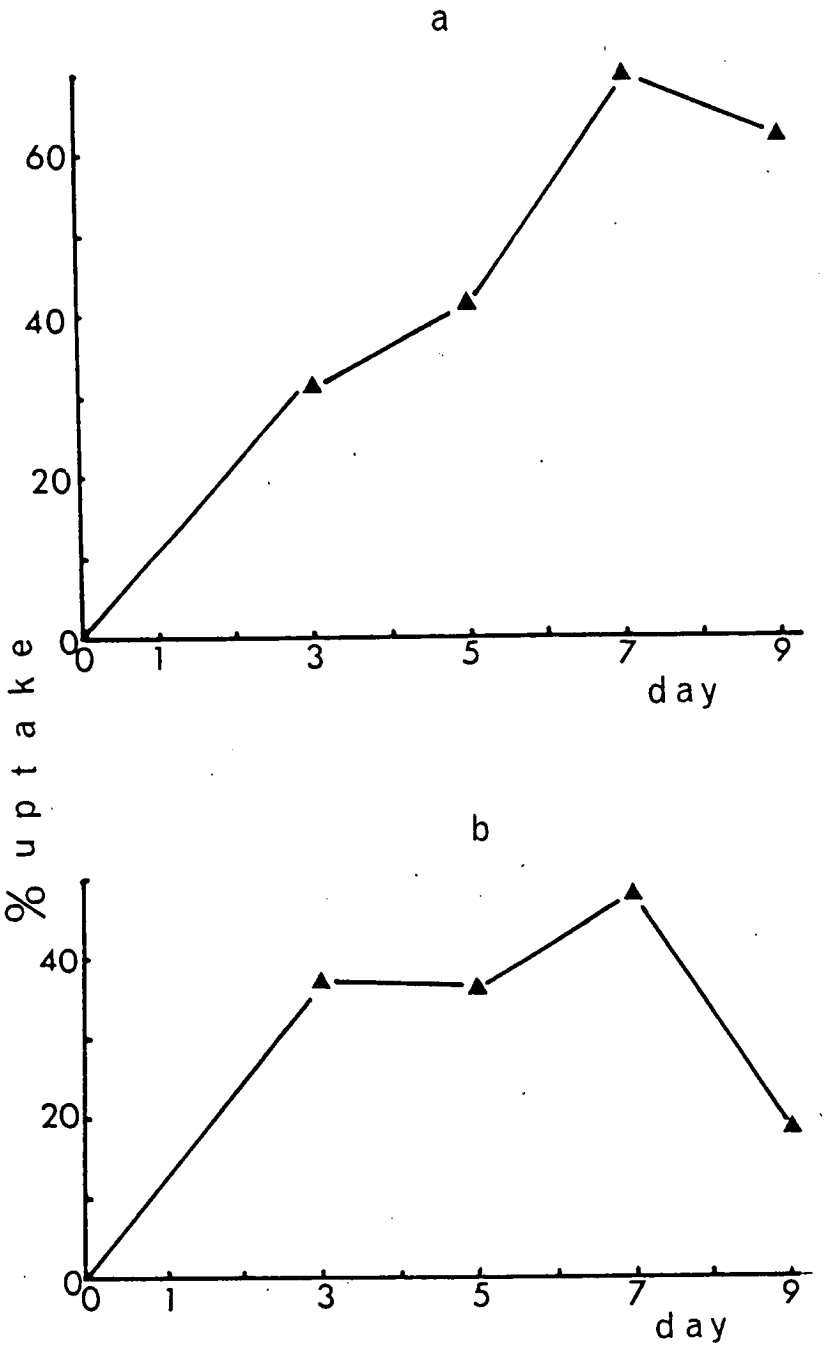



Figure 3.4.8 Nutrient Uptake by Alginate-Immobilised Cells of C. frutescens

(a) Orthophosphate

(b) Ammonia

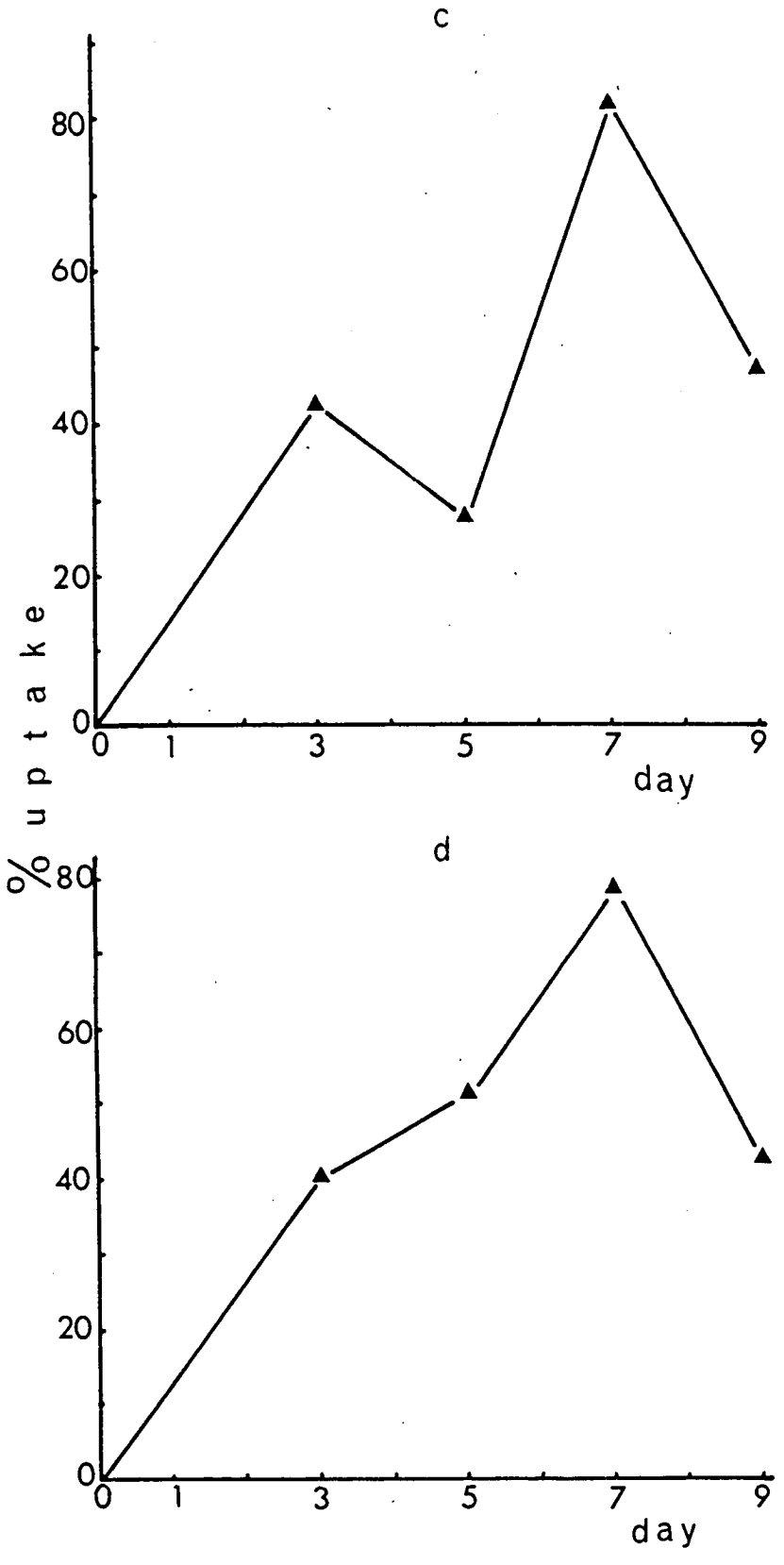



Figure 3.4.8 (continued)

(c) Nitrate

(d) Sucrose

Cell Extract

No compounds were visualised after spraying.

Nutrient Medium Extract

A single large blue spot was visualised on spraying, the $R_f$ value of which ranged from 0.82 - 0.67. It was considered that this was probably mainly capsaicin (or possibly some other capsaicinoid) held back slightly (i.e., having a slightly lower $R_f$ value than the capsaicin standard) due either to the large quantity of capsaicinoid in the spot or to the presence of iso-capric acid in the extract. In order to aid further identification, extracts of the nutrient medium were separated in a second solvent system comprising 100 ml of chloroform : methanol (98 : 2 v/v) (Iwai et al., 1979). The following were therefore loaded onto a polyamide TLC plate : 10 µg of capsaicin standard, 75 µl of medium extract, and 75 µl of medium extract plus 10 µg of capsaicin as an internal standard.

On spraying the dried plate with the $KFeCN^-/FeCl_3$ reagent, there was co-chromatography of all three (U-shaped) spots at $R_f$ value 0.83; the single medium extract spot was enlarged by the presence of the capsaicin internal standard. By comparing the area of all three spots, it was estimated that between 200 and 300 µg of capsaicin were present in 100 ml of nutrient medium.

GC

Gas chromatography of the nutrient medium extract, carried out by Dr. Simpson of the Department of Chemistry, Edinburgh University,

revealed that 100 ml of medium contained approximately 300 µg of capsaicin, estimated by comparing the area of peaks produced by extracted capsaicin with and without an internal standard of capsaicin.

Mass Spectrometry

Mass spectrometry of a sample of nutrient medium, carried out by Dr. Trinnaman of Bush Boake Allen Ltd. indicated that other capsaicinoids, most notably dihydrocapsaicin, were also present in the extract, in a ratio of 3:1 capsaicin : dihydrocapsaicin.

RESULTS OF FLATBED CULTURE

(a) TLC

At the end of the 10-day culture period, 100 µl samples of extracts from 100 ml of nutrient medium were loaded onto polyamide TLC plates, together with two 10 µg capsaicin standards (one internal, one external). The extracts and samples were then run in the chloroform : methanol solvent system, and on spraying the dried plate with the $KFeCN^{-}/FeCl_3$ reagent, three blue spots were visualised, all of $R_f$ values 0.82 - capsaicin therefore appeared to be present, and it was estimated (by comparing the three spot sizes) that the original 100 ml of nutrient medium contained of the order of 200 µg of capsaicin.

(b) GC

Gas chromatography of the flatbed nutrient medium extract, carried out again by Dr. Simpson of the Department of Chemistry, Edinburgh University, revealed that 100 ml of medium contained approximately 250 µg capsaicin (estimated by comparing the areas of peaks produced by extracted capsaicin with and without an internal capsaicin standard).

The results presented above allow a number of conclusions to be drawn. Firstly, C. frutescens cells, like cells of D. innoxia and S. nigrum, were capable of growth while embedded in a gel of calcium alginate. Secondly, capsaicin was produced by and released from the immobilised cells into the nutrient medium. Moreover, the immobilised cells released more capsaicin, on a basis of weight of capsaicin per weight of tissue, than did the cells cultured on the flatbed. The final concentration of capsaicin in the nutrient medium was approximately the same in both culture systems.

The results of Section 3 have shown how cells of D. innoxia and C. frutescens were immobilised in 2% calcium alginate gel. The D. innoxia cultures were capable of accumulating a range of tropane alkaloids, and the addition of 5 mM ornithine to the nutrient medium had a small effect in increasing the levels of alkaloids produced. The alkaloids were not released from the cells. The C. frutescens cultures produced and released capsaicin, demonstrating that the immobilisation of the cells was not inhibitory to the release of the metabolite.

## A BRIEF SUMMARY OF RESULTS PART 4

A column culture system was developed in which cells were immobilised in a gel matrix which was supported by nylon netting. Agar and calcium alginate were used as entrapment matrices, in both of which cells were capable of growth and the production of alkaloids. Cell growth was better in the alginate gel. Immobilised cells of *D. innoxia* and *S. nigrum* did not release alkaloids into the nutrient medium. The cells which were immobilised in calcium alginate may have been limited in their growth by low levels of phosphate, oxygen and (for *C. frutescens*) nitrate. Illumination of *S. nigrum* cultures was necessary for alkaloid production. The feeding of ornithine to immobilised cells of *D. innoxia* had a small effect on the level of alkaloids accumulated and immobilised *C. frutescens* cells produced capsaicin, when supplied with iso-capric acid, to a greater extent (cell weight for weight) than flatbed cells. A similar concentration of capsaicin accumulated in the medium of both culture systems.

Since both immobilised and flatbed cells of *D. innoxia* and *S. nigrum* failed to release alkaloids into the nutrient medium but accumulated them intracellularly, the final part of the 'Results' chapter describes experiments designed to induce alkaloid release.

# RESULTS PART 5

## Attempts To Induce Cells To Release Alkaloids Into The Nutrient Medium

The value of plant cell cultures for the production of specific secondary metabolites would be greatly increased if the cells released the compounds into the nutrient medium. Some species naturally release secondary metabolites; this has been shown to be the case for cells of C. frutescens (Results Part 3, Section 3) and Trigonella foenum-graecum (Radwan and Kokate, 1980). The experiments described above have shown that cells of D. innoxia and S. nigrum did not release their alkaloids, whether grown in liquid suspension culture, on the flatbed apparatus, or immobilised in agar or calcium alginate. The experiments described in this section aimed to induce the release of alkaloids from cells of S. nigrum by physical and chemical treatments.

## 1. THE EFFECTS OF LOW TEMPERATURE ON THE RELEASE OF ALKALOIDS BY S. NIGRUM CELLS

Yoshida and Niki (1979) have demonstrated that when callus of Cornus stolonifera was chilled for at least 48 hours, there occurred an increased permeation of the cells by dopamine and other amines. In this experiment, cells of S. nigrum were chilled to see whether such a treatment would cause the release of alkaloids.

Between 2 g and 2.5 g of stem callus of S. nigrum (which had been maintained for 13 months on full MS medium, and had been allowed to grow for 27 days after the previous subculture) was put into each of six 250 ml Erlenmeyer flasks containing 50 ml of full MS liquid nutrient medium. Three 'control' flasks were allowed to stand (i.e., stationarily, not shaken) under normal callus culture conditions ($25^{o}C \pm 1^{o}C$) and three 'chilled' flasks were kept at

$3^{o}C \pm 1^{o}C$ in a cold room (at an illuminance of approximately 170 $\mu Em^{-2}sec^{-1}$). All flasks were swirled by hand twice a day to ensure aeration of the medium. At day 0 and after 7 days, the percentage viability of the cells was determined, and the cells and medium at day 7 were analysed for the presence of alkaloids.

(a) Viability of the Cells

The results of the cell viability determinations are given in Table 3.5.2. It can be seen that the control cells maintained a high percentage viability (approximately 70%) - the cells therefore were aerated sufficiently. The chilling of cells to $3^{o}C$ did not severely reduce the viability, which was a mean of 64% at the end of the experimental period.

(b) Alkaloid Content of the Cells

The alkaloid content of the cells was estimated by the cell-squash method and the results are given in Table 3.5.1. The cells of the two treatments showed no real difference in the degree to which they accumulated alkaloids, the mean I value for each being approximately 3 (representing approximately 14-16 mg alkaloids/g dry weight callus). Immersion and chilling of the cells did not affect the ability of the cells to accumulate alkaloids.

(c) Alkaloid Content of the Medium

At the end of the 7 day culture period, the nutrient medium (150 ml per treatment) was filtered and reduced in volume, on a vacuum rotary evaporator (at $40^{o}C$) to approximately 1-2 ml. This

Table 3.5.1

Alkaloid content of chilled and unchilled callus of S. nigrum before and after 7 days treatment

| | MEAN I VALUE | | |
|---|---|---|---|
| Replicate | Control Cultures | Chilled Cultures | Day 0 Cultures |
| 1 | 3.0 | 2.8 | 2.9 |
| 2 | 3.1 | 3.0 | 3.1 |
| 3 | 2.8 | 2.8 | 3.1 |
| | $\bar{x}$ = 3.0 | 2.9 | 3.0 |

Table 3.5.2

Percentage viability of chilled and unchilled callus of S. nigrum before and after 7 days treatment

| Replicate | Control Cultures | Chilled Cultures | Day 0 Cultures |
|---|---|---|---|
| 1 | 74.9 | 62.4 | 73.4 |
| 2 | 70.6 | 66.7 | 68.2 |
| 3 | 64.9 | 63.0 | 71.1 |
| | $\bar{x} \pm$ S.E. = 70.1 $\pm$ 0.1 | 64.0 $\pm$ 0.0 | 70.9 $\pm$ 0.0 |

concentrate was then both spotted onto filter paper and dropped (approximately 0.25 ml) onto white enamel, and tested for the presence of alkaloids with Dragendorff's reagent.

The control-culture medium was found to contain no detectable levels of alkaloids; the chilled-culture medium gave a very faint precipitation reaction in solution (i.e., on the white enamel), but there was no visible colour reaction on the filter paper - this suggests that some alkaloids were released by the cells, but only to a very limited extent.

## 2. THE EFFECTS OF CHLOROFORM ON THE RELEASE OF ALKALOIDS BY S. NIGRUM CELLS

Brodelius et al. (1979) discovered that immobilised cells of Catharanthus roseus released alkaloids into the nutrient medium, and it was suggested that this was due to the presence of trace quantities of chloroform accidentally present in the culture apparatus rather than because of any inherent property of the cells.

To investigate this possibility and to assess the usefulness of a solvent as a means of inducing secondary metabolite release to obtain continuous production, chloroform was added to cultures of S. nigrum and the medium was analysed.

Approximately 5 g of stem callus (which had been maintained for 14 months on full MS medium, and allowed to grow for 25 days after the previous subculture) was put into each of six 250 ml Erlenmeyer flasks containing 50 ml of full MS liquid nutrient medium; three 'test' flasks also contained 1 ml chloroform, and all six

were allowed to stand under normal callus conditions for the duration of the experimental period. Each was swirled by hand twice a day to ensure aeration of the medium and dispersion of the chloroform; after 8 days the cells and medium were analysed for the presence of alkaloids, and determinations were made of percentage cell viability.

(a) Alkaloid Content and Viability of the Cells

The alkaloid contents of the cells were estimated by the cell-squash technique (both before and after treatment) and are given in Table 3.5.3, along with the percentage cell viability data. It can be seen that the effects of the chloroform treatment were dramatic - most of the cells treated were killed, and they contained no detectable levels of alkaloids; the control cultures, on the other hand, contained levels comparable to those found in the day 0 cultures. As was found above, immersion in liquid medium for 8 days in itself did not affect the ability of the cultures to accumulate alkaloids, and percentage cell viability was relatively high at between 60% and 70%.

(b) Alkaloid Content of the Medium

The medium (150 ml per treatment) was filtered, and concentrated up to 2 ml and analysed as described for the chilling experiment above. The control culture medium was found to contain no detectable levels of alkaloids; the chloroform-treated culture medium, on the other hand, did contain detectable quantities of alkaloids - a spot of the concentrate gave an I value of 3-4, indicating the presence in the 150 ml of medium of about 3 mg of glyco-alkaloids.

Table 3.5.3

Alkaloid content and viability of chloroform-treated and untreated cells of S. nigrum

| | Mean I Value | | | % Cell |
|---|---|---|---|---|
| | Day 0 | Day 8 | | Viability (Day 8) |
| Replicate | Cells | Treated | Controls | Treated | Controls |
| 1 | 2.4 | ND | 2.7 | 4.2 | 69.2 |
| 2 | 2.8 | ND | 2.8 | 3.0 | 62.9 |
| 3 | 2.8 | ND | 2.0 | 6.1 | 70.4 |
| $\bar{x}$ = | 2.7 | - | 2.8 | 4.3 ± 0.1 | 67.6 ± 0.1 |

Therefore, the effect of chloroform was to kill the cells but cause them to release alkaloids.

## THE EFFECTS OF CHLOROFORM ON THE RELEASE OF ALKALOIDS BY IMMOBILISED CELLS OF S. NIGRUM

The aim of this experiment was to cause the release of alkaloids from immobilised S. nigrum cells by chloroform treatment. By adding chloroform to the medium of the reservoir, where it accumulated in the bottom part of the reservoir tube, it was thereby supplied to the cells at a lower concentration than in the previous experiment. The objective was that the viability of the cultures would be reduced less severely than in the previous experiment.

Six columns were set up as described previously, using cells which had been subcultured in full MS liquid medium for 4 months. All six columns were supplied with 50 ml of full MS liquid medium, but the medium of three 'test' columns contained 1 ml of chloroform (added to the medium reservoir). At the end of a 3 week period of culture (under the usual column culture conditions) the alkaloid content of the medium and cells was investigated as was the cell viability.

### (a) Alkaloid Content and Cell Viability

The results of the determinations of cell alkaloid content (by the cell-squash technique) and viability are given in Table 3.5.4. The chloroform treatment caused a lowering of both cell viability and levels of accumulated alkaloids in the cells compared with the controls (approximately 10% cell viability compared with 54%, and a

Table 3.5.4

Alkaloid content and viability of chloroform treated and untreated immobilised cells of S. nigrum

| | Mean I Value | | | % Cell | |
|---|---|---|---|---|---|
| | Day 0 | Day 21 | | Viability (Day 21) | |
| Replicate | Cells | Treated | Controls | Treated | Controls |
| 1 | 2.5 | 1.1 | 2.9 | 15.3 | 55.1 |
| 2 | 2.6 | 1.3 | 2.7 | 4.1 | 48.2 |
| 3 | 2.6 | 1.1 | 2.7 | 11.5 | 57.9 |
| $\bar{x}$ = | 2.6 | 1.2 | 2.8 | 13.1 ± 1.1 | 54.0 ± 0.1 |

mean I value of about 1 (i.e., about 10 mg alkaloids/g dry weight cells) compared with about 3 (i.e., about 15 mg/g dry weight) respectively).

