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THE EFFECT OF LIGHT ON GROWTH AND ALKALOID SYNTHESIS  
IN CULTURES OF *NICOTIANA* SPP.

by

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Michael C. Hobbs. 1988

## ABBREVIATIONS

ASP	aspartate
ASCORB	ascorbic acid
B <sub>5</sub> medium	Gamborgs B <sub>5</sub> medium
BAP	6-benzylaminopurine
BSA	Bovine serum albumin
butyl-PBD	2-(4'-tetra-butylphenyl)-5-(4''-biphenyl)-1,3,4,-Oxadiazole
°C	degrees centigrade
c (as a prefix)	centi (10 <sup>-2</sup> )
C	<i>circa</i>
CO <sub>2</sub>	carbon dioxide
conc	concentrated
cpm	counts per minute
Cv	cultivar
DTT	DL-dithiothreitol
dpm	disintegrations per minute
E	Einstein(s)
EDTA	ethylenediamine-tetraacetic acid
<i>et al</i>	<i>et alia</i>
eg	for example
F <sub>0</sub>	"constant" fluorescence
fr wt	fresh weight
g	gramme(s)
GC-MS	Gas Chromatography Mass Spectrometry
G-3-P	glyceraldehyde 3 phosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
ie	that is
kinetin	6-furfurylaminopurine
l	litre(s)
LED	light emitting diode
Log	logarithmic
μ (as a prefix)	micro (10 <sup>-6</sup> )
m (as a prefix)	milli (10 <sup>-3</sup> )
M	molar (1mole/litre)
min	minute(s)
MOPS	3-(N-morpholino)propane sulfonilic acid
MS medium	Murashige and Skoog medium
MSS medium	Murashige and Skoog plant salts medium
n (as a prefix)	nano (10 <sup>-9</sup> )

N <sub>2</sub>	nitrogen
NAA	α- naphthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
NaAD	nicotinic acid adenine dinucleotide
NaMN	nicotinic acid mononucleotide
ND	not detectable
NMN	nicotinamide mononucleotide
No	number
O <sub>2</sub>	oxygen
OD	optical density
PD <sub>10</sub>	prepacked sephadex G25 desalting column
pH	negative log of the hydrogen ion concentration
ppm	parts per million
PMSF	phenylmethylsulfonyl fluoride
POPOP	1,4-di-2-(5-phenyloxazolyl)-benzene
PPO	2-5 diphenyloxazole
PRPP	phosphoribosylpyrophosphate
psi	pounds per square inch
PVP	polyvinylpyrrolidone
QaPRT'ase	quinolinic acid phosphoribosyl transferase
Rf	distance moved by a compound relative to a solvent front
rpm	revolutions per minute
SE	standard error
sec	second(s)
SH medium	Schenk and Hildebrandt medium
spp	species (plural)
TLC	Thin Layer Chromatography
UQ	unquantifiable
UV	ultra violet
V	volt(s)
v/v	volume per unit volume as a percentage
W	watt(s)
W medium	Whites medium
w/v	weight per unit volume (grammes/litre)as a percentage
wt	weight
xg	times the force of gravity
X	mean of values
%	percent
+/-	plus or minus

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## ABSTRACT

The aims of this project were to investigate the effects of illumination on growth and synthesis of four major tobacco alkaloids: anabasine, anatabine, nicotine and nornicotine, in *Nicotiana* spp; and to determine whether any effects of light on alkaloid synthesis were photosynthetic or photomorphogenic in nature.

The cultures used in this study were isolated from three tobacco species. Cultures of *N.tabacum* with different degrees of dependence on photosynthesis for the provision of organic constituents were selected, using the chlorophyll fluorescence properties of the constituent cells. As cultures became more photosynthetic, and were provided with less carbon in the medium, the nutrient limiting growth appeared to change from phosphate to fructose, while at the same time the growth period was reduced. The photosynthetic ~~requirements~~ of cultures ranged from cultures that were non-photosynthetic to cultures that grew in medium lacking added carbon.

The alkaloids were extracted from plant and cultured material using a technique developed to enable the extraction of all four main alkaloids simultaneously. The alkaloid content of green illuminated cultures was greatly reduced compared with non-green cultures, and this decreased accumulation did not correlate with photosynthetic activity. Movement of callus cultures of *N.glauca* from the dark into the light promoted chlorophyll synthesis and suppressed anabasine synthesis, although not to the level seen in cultures grown continuously in the light. Anabasine accumulation in these cultures occurred during the stationary phase of the culture cycle.

The inability of green illuminated cultures to accumulate alkaloids was not due to photochemical degradation of the alkaloids, since alkaloid added to cultures was recovered after a period of growth. Nicotine added to cultures was

converted at a high efficiency to nornicotine with no overall change in alkaloid content.

The activity of quinolinic acid phosphoribosyl transferase, an enzyme involved in the synthesis of nicotinic acid a common precursor to all of the nicotine alkaloids, was greater in the roots than the shoots of tobacco plants. This enzyme was not detected in green illuminated cultures of *N.glauca* but in non-green unilluminated cultures of this species there was an activity similar to that in plant roots.

The results of this investigation show that light suppresses alkaloid synthesis in cultures of *Nicotiana* spp and that the activity of quinolinic acid phosphoribosyl transferase is higher in tissues that synthesise the alkaloids than in tissues which do not.

**CHAPTER 1**  
**INTRODUCTION.**

## *NICOTIANA TABACUM* L. (1753).

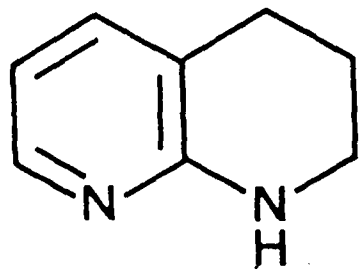
*Nicotiana* is a genus in the Solanaceae, which contains more than 2,000 species. Over half of the species are in the type genus *Solanum* while the remaining species are distributed between over 70 genera. *Nicotiana* is one of the largest of these genera containing in the region of 60 species (Goodspeed 1954). From morphological and metabolic characteristics it has been proposed that the species *N.tabacum* originated on forest margins in mid to low altitude zones of Central and South America (Papenfus & Quin 1984) from the natural hybridization of *Nicotiana sylvestris* Speg. & Comes and *Nicotiana tomentosiformis* Goodsp. (Wernsman & Matzinger 1980). The resultant allotetraploid probably owes its survival to man, since it is only found in association with man (Goodspeed 1954, Papenfus & Quin 1984).

Tobacco has been used by man, for its pharmacological effects, for at least 5,000 years (Morris 1988). In more recent times improvements in the crop have been achieved through plant breeding (Smeeton 1987) and through a greater understanding of the effect of cultural practices (Papenfus & Quin 1984). Further improvement of tobacco quality is hindered by an inability to define quality in measurable terms (Smeeton 1987). With an increased understanding of the pharmacological, olfactory and other effects of individual chemical components of smoke a more defined product can be aimed for. There is then the problem that most quality related attributes have complex inheritance characteristics thus future improvements in tobacco may come through the use of molecular genetical techniques (Smeeton 1987). In order to carry out a structured programme of plant product improvement by such methods an understanding of the metabolism of the plant, and products of interest in particular, is required so that specific regulatory points can be modified in an attempt to obtain the desired end result.

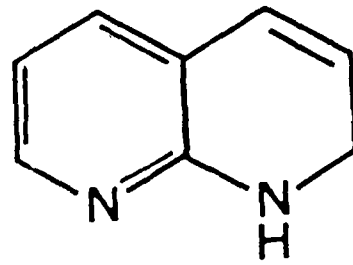
## THE TOBACCO ALKALOIDS AND THEIR SYNTHESIS.

The majority of work undertaken on tobacco has involved the tobacco alkaloids, in particular nicotine. In *N.tabacum* four main alkaloids: anabasine, anatabine, nicotine and nornicotine (Fig 1.1) are synthesised and accumulated (Saitoh *et al* 1985). All of these alkaloids contain a pyridine ring which is formed from nicotinic acid (Leete 1977a). The pyridine ring combines with ring structures derived from: ornithine, lysine and another molecule of nicotinic acid to form nicotine (and subsequently nornicotine), anabasine and anatabine respectively (Fig 1.2) (see Bush 1981).

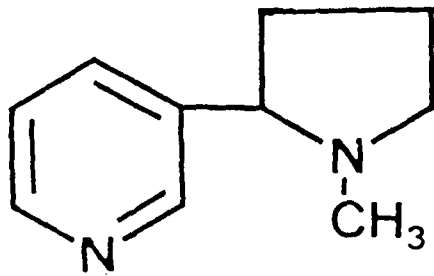
Nicotinic acid is an intermediate in the pyridine nucleotide cycle (PNC) (Fig 1.3) (Gholson 1966, Ryrie & Scott 1969), which is involved in the synthesis and recycling of NAD (White 1982). NAD is synthesised by the addition of a phosphoribosyl group to quinolinic acid, by the enzyme quinolinic acid phosphoribosyl transferase, to form nicotinic acid mononucleotide (NaMN) (Hadwinger *et al* 1963). NaMN is converted to NAD via the intermediate nicotinic acid adenine dinucleotide (NaAD). The majority of enzymes requiring NAD catalyze oxidation-reduction reactions, and do not lead to an overall cellular loss of NAD. However, some enzymes cleave the N-glycosidic bond or the pyrophosphate linkage of NAD. The metabolism of NAD, in this way, leads to the formation of nicotinamide or nicotinamide mononucleotide (NMN) (White 1982). Nicotinamide and NMN are recycled to reform NAD via the PNC (Gholson 1966) with this salvage of NAD metabolites accounting for a large percentage of NAD formation. Nicotinamide can be recycled to NAD via NMN (White 1982). However, the recycling of NAD metabolites via nicotinic acid is of particular interest in relation to this study since nicotinic acid is a precursor of the alkaloids. NMN can be converted via nicotinamide to nicotinic acid. The formation of NAD from nicotinic acid proceeds along the Preiss-Handler pathway, via NaMN and NaAD (Preiss & Handler 1958a,b). Therefore the availability of nicotinic acid for alkaloid synthesis appears to be closely associated with the synthesis of NAD.



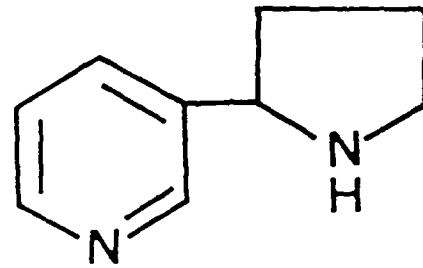
ANABASINE



ANATABINE



NICOTINE



NORNICOTINE

FIGURE 1.1

The tobacco alkaloids.



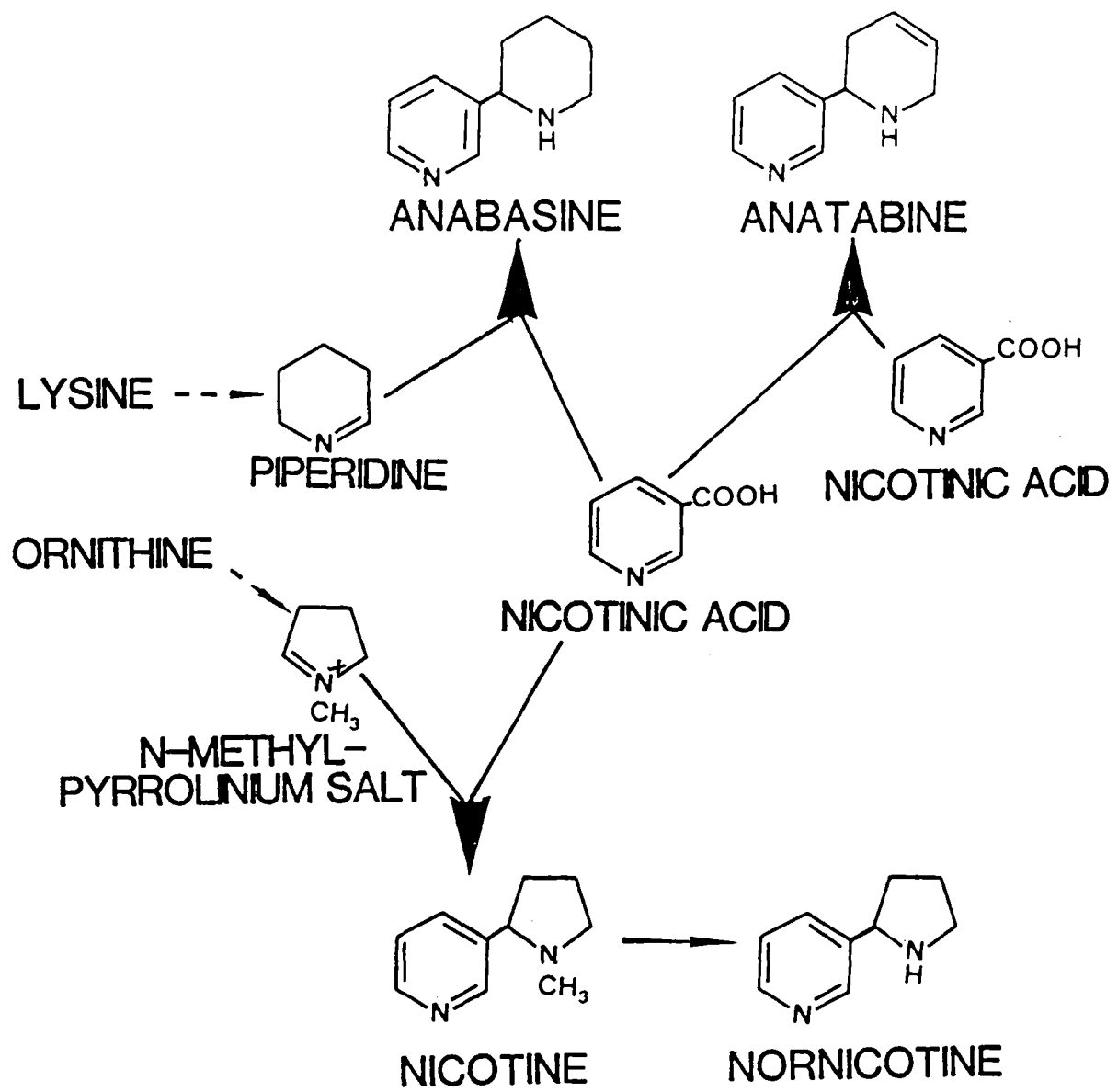


FIGURE 1.2

A schematic representation of the synthesis of the tobacco alkaloids.

**FIGURE 1.3**

**The pyridine nucleotide cycle.**

**G-3-P = Glyceraldehyde 3 phosphate.**

**ASP = Aspartate.**

**Qa = Quinolinic acid.**

**NaMN = Nicotinic acid mononucleotide.**

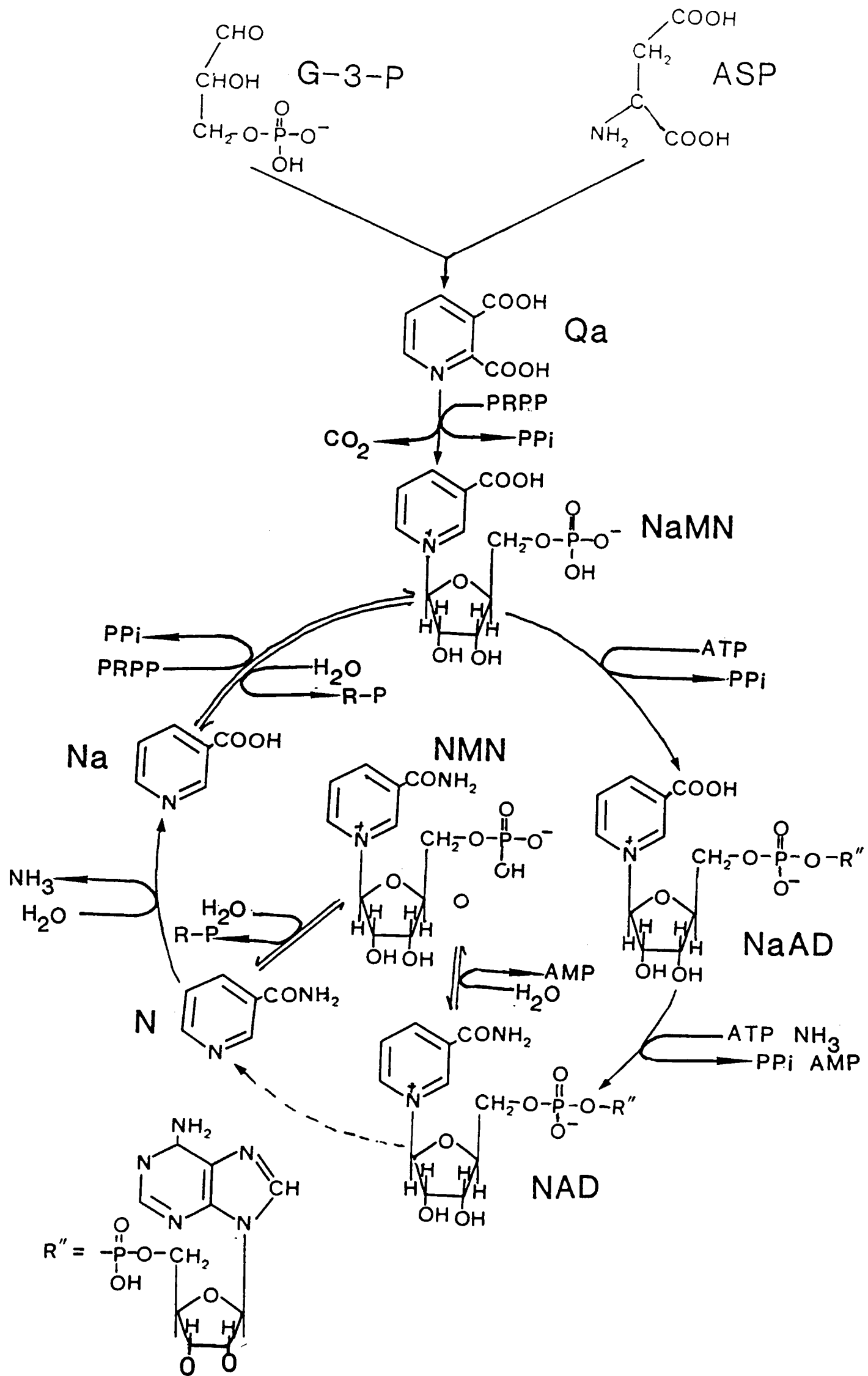
**NaAD = Nicotinic acid adenine dinucleotide.**

**NAD = Nicotinamide adenine dinucleotide.**

**NMN = Nicotinamide mononucleotide.**

**N = Nicotinamide.**

**Na = Nicotinic acid.**



## PRIMARY AND SECONDARY METABOLISM.

The alkaloids fall under the general heading of secondary metabolites, which can be defined as:

"a diverse group of compounds synthesised and often accumulated by plant tissues, which although not essential to the life of the cell in which they are found may give the organism which contains such cells a greater degree of fitness for survival" (Hall 1984) .

NAD and consequently the intermediates in the PNC are essential for the survival of an organism and are therefore involved in primary metabolism, which can be defined as:

"processes that produce essential cellular constituents, that enjoy universal roles in cellular metabolism (eg amino acids, purines, pyrimidines etc)" (Jensen 1986) .

While this distinction between primary and secondary metabolism is useful the boundary between the two areas is nevertheless imprecise (see Haslam 1986). However, with respect to the synthesis of alkaloids in tobacco this distinction between primary metabolism (ie NAD synthesis and recycling) and secondary metabolism (ie alkaloid synthesis) is useful since the synthesis and salvage of NAD is essential for the growth and maintenance of cells while alkaloid synthesis is advantageous to the plant but not essential for its survival.

It is believed that the genes ~~may be~~ determining the metabolic pathways of primary and secondary metabolism are present in <sup>all</sup> living cells of a given species of plant, but are differentially expressed at one or <sup>more</sup> points in the life of the cell (Yeoman *et al* 1980). It has been suggested that the transition from one state (eg growth, which involves primary metabolic processes) to another (eg alkaloid synthesis, via secondary metabolic pathways) is the result of a changes in enzyme activities (Yeoman *et al* 1980). The present evidence links the activation of secondary metabolism to changes in a range of parameters: morphological, concentrations of enzymes and of their substrates, nutrients and external stress. However, the factors that control the onset and regulation of secondary metabolism are generally not well understood (Haslam 1986). In tobacco the synthesis of the alkaloids is dependent on the availability of nicotinic acid which is involved in the recycling of NAD, therefore there is a

close association between primary and secondary metabolism. Thus the synthesis of the alkaloids in tobacco provides an opportunity to investigate the regulation of a primary metabolic process that has a direct bearing on the level of secondary metabolism.

The association between the synthesis of pyridine nucleotides and alkaloid synthesis is complicated by the formation of conjugates of nicotinamide and nicotinic acid (see Barz 1985). Therefore although the amount of nicotinic acid available for alkaloid synthesis is ultimately determined by the synthesis of NaMN from quinolinic acid (the synthetic pathway to NAD) the level of alkaloid synthesis may not necessarily mirror increases in the synthetic activity of the PNC. The conjugates of nicotinic acid have been found to<sup>be</sup> utilized in the synthesis of the alkaloids in the plant (Mizusaki *et al* 1970). A factor which may determine the formation of conjugates of nicotinic acid and nicotinamide is the availability of the non-pyridine ring of the alkaloids. For the synthesis of the alkaloids both the pyridine ring and the non-pyridine ring of the alkaloid must be available and in the same cellular compartment. In the absence of the non-pyridine ring of the alkaloids nicotinic acid and/or nicotinamide may become conjugated so that the cellular concentration of free nicotinic acid does not increase. The conjugation of nicotinic acid and/or nicotinamide may occur since increases in the amount of free nicotinic acid and nicotinamide may disrupt primary metabolism, which is achieved with very low concentrations of metabolites (Haslam 1986). However, the fact remains that for alkaloid synthesis nicotinic acid must be synthesised, which requires the synthetic operation of the PNC.

## **REGULATION OF ALKALOID BIOSYNTHESIS VIA THE PYRIDINE NUCLEOTIDE CYCLE.**

Quinolinic acid phosphoribosyl transferase (QaPRT'ase) catalyzes the 5-phosphoribosyl 1-pyrophosphate-dependent conversion of quinolinic acid to NaMN (Mann & Byerrum 1974a). Therefore the activity of this enzyme could

influence the level of *de novo* synthesis of the pyridine nucleotides. The activity of QaPRT'ase has been found to be higher in plant species that accumulate pyridine alkaloids than in species which do not accumulate the alkaloids (Mann & Byerrum 1974b). The reported low activity of the enzyme in species that do not synthesis the pyridine alkaloids suggests that only small amounts of NAD synthesis are required for primary metabolism, as has previously been suggested (White 1982). Therefore the increased activity of QaPRT'ase in pyridine alkaloid producing species may be associated with the synthesis of the alkaloids. This association between the activity of the enzyme and alkaloid synthesis is supported by the reported increase in QaPRT'ase activity when plants are "topped" (a process that increases nicotine synthesis {Papenfus & Quin 1984}) (Saunders & Bush 1979). Saunders and Bush (1979) also found that QaPRT'ase activity was higher in cultivars of tobacco that had high alkaloid contents and that the activity of the enzyme was lower in cultivars that had low alkaloid contents. It has also been found that the activity of the enzyme is much lower in the leaves of tobacco plants than in the roots (Wagner & Wagner 1984). The reduced activity of QaPRT'ase in leaves of tobacco plants is in agreement with the accepted fact that nicotine in particular, and the alkaloids in general, are synthesised in the roots and subsequently transported to the leaves (Dawson 1942a,b, Dawson & Solt 1959, Tso & Jeffrey 1956). This suggests that metabolic differences may exist between the roots and shoots of plants that allow alkaloid synthesis in the former and not in the latter. One such metabolic difference could be photosynthesis, which may suppress alkaloid synthesis in the leaves.

## **THE RELATIONSHIP BETWEEN PHOTOSYNTHESIS AND ALKALOID BIOSYNTHESIS.**

Photosynthesis in green plants can be defined as:

"the synthesis of carbohydrates from CO<sub>2</sub>, as a source of carbon, and water, as a hydrogen donor, using energy trapped by chlorophyll, catalyzed by various enzymes in the chloroplast (Hendersons Dictionary of Biological Terms 1979)".

Carbon fixed via photosynthesis, in chlorophyllous tissues, is used to support the metabolic activities of the plant. For the export of fixed carbon from the chloroplast carbon fixation must exceed the chloroplasts' own demands for

carbon. The chloroplasts can only fix more carbon by returning newly synthesised intermediates of the Calvin cycle (the series of reactions leading to CO<sub>2</sub> fixation and the subsequent regeneration of ribulose bis-phosphate). Conversely for export the products of CO<sub>2</sub> fixation must be removed from the Calvin cycle. Therefore a delicate balance must be struck between recycling and export (Walker 1976). Fixed carbon is exported from the chloroplasts as sugar-phosphates, in particular as triose phosphates (Sharkey *et al* 1986).

Quinolinic acid is synthesised from glyceraldehyde-3-phosphate (G-3-P) and aspartate (Fleeker & Byerrum 1967). G-3-P is an intermediate in the Calvin cycle (Stryer 1981) and is transported out of the chloroplast in the form of dihydroxyacetone phosphate (DHAP). Once in the cytosol DHAP can be metabolised either to sucrose (the main form in which fixed carbon is transported between cells) (see Heber 1974, Walker 1976), or enter the glycolytic pathway (Stryer 1981). In the cytosol DHAP is readily converted back to G-3-P (Heber 1974). This G-3-P may then be utilized in numerous metabolic processes, one of which is the synthesis of quinolinic acid and subsequently the synthesis of the pyridine nucleotides. Therefore the operation of the PNC is associated with the activities of a further primary metabolic pathway (ie photosynthesis). Consequently the synthesis of the tobacco alkaloids may be influenced by photosynthetic activity, such that alkaloid synthesis only occurs in non-chlorophyllous tissues.

#### **THE PRESENCE OR ABSENCE OF PHOTOSYNTHESIS AS A POSSIBLE EXPLANATION FOR THE ACTIVITY OF QaPRT'ase IN CHLOROPHYLLOUS AND NON-CHLOROPHYLLOUS TISSUES.**

The activity of QaPRT'ase has been reported to be higher in non-chlorophyllous tissues (roots) than it is in chlorophyllous tissues (leaves) of plants that accumulate pyridine alkaloids (Wagner & Wagner 1984). A possible explanation of this is that in leaves a high level of *de novo* synthesis of the pyridine nucleotides would, through its demand for G-3-P, disrupt the delicate balance between photosynthetic carbon fixation and the export of fixed carbon from the chloroplast into the cytosol. Therefore in leaves the activity of QaPRT'ase may be such that it only allows the synthesis of the pyridine nucleotides in quantities that are required by primary metabolism. In the roots

G-3-P is an intermediate in the glycolytic pathway, which metabolises glucose (which is a breakdown product of sucrose transported to the roots from the leaves) into pyruvate with the concomitant production of ATP (Stryer 1981). Therefore the availability of G-3-P in the roots is determined by the availability of, and the rate of breakdown of, sucrose. Since sucrose is the source of energy for growth and cellular maintenance, in the roots, it is possible that as growth decreases the availability of G-3-P for pyridine nucleotide synthesis will increase. The amount of pyridine nucleotides required by the cell is unlikely to change very much throughout the life of that cell hence an increase in *de novo* synthesis of these nucleotides in the roots may enable increased alkaloid synthesis.

The possible role of photosynthesis in determining the degree of alkaloid synthesis, through the regulation of pyridine nucleotide synthesis can be investigated in more detail by using cell cultures of tobacco rather than the plant. One of the advantages that cell cultures offer is that the environment in which the cells are grown can be manipulated to either enhance or suppress secondary metabolism. Also the use of cell cultures allows for the addition and/or removal of compounds that may cause changes in the synthesis of secondary metabolites. A third reason is that an understanding of secondary metabolism *in vitro* may lead to the commercial production of compounds by culture methods

#### **THE CONTROL OF ALKALOID SYNTHESIS IN CELL CULTURES OF NICOTIANA**

There is a large amount of literature in which the factors that control the onset and regulation of secondary metabolism have been discussed (see eg Böhm 1978, Lindsey & Yeoman 1985, Luckner 1980, Mantell & Smith 1983, Yeoman 1987), and several such studies have involved alkaloid synthesis in tobacco (Feth *et al* 1986, Pearson 1978, Ohta & Yatazawa 1978a,b, Shiio & Ohta 1973, Wagner & Wagner 1985, Wagner *et al* 1986a,b). In two of these investigations light has been found to suppress alkaloid synthesis when callus cultures capable of synthesis were transferred from the light into the dark (Pearson 1978, Ohta & Yatazawa 1978a). This is of particular interest when considered together with the reports that alkaloid synthesis occurs mainly in roots (Dawson 1942a,b) and that the activity of the enzyme QaPRT'ase is higher in roots than in leaves, of pyridine alkaloid producing plants (Wagner & Wagner 1984). Therefore the determination of alkaloid synthesis and the activity of QaPRT'ase in different light environments may provide information as to the regulation of the synthesis of the alkaloids.



## THE EFFECT OF LIGHT AND PHOTOSYNTHESIS ON ALKALOID SYNTHESIS IN CELL CULTURES.

It is possible to establish cell cultures with differing degrees of dependence on photosynthesis for the provision of organic constituents (see Dalton & Peel 1983). Using such cultures the effect of increasing illumination on alkaloid synthesis can be investigated while at the same time enabling a determination of the effect of photosynthesis on alkaloid synthesis. If photosynthesis plays a role in decreasing QaPRT'ase activity, and alkaloid synthesis, a gradual decrease in activity and alkaloid content will occur as cultures become more dependent on photosynthesis for the provision of organic constituents. Also by examining the effect of light on species of *Nicotiana* that produce alkaloids other than nicotine it should be possible to determine whether light is affecting the synthesis of the pyridine ring, which is common to all of the alkaloids, or the synthesis of the non-pyridine rings which are specific to each alkaloid. Further information about the effect of light on alkaloid synthesis may be obtained using differentiated root and shoot cultures. In root cultures levels of alkaloid synthesis close to those in the plant have been reported (Hashimoto *et al* 1986, Endo & Yamada 1985, Tabata *et al* 1972) while shoot cultures are reported to be incapable of alkaloid synthesis (Dawson 1946). By determining the alkaloid content of these cultures in the light and the dark the influence of the presence (shoot cultures) or absence (root cultures) of photosynthetic structures and pathways on alkaloid biosynthesis can be estimated.

## AIMS AND OBJECTIVES.

The aims of this project were to examine the production of the four main tobacco alkaloids and to determine the effect of light and photosynthesis on their synthesis, using cell cultures of tobacco. These aims can be broken down to give the following objectives:

1. To establish cultures of *N.tabacum* with different degrees of dependence on photosynthesis for the provision of organic constituents.
2. To compare the accumulation of alkaloids in cultures of *N.tabacum* with or without illumination and with increasing photosynthetic capacity.
3. To compare the accumulation of alkaloids in cultures of *N.glauca* (callus cultures), *N.rustica* (root cultures) and *N.tabacum* (shoot cultures) grown in the light and the dark.
4. To find out if light caused the photochemical degradation of nicotine when it was added to the medium of cell suspension cultures.
5. To determine the kinetics of alkaloid accumulation in callus cultures of *N.glauca* which were grown in different light environments prior to and during the period of the experiment.
6. To confirm previous reports regarding the activity of QaPRT'ase in the roots and shoots of tobacco plants.
7. To determine the effect of light on the activity of QaPRT'ase in cultures of *Nicotiana* grown in either the light or the dark.