(b) Alkaloid Content of the Nutrient Medium

The nutrient medium (150 ml per treatment) was filtered, evaporated (in vacuo at 40°C), and the residue taken up in 3 ml of distilled water and analysed as described for the chilling experiment above. The control culture medium was found to contain no detectable levels of alkaloids; the chloroform-treated culture medium, however, reacted positively with Dragendorff's reagent, a spot of the concentrate registering an I value of 2, indicating the presence of approximately 3.5 mg of Solanum alkaloids per 150 ml medium.

These results therefore confirm those of the previous experiment, namely that chloroform causes alkaloid release from the cells. There was some qualitative difference in effect of the chloroform treatment in this and the previous experiment: the treated immobilised cells contained higher levels of alkaloids than did the callus cells in liquid medium (in which no alkaloids at all were detectable), and the percentage cell viability of the immobilised cells was slightly less severely affected than it was in the callus - this may be a result of the fact that, in the previous experiment, the cells were in direct contact with the chloroform, while in this column experiment, the chloroform sank to the bottom of the medium reservoirs and was available to the cells in, effectively, lower concentrations, being only slightly soluble in water.

## A BRIEF SUMMARY OF RESULTS PART 5

Cells of S. nigrum were subjected to chilling and chloroform treatment, to induce the release of alkaloids. The chilling of cells to $3^{o}C$ caused a small release, but a larger effect was produced by using chloroform. At the concentrations employed, a large reduction in the viability of the cultured occurred, but the results suggest that by using lower concentrations of the solvent, the viability of the cells would not be so severely affected. A shorter duration of chloroform treatment, or the use of less toxic solvents, might prove more effective.

# CHAPTER 4

# DISCUSSION

The synthesis and accumulation of secondary metabolites in whole plants is often closely associated with the differentiation and maturation of organs and tissues. An obvious example is given by the development of tracheidal and vessel elements of the xylem (O'Brien, 1974). Here, the laying down of lignin in the cell wall is the final phase of a differentiation process in which cells, which originated in the procambium, are prepared for the transport of water and solutes. Furthermore, since secondary compounds are often accumulated during specific developmental periods, their synthesis and accumulation may be considered a facet of differentiation (Luckner, 1980).

For example, Pfeifer (1962) has shown that during the flowering stage of Papaver somniferum, all the alkaloids examined increased in quantity, and in this period, which lasted for two weeks, the greatest accumulation in the stems occurred. As maturation proceeded, accumulation in the capsules of morphine, narcotoline and papaverine became predominant, until the fully differentiated habit of the plant, which comprised mature capsules containing the highest levels of morphine alkaloids, was established. Similarly, Verzar-Petri (1966) has demonstrated that in six species of Datura studied over eleven developmental stages, peaks of total alkaloid content occurred at flowering and at the onset of fruit ripening. Iwai et al. (1979) have shown that capsaicinoids in Capsicum frutescens were first detected in fruits 20 days after flowering, and reached a maximal level at about 40 days after flowering. The main site of formation and accumulation of the capsaicinoids was in the placenta of the fruit. These results demonstrate an association between the differentiation and maturation of organs and tissues and the synthesis

and accumulation of secondary compounds. Indeed, the young seeds and fruits of all alkaloid-producing species usually accumulate alkaloids in abundance (Waller and Nowacki, 1978). Moreover, it is conceivable that the observed accumulation of alkaloids and other secondary metabolites in immature organs such as actively growing roots (Solt, 1957). is related to the fact that only a small proportion of the structure may be involved in active division, and that most of the organ has undergone differentiation. Obviously, the fact that a root is recognisable as such demonstrates that differentiation (i.e., both cyto-differentiation and the organisation of cells in the form of an organ) has occurred.

It is, however, the case that the site of accumulation may not also be the site of synthesis, but the metabolites may be transported from one organ to another; consequently, mature or differentiated structures may not accumulate secondary products, although they may have synthesised them. For example, the root systems of Solanaceous plants such as tobacco (Nicotiana tabacum) may accumulate relatively low levels of alkaloids compared with aerial parts; the compounds are mainly synthesised in the roots and are transported from them in the xylem (Waller and Nowacki, 1978). Conversely, mature structures may, in some species, accumulate but not synthesise secondary compounds. Ricinus communis plants can synthesise ricinine in mature leaves, young leaves and stems. Although the mature leaves contain the greatest quantities of ricinine weight for weight, the alkaloid has been shown to be synthesised from radioactively-labelled nicotinic acid most efficiently in (i.e., the highest specific activity was found in) the younger leaves. Moreover, yellowing leaves were found to translocate ricinine to the younger leaves (Waller and Nowacki, 1978).

Despite complications in the interpretation of data imposed by the transport of metabolites, it is apparent that the accumulation of secondary metabolites is greatest in mature, highly differentiated organs and tissues. The relationship between secondary metabolite production and differentiation and growth in cell cultures is now discussed.

## THE RELATIONSHIP BETWEEN ACCUMULATION, DIFFERENTIATION AND GROWTH IN CELL CULTURES

### (a) Growth Rate, Friability and Accumulation of Alkaloids

Results described in Chapter 3, Parts 1 and 2 show that fast-growing and friable callus most often accumulated the lowest levels of alkaloids, whereas callus (of the same species), in which the cells were more compactly associated (or even structurally differentiated, such as S. dulcamara) and were growing relatively slowly accumulated higher levels of alkaloids. Although it was found (Results, Part 1, Section 2) that friable callus of a number of species was composed primarily of actively-growing cells, and Grant and Fuller (1968) similarly found that friable root callus of Vicia faba contained a greater proportion of meristematic cells than did the non-friable type, it is recognised that friability of callus does not necessarily imply that it is growing rapidly (Yeoman and Street, 1977 ) . Nevertheless, fast-growing calluses were usually friable, and accumulated low levels of alkaloids. This observation is reflected in the fact that cell suspension cultures, composed mainly of friable and relatively rapidly-growing cells, tended to accumulate lower levels of alkaloids than did callus

cultures, which grew more slowly and were more compact. The results of the two growth curve experiments (Results, Part 2, Section 1) support this observation. In both the callus cultures and cell suspension cultures, the levels of alkaloids were lowest when the cells were both growing relatively fast and were friable, in the exponential and linear phases of their growth cycles, and greatest when the cells were at their most dense and lowest growth rate, at the stationary phase. The protein/alkaloid-labelling experiment (Results, Part 2, Section 2) demonstrated that in relatively fast-growing and friable cell suspension cultures of D. innoxia, the amount of radioactivity incorporated into alkaloids was lower than in slower-growing and more dense cultures. In the linear phase cells, comparatively more label was present in proteins than in alkaloids, while in the stationary phase cells comparatively more label was present in alkaloids than in proteins. While recognising the intrinsic difficulties in interpreting results of this type of experiment (described in Results, Part 2, Section 2), it may perhaps be concluded that more alkaloids (both qualitatively and quantitatively) are accumulated in slower-growing, more dense cell cultures. A similar result was obtained for phenolics production in cell suspension cultures of Acer pseudoplatanus by Phillips and Henshaw (1977). By inducing an increase in the growth rate and friability of D. innoxia callus by reducing the concentration of the growth regulator 2,4-D, the levels of alkaloids accumulated dropped significantly (Results, Part 2, Section 3). A similar result was obtained by Tabata and Hiraoka (1976), who found that by increasing the growth rate of tobacco callus by increasing the concentration of 2,4-D in the nutrient medium, the levels of nicotine produced by the callus dropped dramatically.

The concept of a positive correlation between slow growth and compactness in cell cultures may be extended to encompass the observation that highly organised embryoid and shoot-like structures were formed in cell suspension cultures of A. belladonna and S. nigrum only late in the growth cycle, and moreover, accumulated relatively high levels of alkaloids (Results, Part 1, Section 2). Similar observations have been made by Thomas and Street (1970).

Nevertheless, there appear to be one or two exceptions to this general relationship between accumulation, growth rate and differentiation. Kaul and Staba (1968) and Kaul et al. (1969) found that undifferentiated cell and tissue cultures of Dioscorea produced relatively high levels of the steroid diosgenin. However, it has been demonstrated (Yeoman et al., 1982) that diosgenin is accumulated maximally, in cell suspension cultures of Dioscorea composita, during the stationary phase of growth, a pattern of accumulation which, in fact, supports the hypothesis. Furthermore, Zenk et al. (1975) found that, by using a nutrient medium which was suboptimal for the growth of suspended cells of Morinda citrifolia, the rate of anthraquinone formation was maximal in the linear phase of growth; accumulation, however, was maximal in the stationary phase. Moreover, the levels produced exceeded those produced by root tissue. However, nutrient media which allowed optimal growth rate inhibited anthraquinone production by more than 80%, and completely undifferentiated cell cultures, composed of about 60% single cells or pairs of cells, showed a complete absence of anthraquinones. Radwan and Kokate (1980) claim that trigonelline production by Trigonella foenum-graecum cell suspension cultures exceeds that by intact seeds, roots and shoots, but further data on the kinetics of production in this system might

aid the interpretation of this finding.

These results therefore are consistent with the view that there is an inverse relationship between both the alkaloid content and compactness or organisation of cultured cells, and their growth rate, as suggested in the Introduction to this thesis (p.9).
A further feature of cells which was also found to be inversely correlated with growth rate,and is an aspect of differentiation, is chlorophyll formation. This is now considered.

(b) The Relationship between Greening and the Accumulation of Alkaloids

In results described in this thesis, the 'greening' of cells was often associated with slow cell growth, with the organisation of cells, and with the accumulation of relatively high levels of alkaloids. This could be due simply to an induction of chlorophyll accumulation, but Laetsch and Stetler (1965) have shown that chloroplast development can be correlated with the growth rate of tobacco callus. They discovered that actively-growing cells produced only partially-differentiated chloroplasts, while older callus, which was past the exponential phase of growth, contained chloroplasts as fully differentiated as those in the mature leaf. It is therefore possible that the 'greening' phenomenon described (Results, Part 1 and 2) was the result of the onset of chloroplast development rather than simply of chlorophyll accumulation.

The results of the growth curve experiment with suspended cells of S. nigrum (Results, Part 2, Section 1) describe the presence in the cultures of green clumps or balls of cells which were very compact : much more so than the friable and pale-coloured smaller

cell aggregates which, especially in the exponential and linear phases of the growth cycle, constituted the majority of the culture. The green and compact cell clumps invariably contained relatively high levels of alkaloids. It is apparent from these results that heterogeneity with respect to greening occurred both within a culture at a particular time (a structural heterogeneity) and at different times throughout a growth cycle (a temporal heterogeneity). Such a view is supported by the empirical observation that cell suspension cultures of S. nigrum, D. innoxia and A. belladonna often turned green if the passage period (i.e., the length of time the cells were grown in a particular flask) was extended beyond the time when they would normally have been subcultured. Such a temporal heterogeneity was similarly observed and quantified in the S. nigrum callus growth curve experiment (Results, Part 2, Section 1). Moreover, by inducing rapid cell growth and friability using 2,4-D (Results, Part 2, Section 3), the chlorophyll content of D. innoxia callus dropped significantly, and increased as the cells were induced to grow more slowly.

From these observations it might be concluded therefore, that the production of green cultures is necessary if cells are to accumulate high levels of alkaloids. Idrisova et al. (1978) have found that the combination of nutrients in the culture medium of D. innoxia callus which allowed the greatest accumulation of alkaloids also resulted in the greatest accumulation of chlorophyll. Although the greening of cultures has been associated in many cases with the accumulation of relatively high levels of alkaloids (Results, Parts 1 and 2), the observation that colourless callus of S. dulcamara was also capable of accumulating high levels suggests that greening

is not an absolute requirement for high yields. Staba and Jindra (1968) have found that there was no difference in the levels of alkaloids produced in chlorophyllous or non-chlorophyllous strains of D. innoxia.

These results do not necessarily suggest, however, that the biogenesis of chlorophyll or chloroplasts (active or partially active) would have no effect on the levels of metabolites produced. Rather, the converse would be expected to hold true, for the chloroplast has a dominant role in regulating cell chemistry, not merely by supplying carbon skeletons for a wide variety of metabolic pathways, but in numerous other ways, including the provision of reducing power, effects on cell pH, the synthesis of enzymes, etc. (see Barber, 1976). Nevertheless, as far as secondary metabolism in cell suspension and tissue cultures is concerned, it seems that slow growth and especially compactness (cell organisation) are primary determining factors. The high-yielding callus of S. dulcamara discussed above contained no green plastids, but it was relatively very slow-growing and compact. Taken to an extreme, this compactness of the cells takes the form of more obvious organisation, with the formation from within the callus of green shoots and roots (found in callus of S. dulcamara and S. nigrum); these organs always originated from compact callus. Similarly, the embryoid and shoot-like structures found in suspension cultures of A. belladonna and S. nigrum were only present late in the growth cycle, usually during extended passage periods, and were never present in friable and rapidly growing cultures.

The relationship between the growth rate and organisation of cultured cells, and their importance in the regulation of alkaloid production, is now considered.

## THE INTER-RELATIONSHIPS BETWEEN GROWTH AND SECONDARY METABOLITE PRODUCTION

Evidence presented both in the results of this thesis and in the literature (described in Chapter 1) indicates a strong correlation between slow growth and increased production of secondary metabolites in cell cultures. The use of inhibitors of growth, particularly of protein and RNA synthesis (see Yeoman et al., 1980) has demonstrated that by slowing down growth, the production of at least some secondary metabolites can be enhanced.

The role of cell growth rate in the regulation of secondary metabolite production is, at present, little more than a subject for speculation. It is known that changes in the activity of a number of enzymes are associated with changes in the growth rate of cell cultures (Davies, 1972; Forrest, 1969; Hahlbrock et al., 1971; Mäder et al., 1975; Nato et al., 1977; Thorpe and Meier, 1973; Westcott and Henshaw, 1976; Zeleneva and Khavkin, 1980). Furthermore, Mäder et al. (1975) demonstrated that, by the artificial inhibition of the growth of Nicotiana tabacum callus, it was possible to induce a peroxidase isoenzyme pattern characteristic of that found at the onset of differentiation.

What then are the inter-relationships between growth and metabolism, and how can these be explained in relation to the obviously important role of cell organisation and differentiation?

Results in this thesis provide evidence for at least two types of mechanism. The first is based on the concept that the growth itself regulates secondary metabolite production only indirectly, by determining the extent of cell aggregation. Second is a kinetic hypothesis, in which different metabolic pathways compete differentially in fast- and slow-growing cells. These two possibilities are now discussed.

## 1. The Relationship between Growth and the Utilisation of Alternative Metabolic Pathways as a Result of Cell Organisation

The possibility that the growth of cells *per se* may be unrelated to the levels of alkaloids accumulated is suggested by, for example, the observation that slow-growing hard brown callus of *Lupinus angustifolius* accumulated lower levels of alkaloids than did faster-growing, but green and clumped, callus which proliferated from the brown callus (Table 3.1.4). It is therefore proposed that culture growth rate may *indirectly* influence the production of secondary metabolites by affecting the degree of organisation or cell-cell contact. If a rapid growth rate results in a more friable, low-yielding culture, as has been suggested above, then the role of cell growth rate may be a crucial one, for there is little doubt that structural differentiation or partial structural differentiation (including cell clumping) in cultures will result in high levels of accumulation (Al-Abta *et al.*, 1979; Bhandary *et al.*, 1969; Brunet and Ibrahim, 1973; Freeman *et al.*, 1974; Hiraoka and Tabata, 1974; Hirotani and Furuya, 1977; Jalal *et al.*, 1979; Krikorian and Steward, 1969; Mackenzie *et al.*, 1974;

Nikolaeva and Vollosovich, 1972; Tabata et al., 1972; Thomas and Street, 1970). In other words, cell aggregation seems to be essential for the production of high-yielding cultures (also suggested by Carceller et al., 1971), and an adequate degree of aggregation may be possible only in slow-growing cultures.

In Results Parts 1 and 2, greening of cultures was found to be associated with the accumulation of relatively high levels of alkaloids. The results indicate however that greening may not be a causative factor in alkaloid production; it is nevertheless associated with high-yielding organised cells and may therefore be a corollary of the organisation of cells as a result of, or permitted by, a reduced growth rate. The production of chloroplasts may nevertheless be important. The alkaloids accumulated in organised and green structures in vitro are often found to be qualitatively and quantitatively similar to those of the intact plant (Hiraoka and Tabata, 1974; Ikuta et al., 1974; Tabata et al., 1972) whereas not only a quantitative but also a qualitative difference has been observed between undifferentiated cultured cells and whole plant alkaloids (Boulanger et al., 1973; Forrest, 1969; Ibrahim et al., 1971; Jalal et al., 1979; Sejourne et al., 1981). This observation, together with the observation that differentiated structures tend to produce chloroplasts, suggests the (perhaps obvious) conclusion that the more similar, physiologically, the cells of a culture are to the cells of a whole plant, then the more likely they are to metabolise (i.e., produce secondary compounds) like a whole plant. In other words, by undergoing organisation and greening, cells tend towards the 'normal' (i.e., intact) plant habit, with the result that they are then capable of a more 'normal'

secondary (and presumably also primary) metabolism. The results in this thesis show that this phenomenon is most likely to occur in relatively slow-growing cultures.

What role does cell aggregation or organisation play in the organisation of metabolism? Since the regulation of alkaloid and other secondary metabolite production seems to be under epigenetic control, a view supported by the growth curve and growth regulator experiments (Results Part 1, Section 2) and the 'drip-area' experiment (Results Part 3, Section 3), then by definition any cytoplasmic changes may result in qualitative or quantitative changes in the production of secondary metabolites. A consideration of the environment of a cell is therefore appropriate.

All cells in the whole plant are surrounded by other cells, but the relative position of a cell with respect to other cells may vary throughout its life history. Moreover, the position of the cell in the plant is determined ultimately by the rate and pattern of division of it and the surrounding cells, and the degree and form of differentiation of that cell is characteristic of its position in the plant body. Thus, the question of the role of cell organisation in the regulation of metabolism can be rephrased to ask, how does the physical environment of a cell affect its metabolism? Obviously this is an enormous topic, but some examples are immediately obvious.

Roberts (1976), for example, has pointed out that although the genome contains all the genetic information necessary for the development of the cell and thence whole plant, it is the cytoplasmic environment which is responsible for the 'cueing' of developmental sequences. This cytoplasmic environment is, of course, dynamic and

under the influence of a wider environment, namely the body of the plant (or in the case of cell cultures, simply the surrounding cells) and also the ambient atmosphere or liquid nutrient medium. Of the 'external' environment, the two most important factors affecting metabolism are the levels of oxygen/carbon dioxide and light. The roles of light in the control of cell growth and metabolism are undoubtedly diverse. Although cultured cells are not usually autotrophic (Laetsch and Stetler, 1965), some are (Nishida et al., 1980; Yamada et al., 1981) and illumination will have obvious consequences. Even in heterotrophic, non-green cultures (such as the immobilised cells of S. nigrum, Results Part 4, Section 2) the role of light will be important : there are present in cells a variety of non-photosynthetic pigment systems which when illuminated may produce a number of physiological effects, including changes in the pattern of cell division and microfibril orientation (see Roberts, 1976). Moreover, certain enzymes are activated by light (e.g., Attridge, 1974 (ascorbic acid oxidase); Beale, 1978 (enzymes of chlorophyll biosynthesis); Grisebach and Hahlbrock, 1974 (flavonoid biosynthesis); Kobayashi et al., 1980 (enzymes of photosynthesis); Murphy and Stumpf, 1979 (fatty acid biosynthesis); Scheibe and Beck, 1979 (malate dehydrogenase); Tobin and Suttie, 1980 (ribulose diphosphate carboxylase); Zucker, 1972, review). The intensity and wavelength of light reaching a cell will be determined in part by its position within the cell mass, and the response of the cell to the stimulus will therefore be affected by the organisation of the tissue (see review by Dale, 1976; Roberts, 1976; Yeoman and Davidson, 1971).

Not only physical but also chemical gradients will exist in organised tissues. A gradient of oxygen concentration will exist

from the inside to the outside of an organised structure or aggregation of cells, with the result that the metabolism and development of a large cell clump, with a small surface area : volume ratio, may be different to that of isolated cells or smaller groups with larger surface area : volume ratios. Dalton and Street (1976) have shown the importance of low oxygen tension in the promotion of chloroplast differentiation in spinach cell suspensions, and high levels of $CO_2$ have been shown to enhance cytodifferentiation in peach mesocarp tissue cultures (Bradley and Dahmen , 1971). Similarly, gradients of growth regulating substances and nutrients will also be set up, again providing potential for differential metabolism between organised and unorganised cells.

The level and type of mechanical pressure to which a cell is subjected will depend on its position relative to surrounding cells. As discussed by Yeoman and Brown (1971) one would therefore expect this to affect metabolism - they examined the effects and consequences of stress on the plane of division and concluded that mechanical pressure can lead to the production of cells with diverse developmental (metabolic) capacities.