**CHAPTER 2**  
**MATERIALS AND METHODS.**

## **2.1 PLANT MATERIAL AND GREENHOUSE CONDITIONS.**

### **2.1.1 Plant Material.**

The species used in this investigation were: *N.tabacum* Cv. Wisconsin-38, *N.glauca*, and *N.rustica* Seeds of *N.tabacum* were collected from plants grown in the greenhouses of the Botany, Department, Edinburgh. Callus cultures of *N.glauca* were a gift from Dr. I Lyons, of the Department of Botany, Birmingham University and root cultures of *N.rustica* were a gift from Dr. S. Haslam of the Department of Biochemistry, Birmingham University.

### **2.1.2 Greenhouse Conditions.**

Seeds were sown at high density in trays on a 1:1 (v/v) mixture of Fisons Levington potting compost and Perlite. The trays were placed in a greenhouse in which the minimum temperature was 16°C with a 16 hour day light regime (day light being supplemented when necessary with light from mercury vapour lamps). Watering was carried out as required to keep the compost moist. After 3 weeks the seedlings were transplanted to 10cm pots and after a further 12 weeks growth to 17.5cm pots in which the plants reached flowering.

## 2.2 CELL AND TISSUE CULTURE.

### 2.2.1 Preparation Of Culture Media.

For *in vitro* studies five basal media were employed. These were Gamborg's B<sub>5</sub> (B<sub>5</sub>) medium (Gamborg *et al* 1968), Murashige and Skoog (MS) medium (Murashige & Skoog 1962), Murashige and Skoog plant salts (MSS) medium (Murashige & Skoog 1962), Schenk and Hildebrandt (SH) medium (Schenk & Hildebrandt 1972) and White's (W) medium (White 1938). The constituents of each of the media are shown in Table 2.2.1. The media were prepared from powdered mixtures supplied by Flow Laboratories Ltd., Irvine, Scotland by dissolving the appropriate amount of the solid mixture (3.875g l<sup>-1</sup> of B<sub>5</sub>, 4.71g l<sup>-1</sup> of MS, 4.606g l<sup>-1</sup> of MSS, 4.46g l<sup>-1</sup> SH and 1.31g l<sup>-1</sup> of W) in distilled water. In all of the media except for MSS 30g l<sup>-1</sup> of sucrose were added as a carbon source. The carbon source used with MSS medium was fructose at concentrations between 0 and 30g l<sup>-1</sup>. Prior to adjusting the pH of the media the appropriate growth regulators and other additional compounds were added as shown in Table 2.2.2. The pH of the medium was adjusted to pH5.6 with either M potassium hydroxide (BDH Ltd., Poole, Dorset) or M hydrochloric acid (BDH) and the medium made up to the required volume with distilled water.

In addition to the above constituents solid media contained 8g l<sup>-1</sup> agar (Oxoid No. 1 Oxoid Ltd., Basingstoke, Hampshire) added after adjustment of the pH.

### 2.2.2 Sterilization And Sterile Technique.

Due to the many problems which arise when plant cells grown *in vitro* become contaminated with microorganisms it was essential to carry out all manipulations under conditions of total asepsis. This required that all media, glassware and equipment that came into contact with the cultured material at any time were sterilized. This was achieved by autoclaving at 121°C for 20min at a steam pressure of 15psi. Seeds germinated on agar also had to be

TABLE 2.2.1

The nutrient constituents of all tissue culture media, used in this investigation, and their concentration in  $\text{mg l}^{-1}$ . Murashige and Skoog (MS) medium, Murashige and Skoog Plant Salts (MSS) medium (supplemented with either sucrose or fructose), Gamborgs B<sub>5</sub> (B<sub>5</sub>) medium, Schenk and Hildebrandt (SH) medium and Whites (W) medium were made up as described in the text.

<sup>1</sup> = Potassium nitrate added to the stock medium at a concentration of  $1750\text{mg l}^{-1}$ .

<sup>2</sup> = To avoid caramelisation fructose was autoclaved as a concentrated solution at 15psi for 10min. The appropriate amount was then aseptically added to sterile medium.

CONSTITUENTS	CONCENTRATION IN MEDIA (mg l <sup>-1</sup> )				
	MS	MSS	W	SH	B <sub>5</sub>
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440	440	-	200	150
Ca(NO <sub>3</sub> ) <sub>2</sub>	-	-	208.5	-	-
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.025	-	0.1	0.025
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.025	0.001	0.2	0.025
FeNa-EDTA	36.7	-	4.59	-	-
FeSO <sub>4</sub> ·7H <sub>2</sub> O	-	27.85	-	15	-
Na <sub>2</sub> -EDTA	-	37.25	-	20	-
H <sub>3</sub> BO <sub>3</sub>	6.2	6.2	1.5	5	3
KCl	-	-	65	-	-
KH <sub>2</sub> PO <sub>4</sub>	170	170	-	-	-
KI	0.83	0.83	0.75	1	0.75
KNO <sub>3</sub> <sup>1</sup>	1900	1900	80	2500	3000
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370	370	720	400	250
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.3	16.9	7	10	13.2
MoO <sub>3</sub>	-	-	0.0001	-	-
Na <sub>2</sub> SO <sub>4</sub>	-	-	200	-	-
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	-	-	18.7	-	169.6
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.25	-	0.1	0.25
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	-	-	-	300	-
NH <sub>4</sub> NO <sub>3</sub>	1650	1650	-	-	-
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	-	-	-	-	134
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	8.6	3	1	2
Biotin	-	0.05	-	-	-
Glutamine	-	200	-	-	-
Glycine	2	0.2	3	-	-
Myo-inositol	100	100	-	1000	100
Nicotinic acid	0.5	0.05	0.5	5	1
Pyroxidine-HCl	0.5	0.05	0.1	0.5	1
Thiamine-HCl	0.1	0.1	0.1	5	10
Sucrose	30000	-	30000	30000	30000
Fructose <sup>2</sup>	-	30000 to 0	-	-	-

	BASAL MEDIA				
	MS	MSS	W	SH	B <sub>5</sub>
NAA (mg l <sup>-1</sup> )	0.30	0.30	0.30	0.30	0.30
BAP (mg l <sup>-1</sup> )	-	0.30	-	-	-
Kinetin (mg l <sup>-1</sup> )	0.15	-	0.15	0.15	0.15
Ascorb (mg l <sup>-1</sup> )	5.00	-	5.00	5.00	5.00

TABLE 2.2.2

The growth regulators and additions made to each of the basal media used for the investigation. The basal media employed were: Murashige and Skoog (MS) medium, Murashige and Skoog Plant Salts (MSS) medium (supplemented with either 3% sucrose or 3% fructose), Schenk and Hildebrandt (SH) medium, Gamborgs B<sub>5</sub> (B<sub>5</sub>) medium and Whites (W) medium. The growth regulators and additions were made up as concentrated solutions and the required amount added.  $\alpha$ -Naphthaleneacetic acid (NAA) (Sigma) was dissolved in a small amount of alcohol and made up to volume with distilled water. 6-Benzylaminopurine (BAP) (Sigma) was dissolved in a small amount of 5M potassium hydroxide and made up to volume with distilled water. 6-furfurylaminopurine (kinetin) (Sigma) was dissolved in a small amount of M sodium hydroxide and made up to volume with distilled water. Ascorbic acid (Ascorb) was dissolved in the required volume of distilled water.



sterilized prior to culture and subsequent operations requiring total asepsis were carried out in a laminar air flow cabinet swabbed out prior to use with ethanol. All instruments required for sterile manipulations were stored in 96% (v/v) ethanol and were flamed immediately before use. Disposable plastic gloves (Triflex, Thetford, Norfolk) sterilized prior to use with ethanol were worn throughout all sterile manipulations.

### **2.2.2.1 Sterilization of seeds.**

Seed was placed in a Pyrex glass tube (25mm diameter X 50mm) and both ends sealed with a double layer of muslin (Reinert & Yeoman 1982). A presterilization was carried out for 1min by complete immersion of the tube in 70% (v/v) ethanol. The tube containing the seed was then transferred to, and totally immersed in, a solution of 30% (w/v) hydrogen peroxide (BDH) containing 1% (v/v) Tween-80 (Sigma Ltd, Poole, Dorset) for 5min (Lockwood & Essa 1984). The tube and seed was then taken through six washes in sterile distilled water. Following this the muslin from the lower end of the tube was removed and the seeds separated from it. This was achieved by placing the muslin in a sterile 9cm Petri dish (Sterilin, Feltham, Middlesex) containing sterile distilled water and allowing the seeds to float off.

### **2.2.3 Initiation And Maintenance Of Cultures.**

#### **2.2.3.1 Culture conditions.**

Temperature	25±2°C
Illumination	80-90µE m <sup>-2</sup> sec <sup>-1</sup> or 300-350µE m <sup>-2</sup> sec <sup>-1</sup> supplied by 58W Pluslux 3500 fluorescent tubes (Philips).
Liquid culture agitation	Continuous rotation in a horizontal plane at 98rpm with an amplitude of 8mm.

### **2.2.3.2 Initiation of callus.**

Sterilized seeds of *Nicotiana tabacum* were placed on solid MS medium containing  $1.87\text{mg l}^{-1}$  NAA and  $0.215\text{mg l}^{-1}$  kinetin in 9cm Petri dishes (Sterilin). After 6–8 weeks in the dark callus had formed directly from the radicle. The callus was excised from the seedling parts and transferred onto fresh medium.

Callus cultures were grown in 9cm polystyrene Petri Dishes containing 15–20ml of solid medium. After inoculation Petri dishes were sealed with a double layer of Parafilm (American Can Company, Greenwich, CT.), to exclude microorganisms and to prevent desiccation.

### **2.2.3.3 Initiation of suspension cultures.**

Suspension cultures were initiated by adding 1–1.5g fresh weight of callus to the liquid medium. Cell suspension cultures were grown in 250ml conical (Erlenmeyer) flasks containing 50ml of liquid medium and covered with a double layer of aluminium foil and grown with constant agitation.

### **2.2.3.4 Maintenance of callus and cell suspension cultures.**

All stock cultures were maintained on solid MS medium. At 2–3 week intervals 1.0–1.5g of callus was transferred to fresh medium using a pair of forceps. When there were detectable phenotypic differences between individual calli in a culture, care was taken to transfer representatives of all the calli types.

The growth of *N.tabacum* cells in liquid culture was rapid therefore it was necessary to transfer cells to fresh medium at 3 week intervals. These cultures often became highly aggregated and it was necessary to break up the

aggregates after transferring 1.0–1.5g wet weight of cells to fresh medium. Cells were transferred and aggregates broken up with a perforated spoon spatula.

#### **2.2.3.5 Maintenance of root cultures.**

Root cultures of *N.rustica* were maintained in 50ml of W medium containing no growth regulators and at pH5.4 in 250ml conical flasks. At 3 week intervals 1.0–1.5cm lengths of root, with lateral branching, were excised and transferred to fresh medium.

#### **2.2.4 Single Cell Cloning.**

Single cell cloning was carried out so that variations in the photosynthetic capabilities of clones could be identified. The variation identified was subsequently utilized in establishing cultures with varying degrees of dependence on photosynthesis for the provision of organic constituents (Section 2.2.5).

##### **2.2.4.1 Establishment of fine suspension cultures.**

Suspension cultures became highly aggregated during the culture cycle, even though aggregates were broken up at each subculture, which meant that the yield of single cells was very low. This yield was increased by sieving the cultures at each subculture through a 600 $\mu$ m nylon mesh (Henry Simon Ltd., Stockport) and inoculating fresh medium with the filtrate. After three passages this process yielded suspension cultures with a significant reduction in the size and number of aggregates, which were suitable for use in single cell cloning.

#### **2.2.4.2 Low density plating.**

Suspension cultures in the late exponential phase of the growth cycle (Section 3.5.1.1) were used for low density plating, since such cells give the largest yield of clones (Thomas & Davey 1975). All of the operations involved in low density plating, except for the determination of cell number, were carried out under conditions of total asepsis. Cell suspension cultures were sieved through a 600 $\mu$ m nylon mesh, to remove large aggregates, and then through a 140 $\mu$ m nylon mesh, which allowed groups of four cells or less to pass through. The number of cells ml<sup>-1</sup> of the filtrate was determined using a Hawksley Crystallite Haemocytometer (grid volume 1.8 $\mu$ l) (Section 2.3.1.2) and from this the total number of cells in the filtrate was estimated. The filtrate was then centrifuged at 750xg for 5min, in order to pellet the cells. The supernatant was discarded and the cells resuspended in a volume of molten MS medium at 30°C containing 0.75% (w/v) agarose (grade 7, Sigma) so as to give C.10<sup>2</sup> cells ml<sup>-1</sup>. Approximately 5ml of the medium containing the cells was poured into 9cm Petri dishes and allowed to set. The plates were then sealed with a double layer of Parafilm before being examined microscopically and the position of single cells and pairs of cells marked. The plates were then placed under constant illumination of 80–90 $\mu$ E m<sup>-2</sup> sec<sup>-1</sup>.

#### **2.2.4.3 Maintenance of single cell clones.**

Five weeks after low density plating, calli that had formed where single or pairs of cells had been marked, were transferred to fresh MS medium with 2% (w/v) sucrose. After a further three culture cycles the calli were transferred to liquid MS medium and maintained in cell suspension culture.

## **2.2.5 The Culture Of Cells With Differing Degrees Of Dependence On Photosynthesis For The Provision Of Organic Constituents.**

In order to investigate the effect of photosynthesis on alkaloid biosynthesis cultures with differing degrees of dependence on photosynthesis for the provision of organic constituents were established. The clones obtained in Section 2.2.4 were screened and appropriate ones transferred to environmental and cultural conditions intended to either enhance or suppress photosynthesis. Cells were selected and cultured such that at one extreme they were totally dependent on a carbon source in the medium (heterotrophic), while at the other extreme carbon dioxide fixed via photosynthesis was the major source of organic constituents (photosynthetic). Between these two extremes cells were partially dependent on carbon in the medium and partially dependent on photosynthesis for obtaining organic constituents (mixotrophic). Mixotrophic cells were screened for photosynthetic capability and selected in order to obtain photosynthetic cells.

### **2.2.5.1 Screening of clones and transfer to conditions favouring either heterotrophic growth or promoting photosynthetic development.**

After several passages through liquid culture the chlorophyll content of the clones was estimated. The least chlorophyllous clones were transferred to MSS medium supplemented with 3% (w/v) fructose, while clones with a higher chlorophyll content were transferred to MSS medium with 2% (w/v) fructose. The former cultures were grown in the dark and the latter under constant illumination. The medium was changed from MS to MSS and the carbon source from sucrose to fructose since the latter in each case has been found to enhance the photosynthetic development of cells in culture (Dalton 1980). The cultures in the dark with 3% fructose have been maintained under these conditions, while those cultures in medium supplemented with 2% fructose, and grown in the light, were constantly screened for photosynthetic capacity. After each screening cultures with the desired photosynthetic characteristics were moved to conditions favouring increased levels of photosynthesis.

### **2.2.5.2 Screening of cell suspension cultures for photosynthetic capacity and transfer of clones with the desired characteristics to conditions favouring increased photosynthetic carbon fixation.**

Each clone was sampled by aseptically removing 2.0–2.5g wet weight of cells between the fourteenth and the sixteenth day after inoculation during the fourth subculture. At this time the cultures were in the stationary phase of the growth cycle (Section 3.5.2.1) when maximal photosynthetic activity is achieved (Bender *et al* 1985, Section 3.5.1.1). Previously, screening for cells suitable for transfer to conditions promoting increased photosynthetic activity has been carried out according to the chlorophyll content of the cultures (Berlyn *et al* 1978, Dalton & Peel 1983, Husemann 1978, Yamada *et al* 1978). However, it was found that chlorophyll content did not necessarily correlate with maximal photosynthetic potential as measured by chlorophyll fluorescence (Appendix 1). The chlorophyll content, photosynthetic rate and fluorescence profiles were determined and cultures with the required characteristics transferred into medium promoting further photosynthetic development. Cultures with the greatest photosynthetic potential were recorded and at the subsequent subculture transferred to medium with the fructose level in the medium reduced by  $2.5\text{g l}^{-1}$ . This process was repeated until a fructose level of 1.0% was achieved. At fructose levels below 1.0% culture growth was greatly reduced and viability very low. To overcome these effects of decreased carbon source in the medium and to select cells capable of growth at lower fructose levels a culture system in which the medium was replenished at regular intervals was employed. This system reduced fluctuations in nutrient levels which occurred in suspension cultures between the end of one culture cycle and the start of the next (Section 3.5.2.2), thus allowing populations of viable cells to accumulate.