Apart from the setting up of physical and chemical gradients, the organisation of cells may affect metabolism by changing the ultrastructural organisation of the cells; this is now briefly discussed. The activity of an enzyme system may be regulated by the availability of substrate or cofactors : the division of the cell into a number of sealed, but potentially permeable, compartments would allow the reactions of particular metabolic pathways to proceed at particular times while others would be restricted, and

a number of examples of this type of control of metabolism are well known. Particular enzymes are known to be associated with particular membrane fractions, most notably with mitochondria and chloroplasts, but also with the plasmalemma and other subcellular organelles such as microbodies, Golgi apparatus and peroxisomes (reviewed by Hanke, 1977). The membranes provide barriers to molecules, and so act in a 'limiting capacity', and also provide surfaces at which molecules can interact. In the latter case the metabolic reactants do not move about randomly and so interact randomly in a free solution, but can be brought together at, as it were, 'one point' in the cell to produce increased concentrations of reactants and permit vectorial enzyme arrangements and reactions. There is little doubt that the endomembrane system of the cell is directly involved, therefore, in the processes of cell differentiation and secondary metabolite production. There are examples of the possible involvement of endomembrane changes (both spatial and permeability changes) in the differentiation of vascular tissues (e.g., Cronshaw, 1974; Northcote, 1968; O'Brien, 1974); the arrangement of the cytoplasm, in these cases, reflects the pattern of structural differentiation of the cell. Although this does not necessarily imply control of differentiation, it is tempting to suggest that the organisation of the different membrane fractions and their associated enzyme systems will act in determining the pattern of development of the cell.

The association of changes in subcellular organisation with changes in cell growth rate and organisation (without, necessarily, any structural differentiation such as secondary wall formation and lignification) is beyond doubt. The difference in the extent of vacuolation of fast- and slow-growing callus cells of A. belladonna

and S. dulcamara were noted in 'Results' Part 1, Section 2, and, similarly, noticeable cytoplasmic changes take place throughout the growth cycle of suspended cells (e.g., Yeoman and Street, 1977 ) . As discussed above, chloroplast development has been correlated with changes in cell growth rate (Laetsch and Stetler, 1965).

This discussion of the possible roles of organisation in the control of metabolism has, therefore, attempted to bring attention to the fact that organised and unorganised cells have different cytoplasmic environments and therefore different patterns of metabolism. It has been proposed that the growth of cells will determine to some extent their organisation and hence ability to produce secondary metabolites.

A second, kinetic, hypothesis for the regulation of metabolism is now discussed.

## 2. The Relationship between Growth and the Utilisation of Alternative Metabolic Pathways as a Result of Kinetic Compartmentalisation

If environmental conditions prevail which are favourable to rapid cell growth, then, obviously, the metabolic pathways required for rapid growth will operate. If, for any reason, rapid growth is blocked, for example by a depletion of one or more essential nutrients, then some 'primary' pathways will become inoperative and 'secondary' pathways, such as alkaloid biosynthesis, will come into play. As discussed in Yeoman et al. (1980, 1982), such a change in metabolism might be a relatively simple diversion of precursors from one set of enzymes, leading to one type of product, to another set

of enzymes, leading to a second type of product. Furthermore, both enzyme systems may be continuously present in the cell and not synthesised de novo, whether or not they are in use (Aitchison, 1977, unpublished data; Banthorpe, 1979, 1980, unpublished data, quoted in Yeoman et al., 1982; Mizukami et al., 1977; Neumann and Mueller, 1971, 1974; Ramawat and Arya, 1979).

This phenomenon has been discussed above as a function of cell organisation and its resultant effects on physical and chemical gradients and subcellular compartmentalisation. This second hypothesis attempts to explain the observed 'switching' between primary and secondary metabolism as not a consequence of physical and chemical organisation, but as a result of competition between alternative metabolic pathways for common precursors. Here it is proposed that alternative biosynthetic pathways, such as those of protein and alkaloid biosynthesis, which appear to work antagonistically, are either utilised or not utilised simply depending on whether one or other of the reactions is kinetically favourable. In a situation where pathways A and B have the same precursors, then if reaction A goes faster than reaction B, most or all of the precursors will be used for the more demanding reaction. The enzymes in pathway B would be redundant until reaction A stops or slows down sufficiently so that precursors accumulate to such a level that reaction B can proceed. So, it can be visualised that in fast-growing cells, the pathways of protein synthesis are highly active, and amino acid precursors are utilised in this process in preference to any secondary pathway. On the slowing down of growth, protein synthesis will be reduced, perhaps as a result of feedback inhibition of enzyme activity, causing a diversion of amino acids into alkaloid

biosynthesis. This type of hypothesis has similarly been proposed by Phillips and Henshaw (1977). They suggest that, in suspended cells of Acer pseudoplatanus, the rate of phenolics synthesis is limited by the rate of protein synthesis, and, in stationary phase cells, the accumulation of phenolics proceeds as the result of the diversion of precursors from protein synthesis to phenolics synthesis. Such a possibility has also been discussed by Davies (1972).

As in the 'cell organisation' hypothesis above, it is suggested that the availability of substrate is a factor in determining the rate of reactions, but the difference between the two lies in the postulate that, in this case, a kinetic separation of enzyme and substrate is involved rather than a separation as the result of physical compartmentalisation. This is obviously a highly simplistic model, which in this form takes no account of differences in $K_M$ values of the enzymes in the alternative pathways. Nevertheless, both hypotheses have attempted to elucidate the relationship between growth and accumulation of secondary metabolites.

How then does this information aid the cell culturist interested in increasing the yields of specific secondary metabolites? An obvious approach to present itself from this work is to manipulate the culture conditions in such a way that the growth of the cells is slowed down, followed by an addition of precursors of the desired compounds and the prevention of feedback inhibition of secondary metabolite biosynthesis, by inducing the export of the compounds from the cells into the surrounding medium (unless this occurs naturally). The approach to these problems undertaken in this thesis is now discussed.

## THE MANIPULATION OF THE ENVIRONMENT

There are a number of problems associated with the traditional techniques for cell culture with regard to the manipulation of the environment of the cells. For example, if the nutrient medium supplying callus or liquid-suspended cells is to be altered, then physical manipulation of the cells is inevitable, and may result in the damage and contamination of the cultures. Furthermore, the addition of possibly toxic precursors to the nutrient medium is limited to low concentrations if the viability of the cultures is to be maintained. Agar culture, however, does have a number of advantages over suspension culture. The cells grow in close association with each other and are physically stationary, with the result that a variety of physical and chemical gradients can be established. Moreover, suspended cells appear to have particularly unusual metabolic activities. For example, Zeleneva and Khavkin (1980) have found that, in callus cells, the activities of a number of enzymes are similar to those in the whole plant, but this was not so for suspended cells. It was concluded that the metabolism of suspended cells is probably unique, and dissimilar to that of the whole plant, due to the peculiarity of their environment. This evidence suggests that suspended cells in general would be unsuitable for the production of high yields of secondary compounds; physically stationary cultures, in which differentiation is a more commonly observed phenomenon, would seem to be a better source of metabolites. Thus, the chemostat, in which the manipulation of the chemical environment can be performed with relative ease, is not an ideal culture technique for this purpose, simply because the cells are suspended and dispersed and are not therefore subjected to the

gradients which seem to be essential for the production of high yields of metabolites.

As stated in the introduction, it was considered that the flatbed- and column-culture systems used in this thesis were particularly suitable for use in the production of secondary metabolites, for four main reasons : (1) the cells grow in close association with each other; (2) the cells are grown in a physically stationary condition, so that gradients will be established; (3) sequential chemical treatments can be performed with relative ease; and (4) large amounts of precursors can be supplied to the cells at low concentrations. It is now possible to evaluate further these culture systems in view of the experimental results obtained.

## THE GROWTH AND MANIPULATION OF CELLS CULTURED ON THE FLATBED

The results discussed so far suggest strongly that, in order to induce cultured cells to produce levels of specific secondary compounds approaching those present in whole plants, it is important that the cells should be allowed to partially differentiate. There are two main characteristics of the flatbed system which contribute specifically to this approach, namely the cells are capable of growing while physically stationary and in close association with each other, and secondly, the composition of the medium can be altered with ease so as to supply an 'inductive' medium; this might lack an essential nutrient or contain different concentrations of growth regulating substances, to artificially reduce the cell growth rate.

Let us now consider the first characteristic, i.e., the growth habit of the cells. The results of the drip-area experiment (Results Part 3, Section 3) have demonstrated well the 'reversible' nature of cells with respect to alkaloid accumulation. Drip-area cells effectively de-differentiated when grown as cell suspensions, but could revert (differentiate) to drip-area cells when returned to the flatbed. Similarly, despite the fact that the flatbed-grown cells were obtained from rapidly growing and friable suspension cultures, they acquired, after a week or so, certain physical and chemical similarities with a callus culture. The first similarity is that mentioned above, namely that the cells grew in closer association than when in liquid culture. Although the extent of the communication between the cells is not known, there was nevertheless observed much more clumping of the flatbed cells than was observed in fast-growing suspension culture. Moreover, the flatbed cultures grew more slowly than did suspension cultures. Because of the physical nature of the flatbed cells, there would be developed in the cultures physical and chemical gradients analagous to those associated with callus cultures, gradients of oxygen and carbondioxide, nutrients, growth regulating substances, light, temperature, gravity and mechanical pressure, resulting in a polarity of the cultures. Most importantly, the flatbed cultures, like callus cultures, accumulated higher levels of alkaloids than did fast-growing friable suspension cultures.

The second feature of the flatbed to be considered is that concerned with the manipulation of the nutrient medium to induce a slowing of the culture growth rate. There has been a vast amount of work carried out on the hormonal and nutritional control of the

production of alkaloids and other secondary metabolites by cultured cells, and this has been reviewed by, for example, Everett et al. (1978) and Floss et al. (1974). In this thesis no such deliberate nutritional manipulation was attempted, other than in the experiment which examined the effects of high and low oxygen levels (Results Part 3, Section 2). Nevertheless, the results described in Results Part 3, show that the flatbed-grown cells did come under nutrient stress, and very rapidly so. In all the flatbed experiments it was found, where examined, that orthophosphate was quickly removed from the nutrient medium, so that by day 2 of culture, for cells of both D. innoxia and S. nigrum, up to between 80 and 90% of the available phosphate had been taken up. It is therefore likely that the growth of the cultures would have been limited by the amount of phosphate in the medium, by a restriction of the synthesis of, in particular, nucleic acids, phospholipids and high-energy phosphates such as ATP. It is likely, therefore, that the growth of the cells would slow down in the early part of the culture period (i.e., in the first three or four days), resulting in an early 'stationary phase'. Not all of the phosphate or other nutrients examined were necessarily utilised immediately, because on what appeared to be cell lysis towards the end of the experimental periods, some was released back into the medium. Ammonia, nitrate and sucrose were never removed from the medium to the extent that they could be considered limiting.
It is possible that the uptake of these three nutrients after about day 4 was a reflection of an increased storage capacity of the cultured cells due to increased vacuolar volumes, often apparent in cells late in their growth cycle (Yeoman and Street, 1977 ) .

This 'nutritional stress' to which flatbed-cultured cells became subjected means that the flatbed system may be particularly useful for secondary metabolite production. Interestingly, Nettleship and Slaytor (1974) have found that of a variety of nutrient-limited media, phosphate-free medium was especially conducive to alkaloid and other secondary metabolite production by callus of Peganum harmala. The flatbed cultures did not appear to be under severe oxygen limitation, for the results of the oxygen limitation experiment (Results Part 3, Section 2) demonstrate that growth rate of aerated cultures was not significantly different to that of cultures grown under the usual conditions. Nevertheless, the results indicate that, by reducing the levels of oxygen available to the cells, it is possible to increase the alkaloid content of the cells, compared with the levels produced in aerated cultures. This phenomenon was associated with a reduced growth rate of the oxygen-limited cells.

The formation of drip-areas in flatbed cultures may be considered as a process of differentiation of the cells. There was evidence of clumping and the switching on of particular secondary metabolic pathways: the drip-areas developed a red/brown colour, and the levels of alkaloids accumulated were higher than in the rest of the culture.

It is difficult to explain satisfactorily this phenomenon from the available data, but one or two interesting points are raised. As briefly mentioned above, the formation of the drip-areas is obviously a readily reversible process, for when the drip-area cells were isolated and grown in liquid suspension they 'lost' the high

yield characteristic. This was regained when the cells were returned to the flatbed. This illustrates well the concept that the synthesis or accumulation of alkaloids is under epigenetic control, and demonstrates a difference in the biosynthetic capabilities of flatbed-grown and suspended cells. This phenomenon may be considered analogous to the regeneration of organs or plantlets from callus. In both situations, aggregated cells (which are more obviously organised in the case of organs) which accumulate relatively high levels of alkaloids, are produced from an undifferentiated mass of tissue. The production of suspension cultures from either regenerated or drip-area cells will result in a decrease in the levels of organisation and alkaloid production, a state which can be reversed by appropriate culture conditions. When considered in this way, the drip-area phenomenon seems slightly less enigmatic.

It is tempting to explain this in terms of the concept of 'physiological or nutrient stress', but there is little supportive evidence. It is not known whether the growth rate of the drip-area cells was any lower than that of the surrounding cells. Certainly, the percentage viability was 10-15% lower, and it might be expected that the growth rate was also reduced. If this was the case, then what was its cause? The results (Part 3, Section 3) demonstrated it was not likely to be a direct result of the mechanical pressure or effects associated with the 'drowning' of cells (i.e., oxygen stress, high levels of carbon dioxide, leaching of nutrients). Nevertheless, it should be noted that in oxygen-stressed flatbeds (Results, Part 3, Section 2) the drip-areas became progressively less distinct throughout an experimental period, suggesting that, even if a low oxygen level was not the direct cause of the formation

of drip areas, it may have a similar physiological effect. This could be interpreted as circumstantial evidence for the role of physiological stress in the production of drip-areas. Moreover, the presence of 5 mM ornithine in the dripping medium might have been expected to slow down the growth rate of the cells. However, it had no effect in increasing the levels of alkaloids in the drip-area cells (Results, Part 3, Section 2), and it is possible that the cells were already stressed. There may have been the imposition of stress associated with the localisation of nutrients in the drip-area, but at present this is impossible to define; perhaps relatively high concentrations of one or more nutrients adversely affected cell growth.

This discussion of the flatbed culture system has confirmed the importance of what were originally considered to be the main advantages of the technique; that is, the importance of promoting cell aggregation and of altering the composition of the nutrient medium to promote nutrient, or otherwise physiological, stress, to limit the growth of the cultures. In order to improve the suitability of the apparatus for scaling up for industrial use, but maintaining the advantages of the flatbed, a column culture system was developed and characterised. This is now discussed.

## THE GROWTH AND MANIPULATION OF IMMOBILISED CELLS

In order to fulfil the 'requirement' of the promotion of cell aggregation, it was considered important that cells should be grown physically stationary, immobilised along the length of a column in a supportive substratum. This would also prevent the packing of

cells at the bottom of the column. The immobilisation technique developed comprised the entrapment of cells inside a gel, which itself was strengthened by a basket-like nylon mesh. This pan-scrubber mesh was found to be non-toxic (Results Part 4, Section 1), and performed its expected function satisfactorily : even after a culture period of 10 days, the structure of the column was maintained. Of the two entrapment gels examined, namely agar and calcium alginate, the latter was the more useful. Although the percentage viability of the cultures was similar, at about 60%, under both immobilisation regimes, the cells grew better in the alginate, as demonstrated by the nutrient uptake data (Results Part 4, Section 2). The difference in the rate of uptake between cells in either gel may have been associated with the fact that the procedure for immobilisation in agar involved the suspension of cells in molten agar (of a temperature of about 40$^{o}$C), which may have damaged the cells. The levels of alkaloids accumulated were approximately the same in cells entrapped in either gel, and were equivalent in quantity to those found in callus or cells grown on the flatbed.

Cells immobilised in calcium alginate appeared to suffer from phosphate limitation by the eighth or tenth day of culture (Results Part 4, Sections 2 and 3). This was not the case for cells immobilised in agar, and such 'nutritional stress' of the cells suggests a greater potential of the alginate columns for secondary metabolite production. The uptake of phosphate was not as rapid as in the flatbed system, indicating that the cells were growing more slowly under the conditions of immobilisation. This may have been a direct result of entrapment, perhaps due to mechanical constraints on cell growth, but it seems more likely to be due to an

oxygen limitation in the columns. The oxygen levels in the medium supplying alginate columns of S. nigrum dropped to 0.014 μmol $O_2$ $ml^{-1}$ medium (i.e., 28% air saturation) after 8 days, a value lower than that found on the flatbed (Results, Part 4, Section 2). Certainly the available free space in the column apparatus was much less than in the flatbed. Since the extinction point (i.e., the concentration of oxygen below which fermentation occurs) is approximately 5% oxygen in the atmosphere, a level of 28% air saturation in the nutrient medium, representing a maximum of 6 or 7% available oxygen, almost certainly implies that the cells were subject to oxygen stress. This apparent oxygen limitation may therefore increase the potential of the column system for secondary metabolite production over that of the flatbed. A proportion of the orthophosphate uptake may have been due to its complexing with calcium ions in the gel matrix, resulting in the formation of calcium phosphate. This reaction might have been expected to weaken the structure of the gel (Ohlson et al., 1979), but no serious disintegration of the column was observed over a 10 day culture period.

Therefore, this discussion has shown that the column culture system, like the flatbed, allows cells to grow in close association and at a slow rate, and therefore satisfies two of the main features valuable for the production of high yields of secondary metabolites. The third main feature, namely the ability to supply cells with large quantities of precursors at low concentrations, is now discussed for both culture systems.

Since cells cultured on the flatbed and column culture systems appear to reach a premature stationary phase of growth, it would

therefore be expected that the addition to the nutrient medium of precursors of secondary metabolites would be particularly effective in enhancing production, assuming that precursors are limiting. Evidence in the literature for the promotion of alkaloid production by the exogenous application of precursors is described in the introduction to this thesis.

The results of the experiment in which ornithine was supplied to S. nigrum cells cultured on agar plates and on the flatbed apparatus (Results Part 3, Section 2) indicate that there is a small increase in the yield of alkaloids. It may be significant that, on the agar plates, a concentration of only 1 mM ornithine caused an obvious retardation of growth of the cells (but with no apparent effect on their viability). When this is considered against the background of the concept of the inverse relationship between growth and accumulation, it seems possible that the higher levels of alkaloids accumulated in the presence of ornithine could have been merely the result of a growth-inhibitory property of the precursor, rather than of a relief of precursor limitation. The smaller difference in effect of precursor treatment in the flatbed-grown cells than in the agar-grown cells may, if this is the case, have been because there was not a large reduction in the nutrient uptake (growth) of precursor-treated and control cultures. This may, in turn, have been because the cultures were growing slowly anyway, as a result of phosphate limitation, suggested in the data in Table 3.3.16 (Results Part 3, Section 2). This observation is consistent with the view that the effects of ornithine on alkaloid content observed in the agar-plate experiment were mediated through growth-inhibitory effects.

The results of the experiment in which immobilised cells of D. innoxia were supplied with 5 mM ornithine (Results Part 4, Section 3) showed that, as was the case for S. nigrum cells cultured on the flatbed, the precursor failed to promote a dramatic increase in the levels of accumulated alkaloids. The TLC data suggest that there was an increase in the quantity of scopolamine produced, a result which is consistent with the results of the experiment in which radioactively-labelled ornithine was found to be incorporated mainly into scopolamine, in the alkaloid fraction of fast- and slow-growing cells (Results Part 2, Section 2). When the results of both these experiments are viewed together, it seems that any increases in the levels of accumulated alkaloids as a result of the addition of ornithine to the medium supplying D. innoxia cells can be accounted for mostly by increases in scopolamine. As was found for the S. nigrum cells grown on the flatbed, 5 mM ornithine caused a small reduction in the nutrient uptake (growth) of the immobilised cells. This may have contributed to the slight promotion of alkaloid content.

The results of these precursor experiments therefore have shown that the feeding of 5 mM ornithine to cells grown on the flatbed and column culture systems resulted in small increases in the alkaloid content of the cells. It is likely that the yields of alkaloids could be increased in three ways. Firstly, the amount of precursor supplied could be increased or the culture period could be extended. Yeoman and Forche (unpublished data) found that ornithine and phenylalanine increased the quantities of tropane alkaloids in D. innoxia suspension cultures, but only after incubation

for three to six weeks. Secondly, the use of precursors closer to the desired product than ornithine would probably be more effective in increasing yields. Tropine or tropic acid have been found to be particularly useful in promoting tropane alkaloid production in cell cultures (Konoshima et al., 1970; Tabata et al., 1972). The third method, mentioned above, is the relief of feedback inhibition mechanisms which would limit the metabolism of precursors. This could be effected by inducing the export of the desired product from the cell into the nutrient medium, and this important point is now discussed in some detail.

The experimental results presented in this thesis have demonstrated that cell cultures of D. innoxia and S. nigrum accumulated alkaloids intracellularly, and did not release them into the nutrient medium. If the activity of any of the enzymes concerned with alkaloid biosynthesis was regulated by a feedback control mechanism, then the inability of cells to export, i.e., reduce the intracellular concentration of, the products, would result in a limited yield of metabolites. In an industrial process the intracellular accumulation of specific secondary compounds would be most undesirable, both for this reason and because harvesting the metabolite would involve killing at least part of the culture.

In order to surmount this problem, experiments were performed to induce alkaloid release from cultured cells (Results, Part 5).

The results of the low temperature experiment demonstrate that only a small quantity of alkaloids was released by the treated cells. Yoshida and Niki (1979) found that partial leakage of amino acids and an abrupt increase in the permeation of added dopamine occurred

in callus of Cornus stolonifera chilled to $0^{o}C$ for 48 hours. However, most of the callus sustained irreversible damage to the respiratory system.

Chloroform treatment of cells caused the release of a greater amount of alkaloids into the medium, but with severe damage to the viability of the cultures. At the end of the experimental periods, the cells contained no alkaloids or only relatively low levels compared with untreated cells, and the release was almost certainly a direct result of cell lysis. The fact that the reduction in cell viability was slightly less severe in the alginate column cultures than in cells in Erlenmeyer flasks suggests that the use of lower concentrations of chloroform, of less toxic solvents, or of shorter treatments would be more useful.

This release of alkaloids by immobilised S. nigrum cells, and the results of the iso-capric acid feeding experiment in which immobilised cells of C. frutescens exported capsaicin into the nutrient medium (Results Part 4, Section 3) demonstrate that calcium alginate is permeable to these metabolites and would probably not inhibit their synthesis by preventing their export from the cells.

The results of the latter experiment also suggest that, once a secondary metabolite is exported into the medium, it should be quickly removed. The nutrient medium of both the flatbed- and column-cultured cells of C. frutescens contained approximately the same concentration of capsaicin , despite the fact that more cells were present on the flatbeds than on the columns. This may be explained in terms of a more efficient conversion of iso-capric acid

to capsaicin by the immobilised cells, but it is also possible that the accumulation of capsaicin in the medium reached a level which inhibited its synthesis in the cells. If this is the case, then a concentration of approximately 2.5 μg capsaicin/ml would be sufficient to inhibit its synthesis, and unless it was extracted from the medium, this would be the upper limit for production, irrespective of the precursor concentration, the duration of the culture period, or the mass of tissue.

The results presented in this thesis therefore suggest a general method of cell culture which would result in the production of relatively high levels of secondary metabolites. This can be summarised as follows.

1. Cells should be grown physically stationary, in close association with each other, to encourage the development of physical and chemical gradients and allow the partial differentiation of the culture. Ideally the culture should produce chloroplasts and turn green, to ensure the development of a metabolism that is close to that of cells of the intact plant. The cultures should be illuminated (as suggested by the results of the illumination experiment, Part 4, Section 2);

2. The composition of the nutrient medium and/or oxygen level should be manipulated to slow down the growth of the culture;

3. Large quantities of precursors should be supplied to the cells at low concentrations; these should be as close, in the biosynthetic pathway, to the desired product as possible;

4. Cells should be used which either naturally export the desired metabolite(s) into the nutrient medium or can be induced to release the metabolite(s).

## FUTURE WORK

The results of this thesis have indicated the importance of the manipulation of the environment of cultured cells in increasing the yields of secondary metabolites. It is hoped that, in the future, a standardised type of manipulation technique will be developed which will allow the 'switching on' of particular secondary biosynthetic pathways in any cultured cell. In the meantime, therefore, work should be carried out in a number of areas, as suggested from the results presented here.

(a) It has been suggested above that the intracellular accumulation of secondary metabolites would severely limit their own production by cell cultures. Therefore, techniques should be developed to induce the release of the compounds into the nutrient medium, but cause minimal effects on the viability of the culture. As suggested in Results, Part 5 and earlier in this chapter, solvents of low toxicity should be sought and tested, or very brief treatments of cells with chloroform should be investigated, for this purpose. A reduction in the calcium level in the nutrient medium might be expected to increase membrane permeability and so induce metabolite release.

(b) The production of capsaicin by cultured cells of Capsicum frutescens would be a useful experimental system in which to investigate the regulation of secondary metabolite production by negative feedback inhibition. For example, by artificially increasing the concentration of capsaicin in the nutrient medium supplying cultured cells, it could be determined whether production is thereby inhibited. Moreover, enzymological studies should be performed to characterise enzymes in the pathway of capsaicin biosynthesis.

(c) Physical treatments which are known to induce the differentiation of cultured cells should be examined as a means to increase secondary metabolite production. These might include high concentrations of carbon dioxide and high temperatures, which enhance, respectively, differentiation in callus of peach mesocarp (Bradley and Dahmen, 1971) and Jerusalem artichoke (Naik, 1965).

(d) The search for 'inductive' nutrient media of defined composition, which permit the increased production of secondary metabolites, should be made. Also, more effective precursors than those used in this thesis should be sought, and might include tropine or tropic acid for tropane alkaloids (see p. 6 ). Ideally, the precursors should be cheap but as close to the desired product as possible.

(e) In order then, to maximise secondary metabolite production, sequential chemical or physical treatments of the cultured cells should be performed as follows :

1. A nutrient medium or physical regime which allows cell growth should be replaced by one which limits growth;

2. Precursors should be supplied to the 'stationary phase' cultures;

3. A nutrient medium which induces the export of the desired secondary metabolite should be supplied to the cultures, to prevent any limitation of production by negative feedback mechanisms;

4. The desired metabolite should be removed from the nutrient medium, again to prevent the operation of negative feedback mechanisms.

The flatbed- and column culture systems are particularly amenable to this sequential manipulation of cell cultures.

(f) The synthesis of alkaloids or other secondary metabolites by immobilised cells under different physical and chemical regimes should be studied by the incorporation of radioactively-labelled precursors, followed by analysis of the products by TLC, auto-radiography and perhaps GC-MS. This will give an accurate analysis of the production of specific secondary metabolites by immobilised cells.

## REFERENCES

AL-ABTA, S., GALPIN, I.J., COLLIN, H.A. (1979), Plant Sci. Letters 16, 129-134 : Flavour compounds in tissue cultures of celery.

ALFERMANN, W.A., MERZ, D., REINHARD, E. (1975), Planta Med. Suppl., 70-78 : Induktion der Anthocyanbiosynthese in Kalluskulturen von Daucus carota.

ALLEN, R.J.L. (1940), Biochem. J. 34, 858-865 : The estimation of phosphorus.

ASHWELL, G. (1957), Colorimetric analysis of sugars in 'Methods in Enzymology III', Ed. Colowick, S.P., Kaplan, N.O., Academic Press.

ATKINSON, B., BLACK, G.M., LEWIS, P.J.S., PINCHES, A. (1979), Biotechnol. Bioeng. 21, 193-200 : Biological particles of given size, shape and density for use in biological reactors.

ATTRIDGE, T.H. (1974), Biochim. Biophys. Acta 362, 258-265 : Phytochrome-mediated synthesis of ascorbic acid oxidase in Sinapis alba.

BARBER, J. (Ed.) (1976) : 'The Intact Chloroplast', Topics in photosynthesis, Vol. 1, Elsevier/North-Holland Biomedical Press, Amsterdam.

BAYLISS, M.W. (1973), Nature (London), 246, 529-530 : Origin of chromosome variation in cultured plant cells.

BEALE, S.I. (1978), Biosynthesis of photosynthetic pigment, pathways and regulation in 'Proc. 4th Int. Congr. Photosynthesis, Ed. Hall, C.O., Coombs, J., Goodwin, T.W., The Biochemical Soc., London.

BEAUDRY, Y., QUILLON, J.P., FRAPPA, J., DELOINCE, R., FONTANGES, R. (1979), Biotechnol. Bioeng. 21, 2351-2358 : Culture of diploid and heteroploid cells on dextran 'microbeads' in suspension : Studies of growth kinetics.

BECKER, H. (1970), Biochem. Physiol. Pflanzen 161, 425-441 : Formation of volatile substances in plant tissue cultures.

BHANDARY, R., COLLIN, H.A., THOMAS, E., STREET, H.E. (1969), Ann. Bot. 33, 647-656 : Root, callus and suspension cultures from Atropa belladonna.

BLAIN, J.A., ANDERSON, J.G., TODD, J.R., DIVERS, M. (1979), Biotechnology Letters 1, 269-274 : Cultivation of filamentous fungi in the disc fermentor.

BLAKELY, L.W., STEWARD, F.C. (1964), Am. J. Bot. 51, 809-820 : Growth and organised development of cultured cells : VII : Cellular variation.

BOULANGER, D., BAILEY, B.K., STECK, W. (1973), Phytochem. 12, 2399-2405 : Formation of edulinine and furoquinoline alkaloids from quinoline derivatives by cell suspension cultures of Ruta graveolens.

BRADLEY, M.V., DAHMEN, W.J. (1971), Phytomorphology 21, 154-164 : Cytohistological effects of ethylene, 2,4-D, kinetin and carbon dioxide on peach mesocarp callus cultured in vitro.

BRODELIUS, P., DEUS, B., MOSBACH, K., ZENK, M.H. (1979), FEBS Letters 103, 93-97 : Immobilized plant cells for the production and transformation of natural products.

BRODELIUS, P., NILSSON, K. (1980), ibid. 122, 312-316 : Entrapment of plant cells in different matrices.

BRUNET, G., IBRAHIM, R.K. (1973), Z. Pflanzenphysiol. 69, 152-162 : Tissue culture of Citrus peel and its potential for flavonoid synthesis.

CARCELLER, M., DAVEY, M.R., FOWLER, M.W., STREET, H.E. (1971), Protoplasma 73, 367-385 : The influence of sucrose, 2,4-D and kinetin on the growth, fine structure and lignin content of cultured sycamore cells.

CARLSSON, J., GABEL, D., LARSSON, E., PONTEN, J., WESTERMARK, B. (1979), In vitro 15, 844-850 : Protein-coated agarose surfaces for attachment of cells.

CHAN, W-N, STABA, E.J. (1965), Lloydia 28, 55-62 : Alkaloid production by Datura callus and suspension tissue cultures.

CHEETHAM, P.S.J., BLUNT, K.W., BUCKE, C. (1979), Biotechnol. Bioeng. 21, 2155-2168 : Physical studies on cell immobilisation using calcium alginate gels.

CHIBATA, I., TOSA, T., SATO, T. (1974), Appl. Microbiol. 27, 878-885 : Immobilised aspartase-containing microbial cells : Preparation and enzymatic properties.

CLARKE, E.G.C. (1970), The forensic chemistry of alkaloids in 'The Alkaloids', Vol 12, Ed. Manske, H.F., Academic Press.

CLOWES, F.A.L. (1976), The root apex in 'Cell Division in Higher Plants', Ed. Yeoman, M.M., Academic Press.

CONSTANTINIDES, A. (1980), Biotechnol. Bioeng. 22, 119-136 : Steroid transformation at high substrate concentrations using immobilised Corynebacterium simplex cells.

CORDUAN, G., REINHARD, E. (1972), Phytochem. 11, 917-922 : Synthesis of volatile oils in tissue cultures of Ruta graveolens.

CRONSHAW, J. (1974), Phloem differentiation and development in 'Dynamic Aspects of Plant Ultrastructure', Ed. Robards, A.W., McGraw-Hill.

DALE, J.E. (1976), Cell division in leaves in 'Cell Division in Higher Plants', Ed. Yeoman, M.M., Academic Press.

DALLYN, H., FALLOON, W.C., BEAN, P.G. (1977), Lab. Practice 26, 773-775 : Method for the immobilisation of bacterial spores in alginate gel.

DALTON, C.C., STREET, H.E. (1976), In vitro 12, 485-494 : The role of the gas phase in the greening and growth of illuminated cell suspension cultures of spinach (Spinacia oleracea L.).

DARLINGTON, C.D., LA COUR, L.F. (1942) : 'The Handling of Chromosomes', Unwin, London.

DAVEY, M.R., FOWLER, M.W., STREET, H.E. (1971), Phytochem. 10, 2559-2575 : Cell clones contrasted in growth, morphology and pigmentation isolated from a callus culture of Atropa belladonna v. lutea.

DAVIES, M.E. (1972), Planta 104, 50-65 : Polyphenol synthesis in cell suspension cultures of Paul's Scarlet Rose.

DAWSON, R.F., OSDENE, T.S. (1972), Rec. Adv. Phytochem. 5, 317-338 : A speculative view of tobacco alkaloid biosynthesis.

DHOOT, G.K., HENSHAW, G.G. (1977), Ann. Bot. 41, 943-949 : Organisation and alkaloid production in tissue cultures of Hyoscyamus niger.

DOBBERSTEIN, R.H., STABA, E.J. (1968), Proc. Am. Soc. Pharmacog. 9th Ann. Meeting : Ipomoea, Rivea and Agyreia suspension tissue cultures : Influence of various chemical factors on indole alkaloid production.

DURAND, G., NAVARRO, J.M. (1978), Proc. Biochem. 13, 14-23 (1978 ) : Immobilised microbial cells.

EVERETT, N.P., WANG, T.L., STREET, H.E. (1978), Hormone regulation of cell growth and development in vitro in 'Frontiers of Plant Tissue Culture', Proc. 4th Int. Congr. Plant Tissue and Cell Cultures, Ed. Thorpe, T.A., The International Association for Plant Tissue Cultures.

FARNSWORTH, N.R., PILEWSKI, N.A., DRAUS, F.J. (1962), Lloydia 25, 312-319 : Studies of false-positive alkaloid reactions with Dragendorff's reagent.

FISHER, R.A., YATES, F. (1963) : 'Statistical Tables for Biological Agricultural and Medical Research', 6th Edn., Oliver and Boyd, London.

FLOSS, H.G., ROBBERS, J.E., HEINSTEIN, P.F. (1974), Rec. Adv. Phytochem. 8, 141-178 : Regulatory control mechanisms in alkaloid biosynthesis.

FORREST, G.I. (1969), Biochem. J. 113,765-72 : Studies on the polyphenol metabolism of tissue cultures derived from the tea plant (Camellia sinensis L.).

FREEMAN, G.G., WHENHAM, R.J., MACKENZIE, I.A., DAVEY, M.R. (1974), Plant Sci. Letters 3, 121-125 : Flavour components in tissue cultures of onion (Allium cepa).

FURUYA, T., IKUTA, A., SYONO, K. (1972), Phytochem 11, 3041-3044 : Alkaloids from callus tissue of Papaver somniferum.

GRANT, M.E., FULLER, K.W. (1968), J. Exp. Bot. 19, 667-680 : Tissue culture of root cells of Vicia faba.

GRISEBACH, H., HAHLBROCK, K. (1974), Rec. Adv. Phytochem. 8, 21-52 : Enzymology and regulation of flavonoid and lignin biosynthesis in plants and plant cell suspension cultures.

HACKEL, U., KLEIN, J., MEGNET, R., WAGNER, F. (1975), European J. Appl. Microbiol. 1, 291-293 : Immobilisation of microbial cells in polymeric matrices.

HADDON, L.E., NORTHCOTE, D.H. (1975), J. Cell Sci. 17, 11-26 : Quantitative measurement of the course of bean callus differentiation.

HAHLBROCK, K., KUHLEN, E., LINDL, T. (1971), Planta 99, 311-318 : Anderungen von Enzymaktivitaten wahrend des Wachstums von Zell-suspensionkulturen von Glycine max : Phenylalanin ammonia-lyase und p-cumurat : CoA-ligase.

HANKE, D. (1977), The endomembrane system and the integration of cellular activities in 'The Molecular Biology of Plant Cells', Ed. Smith, H., Botanical Monographs, Vol. 14, Blackwell Scientific Publications.

HARBORNE, J. B. (1973) : 'Phytochemical Methods', Chapman and Hall, London.

HAVILAH, E.J., WALLIS, D.M., MORRIS, R., WOOLNOUGH, J.A. (1977), Lab. Practice, 26, 545-547 : A micro-colorimetric method for determination of ammonia in kjeldahl digests with a manual spectrophotometer.

HESLOP-HARRISON, J., HESLOP-HARRISON, Y. (1970), Stain Technol. 45, 115-120 : Evaluation of pollen viability by enzymatically-induced fluorescence : Intracellular hydrolysis of fluorescein diacetate.

HIRAOKA, N., TABATA, M. (1974), Phytochem. 13, 1671-1675 : Alkaloid production by plants regenerated from cultured cells of Datura innoxia.

HIROTANI, M., FURUYA, T. (1977), ibid 16, 610-611, Restoration of cardenolide synthesis in redifferentiated shoots from callus cultures of Digitalis purpurea.

HULTIN, E. (1966), Acta Chem. Scand. 20, 1588-1592 : Thin layer chromatography of plant extracts : IV : Alkaloids.

HULTIN, E., TORSELL, K. (1965), Phytochem. 4, 425-433 : Alkaloid screening of Swdish plants.

IBRAHIM , R.K., THAKUR, M.L., PERMANAND, B. (1971), Lloydia 34, 175-182 : Formation of anthocyanins in callus tissue cultures.

IDRISOVA, L.S., BEREZNEG, L.N. (1978), Khim.-Farm. ZH. 12, 97-101 : Optimisation of some physiological processes in a Datura innoxia tissue culture.

IKUTA, A., SYONO, K., FURUYA, T. (1974), Phytochem. 13, 2175-2179 : Alkaloids of callus tissues and redifferentiated plantlets in the papaveraceae.

IWAI, K., SUZUKI, T., FUJIWAKE, H. (1979), Agric. Biol. Chem. 43, 2493-2498 : Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues, in Capsicum annum, v. annuum cv. karayatsubusa at different growth stages after flowering.

JACK, T.R., ZAJIC, J.E. (1977), Adv. Biochem. Eng. 5, 125-145 : The immobilisation of whole cells.

JALAL, M.A.F., OVERTON, K.H., RYCROFT, D.S. (1979), Phytochem, 18, 149-151 : Formation of three new flavones by differentiating callus cultures of Andrographis paniculata.

JOHNSON, D.E., CIEGLER, A. (1969), Arch. Biochem. Biophys. 130, 384-388 : Substrate conversion by fungal spores entrapped in solid matrices.

JOHNSON, R.D., WALLER, G.R. (1972), Abstr. Pap. 164th Meet., Amer. Chem. Soc. Biol. 227.

KAUL, B., STABA, E.J. (1968), Lloydia 31, 171-179 : Dioscorea tissue cultures : I : Biosynthesis and isolation of diosgenin from Dioscorea deltoidea callus and suspension cells.

KAUL, B., STOHS, S.J., STABA, E.J. (1969), ibid. 32, 347-359 : Dioscorea tissue cultures : III : Influence of various factors in diosgenin production by Dioscorea deltoidea callus and suspension cultures.

KENNEDY, J.F., BARKER, S.A., HUMPHREY, J.D. (1976), Nature (London) 261, 242-244 : Microbial cells living immobilised on metal hydroxides.

KHANNA, P., JAIN, S.C. (1972), Ind. J. Exp. Biol. 10, 248-249 : Effect of nicotinic acid on growth and production of trigonelline by Trigonella foenum-graecum tissue cultures.

KHANNA, P., JAIN, S.C. (1973), Lloydia 36, 96-98 : Diosgenin, gitogenin and tigogenin from Trigonella foenum-graecum tissue cultures.

KIERSTAN, M., BUCKE, C. (1977), Biotechnol. Bioeng. 14, 387-397 : The immobilisation of microbial cells, subcellular organelles and enzymes in calcium alginate gels.

KOBAYASHI, H., ASAMI, S., AKAZAWA, T. (1980), Plant Physiol. 65, 198-203 : Development of enzymes involved in photosynthetic carbon assimilation in greening seedlings of maize (Zea mays).

KONOSHIMA, M., TABATA, M., YAMAMOTO, H., HIRAOKA, N. (1970), Yakugaku Zasshi 90, 370-377 : Growth and alkaloid production of Datura tissue cultures.

KRIKORIAN, A.D., STEWARD, F.C. (1969), Biochemical differentiation : The biosynthetic potentialities of growing and quiescent tissue in 'Plant Physiology, a Treatise', Vol. VB : Analysis of growth : The response of cells and tissues in culture, Ed. Steward, F.C., Academic Press.

LAETSCH, W.M., STETLER, D.A. (1965), Am. J. Bot. 52, 798-804 : Chloroplast structure and function in cultured tobacco tissue.

LARSSON, P.O., OHLSON, S., MOSBACH, K. (1976), Nature (London) 263, 796-797 : New approach to steroid conversion using activated immobilised microorganisms.

LEVINE, D.W., WONG, J.S., WANG, D.I.C., THILLY, W.C. (1977), Somatic Cell Gen 3, 149-155 : Microcarrier cell culture : New methods for research-scale application.

LUCKNER, M. (1980), Expression and control of secondary metabolism in 'Secondary Plant Products', Encyclopaedia of Plant Physiology Volume 8, Ed. Bell, E.A., Charlwood, B.V., Springer-Verlag.

MACKENZIE, I.E., DAVEY, M.R., FREEMAN, G.G. (1974) : Structure, differentiation and flavour production of onion callus isolates in 3rd Int. Congr. Plant Tissue and Cell Culture, Leicester, Abstract 255.

MADER, M., MUNCH, P., BOPP, M. (1975), Planta 123, 257-265 : Regulation of peroxidase patterns during shoot differentiation in callus cultures of Nicotiana tabacum.

MEINS, F., BINNS, A. (1977), Proc. Natl. Acad. Sci. USA 74, 2928-2932 : Epigenetic variation of cultured somatic cells : Evidence for gradual changes in the requirement for factors promoting cell division.

MEYER-TEUTER, H., REINERT, T. (1973), Protoplasma 78, 273-283 : Correlation between rate of cell division and loss of embryogenesis in long term tissue cultures.

MIZUKAMI, H., KONOSHIMA, M., TABATA, M. (1977), Phytochem. 16, 1183-1186 : Effect of nutritional factors on shikonin derivative formation in Lithospermum callus cultures.

MIZUKAMI, H., KONOSHIMA, M., TABATA, M. (1978), ibid 17, 95-97 : Variation in pigment production in Lithospermum erythrorhizon callus cultures.

MOHAN, R.R., LI, N.N. (1975), Biotechnol. Bioeng. 17, 1137-1156 : Nitrate and nitrite reduction by liquid membrane-encapsulated whole cells.

MOORE, T.C. (1974) : 'Research Experiments in Plant Physiology : A Laboratory Manual', Springer-Verlag.

MOSBACH, K., LARSSON, P.-O. (1970), Biotechnol. Bioeng. 12, 19-27 : Preparation and application of polymer-entrapped enzymes and microorganisms in microbial transformation processes with special reference to steroid 11-β-hydroxylation and Δ'-dehydrogenation.