### **2.2.5.3 The culture of cells in a "flat-bed" system.**

A modified form of the "flat-bed" system employed by Lindsey and Yeoman (1983a) was used when culturing cells at fructose levels below 1.0%. The principal features of this system were that the cells were physically stationary and that the medium supplying the cells was replenished at regular intervals.

#### **i. Construction of the "flat-bed" apparatus.**

Cells were seated on a polypropylene fabric matting substratum ("Fresh mat" Access Ltd., Crick, Northampton) which was contained within a glass culture vessel (a circular dish plus lid, containing two 1cm diameter holes, of dimensions 9cm diameter X 5cm with a volume of approximately 350ml) (Fig 2.2.1). The holes in the culture vessel lid were sealed with No. 20 subbaseals (Gallenkamp) and through each seal two stainless steel needles (5cm X 1mm internal diameter) were inserted. One of the needles was connected to a Millipore air filter (Millipore SA. Molsheim, France). The other needle was connected with silicon rubber tubing (1mm internal diameter X 3mm external diameter) (Esco Rubber Ltd., Feltham, Middlesex) via a LKB Multiperpex 2115 pump (LKB, Croydon) to either an inlet or an outlet medium reservoir (in each case a 1l Schott flask with stainless steel tubes inserted through the lid). The medium reservoirs were maintained at atmospheric pressure via Millipore air filters.

#### **ii. The culture of cells in the "flat-bed" apparatus.**

The apparatus described above was sterilized by autoclaving at 121°C (15psi steam pressure) for 20min and all subsequent operations carried out under conditions of total asepsis.

Aggregates of cells, 0.5–1.0cm in diameter, were transferred from

FIGURE 2.2.1

The construction of the "flat-bed" culture system used for the maintenance of photosynthetic cultures of *N.tabacum*.

F = Millipore Millex-FG<sub>50</sub> filter unit.

P = LKB Peristaltic pump.

R<sub>1</sub> = Inlet reservoir.

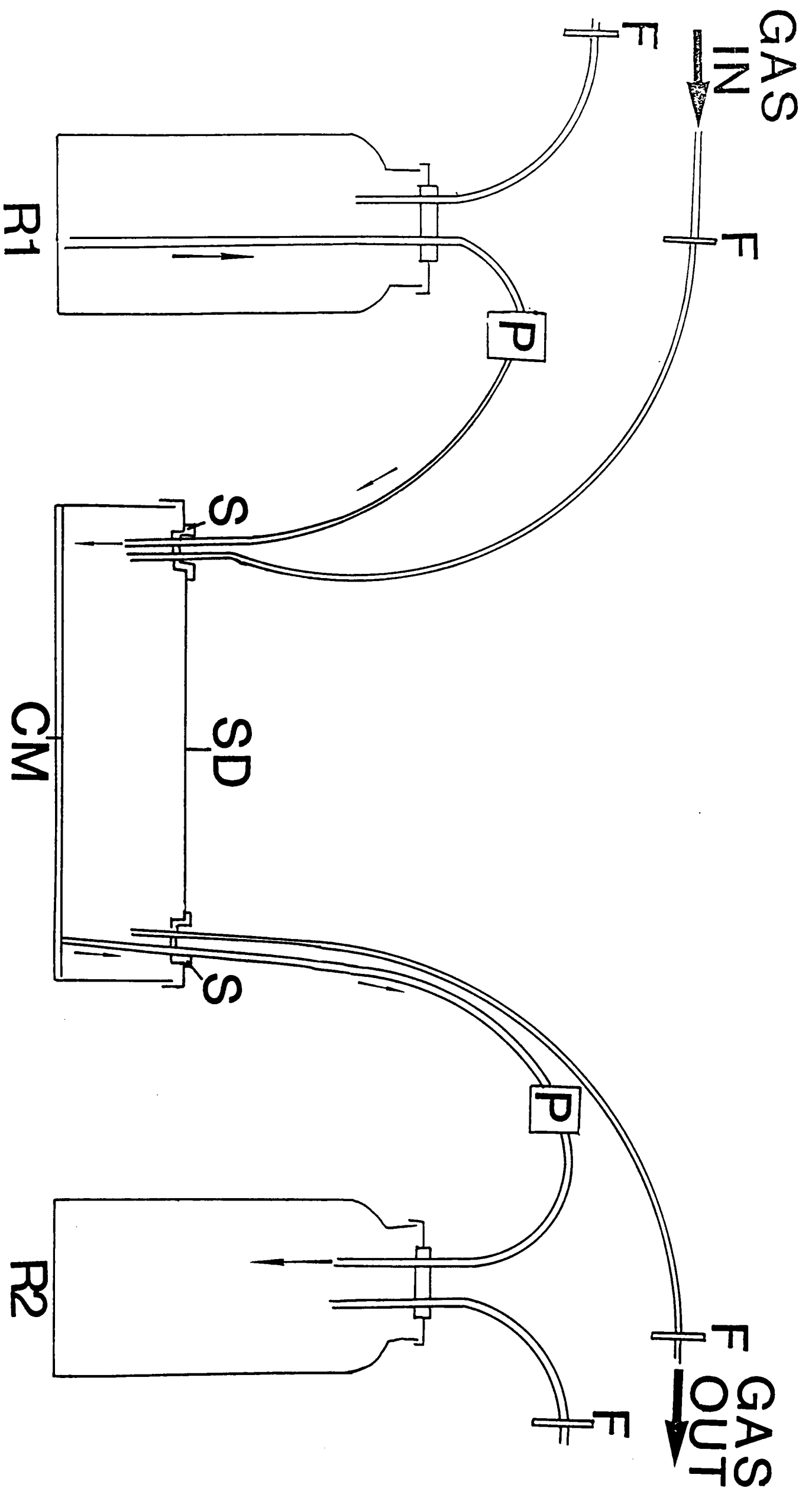
R<sub>2</sub> = Exhaust reservoir.

S = Number 20 Suba-seal.

SD = Spirit dish.

CM = Capillary matting.





cell suspension cultures supplemented with 1.4% fructose onto the polypropylene substratum. To each culture vessel 15ml of fresh MSS medium with 1.4% fructose was added. The medium moved through the fabric by capillary action and thus supplied the cells. Once every six hours the pump was activated by a time switch which replenished the medium in the culture chamber over a 15min period. It was necessary to replace the flasks containing fresh and spent medium at four weekly intervals.

### iii. Establishment of photosynthetic cells.

Cells were grown in the "flat-bed" system at a constant fructose level of 1.0% until the culture vessel was approximately half full of cells. At this point the fructose level in the medium was reduced by 0.25%. Ten to fourteen days after the reduction in carbon concentration surviving cells were separated from dead cells <sup>using sterile forceps</sup> and transferred to fresh culture chambers. By repeating this process the fructose level was reduced to 0.5%. However, below a fructose level of 0.5% cell death reached levels that appeared to have a deleterious effect on any cells capable of survival. In order to overcome this problem small groups of cells from the "flat-bed" system were transferred onto solid MSS medium with no fructose. By using small groups of cells the effect of cell death on cells capable of survival was minimized. After 12 weeks growth during which time the calli were twice transferred to fresh medium, surviving calli were used to reinoculate the "flat-bed" culture system. To enhance growth the gaseous atmosphere in the chamber was adjusted to 2% carbon dioxide, 10% oxygen and 88% nitrogen so as to promote photosynthetic pigment synthesis and photosynthesis, while suppressing photorespiration (Dalton & Street 1976). The adjustment of the atmosphere was achieved by supplying the required mixture of gases (obtained from BOC Special Gases, London) through one of the Millipore air filters connected to the culture chamber and allowing the other Millipore air filter to act as an outlet for exhaust gases. The flow rate of the gas was  $8\text{ml hr}^{-1}$  which allowed fluctuations of up to 1% (as calculated from culture vessel volume, culture weight and maximum photosynthetic and respiration rates) in the constituent gases.

## 2.3 CHARACTERIZATION OF CALLUS AND CELL SUSPENSION CULTURES.

### 2.3.1 Measurement of Culture Growth.

#### 2.3.1.1 Measurement of culture fresh weight.

The fresh weight of callus cultures was measured after separating the cells from the solid medium with a pair of forceps. Suspended cells were weighed after the bathing medium had been removed by filtration through Whatman filter paper, No.1, at reduced pressure using a Buchner funnel.

#### 2.3.1.2 Determination of cell number.

The cell population density of suspended cells was estimated with a Hawksley Crystallite Haemocytometer (grid volume 1.8 $\mu$ l). Due to the highly aggregated nature of cell suspension cultures it was necessary to determine the fresh weight per ml and then to incubate a known weight in a 10% aqueous solution of chromium trioxide (BDH) for 16hours. After incubation six grids were counted for each sample and the mean used to calculate the density of the suspended cell population, the formulae for which were:

$$C_n = \frac{W_t \cdot C_a}{W_a}$$

where:  $W_t$  = the total culture weight (g)  
 $W_a$  = the weight (g) digested in chromium trioxide  
 $C_a$  = the cell number in  $W_a$ (g), calculated as:

$$C_a = \frac{V_c \cdot C_h}{V_h}$$

where:  $V_c$  = the volume (ml) of chromium trioxide  
 $V_h$  = the volume (ml) of the haemocytometer  
 $C_h$  = the mean cell number counted with the haemocytometer.

## 2.3.2 Photosynthetic Characterization.

### 2.3.2.1 Measurement of chlorophyll content.

Suspension cultured cells were separated from their medium by filtration through Whatman No.1 filter paper under reduced pressure while calli were lifted off their medium with a pair of forceps. The chlorophyll content was estimated according to the method of Harborne (1976). 1.5–2.0g fresh weight of cultured cells (for leaves 0.1–0.2g) was ground using a pestle and mortar in 5ml of 80% (v/v) acetone (BDH) and placed on ice for 30min. The homogenate was then filtered under reduced pressure through a glass microfibre filter (Whatman) using a Millipore filtration unit (Millipore SA, Molsheim, France). The residue was reground in 5ml 80% acetone and refiltered. The filtrates were pooled and made up to 10ml with 80% acetone and the optical density measured against an 80% acetone blank at 645 and 663nm using a Pye Unicam SP8-100 UV spectrophotometer. The chlorophyll concentration,  $C_c$   $\mu\text{g ml}^{-1}$ , was calculated as:

$$C_c = 20.2A_{645} + 8.02A_{663}$$

where:  $A_{645}$  = the optical density at 645nm.  
 $A_{663}$  = the optical density at 663nm.

and the chlorophyll content per g fresh weight,  $C_w$  ( $\mu\text{g g}^{-1}$ ), was calculated as:

$$C_w = \frac{C_c \cdot V_e}{W_e}$$

where:  $V_e$  = the final extraction volume (ml)  
 $W_e$  = the fresh weight (g) extracted.

### 2.3.2.2 Determination of the respiration rate and the rate of photosynthesis.

In order to assess the contribution of photosynthesis to the provision of organic constituents in cultured cells the photosynthetic rate was determined. It was also necessary to measure the respiration rate since oxygen uptake due to respiration during measurements of photosynthetic oxygen evolution could give a false impression of the actual photosynthetic capacity of cells.

An oxygen electrode (Rank Brothers, Cambridge) connected to a Servoscribe 1S chart recorder (Belmont Instruments, Glasgow) set at 1mV was used to measure the rate at which oxygen was either taken up by cells (respiration), or evolved by cells (photosynthesis). The oxygen electrode was set up in the manner described by Coombs and Hall (1982). On each occasion the equipment was calibrated as ~~is~~ described below.

The required volume of distilled water (usually 5ml) was placed in the chamber of the oxygen electrode and stirred vigorously, for 30min, by means of a magnetic follower. The concentration of oxygen dissolved in the distilled water at ambient temperature (25°C) was then assumed to be  $240\text{nmolml}^{-1}$ . The level of the chart recorder was set at a maximum by adjusting the input voltage to the electrode. The dissolved oxygen was then removed from the water by adding 2–5mg of sodium dithionite (BDH) and the zero position on the chart recorder set. The process was repeated until a full scale deflection was achieved for  $0\text{--}240\text{nmolO}_2\text{ml}^{-1}$ . Measurements of photosynthetic rates were made under <sup>light</sup> $\lambda$  saturating conditions and therefore do not represent the levels obtained under normal growth conditions.

#### i. Determination of respiration rate.

The rate of oxygen uptake by cells was determined by placing 0.2–0.3g fresh weight of cells in 5ml of air saturated *medium* (assumed to have an oxygen concentration of  $240\mu\text{moles l}^{-1}$  at 20°C) in the oxygen electrode chamber. The chamber was sealed and made light tight and the chamber

contents constantly mixed by means of a magnetic follower. The dissolved oxygen concentration in the water in the chamber was then recorded over a 15–20min period. The respiration rate,  $R_r$ ,  $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ , was calculated using the following equation:

$$R_r = \frac{(D_0 - D_1) \cdot V_c \cdot 240}{(T_1 - T_0) \cdot D_t \cdot W_c}$$

where:

- $D_0$  = the reading on the chart recorder (mm) at  $T_0$ .
- $D_1$  = the reading on the chart recorder (mm) at  $T_1$ .
- $V_c$  = the volume (ml) of water in the oxygen electrode chamber.
- $T_0$  = time zero at the start of the analysis.
- $T_1$  = the time (min) at the end of the analysis.
- $D_t$  = the distance (mm) for a full scale deflection on the chart recorder.
- $W_c$  = the fresh weight (g) of cells added to the electrode chamber.

## ii. Determination of the rate of photosynthesis.

The rate of photosynthesis was measured as the rate of oxygen evolution into the water in the oxygen electrode chamber by a sample of cells under conditions that were saturating for photosynthesis. The photosynthetic rate was measured immediately after the respiration rate using the same sample. On completion of the measurement of the respiration rate the electrode chamber was illuminated at a photon flux density of  $400 \mu\text{E m}^{-2} \text{ sec}^{-1}$ , supplied by an argaphoto B 500W bulb (Philips). Under these conditions the dissolved oxygen concentration in the water in the electrode chamber was recorded over a 15–20min period. The net photosynthetic rate,  $P_n$  ( $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ ), was calculated as:

$$P_n = \frac{(D_1 - D_0) \cdot V_c \cdot 240}{(T_1 - T_0) \cdot D_t \cdot W_c}$$

where:  $D_0$  = the reading on the chart recorder (mm)  $T_0$ .  
 $D_1$  = the reading on the chart recorder (mm)  $T_1$ .  
 $V_c$  = the volume (ml) of water in the oxygen electrode chamber.  
 $T_0$  = time zero at the start of the analysis.  
 $T_1$  = the time (min) at the end of the analysis.  
 $D_t$  = the distance (mm) on the chart recorder for a full scale deflection.  
 $W_c$  = the fresh weight (g) of cells added to the electrode chamber.

Gross photosynthesis,  $P_g$  ( $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ ) was calculated as:

$$P_g = R_r + P_n$$

where:  $R_r$  = the respiration rate ( $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ )  
 $P_n$  = the net photosynthetic rate ( $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ )

Photosynthetic competence,  $P_c$ , was expressed as:

$$P_c = \frac{P_g}{R_r}$$

where:  $P_g$  = the gross photosynthetic rate ( $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ )  
 $R_r$  = the respiration rate ( $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$ )

Growth through the fixation of  $\text{CO}_2$  (photosynthetic growth) can only be achieved in cultures in which the rate of photosynthesis is above the compensation point (the point at which oxygen evolution due to photosynthesis is equal to oxygen uptake due to respiration) ie. where  $P_r/R_r$  is greater than 1. In some cases photosynthetic conditions caused an increase in oxygen uptake rather than an increase in oxygen evolution. In these cases the photosynthetic rate was expressed as a negative figure and therefore  $P_c$  was negative.

### 2.3.3 Detection and Analysis of Chlorophyll Fluorescence.

The phenomenon of chlorophyll fluorescence was used to assess the photosynthetic capacity of cells grown *in vitro* according to their ability to utilize a defined level of photosynthetically active radiation (PAR). Chlorophyll fluorescence occurs when electrons excited by captured photons of light cannot be utilized in a photochemical event and therefore return to their ground state accompanied by an emission of energy, seen as fluorescence. The time period between electron excitation and its return to the ground state is in the order of  $10^{-9}$  sec (Gregory 1977) and thus the primary photochemical event must occur in a time less than this if the captured photon of light is to be utilized in photosynthesis.

The development of a system for the detection of chlorophyll fluorescence from cultured cells is described in Section 3.1, and the characteristics of fluorescence transients produced by photosynthetically active tissue are described in Section 3.1.1.1, 3.1.1.2 and 3.1.3.1.

Cultured cells were separated from their medium and blotted dry with Whatman No. 1 filter paper before approximately 2g fresh weight were placed in the sample well of the apparatus (Fig 2.3.1). The cover containing a low heat light source (Hansatech Ltd., Kings Lynn), giving a photon flux density of  $300\mu\text{E m}^{-2} \text{sec}^{-1}$  at 640nm within 100nsec of activation, and a photodiode (Hansatech Ltd.), protected by a 740nm interference cut-off filter, was placed over the sample well. Chlorophyll fluorescence with a wavelength of greater than 740nm (the wavelength of energy<sup>9</sup> re-emitted as fluorescence is greater than the excitation wavelength due to energy losses) was detected by the photodiode. The fluorescence detected was converted into an electrical signal, by the photodiode, proportional to the level of fluorescence detected. This electrical signal was amplified before capture on a Gould 20MHz Digital Storage Oscilloscope, in the DC mode, set at an appropriate sensitivity. After a period of 5min without illumination the cells were either given a 2.5sec flash of light followed by continuous illumination during which the fluorescence emission was recorded at a time-base of  $0.5\text{sec cm}^{-1}$ , or illuminated continuously and the fluorescence transient recorded at a time-base of  $10\text{msec cm}^{-1}$ . After capture



**FIGURE 2.3.1**

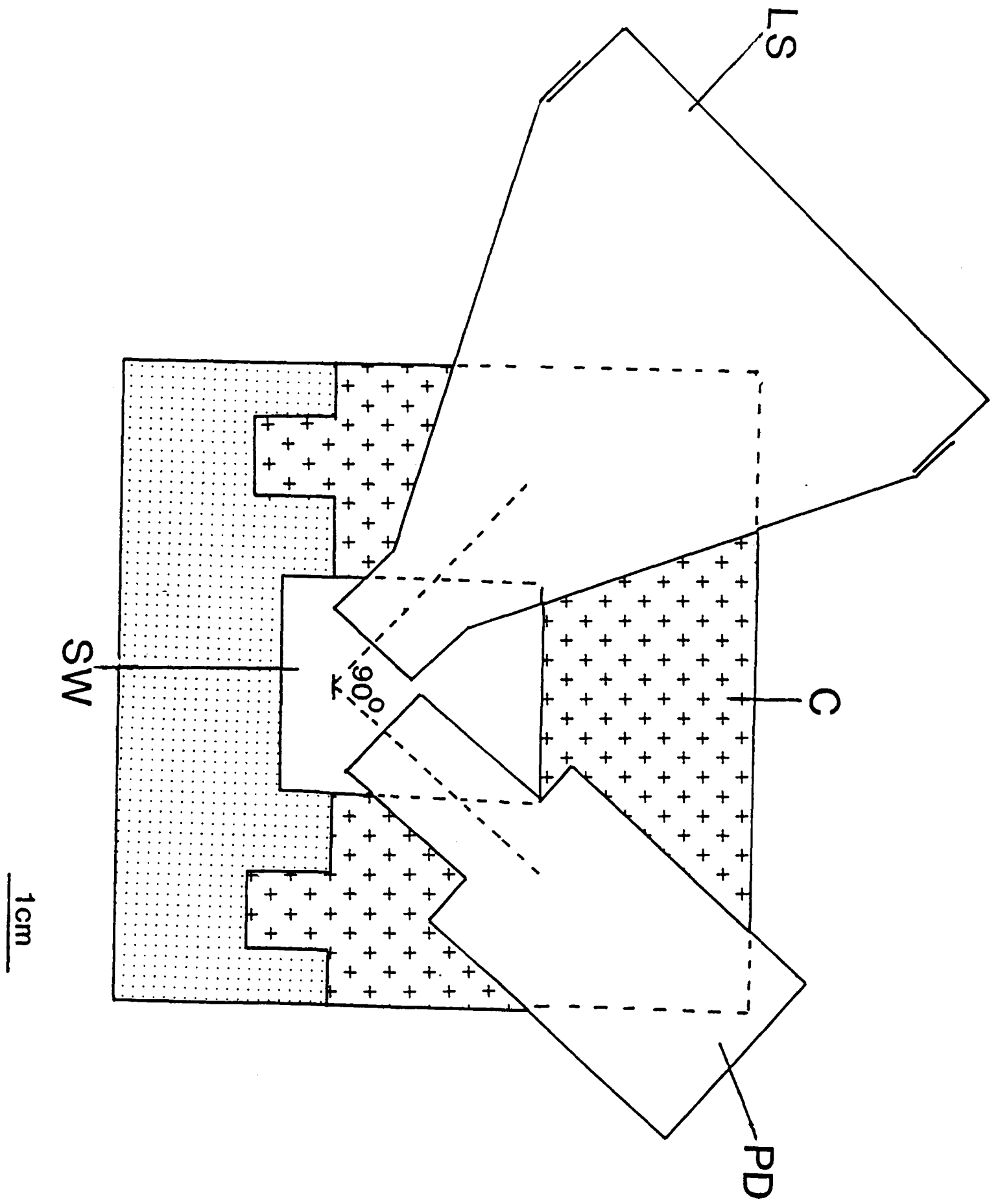
The construction of the chlorophyll fluorescence detection system for the detection of chlorophyll fluorescence from cultured cells.

**LS = LED light source.**

**C = Non-reflective grey PVC casing.**

**PD = Photodiode protected by a 740nm interference cut-off filter.**

**SW = Sample well.**



fluorescence profiles were plotted out on a chart recorder set at 1mV, linked to the oscilloscope, using an oscilloscope plot speed of 10sec cm<sup>-1</sup> and a chart speed of 6cm min<sup>-1</sup> on the chart recorder.

### **2.3.4 Analysis Of The Uptake Of Four Of The Major Nutrients From The Culture Medium.**

The uptake of fructose by cell suspension cultures grown with differing levels of fructose supplementation was examined throughout a growth cycle to determine the kinetics of its uptake by the cells. The uptake of ammonium, nitrate and phosphate was also examined so as to establish whether or not altering the fructose concentration in the medium had an effect on their uptake.

Each determination was by a spectrophotometric method and all optical densities were measured on a Pye-Unicam SP8-100 UV spectrophotometer. The methods employed show minimal interference with the colour reactions resulting from the presence of ions and nutrients other than the nutrient under analysis. Any such interference was minimized by using full MSS medium minus the nutrient under analysis as the blank for zeroing the spectrophotometer and for diluting nutrient samples where required.

Nutrient medium was retained after separation from the cells, on each sampling day during the period of investigation, and stored at -40°C until the completion of the time course. At the end of the experiment the medium retained was analysed for fructose, phosphate, ammonium and nitrate.

#### **2.3.4.1 Fructose determination.**

The fructose concentration in the medium was determined using the Anthrone method (Ashwell 1957). Anthrone reagent was prepared by dissolving 2<sup>0</sup><sub>g</sub> of anthrone (Sigma) in 1l of concentrated sulphuric acid (BDH).

The sample (2µl of medium made up to 1ml with MSS medium minus fructose) was thoroughly mixed with 2ml of anthrone reagent at 0–5°C before being heated at 90°C for 10min. After cooling to room temperature the optical density was measured at 620nm. A calibration curve for standard fructose solutions is shown (Fig 2.3.2).

#### **2.3.4.2 Orthophosphate determination.**

The orthophosphate concentration in the medium was determined according to the method of Allen (1940). Amidol reagent was prepared by dissolving 0.2g of 2,4-diaminophenol hydrochloride (amidol) (BDH) and 4.0g of sodium metabisulphite (BDH) in 20ml of distilled water and filtered prior to use.

The sample (0.2ml of medium made up to 1ml with MSS medium minus phosphate) was acidified by the addition of 0.4ml of 60% perchloric acid (BDH). To this solution was added: 4ml of distilled water, 0.4ml of amidol reagent and 0.2ml of 8.3% (w/v) ammonium molybdate (BDH). After vortexing the mixture was left to stand at room temperature for 30min before the optical density at 620nm was measured. A calibration curve for standard orthophosphate solutions is shown (Fig 2.3.3).

#### **2.3.4.3 Ammonium determination.**

The method of Havilah *et al* (1977), which uses two reagents, was used in determining the ammonium concentration in the medium. Salicylate reagent was prepared by dissolving 34g of sodium salicylate (BDH) and 0.24g of sodium nitroprusside (BDH) in 500ml of distilled water and subsequently making the volume up to 1l with distilled water. Cyanurate reagent was prepared by dissolving 0.25g of sodium dichloroisocyanurate (BDH) in 200ml of distilled water and adding 15ml of 40% (w/v) sodium hydroxide (BDH) before making the volume up to 1l with distilled water.

FIGURE 2.3.2

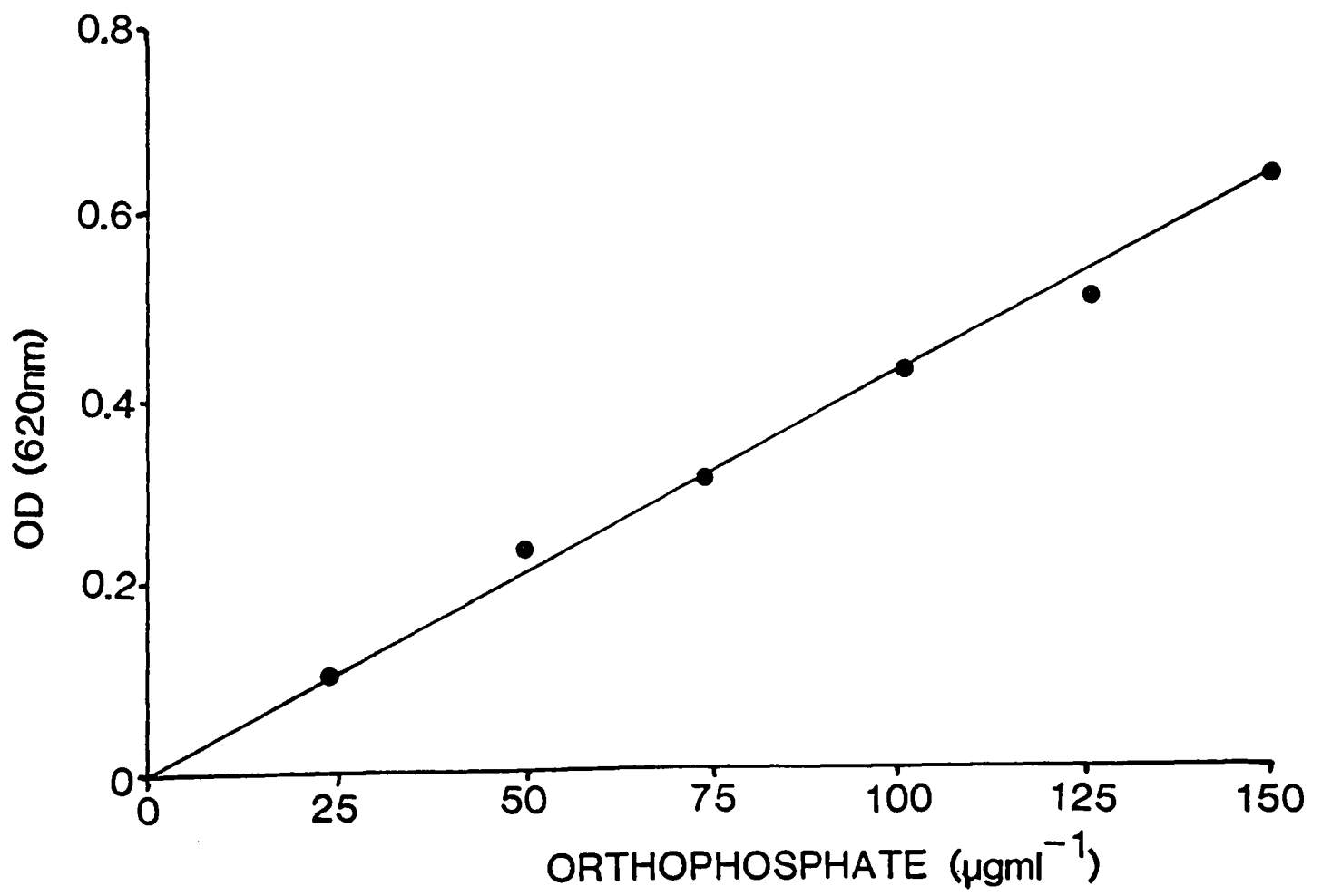
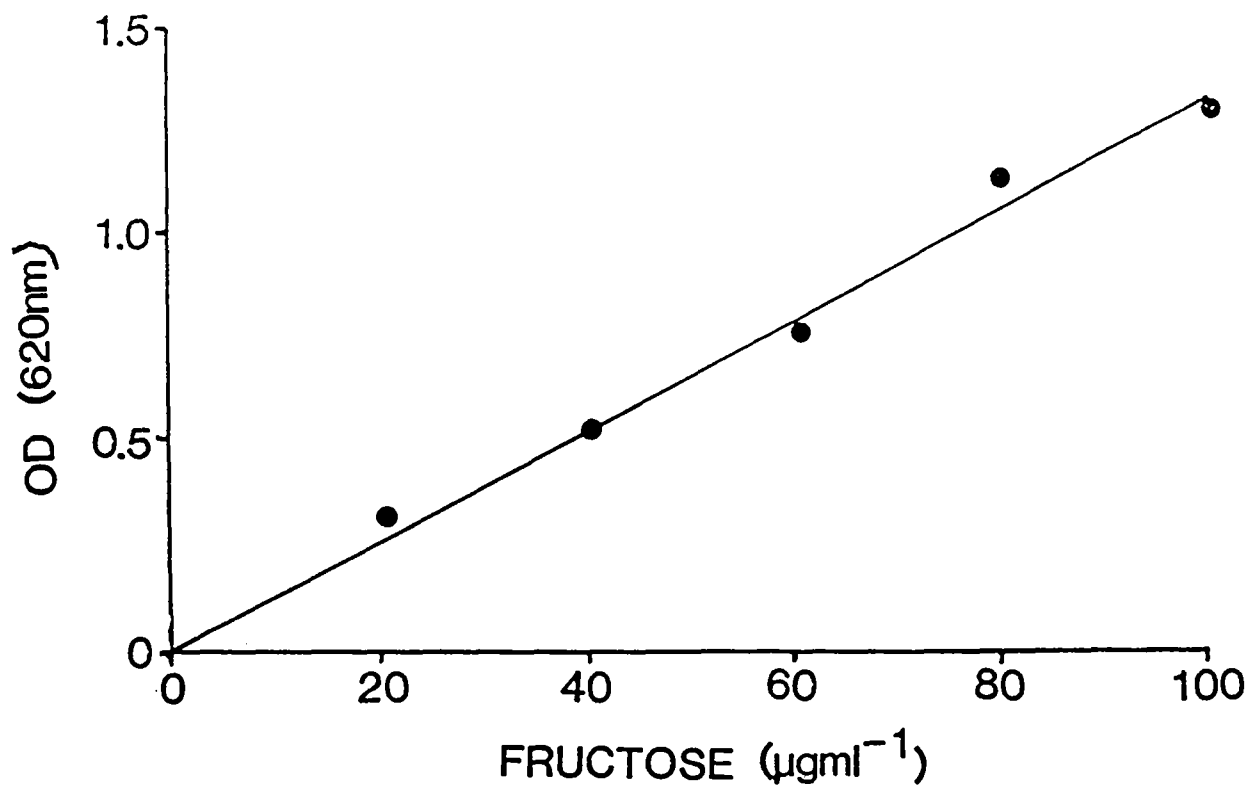
Fructose calibration curve using the Anthrone method.