MOSBACH, K., MOSBACH, R. (1966), Acta Chem. Scand. 20, 2807-2810 : Entrapment of enzymes and microorganisms in synthetic cross-linked polymers and their application in column techniques.

MURASHIGE, T., SKOOG, F. (1962), Physiol. Plant. 15, 473-497 : A revised medium for rapid growth and bioassays with tobacco tissue cultures.

MURPHY, D.J., STUMPF, P.K. (1979), Plant Physiol. 63, 328-335 : Light-dependent induction of polyunsaturated fatty acid biosynthesis in greening cucumber cotyledons.

NAIK, G.G. (1965), M.Sc. Thesis, Edinburgh University : Studies on the effects of temperature on the growth of plant tissue cultures.

NATO, A., BAZETOUX, S., MATHIEU, Y. (1977), Physiol. Plant. 41, 116-123 : Growth rate of cells affects enzyme activity.

NETTLESHIP, L., SLAYTOR, M. (1974), J. Exp. Bot. 25, 114-123 : Adaptation of Peganum harmala callus to alkaloid production.

NEUMANN, D., MUELLER, E. (1971), Biochem. Physiol. Pflanzen. 162, 503-513 : Beitrage zur Physiologie der Alkaloide : V. Alkaloidbildung in Kallus - und Suspensionculturen von Nicotiana tabacum.

NEUMANN, D., MUELLER, E. (1974), ibid 165, 271-282 : Formation of alkaloids in callus cultures of Macleaya.

NIKOLAEVA, L.A., VOLLOSOVICH, A.G. (1972), Rastit. Resur. 8, 188-192 : Effect of auxins on the morphology of organogenesis in tissue cultures of some medicinal plants and biosynthesis of tropane and indole alkaloids.

NILSSON, K., MOSBACH, K. (1980), Febs Letters 118, 145-150 : Preparation of immobilised animal cells.

NISHI, A., YOSHIDA, A., MORI, M., SUGANO, N. (1974), Phytochem. 13, 1653-1656 : Isolation of variant carrot cell lines with altered pigmentation.

NISHIDA, K., SATO, F., YAMADA, Y. (1980), Plant and Cell Physiol. 21, 47-55 : Photosynthetic carbon metabolism in photoautotrophically and photomixotrophically cultured tobacco cells.

NORTHCOTE, D.H. (1968), The organisation of the endoplasmic reticulum, the golgi bodies and microtubules during cell division and subsequent growth in 'Plant Cell Organelles', Ed. Pridham, J,B., Academic Press.

O'BRIEN, T.P. (1974), Primary vascular tissues in 'Dynamics of Plant Ultrastructure', Ed. Robards, A.W., McGraw-Hill.

OGINO, T., HIRAOKA, N., TABATA, M. (1978), Phytochem. 17, 1907-1910 : Selection of high nicotine-producing cell lines of tobacco callus by single cell cloning.

OHLSON, S., LARSSON, P.-O., MOSBACH, K. (1979), European J. Appl. Microbiol. Biotechnol. 7, 103-110 : Steroid transformation by living cells immobilized in calcium alginate.

OHMIYA, K., OHASHI, H., KOBAYASHI, T., SHIMIZU, S. (1977), Appl. Env. Microbiol. 33, 137-146 : Hydrolysis of lactose by immobilised micro-organisms.

PARKER, R.E. (1973), 'Introductory Statistics for Biology', Studies in Biology No. 43, Edward Arnold.

PFEIFER, S. (1962), Pharmazie 17, 467-479 : Mohn-arnzeipflanze seit mehr als zweitausend Jahren.

PHARMACOPOEIA OF JAPAN (1971), 8th Edn., Part 1, Society of Japanese Pharmacopoeia, Yakuji Nippo Ltd., Tokyo.

PHILLIPS, R., HENSHAW, G.G. (1977), J. Exp. Bot. 28, 785-794 : The regulation of synthesis of phenolics in stationary phase cell cultures of Acer pseudoplatanus L.

RADWAN, S.S., KOKATE, C.K. (1980), Planta 147, 340-344 : Production of higher levels of trigonelline by cell cultures of Trigonella foenum-graecum than by the differentiated plant.

RAMAWAT, K.G., ARYA, H.C. (1979), Phytochem. 18, 484-485 : Effects of amino acids on ephedrine production in Ephedra gerardiana callus cultures.

RANDERATH, K. (1968), 'Thin Layer Chromatography', 2nd Edn., Academic Press.

REINHARD, E., CORDUAN, G., VOLKS, O.H. (1968), Planta Med. 16, 9-16 : Tissue cultures of Ruta graveolens.

ROBERTS, L.W. (1976), 'Cytodifferentiation in Plants', Cambridge Univ. Press.

SAINI, R., VIETH, W.R. (1975), J. Appl. Chem. Biotechnol. 25, 115 : Reaction kinetics and mass transfer in glucose isomerisation with collagen-immobilised whole microbial cells.

SAIRAM, T.V., KHANNA, P. (1971), Lloydia 34, 170-171 : Effect of tyrosine and phenylalanine on growth and production of alkaloids in Datura tatula tissue cultures.

SCHEIBE, R., BECK, E. (1979), Plant Physiol. 64, 744-748 : On the mechanism of activation by light of the NADP-dependent malate dehydrogenase in spinach chloroplasts.

SEJOURNE, M., VIEL, C., BRUNETON, J., RIDEAU, M., CHENIEUX, J.C. (1981), Phytochem. 20, 353-355 : Growth and furoquinoline alkaloid production in cultured cells of Choisya ternata.

SOLT, M.L. (1957), Plant Physiol. 32, 480-484 : Nicotine production and growth of excised tobacco root cultures.

SPEAKE, T., McCLOSKEY, P., SMITH, W., SCOTT, T., HUSSEY, H. (1964), Nature (London) 201, 614-615 : Isolation of nicotine from cell cultures of Nicotiana tabacum.

SPIER, R.E., WHITESIDE, J.P. (1976a), Biotechnol. Bioeng. 18, 649-657 : The production of foot-and-mouth disease virus from BHF 21C 13 cells grown on the surface of glass spheres.

SPIER, R.E., WHITESIDE, J.P. (1976b), ibid 18, 659-667 : The production of foot-and-mouth disease virus from BHF 21 C 13 cells grown on the surface of deae sephadex A50 beads.

STABA, E.J., JINDRA, A. (1968), J. Pharm. Sci. 57, 701-704 : Datura tissue cultures : Production of minor alkaloids from chlorophyllous and non-chlorophyllous strains.

SUNDERLAND, N. (1977), Nuclear Cytology in 'Plant Tissue and Cell Culture', 2nd Edn., Ed. Street, H.E., Blackwell Scientific Publications.

TABATA, M., HIRAOKA, N. (1976), Physiologia Pl. 38, 19-23 : Variation of alkaloid production in Nicotiana rustica callus cultures.

TABATA, M., OGINO, T., YOSHIOKA, K., YOSHIKAWA, N., HIRAOKA, N. (1978) in 'Frontiers of Plant Tissue Culture', Proc. 4th Int. Congr. Plant Tissue and Cell Culture, Ed. Thorpe, T.A., The International Association for Plant Tissue Culture.

TABATA, M., YAMAMOTO, H., HIRAOKA, N., KONOSHIMA, M. (1972), Phytochem. 11, 949-955 : Organisation and alkaloid production in tissue cultures of Scopelia parviflora.

TAKAHASHI, M., YAMADA, Y. (1973), Agric. Biol. Chem. 37, 1755-1757 : Regulation of nicotine production by auxins in tobacco cultured cells in vitro.

THOMAS, E., STREET, H.E. (1970), Ann. Bot. 34, 657-669 : Organogenesis in cell suspension cultures of Atropa belladonna L. and Atropa belladonna cultivar Lutea doll.

THORPE, T.A., MEIER, D.A. (1973), Phytochem. 12, 493-497 : Sucrose metabolism during tobacco callus growth.

TOBIN, E.M., SUTTIE, J.L. (1980), Plant Physiol. 65, 641-647 : Light effects on the synthesis of RUDPC ase in Lemna gibba L. G-3.

TODA, K., SHODA, M. (1975), Biotechnol. Bioeng. 17, 481-497 : Sucrose inversion by immobilised yeast cells in a complete mixing reactor.

TOWNSLEY, P.M. (1974), J. Inst. Can. Sci. Technol. Aliment. 7, 76-78 : Chocolate aroma from plant cells.

VAN WEZEL, A.L. (1967), Nature (London) 216, 64-65 : Growth of cell strains and primary cells on micro-carriers in homogeneous culture.

VAN WEZEL, A.L. (1976), Dev. Biol. Standard 37, 143-147 : The large-scale cultivation of diploid cell strains in microcarrier culture. Improvement of microcarriers.

VERZAR-PETRI, G. (1966), Pharmazie 21, 48-54 : Ontogenetische Veranderungen des Alkaloidgehaltes einiger Datura-arten.

WALLER, G.R., NOWACKI, E.K. (1978), 'Alkaloid Biology and Metabolism in Plants', Plenum Press.

WEST, F.R., MIKA, E.S. (1957), Bot. Gaz. 119, 50-54 : Synthesis of atropine by isolated roots and root callus cultures of Belladonna.

WESTCOTT, R.J., HENSHAW, G.G. (1976), Planta 131, 67-73 : Phenolic synthesis and phenylalanine ammonia-lyase activity in suspension cultures of Acer pseudoplatanus L.

WIDHOLM, J.M. (1972), Stain Technol. 47, 189-194 : The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells.

WOOLLEY, J.T., HICKS, J.P., HAGEMAN, R.H. (1960), J. Agric. Fd. Chem. 8, 481-482 : Rapid determination of nitrate and nitrite in plant material.

YAMADA, Y., IMAIZUMI, K., SATO, F., YASUDA, T. (1981), Plant and Cell Physiol. 22, 917-922 : Photoautotrophic and photomixotrophic culture of green tobacco cells in a jar-fermenter.

YEOMAN, M.M., BROWN, R. (1971), Ann. Bot. 35, 1001-1012 : Effects of mechanical stress on the plane of division in developing callus cultures.

YEOMAN, M.M., DAVIDSON, A.W. (1971), Ann. Bot. 35, 1085-1100 : Effect of light on cell division in developing callus cultures.

YEOMAN, M.M., FAGANDINI, D.A.A., CHILDS, A.F. (1978), British Patent Application Number 32185/78 : Callus culture in nutrient flow.

YEOMAN, M.M., LINDSEY, K., MIEDZYBRODZKA, M.B., McLAUGHLAN, W.R. (1982, in press) in 'Differentiation in vitro', Brit. Soc. Cell Biol. Symp., Ed. Yeoman, M.M., Truman, D., Cambridge Univ. Press.

YEOMAN, M.M., MIEDZYBRODZKA, M.B., LINDSEY, K., McLAUCHLAN, W.R. (1980) in 'Plant Cell Cultures : Results and Perspectives', Ed. Cifferi, O., Parisi, B., Elsevier/North-Holland, Amsterdam.

YEOMAN, M.M., STREET, H.E. (1977), General Cytology of Cultured Cells in 'Plant Tissue and Cell Culture', 2nd Edn., Ed. Street, H.E., Botanical Monographs, Vol 11, Blackwell Scientific Publications.

YOSHIDA, S., NIKI, T. (1979), Plant and Cell Physiol. 20, 1237-1242 : Cell membrane permeability and respiratory activity in chilling-stressed callus.

ZELENEVA, I.V., KHAVKIN, E.E. (1980), Planta 148, 108-115 : Rearrangement of enzyme patterns in maize callus and suspension cultures : Is it relevant to the changes in the growing cells of the intact plant?

ZENK, M.H., EL-SHAGI, H., ARENS, H., STOCKIGT, J., WEILER, E.W., DEUS, B. (1977) in 'Plant Tissue Culture and its Bio-Technological Application', Ed. Barz, W., Reinhard, E., Zenk, M.H., Springer-Verlag, Berlin.

ZENK, M.H., EL-SHAGI, H., SCHULTE, U. (1975), Planta Med. Suppl. 79-101 : Anthraquinone production by suspension cultures of Morinda citrifolia.

ZUCKER, M. (1972), Ann. Rev. Plant Physiol. 23, 133-156 : Light and Enzymes.

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# THE SYNTHETIC POTENTIAL OF CULTURED PLANT CELLS

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Many of the substances used in the pharmaceutical, food, flavour and perfume industries originate from plants, and although the trend over the last fifty years has been towards chemical synthesis, plants still remain an important source of many of these compounds, for a number of reasons.

(1) The compounds are difficult and/or costly to synthesise.

(2) Complex mixtures, e.g. rose oil, cannot be constituted successfully by man.

(3) Isolation from a natural source circumvents many of the regulations which must be satisfied before a chemically synthetic compound can be used commercially as a food or drink additive.

(4) Chemical synthesis may, depending on the compound, result in a mixture of isomers which cannot be separated on a commercial scale. This is of obvious importance when the major desirable property resides with one isomer.

## ORIGINS OF COMMERCIALLY USEFUL COMPOUNDS FROM PLANTS

The majority of commercially useful substances originate from plants grown in tropical and sub-tropical regions of the world and the availability and cost of these materials is frequently affected by political and economic considerations in the countries of origin. For example, over a period lasting many years most of the world supply of diosgenin, a precursor of a range of steroid pharmaceuticals came from Mexico and Central America. The plant of origin, *Dioscorea* (various species were used), grew wild and the tubers were gathered and processed locally. Attention was turned to growing *Dioscorea* as a crop only when the supply of wild plants was becoming exhausted and so far has not been successful. Now another plant, *Agave*, grown in Australia provides another steroidal precursor which is replacing diosgenin. It is clearly desirable that an adequate supply of these materials is available

to the chemical industry but at the right price!

## ACCUMULATION OF DESIGNATED COMPOUNDS BY PLANT CULTURES

The success of the fermentation industry in harnessing micro-organisms to produce designated chemicals in abundance provided an attractive precedent for the plant cell and tissue culturist. It is now nearly thirty years since the first experiments were performed with callus cultures in which attempts were made to induce the accumulation of specific secondary metabolites. The initial approach was logical and consisted of removing and culturing organs or tissues from a plant known to synthesise and accumulate substantial amounts of one or more commercially useful compounds. It was hoped that the cultured organs or tissues would retain the synthetic abilities of the intact plant. However, these cultures tended to grow rapidly producing large numbers of cells and substantial amounts of dry matter but only insignificant amounts of the designated compounds. Frequently the substances, produced only in trace amounts, were different from those found in the intact plant and of less commercial value. Such studies have provided a substantial literature[1,2] in which a wide range of tissues, cells and organs have been tested for the ability to accumulate compounds characteristic of their parents but with surprisingly little success. However, a number of general points emerge from a survey of the literature which may help us towards our goal to produce designated compounds in abundance from plant cell cultures. These are:

(1) Rapidly growing cultures accumulate cells and *not* secondary products.

(2) Conversely, mature cultures which exhibit a marked amount of cellular differentiation tend to accumulate secondary products, normally in very small amounts and often different from that that can be isolated from the intact plant.

(3) Some cultures, and these are in a small minority, can accumulate moderate amounts of a designated compound. Occasionally this may exceed 1% of cell dry weight[3].

As a rule plants tend to accumulate secondary metabolic products in parts which are no longer engaged in active growth, e.g. in older leaves, fruits and seeds. The actively growing parts of

the plant, meristems, enlarging regions, and early stages in fruit and seed development do *not* contain more than traces of the compound characteristic of a particular plant. It is therefore *not* surprising that actively growing cell cultures are *not* a good source of the secondary product and that these substances tend to accumulate as the cultures approach senescence.

## RELATIONSHIP BETWEEN ACCUMULATION OF SECONDARY METABOLITES, GROWTH AND DIFFERENTIATION

There is in the literature a body of evidence which supports the view that an inverse relationship exists between secondary metabolite production and the growth rate of tissue and cell cultures. In 1969 Bhandary *et al.*[4] examined the accumulation of atropine in callus derived from *Atropa belladonna* and found that actively growing, essentially undifferentiated callus did not produce detectable amounts of the alkaloid. In contrast West and Mika[5] have shown that *A. belladonna* callus cultured in conditions incompatible with cell proliferation and conducive to cell differentiation accumulated small amounts of atropine. Thomas and Street[6] have also shown that cellular organisation is necessary for alkaloid production in *A. belladonna* suspension cultures. Nikolaeva and Vollosovich[7] similarly found that the production of tropane alkaloids by *Datura* spp. and indole alkaloids by *Rauwolfia serpentina* tissue cultures was restricted to those conditions in which the level of auxin permitted organogenesis. Tabata *et al.*[8] noted that roots regenerated from callus cultures of *Scopolia parviflora* (unlike the disorganised tissues) accumulated the pattern of alkaloids characteristic of the intact plant and showed that if the 'undifferentiated' callus was transferred to a medium conducive to root development alkaloid accumulated to a level of 0.08%. Similarly the production of the flavour components of onion (*Allium cepa*) occurred in tissue cultures to intensities approaching that of fresh onion but only in cultures in which roots had differentiated[9]. It was also found[10] that the nicotine content of tobacco callus decreased to trace amounts in cultures which had lost the ability to regenerate roots. Light regimes inducing tissue differentiation and lignification also stimulate flavonoid production in *Citrus* tissue cultures[11].

The appearance of specialised cells in a variety of tissue cultures has been correlated with the appearance of specific secondary compounds[12,13,14,15].

Secondary metabolite production and differentiation have also been shown to be closely related to that part of the growth cycle in which the rate of cell division has slowed down, i.e. at the end of the logarithmic phase and at the beginning of the stationary phase. Thomas and Street[6] found that morphogenesis in cell suspension cultures of *A. belladonna* was favoured late in the growth phase and was increased by delaying sub-culture. Similar observations have been made by Lindsey[16] with *A. belladonna* and *Solanum nigrum* suspensions. In *Glycine max* cell cultures large changes in the activity of phenylalanine ammonia lyase (PAL) and p-coumarate:CoA ligase occur just prior to the stationary phase, indicating the initiation of some facets of secondary metabolism[17]. Speake *et al.*[18] showed that *Nicotiana tabacum* suspension cultures accumulated maximum levels of alkaloids at the end of the growth phase, a result identical to that for caffeine production in tea (*Camellia sinensis*) callus cultures[19]. A similar pattern of accumulation was reported by Townsley[20], who followed the levels of a chocolate aromatic product in cell cultures of *Theobroma cacao*; accumulation occurred only at the mature-death stage of the growth curve. The production of trigonelline and diosgenin by *Trigonella foenum-graecum* tissue cultures seems also to be inversely proportional to the growth index[21,22]. Takahashi and Yamada[23] discovered that, in the presence of auxin (either IAA or 2,4-D) there seemed to be an antithetical relationship between the growth rate of tobacco callus and nicotine production by that callus, and it was suggested that phytohormones may control alkaloid production by affecting the growth rate of the cells. Similar results were obtained by Konoshima *et al.*[24].

In 1973 Meyer-Teuter and Reinert[25] altered the normal pattern of embryo formation in carrot (*Daucus carota*) tissue cultures on an inductive medium by effecting a retardation of growth, either by low temperatures or by transfer to a non-inductive medium. In both cases partial inhibition of growth resulted in a prolonged period of competence of the cultures for embryo formation. More-

over, a close inverse relationship was found to exist between the rate of cell division and the percentage of cultures forming embryos.

It may therefore be concluded that as plant cells slow down their rate of growth, the production of at least some types of secondary metabolites appears to increase concomitantly with an increase in cell and tissue differentiation and organisation. This situation may be summarised by the simple formula

$$\text{SECONDARY METABOLITE PRODUCTION AND CELL DIFFERENTIATION} \propto \frac{1}{\text{GROWTH RATE}}$$

In other words, either as a response to slow cell growth, or as a response elicited by those hormonal and other environmental conditions inducing slow cell growth, there seems to be a switch in cell chemistry, i.e. a movement away from primary towards secondary metabolism.

## THE SWITCH AWAY FROM PRIMARY TO SECONDARY METABOLISM

It is believed that the genes determining the metabolic pathways of primary and secondary metabolism are present in all living cells of a plant, but are differentially derepressed at one or more points in the history of a particular cell. It may be suggested that the transition from one stable condition (for example primary metabolism and active growth) to another (such as alkaloid synthesis and accumulation, and slow cell growth) is the result of a change in emphasis of enzyme utilisation; metabolites involved in, for example, primary protein synthesis could be 'diverted' down a different biochemical pathway, such as one leading to alkaloid synthesis.

Ramawat *et al.*[26,27] have demonstrated that alkaloid synthesis in *Ephedra gerardiana* callus cultures starts in those conditions which preclude protein synthesis. It was found that as auxin and nitrogen were depleted from the culture medium, protein synthesis stopped and ephedrine synthesis commenced. They suggested that at high auxin and high nitrogen concentrations, phenylalanine, methionine and glycine are converted to proteins, but as they become exhausted, and protein synthesis stops these amino acids are used for alkaloid biosynthesis. Similarly, Muzukami *et al.*[28] discovered that a high level of nitrogen inhibited or retarded the formation of shikonin derivatives in *Lithospermum erythorhizon*

callus cultures, and streptomycin sulphate known to inhibit protein synthesis, stimulated the biosynthesis of these secondary metabolites.

Neumann and Mueller[29,15] demonstrated that Actinomycin D, an inhibitor of gene transcription, and D-threo chloramphenicol, which inhibited growth in *Macleaya* tissue cultures and increased the free amino acid pool in *Nicotiana* tissue cultures, both stimulated alkaloid production. The activity of ornithine decarboxylase, an enzyme known to participate in a number of alkaloid biosynthetic pathways, can be stimulated by the presence of Actinomycin D; moreover, this effect was much greater in non-growing cells than in growing cells[30].