*2  $\mu$ l taken from an  $x \mu\text{g ml}^{-1}$  solution*

FIGURE 2.3.3

Phosphate calibration curve.

*c. 2 ml taken from an  $x \mu\text{g ml}^{-1}$  solution*



To the sample (2µl of medium made up to 1ml with MSS medium minus ammonia) was added 5ml of salicylate reagent and 5ml of cyanurate reagent. After vortexing the solution was left to stand for 30min before the optical density was measured at 660nm. A calibration curve for standard ammonium solutions is shown (Fig 2.3.4).

#### **2.3.4.4 Nitrate determination.**

For the determination of nitrate in the medium the method of Wooley *et al* (1960) was employed. An intimate powder mixture of: 100g of barium sulphate, 75g of citric acid, 12g of manganese sulphate.4H<sub>2</sub>O, 4g of sulphanilic acid, 2g of powdered zinc (all obtained from BDH) and 2g of 1-naphthylamine (Sigma) was prepared at least 15 days prior to use. Immediately before use an acetic acid reagent consisting of 20% (v/v) acetic acid (BDH) containing 0.2ppm copper, as copper sulphate (BDH), was prepared.

To the sample (20µl of medium made up to 1ml with MSS medium minus nitrate) was added 9ml of acetic acid reagent. To this solution was added 0.8g of the powder and the mixture shaken for 15sec before being left to stand for 3min followed by shaking for a further 15sec. After standing for a further 3min the solution was centrifuged at 1,000xg for 3min and the optical density of the supernatant measured at 520nm. A calibration curve for standard nitrate solutions is shown (Fig 2.3.5).

FIGURE 2.3.4

Ammonium calibration curve.

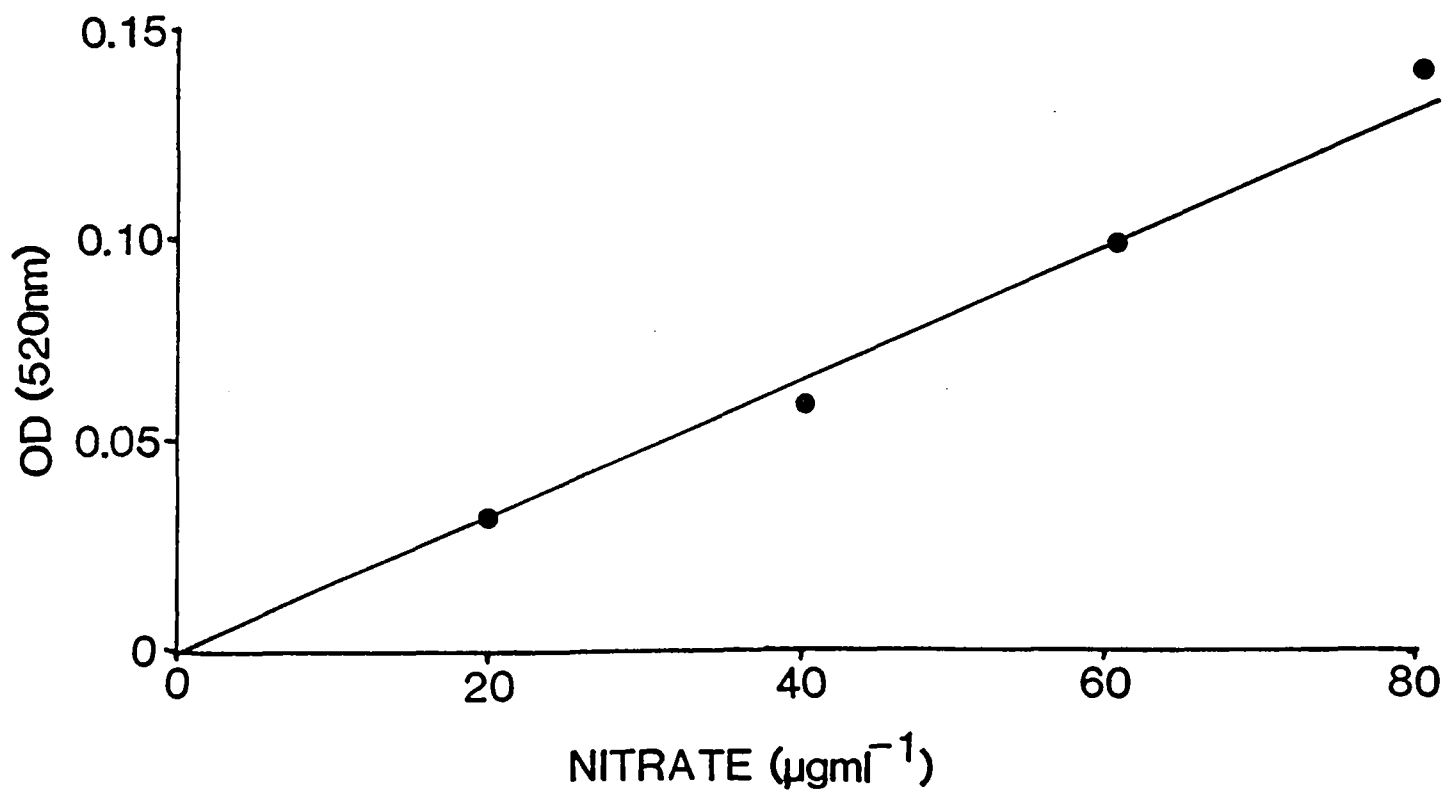
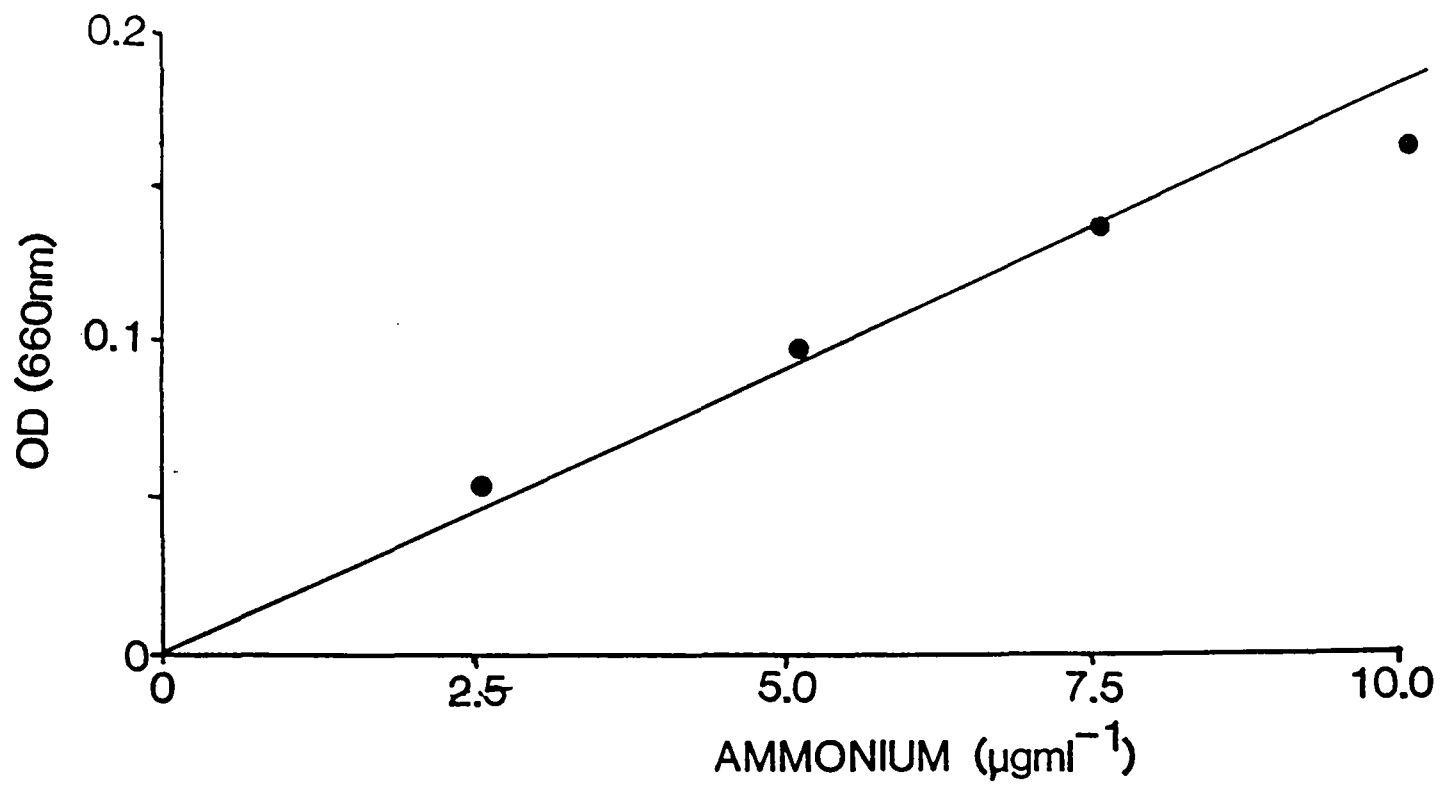
$2\mu$  taken from an  $x \mu\text{g ml}^{-1}$  solution.

FIGURE 2.3.5

Nitrate calibration curve.

$20\mu$  taken from an  $x \mu\text{g ml}^{-1}$  solution.





## **2.4 DETECTION AND ANALYSIS OF CHLOROPHYLL FLUORESCENCE FROM ISOLATED CHLOROPLASTS.**

During the development of chlorophyll fluorescence analysis systems and subsequent experimental investigations of photosynthesis, using this technique, isolated pea chloroplasts have frequently been the material studied. Since isolated pea chloroplasts provide a well defined material, with respect to chlorophyll fluorescence, they were used during the development of the chlorophyll fluorescence analysis system for cultured cells, as well as providing fluorescence profiles that were used as standards.

The fluorescence profiles of isolated tobacco chloroplasts were also analysed in order to determine whether or not there were any differences between the fluorescence profiles from isolated chloroplasts of these two species. Chloroplasts were also isolated, and analysed, from mixotrophic cell cultures. Cells capable of growth under conditions favouring mixotrophic growth had a high enough chlorophyll content to make it feasible to extract chloroplasts for fluorescence analysis. This enabled a direct comparison of fluorescence profiles obtained from whole cells and the profiles obtained from chloroplasts isolated from those cells.

### **2.4.1 Isolation Of Chloroplasts.**

All of the steps in the isolation of chloroplasts were carried out at 0-4°C according to the method of Walker (1971) . The material used was either: 7 to 10 day old pea seedling leaves, or the leaves of 4 month old tobacco plants, or cells grown in suspension culture for 19days in MSS medium supplemented with 1.4% fructose.

Leaves (25g fresh weight) or cultured cells (50g fresh weight) were homogenised for 10sec with a Polytron in 100ml of buffer. The buffer at pH7.6 consisted of: 0.33M sorbitol, 0.05M HEPES, 5mM magnesium chloride, 1mM cystine, 1mM EDTA, 0.2% (w/v) PVP and 0.1% (w/v) BSA, all chemicals were obtained from Sigma, except magnesium chloride which was obtained from BDH. The homogenate was strained through two layers of muslin and the

resulting filtrate strained through a further eight layers of muslin. The filtrate was centrifuged at 1,250xg for 1min and the supernatant discarded. The pellet was resuspended in 2ml of the buffer, using a glass rod capped with cotton wool, and stored on ice until required.

#### **2.4.2 Determination Of The Chlorophyll Content Of Isolated Chloroplasts.**

The chlorophyll content of the isolated chloroplasts was determined according to the method of Walker (1971) . 50 $\mu$ l of the chloroplast preparation was mixed with 10ml of 80% (v/v) acetone (BDH) and vortexed for 3min. The solution was then filtered through a Whatman No. 1 filter paper and made up to 10ml with 80% acetone. The optical density of the filtrate was measured against an acetone blank at 652nm in a Pye-Unicam SP8- 100 UV. sepectrophotometer. The chlorophyll content,  $C_c$  (mg ml<sup>-1</sup>), was calculated as:

$$C_c = A_{652} \times 11.11$$

where:  $A_{652}$  = the optical density at 652nm.

#### **2.4.3 Chlorophyll Fluorescence Detection.**

A volume containing 100 $\mu$ g of chlorophyll was taken from the chloroplast preparation and placed in a 4ml fluorescence cuvette (Hughs and Hughs) along with 3ml of buffer. The buffer at pH7.6 consisted of: 0.1M sorbitol, 0.05M HEPES (both from Sigma), 5mM magnesium chloride and 5mm sodium chloride (both from BDH). The cuvette was placed in the grey PVC holder (Fig 2.4.1) so that one face was parallel to the LED light source and a face at 90<sup>o</sup> to this was parallel to the photodiode. Once in place the chloroplasts in the cuvette were kept suspended by the action of a magnetic follower. Having placed the cuvette in the fluorescence detector holder the chloroplast suspension was incubated and illuminated in the same manner as cultured cells (Section 2.3.3).

**FIGURE 2.4.1**

**The construction of the system for the detection of chlorophyll fluorescence emitted from isolated chloroplasts.**

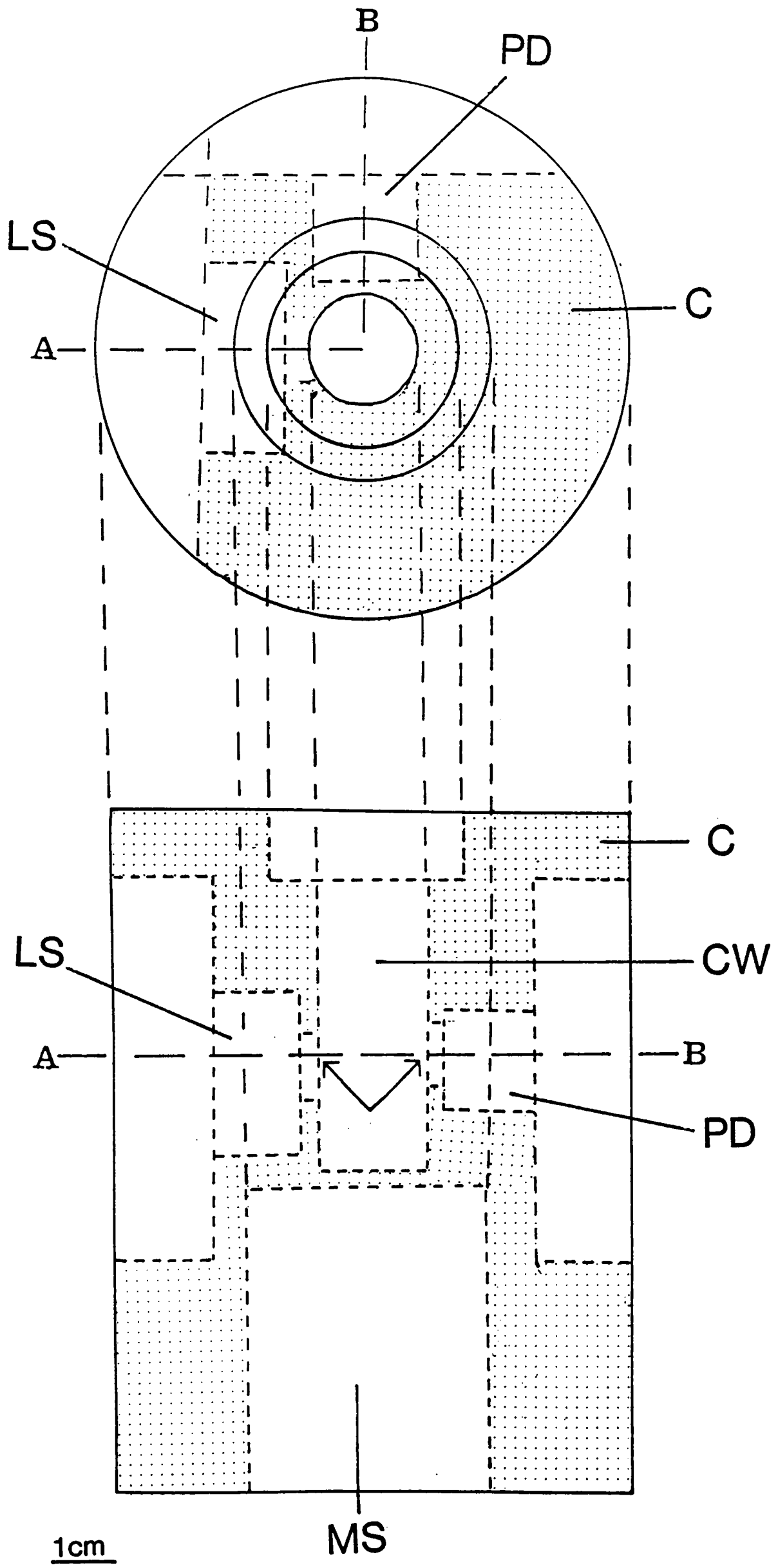
**LS = LED light source.**

**PD = Photodiode protected by a 740nm interference cut-off filter.**

**CW = Fluorescence cuvette well.**

**C = Non reflective grey PVC casing.**

**MS = Magnetic stirrer.**



## 2.5 CHEMICAL ANALYSIS OF THE TOBACCO ALKALOIDS.

### 2.5.1 Extraction Of The Tobacco Alkaloids.

The extraction of the pyridine alkaloids: nicotine, nornicotine, anabasine and anatabine from tobacco plants and cell cultures was achieved using a method modified from that of Hamill *et al* (1986). The development of this method is described in detail in Section 3.2.

Cultured cells, or roots, were separated from their bathing medium by filtration under reduced pressure through a Whatman No. 1 filter paper. Both the cells and the medium were then analysed for alkaloids.

#### 2.5.1.1 Extraction of the tobacco alkaloids from plant material and cultured cells.

Plant tissue (4–5g fresh weight), cultured cells (9–10g fresh weight) or cultured roots (1–2g fresh weight) were ground using a pestle and mortar with 15ml of 2% (v/v) ammonium hydroxide (30%<sup>w/v</sup> ammonia solution from BDH). To the homogenate an equal volume of chloroform (BDH) was added and mixed. The extract was left to stand for 5min before cell debris was removed by filtration under reduced pressure through a Whatman No. 1 filter paper. The phases of the filtrate were separated, using a separating funnel, and the aqueous phase re-extracted with a further two volumes of chloroform. The chloroform extract was evaporated to dryness at 30°C using a rotary evaporator (Buchi, Switzerland). The residue was re-suspended in 10ml of 0.3% (v/v) sulphuric acid (BDH) on ice for 30–60min. The sample was transferred to a separating funnel and the pH adjusted to pH10.8 with 20% (v/v) ammonium hydroxide followed by extraction with 3 X 15ml of chloroform. Dissolved water was removed from the chloroform extract by the addition of 0.5–1.0g of anhydrous sodium sulphate (BDH), which was subsequently removed by filtration through a glass microfibre filter (Whatman) under reduced pressure using a Millipore filtration unit (Millipore SA., Molsheim, France). The residual sodium sulphate was washed with 5ml of chloroform before the filtrate was

evaporated to dryness as previously. After evaporation to dryness the residue was resuspended in 1ml of HPLC grade methanol (BDH).

#### **2.5.1.2 Extraction of the tobacco alkaloids from culture medium.**

Medium was retained after separation from suspended cells and the pH adjusted to pH10.8 with 20% (v/v) ammonium hydroxide. The alkaline medium was then extracted with 3 X 50ml of chloroform and the resulting chloroform extract treated in the same way as the cellular samples above.

#### **2.5.2 Analysis Of The Alkaloids By Thin Layer Chromatography.**

Thin layer chromatography (TLC) was used to perform qualitative analysis of extracts since it allowed a sample volume of up to 100 $\mu$ l to be analysed.

##### **2.5.2.1 Thin layer chromatography.**

Plastic backed TLC plates (20 X 20cm) coated with silica gel 60 to a thickness of 0.2mm (Merck, Dassel, W. Germany) were used as the adsorbent. A solvent system of chloroform : 96% (v/v) ethanol in the ratio 4:1 (Randerath 1968) was used to separate the alkaloids. After loading an appropriate volume of either sample or standard alkaloid solution the plates were *developed* for 2-2.5hours in a sealed tank (Shandon) which had been equilibrated for 1-2hours. After *development* was complete the plates were dried before being sprayed with Iodoplatinate reagent (Section 2.5.2.2) using a Humbrol <sup>P</sup>Spray Gun.

Alkaloids in the extract were identified by their *comigrated* with authentic standards (a gift from Rothmans International Services which is gratefully acknowledged), which were run in parallel with the extracts on each plate (Fig 2.5.1).

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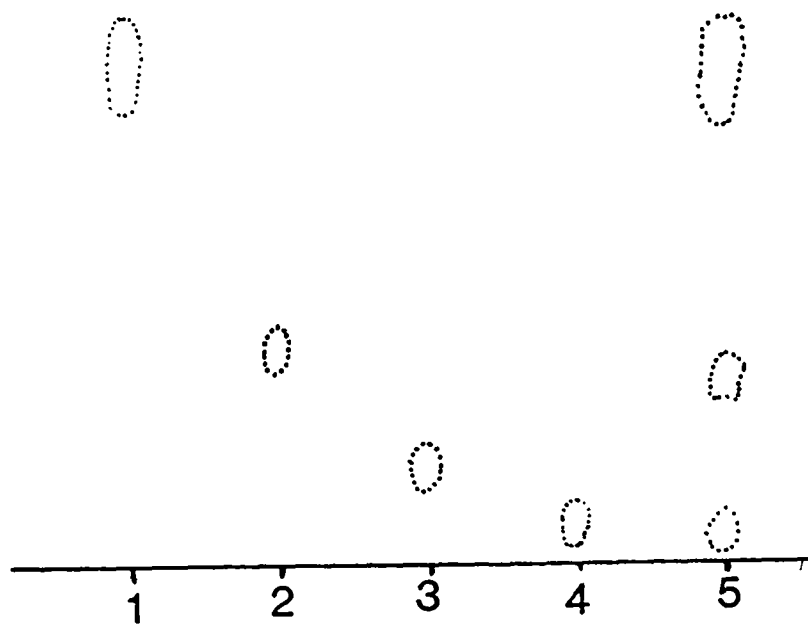


FIGURE 1.5.1

The *migration* of the tobacco alkaloids on a Silica TLC plate with chloroform: 96% (v/v) ethanol (4:1). The alkaloids were detected by spraying the *developed* plate with iodoplatinate reagent.

- 1 = Nicotine.
- 2 = Anatabine.
- 3 = Anabasine.
- 4 = Nornicotine.
- 5 = 50µl of a tobacco leaf extract.



### **2.5.2.2 Iodoplatinate reagent for the detection of alkaloids on TLC plates.**

Iodoplatinate reagent was prepared by adding 10ml of 55% (w/v) aqueous platinum chloride (BDH) and 5ml of concentrated hydrochloric acid (BDH) to 240ml of 2% (w/v) aqueous potassium iodide (BDH). The reagent was filtered through a Whatman No. 1 filter paper and stored at 5°C until required.

On spraying ~~developed~~ plates with the reagent any of the major four alkaloids present were immediately detectable. All four of the alkaloids appeared as purple-grey spots, on a pink-brown background, which faded rapidly in the light.

### **2.5.3 Analysis Of The Alkaloids By High Performance Liquid Chromatography.**

High performance liquid chromatography (HPLC) was used for the qualitative and quantitative analysis of the four major pyridine alkaloids of tobacco. The maximum volume analysed by this method was 20µl therefore samples were also analysed by TLC, where over 100µl could be loaded, so as to detect trace amounts of the alkaloids.

#### **2.5.3.1 HPLC analysis of samples.**

Samples and solvents were prepared as described in section 2.6.1 and 2.6.2 respectively and the HPLC system was as described in Section 2.6.3. The column was eluted at 1ml min<sup>-1</sup> with a mobile phase consisting of methanol and a buffer, which contained: 0.2% (v/v) orthophosphoric acid (BDH) in water adjusted to pH7.5 with triethylamine (BDH) (Saunders & Blume 1981), according to the gradient system shown in Fig 2.5.2 (Cooke 1987 personal communication). Detection of eluted alkaloids was at 260nm. A chromatogram of a mixture of authentic tobacco alkaloid standards is shown in Fig 2.5.3.

### FIGURE 2.5.2

The gradient system used to elute the tobacco alkaloids off a C<sub>18</sub> reverse phase HPLC column.

Mobile phase A = Methanol

Mobile phase B = 0.2% (v/v) orthophosphoric acid buffered to pH7.5 with triethylamine.

### FIGURE 2.5.3

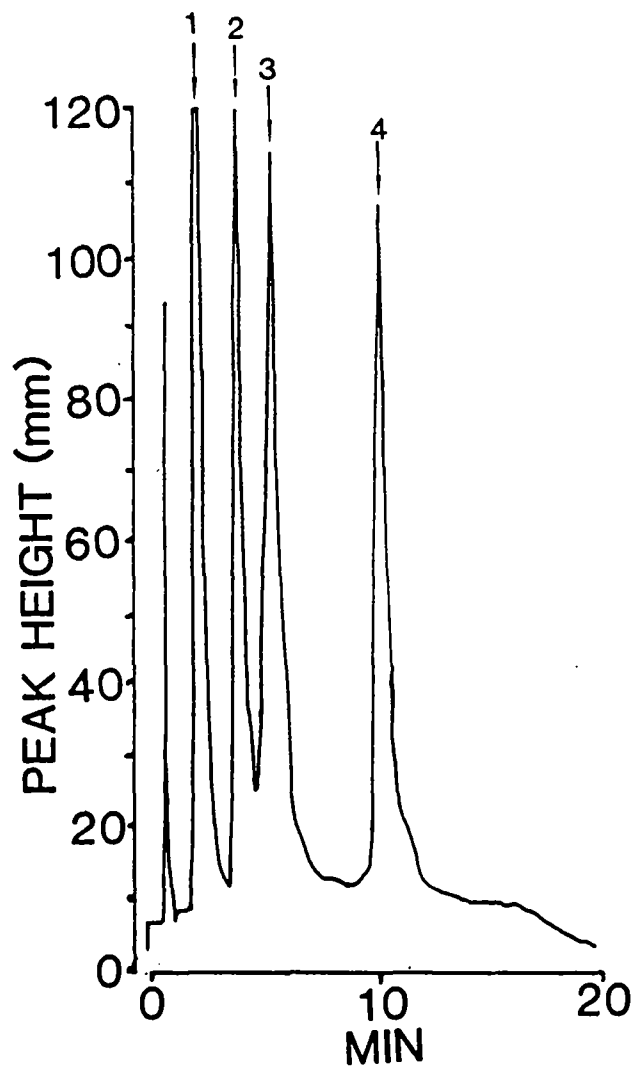
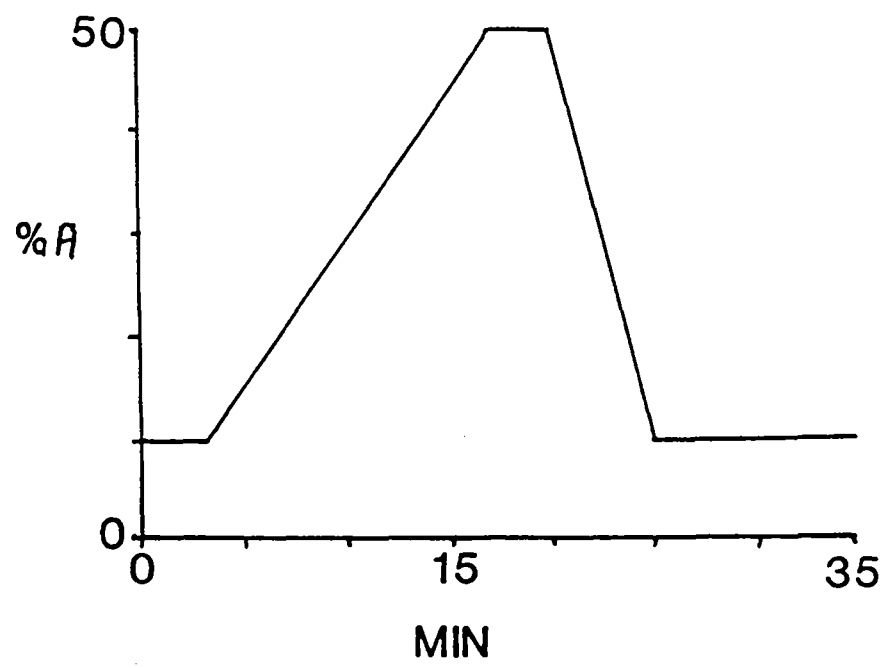
A chromatogram showing the elution of a 20µl injection of a solution containing 2.5µg of each of the main tobacco alkaloids. The alkaloids were eluted from a C<sub>18</sub> reverse phase column with a gradient system based on methanol and a weak phosphoric acid buffer. The alkaloids were detected by their UV absorbance at 260nm.

1 = Nornicotine.

2 = Anabasine.

3 = Anatabine.

4 = Nicotine.



A given extract was analysed, as described above, and a chromatogram obtained (Fig 2.5.4a) and in this case the nicotine peak identified by its retention time. After the addition of exogenous nicotine to the sample the chromatogram shown in Fig 2.5.4b was produced. It can be seen that the area of the peak marked in the first chromatogram has increased thus confirming the peak as nicotine. The identity of alkaloid peaks was confirmed further by comparison of the UV. spectra of peaks with the spectra of alkaloid standards using a Hewlett Packard 1090 liquid chromatograph fitted with a binary DR5 solvent delivery system and autosampler and used in conjunction with a HP1040 diode array detection system (Hewlett Packard, Reading).

The amount of alkaloid present in a given extract was calculated by measuring the height of the peak with a retention time of the given alkaloid, on the chromatogram. Calibration curves were prepared by adding a known amount of alkaloid to a tissue that did not contain alkaloids and extracting. The extracts produced were analysed by HPLC and the peak heights for different initial concentrations of alkaloid plotted against initial alkaloid concentration (Fig 2.5.5). These calibration curves were then used in calculating the alkaloid content of tissues.

#### **2.5.4 Purification Of An Unknown Peak For Subsequent Analysis**

On analysis of alkaloid extracts by HPLC it was possible to detect all four major alkaloids. However, in the majority of cases there was a fifth peak that had a retention time between those of anatabine and nicotine. Since the extraction procedure was specific for alkaloids or compounds that behaved in a similar manner to alkaloids the peak was purified in order to determine whether or not it was of alkaloid origin.