SELECTION AND MANIPULATION OF CULTURES FOR 'HIGH YIELD'

There are three major approaches that may be used to produce a culture which will yield appreciable amounts of a designated secondary product, (1) the screening of a large number of 'wild type' cells to find a high yielding strain, (2) the production of mutants by the use of mutagenic treatments, followed by the selection of high yielding cell lines[31], and (3) the manipulation of culture conditions to secure optimal yield.

(1) Screening for 'high yield' strains

Here the assumption has been made that productivity in cell cultures is directly related to productivity in the mature tissues of the plant. Accordingly high yielding tissues, i.e. those in which the designated substance accumulates, which may not be the tissue in which synthesis occurs, have been used as a source of callus. Research in our laboratory on capsaicin production with *Capsicum frutescens* has failed to reveal a correlation between the tissue in the plant where accumulation takes place and the yield of a culture derived from that tissue. For example, explants from the mature fruit, which is the only site of accumulation of capsaicin in *Capsicum frutescens*, yield callus cultures which are no more productive for the designated product than cultures initiated from immature fruits, stems, roots or leaves. However, the possibility exists that high yielding cultures may be isolated from a variety of tissues of high yielding plants. Clearly, the screening of callus cultures from a wide range of plants presents the investigator with a potentially

valuable but time consuming approach. Here an effective and rapid means of screening large numbers of cultures is extremely important.

(2) Production and selection of mutants

Considerable interest has been shown in the production of mutants from plant cell cultures using established microbiological techniques[31]. This approach has great potential, but so far mutants for the production of designated materials have not appeared. Indeed the total number of 'non-leaky' mutant lines available is as yet very small.

(3) Manipulation of culture conditions for optimum yield

Here the usual approach has been to supply a culture previously shown to possess the ability to synthesise and accumulate the designated product with a range of mineral salts, plant growth substances and a primary carbon source in the hope that the secondary metabolite will accumulate in substantial amounts. However, although the material contains the genetic information necessary to set up the synthetic machinery, in practice the *full* expression of this potential is not realised. Attempts to increase the yield of a particular substance by varying the concentrations and balance of growth substances, increasing or decreasing the temperature, manipulating the light intensity and regime have not been particularly successful[32]. Such empirical approaches may on occasions succeed; however, what is really needed is the characterisation of metabolism of the required compound and of the properties of the enzyme(s) involved in its synthesis and degradation, with the ultimate hope of being able to selectively promote production and block degradation *in vivo* and thereby increase net yield.

Let us assume that the particular secondary product of interest X, is on a biosynthetic pathway of the type

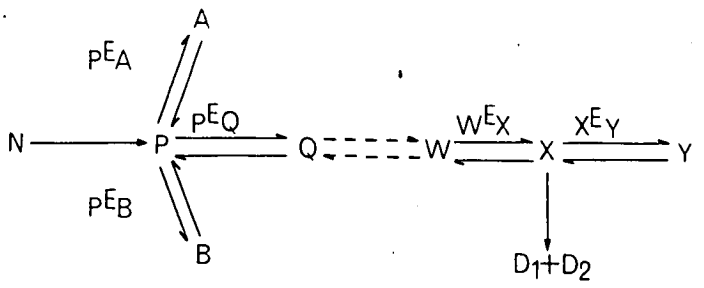



Fig. 1. Model Pathway

Where W is its immediate precursor; P is a general metabolic intermediate which can yield Q, the first intermediate specifically on the pathway of synthesis of X: there are several possible factors which might limit the accumulation of X.

(1) The overall level of metabolism.

(2) Factors specifically restricting entry of material into the pathway leading to X, e.g. (a) alternative metabolism of P, $P \rightleftharpoons A$, $P \rightleftharpoons B$; (b) low activity of the enzyme $^{P}E_{Q}$, or limiting activity of any subsequent enzyme on the pathway leading to X.

(3) Further modification of X ($X \rightleftharpoons Y$) or its breakdown ($X \rightarrow D_1 + D_2$).

Attempts to increase the yields of particular secondary products have used simple empirical methods whereby culture conditions have been varied and the effect on accumulation of X noted. Such an approach is appealing in its simplicity and would probably provide the first step in any similar investigation, but has not generally yielded satisfactory results in the past. Clearly the problem needs approaching in somewhat greater depth, and two immediately obvious aims are suggested in terms of the above scheme. (1) To use conditions under which the contributions of steps $P \rightarrow A$, $P \rightarrow B$, $P \leftarrow Q$, $X \rightarrow Y$ and $X \rightarrow D_1 + D_2$ are minimal. (2) As a corollary, to use conditions under which the contribution of steps $P \rightarrow Q \dashrightarrow W \rightarrow X$ are maximal.

For instance, it should be possible to increase the flow of material along this pathway by supplying, as a substrate, Q, the first precursor specific to the pathway. Then simple equilibrium kinetics would predict that the reaction $P \leftarrow Q$ would be inhibited

by the additional presence of an excess quantity of P. Alternatively if it was decided to supply the general intermediate P, then the diversion of P into other pathways might be inhibited by the simultaneous provision of A and B. Whether such an approach would be practical in terms of the amounts of P, A and B needed to produce a significant effect would depend on the *in vivo* equilibrium positions of the reactions. It would not be expected that a similar approach would be practical in reducing the further metabolism of X, because of the probable high free energy decrease in the reaction $X \longrightarrow D_1 + D_2$, and the cost of supplying appreciable quantities of Y (assuming Y is not readily available commercially).

Using this approach we have investigated the synthesis and accumulation of capsaicin by callus and cell cultures of *Capsicum frutescens*.

## ACCUMULATION OF CAPSAICIN BY CALLUS AND CELL CULTURES OF *CAPSICUM FRUTESCENS*

Evidence from the literature suggests that capsaicin is formed from phenylalanine and valine by two pathways which come together ultimately to form capsaicin from vanillylamine and 8-methyl nonenoic acid (see Fig. 2). Phenylalanine has been shown to be the precursor of the $C_6$-$C_1$ moiety of the capsaicin molecule[33] and it is thought that this proceeds via hydroxylated cinnamic acids as is the case with lignin biosynthesis[34]. Studies on the incorporation of labelled pcoumaric, ferulic and caffeic acids,, and of vanillylamine, tend to support this view. It has also been suggested that the formation of capsaicin diverges from the lignin biosynthetic pathway after the formation of caffeic acid and proceeds via protocatechuic aldehyde[35], or alternatively from ferulic acid which can proceed directly to vanillin if the methylation of the hydroxyl group at the C-3 position occurs prior to cleavage of the side chain[33].

Radioactive feeding experiments suggest that valine is the precursor of the ten-carbon moiety of capsaicin formed via isobutyryl coenzyme-A and 3 acetate units[35]. If this pathway is considered in terms of the general model (see Fig. 1), $X \longrightarrow Y$ and $X \longrightarrow D_1 + D_2$ can be regarded as the further metabolism of capsaicin and its breakdown. These two stages may subsequently be ignored because

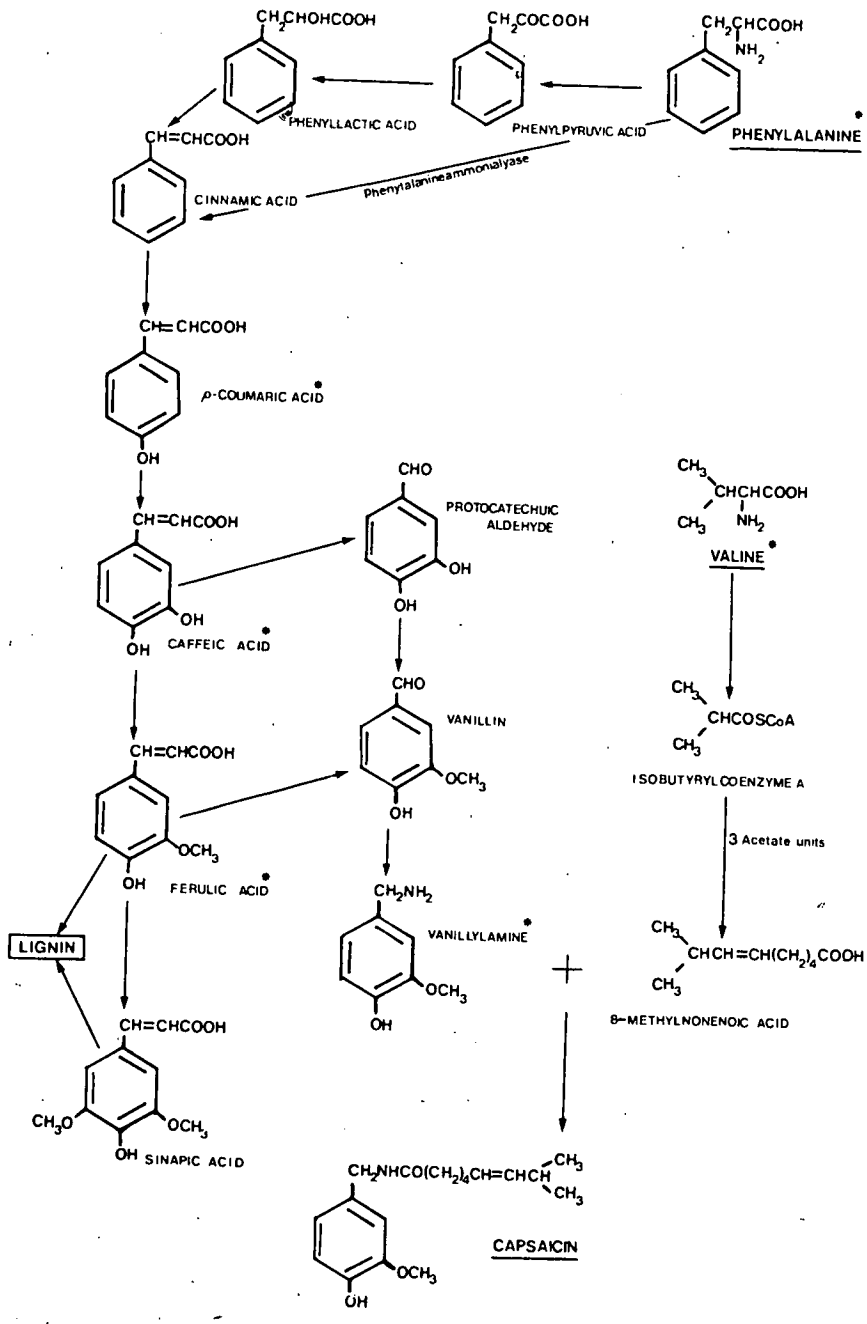



Fig. 2. Proposed biosynthetic pathway for capsaicin from phenylalanine and valine.

no appreciable loss of radioactivity is found when either suspension cultures or callus cells are incubated with radioactive capsaicin[36]. Similarly in the pepper fruit capsaicin also behaves as a stable end product[35].

Returning to the general model (Fig. 1) the step P⟶A may be regarded as the incorporation of either phenylalanine or valine into protein and P⟶B may be regarded as the competition for phenylalanine by the lignin pathway, with some modification of the model(see Fig. 3).

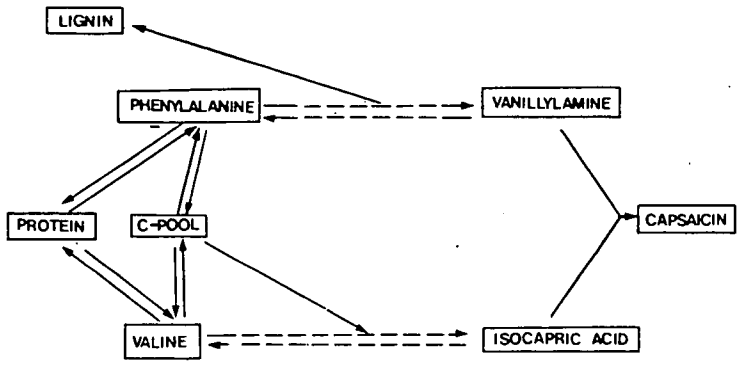
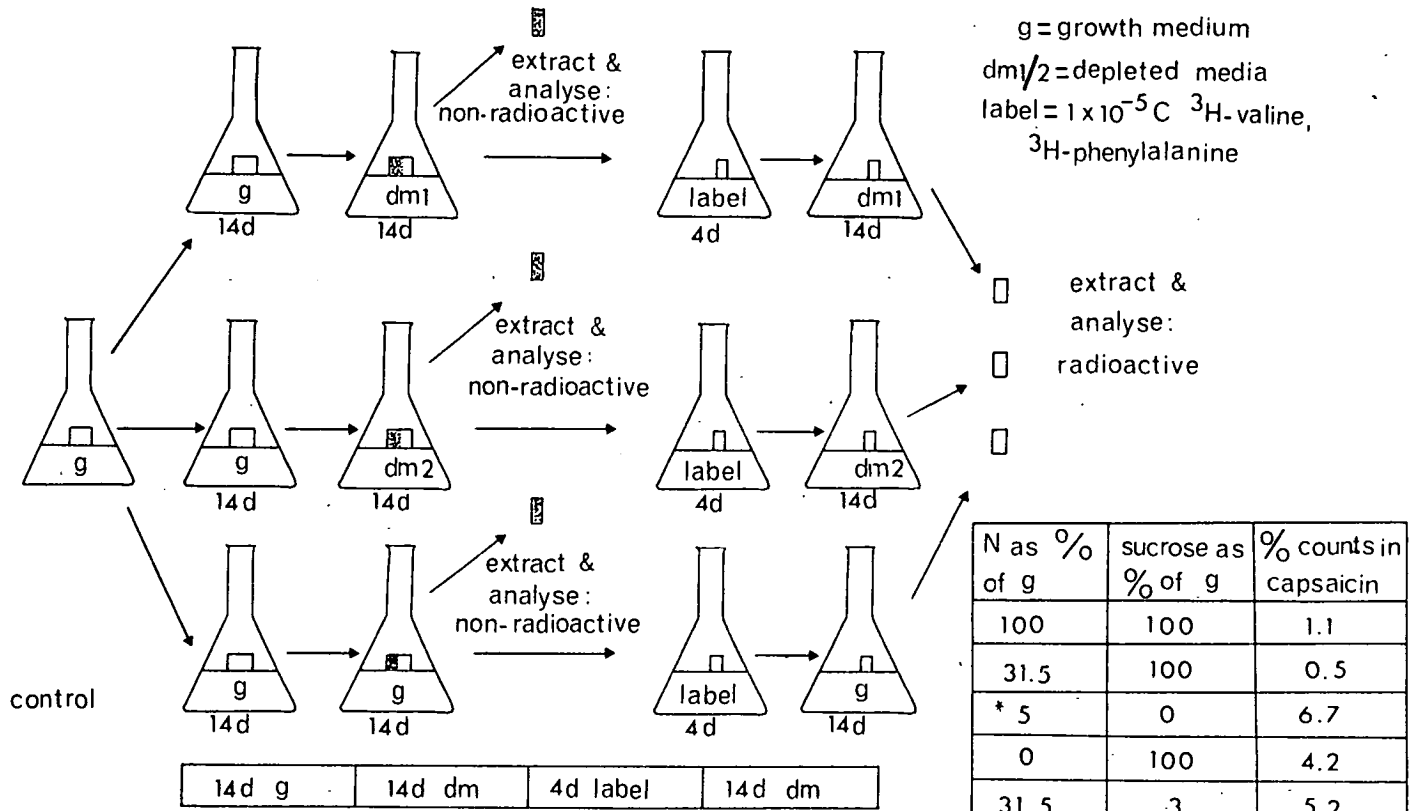



Fig. 3. Modified model for capsaicin accumulation based on Fig. 1 (see text).

From this it is clear that in order to increase the production of capsaicin it is necessary to channel precursors away from the primary metabolic pathways of protein and lignin synthesis and into the capsaicin pathway. However, the situation with respect to the competition for phenylalanine by the enzymes of the lignin pathway is more complicated as capsaicin synthesis only diverges from this at a late stage of the pathway. Accordingly attention was concentrated on the limitation of protein synthesis.

Initially a radioactive assay method was used because this proved to be the most suitable method to detect the vanishingly small amount of capsaicin present in the rapidly growing cultures. The method involved supplying the cultures with $^{3}$H-phenylalanine and $^{3}$H-valine, dropwise, over a period of 4 days and, after 14 days incubation, extraction of the callus culture using chloroform.




| N as % of g | sucrose as % of g | % counts in capsaicin |
|---|---|---|
| 100 | 100 | 1.1 |
| 31.5 | 100 | 0.5 |
| * 5 | 0 | 6.7 |
| 0 | 100 | 4.2 |
| 31.5 | 3 | 5.2 |

Fig. 4. Diagram showing how pepper callus tissue may be manipulated to increase the incorporation of radioactive valine and phenylalanine into capsaicin (see text).

The extracts were then analysed by thin layer chromatography, and radioactive capsaicin from the calluses was identified by co-chromatography with a capsaicin standard. The amount of radioactivity incorporated was determined by a liquid scintillation technique and this was assumed to be proportional to the amount of capsaicin, with obvious reservations.

Experiments using cycloheximide as an inhibitor of protein synthesis have proved successful in increasing the incorporation of labelled phenylalanine and valine into capsaicin[36]. Obviously this cannot be seriously considered as an effective means of enhancing the production of capsaicin because cycloheximide has a severely detrimental effect on the cultures. An alternative means of restricting protein synthesis is by a reduction in the nutrient supply, especially nitrogen and sucrose. Using the protocol outlined in Fig. 4 cultures were transferred to depleted media (dm) and then supplied with radioactive phenylalanine and valine over a period of four days. The cultures were then incubated for a further fourteen days on dm, extracted and analysed as described earlier. It can be seen from the summarised results presented in Fig. 4 that it is possible to increase the incorporation of radioactivity into capsaicin by manipulation of the N and sucrose levels. The most effective treatment was one in which 5% of the total N was used without sucrose.

Manipulation of cultures on agar is very clumsy and the sequential reduction of nutrients also involves disturbing the callus. To overcome these problems and to increase the uniformity of the supply of precursors, a liquid culture system was subsequently used.

The nutrient stress experiments previously performed on agar (see Fig. 4) were repeated using this culture system. A comparison was made between cultures maintained throughout on full Murashige and Skoog medium (Flow Laboratories) and those with medium deficient in N and sucrose. Essentially the same results were obtained with an appreciable increase in incorporation into capsaicin after incubation with a nutrient deficient medium.

It seems clear from the previous experiments that the amino acid precursors phenylalanine and valine may be directed into capsaicin by treatments which retard protein synthesis and slow down growth.

TABLE 1

Capsaicin Accumulation in Response to the Addition of Precursors

| Weeks | Medium | Vanillylamine (mM) | Isocapric Acid (mM) | Phenylalanine (mM) | Valine (mM) | Sample | µg Capsaicin per callus | Capsaicin as a % of dry weight | % Conversion |
|---|---|---|---|---|---|---|---|---|---|
| 2 | MS | 5 | 5 | | | Callus | ND | | |
| | | | | | | Medium | 112 | | |
| 2 | dm* | 5 | 5 | | | Callus | ND | | |
| | | | | | | Medium | 42 | | |
| 2 | dm* +50mM $PO_4$ | 5 | 5 | | | Callus | ND | | |
| | | | | | | Medium | 74 | | |
| 2 | dm* | | | | | Callus | ND | | |
| | | | | | | Medium | ND | | |
| 2 | MS | 5 | 5 | | | Callus | | ND | |
| | | | | | | Medium | | .002 | .09 |
| 2 | MS | | 5 | 5 | | Callus | | ND | |
| | | | | | | Medium | | .004 | .14 |
| 2 | MS | 5 | | | 5 | Callus | | ND | |
| | | | | | | Medium | | .0006 | .02 |
| 2 | MS | | 5 | | | Callus | | .0008 | .026 |
| | | | | | | Medium | | .004 | .12 |

*see Fig. 4

ND = not detectable

However, the amounts of capsaicin accumulated are very small. In an attempt to boost the level of capsaicin in the pepper cultures it was decided to supply vanillylamine and isocapric acid (the probable immediate precursors) using the liquid culture system. In these experiments the capsaicin content was estimated using gas-liquid chromatography. A preliminary experiment was carried out in which the callus was incubated with 5mM vanillylamine and 5mM isocapric acid using different growth media (see Table 1). In this experiment it was not possible to detect capsaicin in the callus extracts, but chloroform extracts of the medium showed the presence of capsaicin in amounts exceeding those previously found using nutrient stress alone. Allowing for differences in the size of the innoculum there is no significant difference between the three treatments which were supplied with vanillylamine and isocapric acid. As can be seen from Figs. 2 and 3 vanillylamine is probably the immediate presursor of the $C_6$-$C_1$ moiety of capsaicin whilst 8-methyl nonenoic acid (similar to isocapric acid) is the other immediate precursor. It is obvious from the model (see Fig. 1) that supplying the immediate precursors eliminates the competion for the more general precursors removing any stimulatory includence (for capsaicin accumulation) of the nutrient deficient medium over the full growth medium.

Supplying vanillylamine and isocapric acid raises the level of capsaicin considerably above that previously achieved with amino acid precursors except that it is mainly present in the medium. Even with further purification of the callus extract it was not possible to detect capsaicin in the callus extract at levels approaching that of the medium. Why capsaicin should be found preferentially in the medium is not known but could it be released by dying cells or even actively exported into the medium; this would compare with the fruit where capsaicin is localised in secretory vesicles[37].

Further experiments carried out using combinations of precursors to establish whether the limiation on production lies between phenylalanine ⟶ vanillylamine or valine ⟶ isocapric acid show (Table 1) that as long as isocapric acid is present then capsaicin is produced, but if only valine is supplied then the production of capsaicin is an order of magnitude less, indicating

that the rate limiting step for the production of capsaicin does not lie between phenylalanine and vannillylamine but is probably between valine and isocapric acid. Further experiments are in progress repeating some of the observations and extending them to ascertain whether the amount of capsaicin produced can be raised by increasing either the incubation time or the amounts of precursors supplied.

A meausre of the success of this approach using nutrient stress together with precursor feeding may be judged from the fact that it has been possible to increase the amount of capsaicin in pepper cultures from ng to 10X µg quantities. A further improvement (c. 100 fold) to mg quantities will make the callus as productive as the Chilli pepper fruit!

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REFERENCES

1. Alfermann, A. W. and Reinhard, E. (1978) Proc. Int. Symp. on Plant Cell Culture, Gesellschaft für Strahlen-und Umweltforschung, mbH, München.
2. Barz, W., Reinhard, E.and Zenk, M. (1977) Plant Tissue Culture and its Biotechnological Application, Springer-Verlag, Berlin, Heidelberg, New York.
3. Staba, E. J. (1977) Plant Cell, Tissue and Organ Culture, Springer-Verlag, Berlin, Heidelberg, New York, pp. 694-702.
4. Bhandary, R., Collin, H. A., Thomas, E. and Street, H. E. (1969) Ann. Bot., 33, 647-656.
5. West, F. R. and Mika, E. A. (1957) Bot. Gaz., 119, 50-54.
6. Thomas, E. and Street, H. E. (1970) Ann. Bot., 34, 657-669.
7. Nikolaeva, L. A. and Vollosovich, A. G. (1972) Rastit. Resor., 8, 188-192.
8. Tabata, M., Yamamoto, Hiraoka, N. and Konoshima, M. (1972). Phytochem., 11, 949-955.
9. Freeman, G. G., Whenham, R. J., Mackenzie, I. A. and Davey, M. R. (1974) Plant Sci. Letters, 3, 121-125.
10. Tabata, M. and Hiraoka, N. (1976) Physiologia Pl., 38, 19-23.
11. Brunet, G. and Ibrahim, R. K. (1973) Z. Pflanzenphysiol., 69, 152-162.
12. Reinhard, E., Corduan, G. and Volks, O. H. (1968) Planta Med., 16, 9-16.

13. Becker, H. (1970) Biochem. Physiol. Pflanzen., 161, 425-441.

14. Corduan, G. and Reinhard, E. (1972) Phytochem., 11, 917-922.

15. Neumann, von D. and Mueller, E. (1974) Biochem. Physiol. Pflanzen., 165, 271-282.

16. Lindsey, K. (1979) Unpublished observations.

17. Hahlbrock, K., Kuhlen, E. and Lindl, T. (1971) Planta, 99, 311-318.

18. Speake, T., McCloskey, P., Smith, W., Scott, T. and Hussey, H. (1964) Nature, Lond., 201, 614-615.

19. Ogutuga, D. and Northcote, D. H. (1970) J. exp. Bot., 21, 258-273.

20. Townsley, P. M. (1974) J. Inst. Can Sci. Technol. Aliment., 7, 76-78.

21. Khanna, P. and Jain, S. C. (1972) Indian Jour. Exp. Biol., 10, 248-249.

22. Khanna, P. and Jain, S. C. (1973) Lloydia, 36, 96-98.

23. Takahaski, M. and Yamada, Y. (1973) Agric. Biol. Chem., 37, 1755-1757.

24. Konoshima, M., Tabata, M., Yamamoto, H. and Hiraoka, N. (1970) Yakugaku Zasshi, 90, 370-377.

25. Meyer-Teuter, H. and Reinert, J. (1973). Protoplasma, 78, 273-283.

26. Ramawat, K. G. amd Arya, H. C. (1979) Phytochem., 18, 484-485.

27. Ramawat, K. G. (1979) PhD Thesis, University of Jodhpur, Jodhpur.

28. Muzukami, H., Konoshima, M. and Tabata, M. (1977) Phytochem., 16, 1183.

29. Neumann, D. and Mueller, E. (1971) Biochem. Physiol. Pflanzen, 162, 503-513.

30. Yamasaki, Y. and Ichihara, A. (1976) J. Biochem. 80, 557-562.

31. Maliga, P. (1976) Cell Genetics in Higher Plants, Akadémai Kiado, Budapest, pp. 59-76.

32. Butcher, D. N. (1977) Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, Springer-Verlag, Berlin, Heidleberg, New York, pp. 668-693.

33. Bennet, D. J. and Kirby, G. W. (1968) J. Chem. Soc. (c), 422.

34. McCalla, D. R. and Neish, A. C. (1959) Can. J. Biochem. and Physiol., 37, 537-547.

35. Leete, E. and Loudon, M. C. L. (1968). J. Am. Chem. Soc., 90:24, 6837-6841.

36. Aitchison, P. A. (1976) Unpublished observations.

37. Furuya, T. and Hashimoto, K. (1954) J. Pharmac. Soc. Japan, 74, 771-772.

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# Accumulation of secondary products as a facet of differentiation in plant cell and tissue cultures

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## INTRODUCTION

The complex metabolic changes which accompany the differentiation of cells in plants bring into operation, or initiate, biosynthetic pathways which result in the accumulation of new compounds. For example, the synthesis and accumulation of lignin by differentiating tracheidal and vessel elements of the xylem (Northcote, 1974) marks the termination of a differentiation process which began much earlier in the expanding cells of the procambium and which prepares the differentiated xylem for water and salt transport. Many of the substances accumulated, such as lignin and chlorophyll, are common, with few exceptions, to all higher plants. In contrast, other compounds appear to be characteristic of a species, or a genus, for example, ricin in *Ricinus communis*, the castor bean and diosgenin in certain species of the genus *Dioscorea*, the yams. Both groups of compounds, the general and the more or less specific, are the products of secondary metabolism and are described as secondary to distinguish them from primary compounds which are the products of primary metabolism. Although it is difficult to distinguish with certainty between primary and secondary metabolism it would appear that it is the products of secondary metabolism which are largely associated with the process of differentiation (Luckner, 1972; Böhm, 1980). Indeed, secondary products are normally found in highly differentiated parts of plants and constitute an important facet of differentiation. Secondary products may be formed as a consequence of differentiation or vice versa, so that a study of secondary metabolism should be expected to yield essential facts about the process of differentiation.

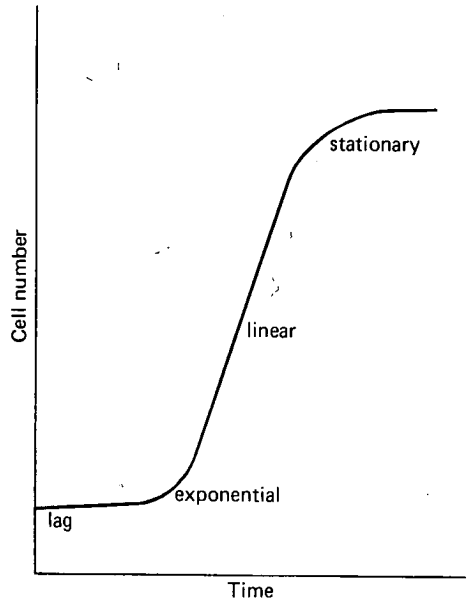


Fig. 1. The generalised growth of a callus or cell culture (shown here in terms of an increase in cell number). Differentiation and the accumulation of secondary metabolites tend to occur maximally when the growth rate decreases, at or approaching the stationary phase.

## USE OF CELL CULTURES FOR THE STUDY OF SECONDARY METABOLISM

Cell cultures from higher plants would appear to be good systems for the study of secondary metabolism because of the ease with which the physical and chemical environment of the cultured cells can be closely controlled and monitored (Barz, Reinhard & Zenk, 1977). Generally, however, it has been found difficult to encourage cultures of plant cells to mimic the synthetic capabilities of the plants from which the culture originated, although there are exceptions (Butcher, 1977; Aitchison & Yeoman, 1977; Staba, 1980; Yeoman, Miedzybrodzka, Lindsey & McLauchlan, 1980). The basis of all studies on the accumulation of secondary products by tissue cultures is that the callus induced from a tissue fragment is sub-cultured to provide either more callus or a cell suspension culture. Although such cultures may appear to be reasonably homogeneous, they are not, and represent mixtures of cells of different shapes, sizes and synthetic capabilities (Yeoman & Forche, 1980). During each period of sub-culture the heterogeneous cell popu-

lation proliferates and the products differentiate freely, producing a variety of cells with different morphologies. If simple growth kinetics are applied to any tissue in culture it can be clearly seen that differentiation follows division in a regular sequence (see Fig. 1) so that the greatest number of differentiated cells is present towards the end of the culture period when cell division has ceased. The transfer of a fragment of this callus to a fresh medium induces a new round of division and differentiation and this constitutes a growth cycle. The growth rate, as measured by the increase in cell number, is high at the beginning of sub-culture and slows down during the growth cycle reaching a low value when differentiation is occurring most extensively. It is during this phase of decreasing growth rate and increasing differentiation that accumulation of secondary products normally takes place. This point can be illustrated from work in this laboratory in which cells of *Dioscorea composita* were grown in suspension culture. It can be seen clearly from Fig. 2 that growth of the cells precedes the accumulation of diosgenin. This agrees well with the situation in many intact plants where accumulation occurs in differentiated structures in which growth is slow or has been terminated. However, here it is relevant to point out an important difference between the culture and the intact plant, which concerns the cytogenetical stability of the constituent cells. The instability of plant cells in culture is well known (Bayliss, 1980) and contrasts with the general stability of the constituent cells of the intact plant. During repeated sub-culture various changes occur in cells which modify the ability of these cells to differentiate. Often sub-culture over an extended period leads to a loss in the ability of a particular cell culture to differentiate in a particular way and may diminish the ability of the cells to synthesise and accumulate a particular secondary product.

A detailed consideration of the literature (see Butcher, 1977; King & Street, 1977; Staba, 1980) shows that rapidly growing cultures tend to accumulate cells and not secondary products, and conversely, mature, highly differentiated cells tend to accumulate secondary products. This suggests quite strongly that an inverse relationship exists between the accumulation of a secondary product (differentiation) and growth (accumulation of cells and primary cellular materials). If an intimate relationship between growth and the accumulation of a secondary product, which the theory predicts, actually exists, then it should be possible to terminate cell division and cell growth prematurely and initiate the accumulation of a particular secondary compound earlier

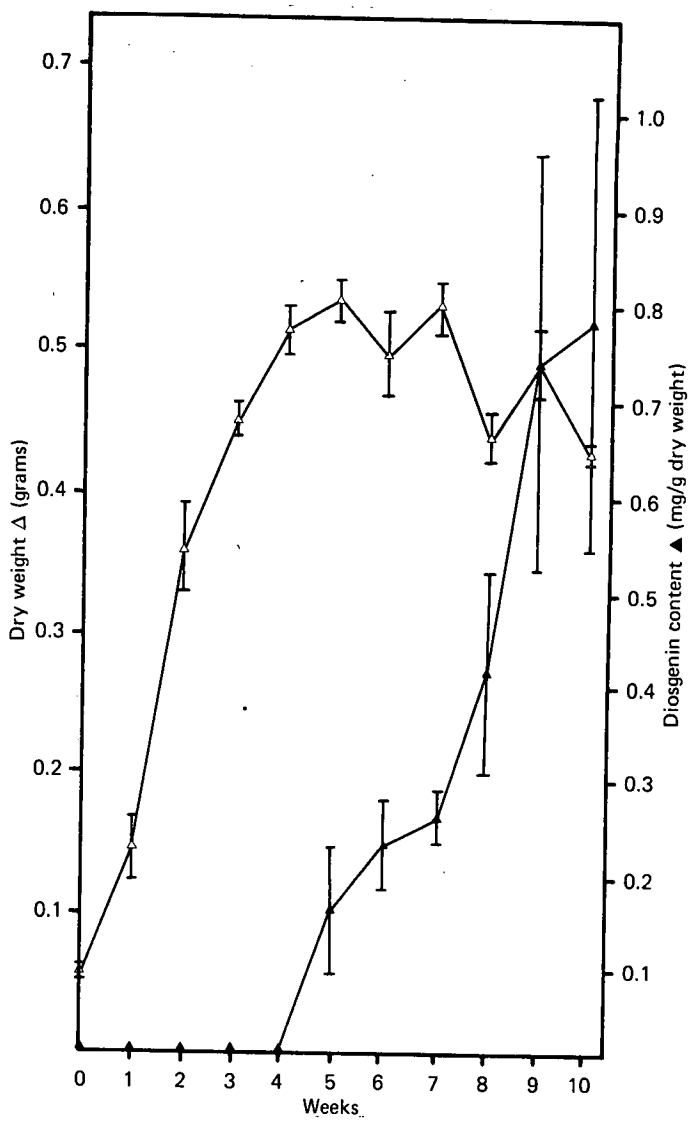


Fig. 2. The pattern of growth (measured as dry weight △) and accumulation of diosgenin (▲) during a growth cycle of *Dioscorea composita* cell suspension cultures. Cells were grown in 60 ml MS medium supplemented with $1.4 \times 10^{-5}$M 2,4-D, in 250 ml Erlenmeyer flasks, which were rotated at 24 ± 1 °C in continuous white light (approximately 200 lux). The vertical bars represent standard errors.

Table 1. *Relationship between alkaloid content, appearance and growth rate of the callus*

| Species | Appearance | Growth rate | Alkaloid content mg/g dry weight |
|---|---|---|---|
| *Datura innoxia* | pale cream, friable | fast | 0.10 |
| *Datura innoxia* | green, compact | slow | 0.40 |
| *Datura stramonium* | pale green | moderate | 0.50 |
| *Datura stramonium* | green/brown | slow | 1.00 |
| *Datura clorantha* | dark brown, friable | moderate | 0.10 |
| *Datura clorantha* | green compact | slow | 1.20 |
| *Solanum dulcamara* | brown | very slow | 0.25 |
| *Solanum dulcamara* | green compact | slow | 1.00 |

Callus sampled at the end of a 28 day culture period.

than it would have occurred in a normal growth situation. It should also be possible to follow the switch from primary to secondary metabolism by the use of a radioactive precursor for the product. Phillips & Henshaw (1977) have successfully switched from protein synthesis (PRIMARY METABOLISM) to the synthesis of polyphenols (SECONDARY METABOLISM) by manipulating the phosphate levels in suspension cultures of sycamore (*Acer pseudoplatanus* L.) and have followed this switch using labelled phenylalanine. They have also shown that this switch can be influenced by other nutrients such as nitrogen, or growth substances.

## RELATIONSHIP BETWEEN ALKALOID CONTENT, APPEARANCE AND GROWTH OF CALLUS

Lindsey (1979, unpublished data) in an extended investigation has studied the relationships which exist between alkaloid content, appearance and growth characteristics of callus cultures of a range of Solanaceous species (see Table 1). It is clear from the data presented that green calluses display a higher alkaloid content than cultures devoid of chlorophyll. This is also true for a range of leguminous species including *Trigonella balansae, T. corniculata, Lupinus angustifolius, L. luteus* and *L. mutabilis*. An additional point which provides further support for the conclusion that secondary product accumulation is inversely proportional to growth can also be seen from Table 1. Pale friable

calluses which are actively growing and poorly differentiated show low levels of alkaloids even in the post-proliferation stage; in contrast slow-growing green cultures accumulate the highest levels of alkaloids.

## EFFECTS OF INHIBITORS ON SECONDARY PRODUCT ACCUMULATION, PROTEIN SYNTHESIS AND GROWTH OF CALLUS

Further evidence in support of the general conclusion that secondary product accumulation is inversely proportional to growth is presented in Table 2. Mizukami, Konoshima & Tabata (1977) have demonstrated that cell cultures of *Lithospermum* can be stimulated to accumulate shikonin derivatives by the addition of streptomycin sulphate, an inhibitor of RNA and protein synthesis. Similar effects have been reported by Neumann & Müller (1971) who have shown that cycloheximide inhibits protein synthesis and therefore growth, but does not affect alkaloid accumulation by callus and suspension cultures of *Nicotiana tabacum*. D-threo-chloramphenicol inhibited growth of the culture, and stimulated free amino acid content as well as alkaloid production. In a more recent paper Neumann & Müller (1974) have shown that actinomycin D promotes alkaloid accumulation in callus cultures of *Macleaya cordata* while cycloheximide inhibits growth without stimulating alkaloid accumulation. Both of these antibiotics inhibit protein synthesis in callus cells of *Macleaya*. Aitchison (1977, unpublished data) working with callus cultures of *Capsicum frutescens* has shown that cycloheximide, at concentrations which inhibit protein synthesis, prevented the incorporation of radioactive phenylalanine and valine into callus cells, but stimulated the incorporation of these amino acids into capsaicin, a secondary product characteristic of the fruit of the plant (Chilli pepper) (see Table 3). From these somewhat limited data (Table 2) it may be tentatively concluded that inhibitors of RNA synthesis tend to stimulate secondary product accumulation while inhibitors of protein synthesis may stimulate secondary product formation or have no effect, certainly at the range of concentrations employed. In all cases the inhibitors of RNA and protein synthesis prevent growth although the long-term effects of substances such as actinomycin D, cycloheximide and D-threo-chloramphenicol are difficult to interpret and these results must be approached with some caution. Despite these reservations it seems reasonable to conclude that

Table 2. *Effects of inhibitors on secondary product accumulation, protein synthesis and growth of callus*

| Species | Inhibitor | Compound | Effect | | | Reference |
|---|---|---|---|---|---|---|
| | | | Compound | Protein synthesis | Growth | |
| *Lithospermum erythrorhizon* | Streptomycin sulphate | shikonin derivatives | ↑ | ↓ | ↓ | Mizukami *et al.* (1977) |
| *Macleaya cordata* | Actinomycin D | alkaloid | ↑ | ↓ | ↓ | Neumann & Müller (1974) |
| *Macleaya cordata* | Cycloheximide | alkaloid | → | ↓ | ↓ | Neumann & Müller (1974) |
| *Nicotiana tabacum* | D-threo-chloramphenicol | alkaloid | ↑ | ↓ | ↓ | Neumann & Müller (1971) |
| *Nicotiana tabacum* | L-threo-chloramphenicol | alkaloid | → | → | → | Neumann & Müller (1971) |
| *Nicotiana tabacum* | Cycloheximide | alkaloid | → | ↓ | ↓ | Neumann & Müller (1971) |
| *Capsicum frutescens* | Cycloheximide | capsaicin | ↑ | ↓ | ↓ | Aitchison 1977 (unpublished data) |

↑ indicates an increase in the level or a stimulation.
↓ indicates a decrease in the level or a depression.
→indicates no effect.

Table 3. *A summary of salient points relating to the incorporation of $^{14}C$ valine and $^{14}C$ phenylalanine into the capsaicin isolated from callus of* Capsicum frutescens *(see Yeoman* et al., *1980)*

| | |
|---|---|
| 1 | $^{14}C$ valine and $^{14}C$ phenylalanine when applied together or separately to actively growing cultures are incorporated into capsaicin at *very* low levels. Most of the recovered radioactivity is in protein. |
| 2 | $^{14}C$ valine and $^{14}C$ phenylalanine when applied together or separately to callus cultures in which the rate of growth has been *slowed down* by reducing the level of nitrogen and/or phosphorus in the medium are incorporated into capsaicin at *much higher* levels. |
| 3 | Application of cycloheximide to actively growing cultures blocks protein synthesis and increases the level of incorporation of $^{14}C$ valine and $^{14}C$ phenylalanine into capsaicin. |

in several instances the accumulation of a particular compound may be stimulated by conditions in which protein synthesis is prevented, which suggests that the metabolic machinery essential for the synthesis of that compound is present in the cells at the time of application of the inhibitor and is sufficient to support synthesis of the designated secondary compound.

## DO CELLS CONTAIN THE ENZYMIC MACHINERY TO SYNTHESISE SECONDARY PRODUCTS PRIOR TO ACCUMULATION?

The accumulation of a particular compound occurs when synthesis exceeds degradation. An increased rate of synthesis accompanied by a smaller increase or a cessation in degradation will lead to an accumulation; similarly in a condition where the rate of synthesis remains unchanged, but the rate of degradation is reduced, accumulation will also be observed. In cells the product may be transported away from the site of synthesis to a vacuole or exported to other cells or outside the organism. Here removal may be considered as synonymous with degradation. Clearly, the particular compound will not be produced in the absence of the biosynthetic pathway. From this it would appear that cultured cells which do not accumulate a particular compound during the growth phase will do so only when growth has slowed down or stopped completely; presumably because the growing cells do not possess the appropriate biosynthetic pathway. Further, it would

Table 4. *A summary of the evidence from the work of Banthorpe on monoterpene synthesis (1979, 1980, unpublished data) supporting the general supposition that biosynthetic pathways for designated secondary metabolites are present in the cells of callus cultures which are not accumulating these compounds (see text for full explanation)*

| | |
|---|---|
| 1. | Callus cultures of *Ocimum basilicum, Tanacetum vulgare, Rosmarinus officinalis* and *Lavandula spica* do not accumulate monoterpenes or terpene glycosides. |
| 2. | Plants of the above-named species do accumulate monoterpenes and terpene glycosides. |
| 3. | Cell-free extracts from both the plant and the callus cultures have the ability to sustain the synthesis of monoterpenes (geraniol, nerol, and α pinene, β pinene, 1,8-cineole and carvone) from isopentenyl pyrophosphate (IPP) which supports the concept that the complete enzyme machinery is present in the callus for monoterpene synthesis. |

follow that as growth slows down and the cells differentiate there is a controlled sequential read-out of the information in the genome which leads to the synthesis of enzymes which are organised into a biosynthetic pathway and the compound is synthesised and accumulated. It is therefore important before proceeding further to determine from the available evidence whether the complete biosynthetic pathways are present or absent from cells not accumulating a particular compound.

The simplest and perhaps the best approach is to discover whether cell-free preparations from cultured cells, which do not accumulate a particular compound, can incorporate a radioactive precursor, far removed from the product, into the designated compound. Here, the work of Banthorpe at the Department of Chemistry at University College, London University, is conclusive. Banthorpe (1979, 1980, unpublished data) (see Table 4) has for some time been examining the ability of established callus cultures from *Ocimum basilicum* (basil), *Tanacetum vulgare* (tansy), *Rosmarinus officinalis* (rosemary), and *Lavandula spica* (lavender) to synthesise and accumulate monoterpenes. None of these cultures accumulated or secreted detectable amounts of monoterpenes, or as far is known, terpene glycosides. However, cell-free extracts of each of these cultures, prepared by three different techniques (see Banthorpe, Bucknall, Doonan, Doonan & Rowan, 1976), could sustain the biosynthesis of monoterpenes (ge-

raniol, nerol, α pinene, β pinene, 1,8-cineole and carvone) from $^{14}C$ isopentenyl pyrophosphate (IPP). Also the observed enzyme levels of the steps of the biosynthetic pathway were *20–4000* fold greater than the enzyme activities that could be extracted, using the same techniques, from the intact plant which displayed the same pattern of products. Additionally in experiments with *Rosa* and Jasmine species Banthorpe prepared active cell-free extracts from calluses which sustained similar reactions to the intact plant but the ratio of enzyme activity, callus/plant was only 0.5–1.5. In all of these studies with cell-free extracts it is important to realise that the lower activities observed with material from the intact plant may reflect a destruction of activity or deactivation of enzymes by complexing of proteins to phenols and quinones. Here it is relevant to point out that the callus extracts contained a lower concentration of phenolic compounds than the intact plant. Recent experiments in this laboratory on alkaloid accumulation in cell suspension cultures of *Solanum nigrum* (Perez-Frances 1980, unpublished data) have demonstrated that cell-free extracts of a culture in the exponential phase of growth not accumulating hyoscyamine, will sustain the conversion of $^{14}C$ ornithine to hyoscyamine. Perez-Frances also showed that exponentially growing cell cultures of *S. nigrum* would incorporate $^{14}C$ ornithine into hyoscyamine demonstrating the operation of the biosynthetic pathway in these cells.

The results obtained in investigations with callus and cell cultures of *Capsicum frutescens* in this laboratory (Yeoman *et al.*, 1980) also support the general idea that the enzymic machinery for the synthesis of a particular secondary compound, in this case capsaicin, is present within cells not accumulating capsaicin. Callus tissue, either freshly isolated or which has been in culture for four years, will not accumulate capsaicin during the course of a growth cycle on an agar growth medium. Although capsaicin cannot be detected in the culture by sensitive analytical procedures (e.g. GLC) the compound can be labelled by feeding the callus with $^{3}H$ or $^{14}C$ phenylalanine or valine (see Table 3). Extracts of Chilli pepper callus from experiments with these labelled amino acids contain radioactively marked capsaicin and dihydrocapsaicin. These compounds can be detected as radioactive areas on TLC plates and will co-chromatograph with pure samples of these compounds in a number of solvent systems. It is estimated, but this must be taken as very approximate, that the level of capsaicin in the cells is of the order of $10^{-10}$M. However, the level of label in

capsaicin can be raised substantially by manipulating the conditions in which the cultures are maintained (Yeoman *et al.*, 1980) or by the use of inhibitors (Table 3). In conditions of 'nutrient stress' in which most of the phosphate has been utilised and precursors are present (the cells are growing very slowly) the yield of capsaicin can be increased spectacularly to milligram levels which is comparable with the concentration present in the mature fruit of the Chilli pepper (2–3 mg).

The results of an experiment in which phenylalanine, valine, vanillylamine and iso-capric acid were fed in various combinations to callus cultures of *Capsicum frutescens* are shown in Table 5. The experiments were conducted using a 'flat-bed' culture apparatus. The principal feature of this culture system is the movement of a liquid nutrient medium across stationary cells. The medium drips from a reservoir into a culture vessel containing cells seated on a synthetic fabric substratum, moves by capillary action across the fabric, and is thence pumped from the vessel back into the reservoir. Such a system allows sequential chemical treatments and additions of relatively large quantities of precursors at low concentration. This culture situation is one in which cell differentiation takes place at rates comparable to that of callus on an agar medium. It can be seen from the data summarised in Table 5 that capsaicin cannot be detected in either callus or medium unless precursors are supplied to the cells. The highest yields were obtained in the presence of iso-capric acid, a precursor close to the end of the biosynthetic pathway (see Yeoman *et al.*, 1980). The presence of vanillylamine which might be expected to further increase the yield decreased the overall amount of capsaicin. This may be due to its toxicity or some antagonistic effect. The best yield was comparable to that found in the mature fruit, 2.3–2.5 mg of capsaicin/g dry weight. The general conclusion from this work with the Chilli pepper is that the slowing down of growth (phosphate is depleted within a few days) and the provision of precursors, results in the accumulation of capsaicin in the medium. In this case the induction of the biosynthetic pathway by the addition of precursors is possible but unlikely. Two pieces of evidence support this interpretation. The first is that in earlier experiments with *Capsicum* callus (Yeoman *et al.*, 1980) very small amounts of $^{3}H$ or $^{14}C$ valine or phenylalanine are rapidly incorporated into capsaicin; although enzyme induction is possible, it seems unlikely at the concentrations used. Secondly, in experiments with cultures from *Solanum nigrum* (Prez-Frances 1980, unpublished data), $^{14}C$ ornithine fed to intact cells, or a cell-free extract, was rapidly incorporated into

Table 5. *Levels of capsaicin accumulated in callus cultures of* Capsicum frutescens

| | Source | Vanillylamine | Iso-capric acid | Phenylalanine | Valine | Incubation time (weeks) | µg per dish | % maximum theoretical yield |
|---|---|---|---|---|---|---|---|---|
| 1 | Callus | — | — | — | — | 2 | ND | — |
| | Medium | | | | | | | |
| 2 | Callus | — | 5 mM | 5 mM | — | 2 | ND | — |
| | Medium | | | | | | 75 | 0.14 |
| 3 | Callus | — | 5 mM | — | — | 3 | ND | — |
| | Medium | | | | | | 2371 | 4.20 |
| 4 | Callus | 10 mM | 10 mM | — | — | 3 | ND | |
| | Medium | | | | | | 597 | 0.47 |
| 5 | Callus | 5 mM | — | — | — | 3 | ND | — |
| | Medium | | | | | | 171 | 0.26 |
| 6 | Callus | 5 mM | — | — | 5 mM | 2 | ND | — |
| | Medium | | | | | | 11 | 0.02 |

ND, Not Detectable.

hyoscyamine; this alkaloid could not be detected in these cultures without ornithine using conventional analytical procedures. Here the biosynthetic pathway from ornithine to hyoscyamine is present in cultures not accumulating the alkaloid. It is also interesting, and perhaps important, to note that if the detached fruits of *Capsicum annuum* var. grossum, the sweet pepper, which does *not* naturally accumulate capsaicin or dihydrocapsaicin, are supplied with iso-capric acid and vanillylamine, dihydrocapsaicin and related capsaicinoids can be detected at a level of 7.2 μg/fruit after six days. It is also highly significant that cell-free extracts of these sweet pepper fruits will synthesise dihydrocapsaicin from a reaction mixture containing vanillylamine and iso-capric acid (Iwai, Suzuki, Lee, Kobashi & Oka, 1977). Clearly here is another example where at least part of the biosynthetic pathway for capsaicin is present in a structure which does not naturally accumulate the compound.

## A WORKING HYPOTHESIS

The evidence presented, although fragmentary, suggests that the inability of at least some cultures of plant cells to accumulate a designated compound or compounds characteristic of the species may *not* be due to the absence of the complete biosynthetic pathway(s) for that compound(s) from the cells. Although there may be exceptions the general rule appears to be that if the chemical milieu encourages growth then growth will occur and when growth ceases or is stopped, because of a shortage or withdrawal of an essential nutrient or nutrients, a metabolic shunt will occur in which biosynthetic pathways are modified, brought into play and new compounds are produced. This accumulation occurs, as in the intact plant at the expense of growth and proliferation, and the addition of precursors for the particular metabolite may encourage synthesis and accumulation, particularly at the 'down-turn' of growth.

At first, we (see Yeoman *et al.*, 1980) were in favour of a 'plumbing analogy' in which the primary and secondary pathways were always present and switching off or reducing the flow of metabolites through the primary pathways leading to protein synthesis and growth would divert certain essential key metabolites into the secondary pathways. However, the argument can be raised that if the flow of metabolites through the primary pathways is proceeding at a *maximum* rate then the provision of a block (slowing down or cessation of growth), would

not increase the rate of flow through the 'narrower pipes' of the secondary pathways. Clearly the hypothesis lacks switches or perhaps more appropriate to the plumbing analogy 'valves'.

From the evidence available it would appear that at least in some culture systems the complete biosynthetic pathway for the particular metabolite in question is present in the cells at times when the culture is *not* accumulating the secondary product and that the addition of a precursor to a cell-free extract will result in the sustained production of the product. This, together with the suggestion that growth is inversely proportional to secondary product accumulation, would suggest that the pathways are present but inoperative and that a switch brings the existing pathway(s) into operation. If we can indulge ourselves with a further analogy it is rather like the switching of points on a railway system which diverts the train from the main to the branch line! Both sets of tracks are present and it is the setting of the points which determines the destination of the train.

Clearly the nature and extent of the switching is crucial. The simplest concept would be to invoke a small number of key enzymes which control the flux of a few important metabolites through various secondary pathways. One candidate immediately springs to mind, phenylalanine as the important metabolite and phenylalanine ammonia lyase (PAL) as the key enzyme. Phenylalanine is a molecule which stands at an important cross-roads in metabolism; it is used extensively in protein synthesis and for a wide range of polyphenolic compounds. Labelled phenylalanine fed to actively growing cultures of *Capsicum frutescens* is rapidly incorporated into protein; however the same compound fed to cultures which have ceased to grow but begun to differentiate will be incorporated into a range of polyphenolic compounds and capsaicin. It has been observed by Davies (1971, 1972) that PAL activity increases very considerably in cell cultures of a *Rosa* sp. immediately before the accumulation of polyphenolics begins. Similar observations have been made by Hahlbrock, Kuhlen & Lindl (1971) who have shown a large increase in PAL activity in dark grown batch cell cultures of *Glycine max* at the end of the growth period. These have been interpreted as indicative of the opening up of aspects of secondary metabolism. PAL is an enzyme which can be induced by light and an increase in the activity of PAL is usually followed by an increase in the formation of a particular range of polyphenolic compounds. Isoenzymes of PAL have not yet been found in cell cultures (Zaprometov, 1978). The synthesis and accumulation of many flavo-

noid compounds are light-dependent, particularly in cultured cells. The light-dependent regulation of biosynthesis of cinnamic acids and some classes of flavonoids at the enzymic level has been studied extensively by Hahlbrock (1977). He has shown that in cell cultures of parsley (*Petroselinum hortense*) and soybean (*Glycine max*), there are two integrated enzyme groups which respond to illumination in different ways. One of the groups is represented by the enzymes of the phenylpropanoid pathway, the other of the flavonoid pathway. Together the two groups of enzymes catalyse the conversion of phenylalanine and several other substrates derived from intermediary metabolism to a variety of flavone and flavonol glycosides (Hahlbrock, 1977; Ebel & Hahlbrock, 1977; Hahlbrock, Betz, Gardiner, Kreuzaler, Matern, Ragg, Schäfer & Schröder, 1978). The first group comprises the three enzymes of general phenylpropanoid metabolism beginning with PAL, whereas the enzymes of the flavonoid glycoside pathway are members of the second group. Clearly, PAL fills the role of a key enzyme which can be switched quite easily and divert an essential metabolite into secondary metabolism.

The essential features of the ideas developed in this article are summarised in Fig. 3. Schemes 1 and 2 represent two possible explanations. The pathways concerned with primary metabolism are lightly stippled, those with secondary metabolism heavily shaded. In both schemes A represents no synthesis of a designated secondary compound and B synthesis of a designated secondary compound. In scheme 1 the option is presented where the biosynthetic pathways are present but inoperative until switched by a key enzyme, on the left A is present but closed and inoperative, on the right B is present, switched on and operative. In scheme 2 the option is presented in which the biosynthetic pathway for a designated compound is absent, and therefore inoperative (A) and subsequently the complete pathway is produced *de novo* and when present is operative (B). The evidence presented in this article although incomplete is consistent with scheme 1; however considerable caution must be exercised in applying this model to all situations in which secondary products are accumulated, especially where the inverse relationship between the accumulation of the designated secondary product and growth is unclear. Like most models it is simplistic and is put forward only as a working hypothesis to encourage further experimentation in a difficult and complex area of research. The central idea of the control of some aspects of differentiation in plants by key switch enzymes which can bring biosynthetic

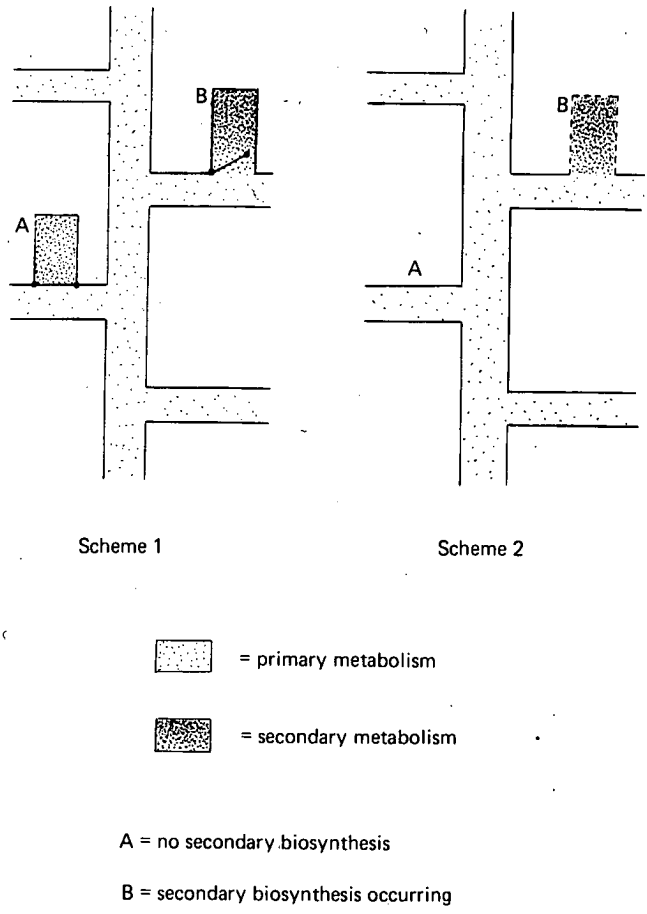


Fig. 3. A diagrammatic representation of two schemes providing an explanation of how a switch may occur between primary and secondary metabolism in cultured plant cells.

In both schemes A represents a state in which there is no synthesis of a designated secondary compound and B a state in which synthesis occurs. The lightly stippled areas are pathways of primary metabolism and the shaded areas pathways of secondary metabolism. (See text for full explanation of model.)

pathways already present rapidly into operation has some merit. Plants are organisms which are unable to move about, they have evolved against a changing environment and the successful individuals are those which respond most effectively to the prevailing physical conditions. For example, it is the nature of the metabolic machinery within the green plant which determines the speed of response and therefore a control system in which a limited number of key regulatory enzymes are involved would be of selective advantage. In contrast, the *de novo*

production of a complete biosynthetic pathway would delay the response and jeopardise survival.

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## REFERENCES

AITCHISON, P. A. & YEOMAN, M. M. (1977). Growth patterns in tissue (callus) cultures. In *Plant Tissue and Cell Culture*, ed. H. E. Street, pp. 267–306, *Botanical Monographs 11*. Oxford: Blackwell.

BANTHORPE, D. V., BUCKNALL, G. A., DOONAN, H. J., DOONAN, S. & ROWAN, M. G. (1976). Biosynthesis of geraniol and nerol in cell-free extracts of *Tanacetum vulgare*. *Phytochemistry*, **15**, 91–100.

BARZ, W., REINHARD, E. & ZENK, M. H. (1977). *Plant Tissue Culture and its Biotechnological Application*. Berlin: Springer-Verlag.

BAYLISS, M. W. (1980). Chromosomal variation in plant tissues in culture. In *Perspectives in Plant Cell and Tissue Culture*, ed. I. K. Vasil, pp. 113–44, *International Review of Cytology, Supplement IIA*. New York: Academic Press.

BÖHM, H. (1980). The formation of secondary metabolites in plant tissue and cell cultures. In *Perspectives in Plant Cell and Tissue Culture*, ed. I. K. Vasil, pp. 183–208, *International Review of Cytology, Supplement IIB*. New York: Academic Press.

BUTCHER, D. N. (1977). Secondary products in tissue cultures. In *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture*, ed. J. Reinert & Y. P. S. Bajaj, pp. 668–93. Berlin: Springer-Verlag.

DAVIES, M. (1971). Multisample enzyme extraction from cultured plant cell suspensions. *Plant Physiology, (Lancaster)*, **47**, 38–42.

DAVIES, M. (1972). Polyphenol synthesis in cell suspension cultures of Paul's Scarlet Rose. *Planta*, **104**, 50–65.

EBEL, J. & HAHLBROCK, K. (1977). Enzymes of flavone and flavonol-glycoside biosynthesis. *European Journal of Biochemistry*, **75**, 201–9.

HAHLBROCK, K. (1977). Regulatory aspects of phenylpropanoid biosynthesis in cell cultures. In *Plant Tissue Culture and its Biotechnological Application*, ed. W. Barz, E. Reinhard & M. H. Zenk, pp. 95–111. Berlin: Springer-Verlag.

HAHLBROCK, K., BETZ, B., GARDINER, S. E., KREUZALER, F., MATERN, U., RAGG, H., SCHÄFER, E. & SCHRÖDER, J. (1978). Enzyme induction in cultured cells. In *Frontiers of Plant Tissue Culture*, ed. T. A. Thorpe, pp. 317–24. Calgary: IAPTC.

HAHLBROCK, K., KUHLEN, E. & LINDL, T. (1971). Anderungen von Enzymaktivitaten während des Wachstums von Zellsuspensionskulturen von *Glycine max*: phenylalanin ammonium und p-cumarat: CoA Ligase. *Planta*, **99**, 311–18.

IWAI, K., SUZUKI, T., LEE, K., KOBASHI, M. & OKA, S. (1977). *In vivo* and *in vitro*

formation of dihydrocapsaicin in sweet pepper fruits, *Capsicum annuum* L. var. grossum. *Agricultural and Biological Chemistry*, **41**, 1877–82.

KING, P. J. & STREET, H. E. (1977). Growth patterns in cell cultures. In *Plant Tissue and Cell Culture*, ed. H. E. Street, pp. 307–87, *Botanical Monographs 11*. Oxford: Blackwell.

LUCKNER, M. (1972). *Secondary Metabolism in Plants and Animals*. London: Chapman & Hall.

MIZUKAMI, H., KONOSHIMA, M. & TABATA, M. (1977). Effect of nutritional factors on shikonin derivative formation in *Lithospermum* callus cultures. *Phytochemistry*, **16**, 1183–6.

NEUMANN, VON D. & MÜLLER, E. (1971). Beiträge zur Physiologie der Alkaloide. *V* Alkaloidbildung in Kallus und Suspensionskulturen von *Nicotiana tabacum* L. *Biochemie und Physiologie der Pflanzen*, **162**, 503–13.

NEUMANN, VON D. & MÜLLER, E. (1974). Beiträge zur Physiologie der Alkaloide. *IV* Alkaloidbildung in Kalluskulturen von *Macleaya*. *Biochemie und Physiologie der Pflanzen*, **165**, 271–82.

NORTHCOTE, D. H. (1974). *Differentiation in Higher Plants. Oxford Biology readers, 44*, ed. J. J. Head. Oxford University Press.

PHILLIPS, R. & HENSHAW, G. G. (1977). The regulation of synthesis of phenolics in stationary phase cell cultures of *Acer pseudoplatanus* L. *Journal of Experimental Botany*, **28**, 785–94.

STABA, E. J. (1980). *Plant Tissue Culture as a Source of Biochemicals*. Florida: CRC Press.

YEOMAN, M. M. & FORCHE, E. (1980). Cell proliferation and growth in callus cultures. In *Perspectives in Plant Cell and Tissue Culture*, ed. I. K. Vasil, pp. 1–24, *International Review of Cytology, Supplement IIA*. New York: Academic Press.

YEOMAN, M. M., MIEDZYBRODZKA, M. B., LINDSEY, K. & MCLAUCHLAN, W. R. (1980). The synthetic potential of cultured plant cells. In *Plant Cell Cultures: Results and Perspectives*, ed. F. Sala, B. Parisi, R. Cella & O. Ciferri, pp. 327–43. Amsterdam: Elsevier/North-Holland.

ZAPROMETOV, M. N. (1978). Enzymology and regulation of the synthesis of polyphenols in cultured cells. In *Frontiers of Plant Tissue Culture*, ed. T. A. Thorpe, pp. 335–43. Calgary: IAPTC.