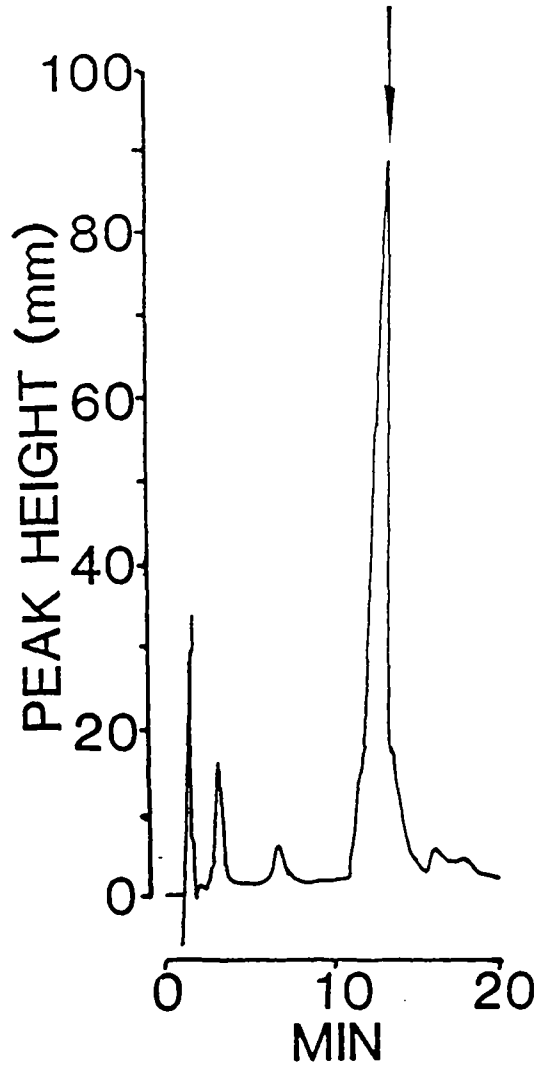
**FIGURE 2.5.4**

Chromatograms of an extract from leaves of *N.tabacum*. The peak corresponding to nicotine is arrowed.

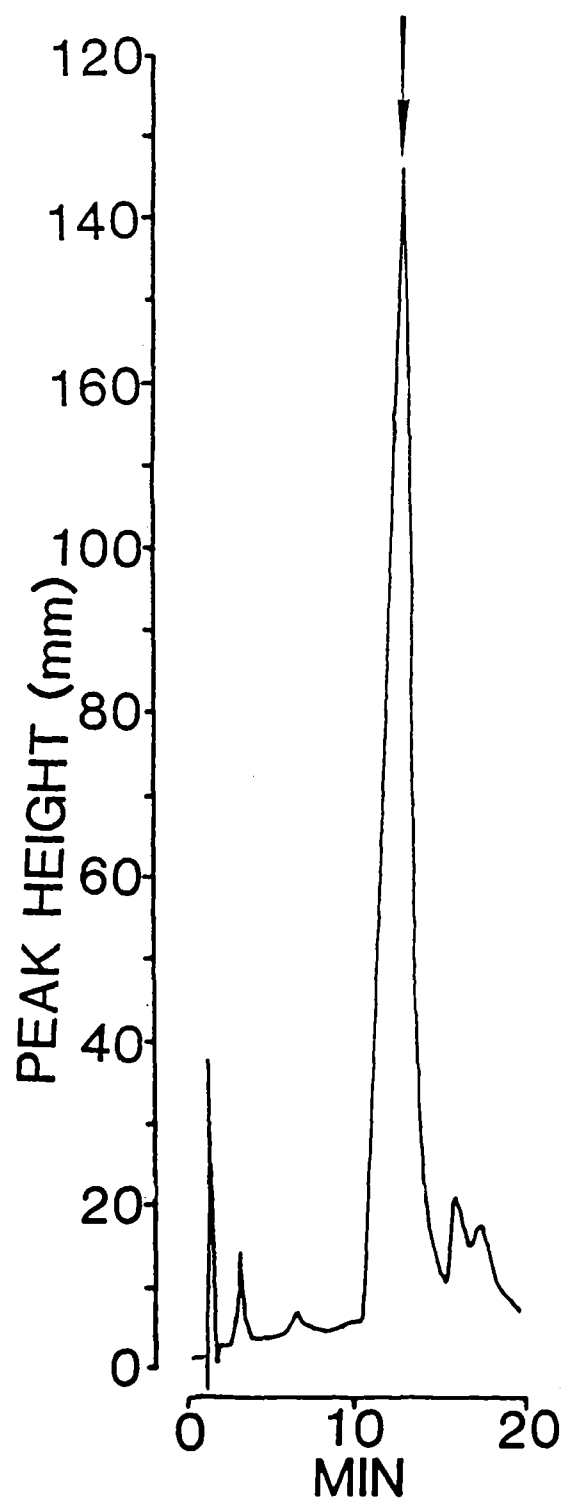
a. = A 20 $\mu$ l injection of a leaf extract.

b. = A 20 $\mu$ l injection of the leaf extract used in (a.) plus 1 $\mu$ g of nicotine.

a



b



**FIGURE 2.5.5**

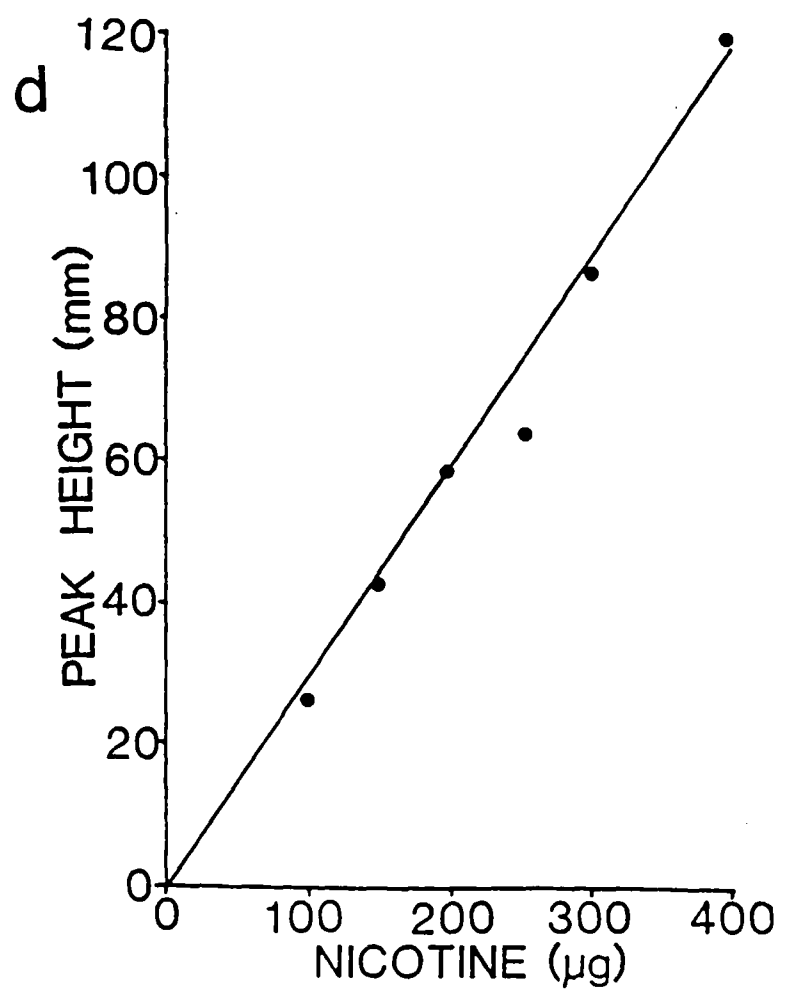
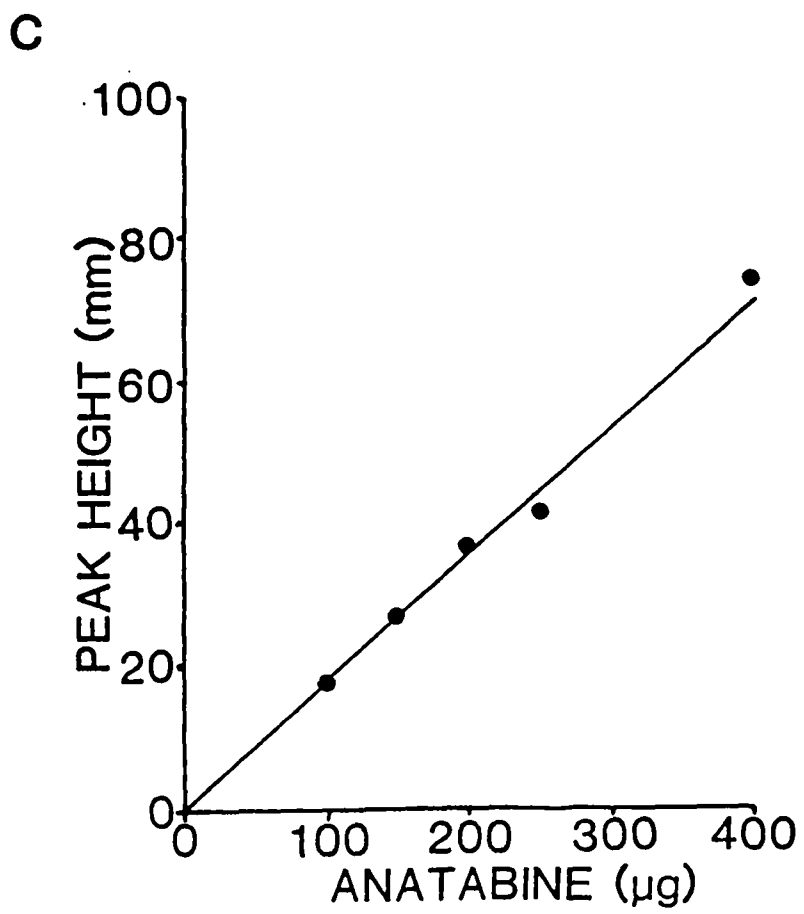
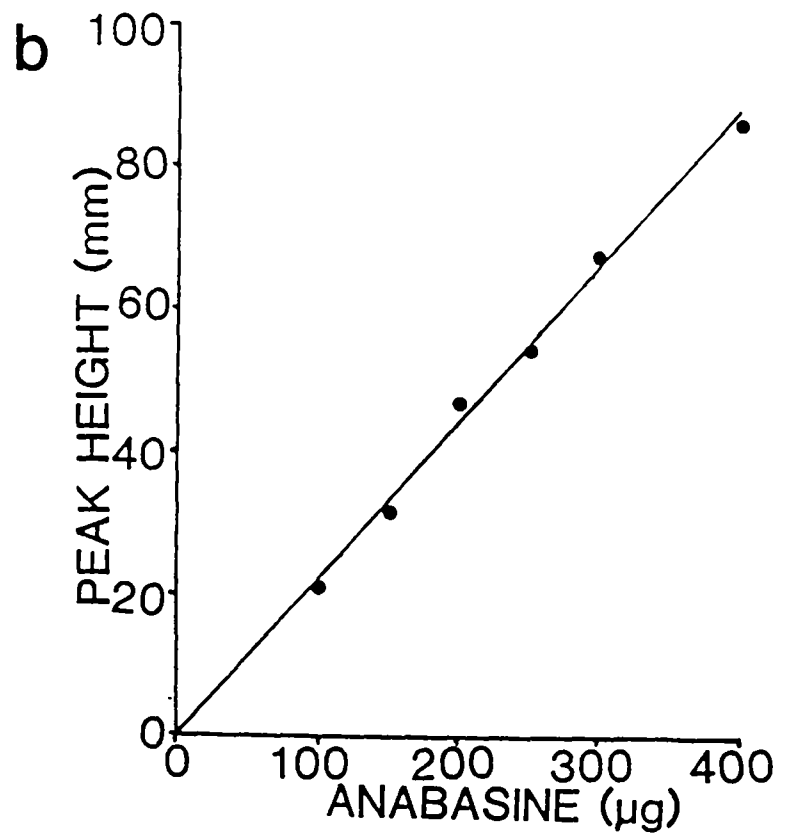
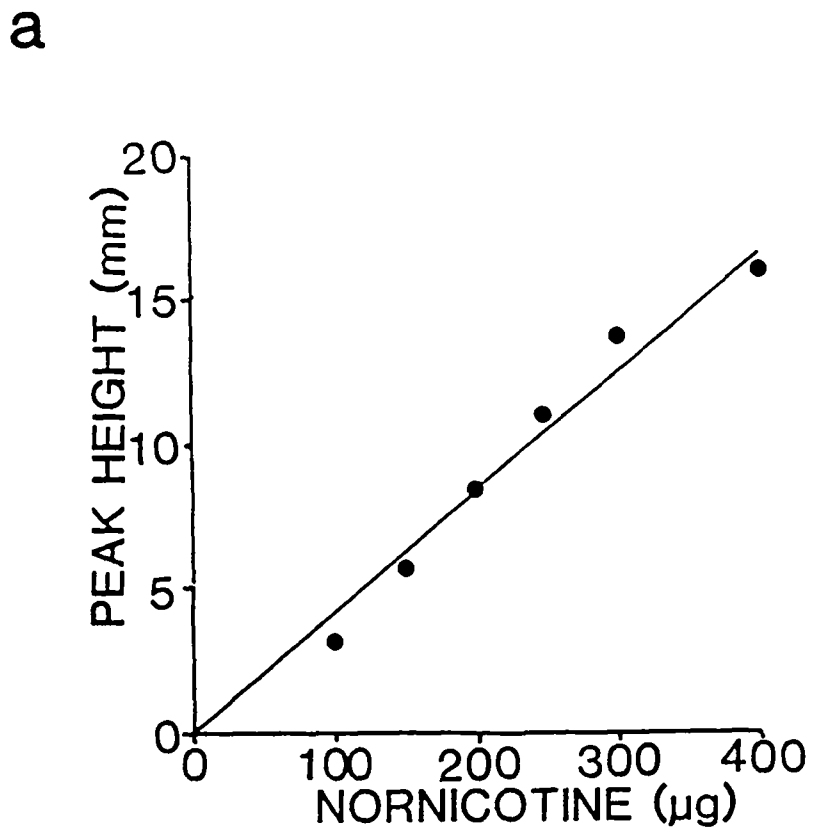
HPLC calibration curves for the four main tobacco alkaloids. Different amounts of each alkaloid were added to tissue that did not contain any alkaloid and extracted. The peak heights on the chromatograms produced with these extracts were then plotted against the initial alkaloid concentration.

a. = Nornicotine.

b. = Anabasine.

c. = Anatabine.

d. = Nicotine.





#### **2.5.4.1 Extraction from cultured material.**

Approximately 50g of suspension cultured cells, grown in the dark for 26 days with a MSS based bathing medium, were homogenised using a Waring blender in 75ml of 2% (v/v) ammonium hydroxide. To the homogenate 75ml of chloroform was added and the extract left to stand for 5min. Cell debris was removed by filtration through a Whatman No.1 filter paper under reduced pressure. The phases of the filtrate were separated and the aqueous phase reextracted with a further 2 X 75ml of chloroform. The chloroform extract was then treated as previously (Section 2.5.1.1).

#### **2.5.4.2 HPLC analysis of the extract.**

The extract was diluted fourfold and prepared for analysis as described in Section 2.6.2. HPLC analysis was carried out using a Hewlett Packard HP1090 liquid chromatograph with stationary and mobile phases as described in Section 2.5.3.1. An injection volume of 20 $\mu$ l was used in order to determine the retention times of the alkaloids and the unknown peak. Using a HP1040 diode array detection system, fitted to the HP1090, it was possible to examine the UV spectra of the unknown peak.

Having determined the retention times of the alkaloids and the unknown and obtained a UV spectrum for the latter the Gilson HPLC system in conjunction with a LKB fraction collector were used to collect the fraction of the column eluate containing the unknown peak, as described in Section 2.6.4. This processes was repeated until all of the extract had been injected onto the column.



#### **2.5.4.3 Extraction of the unknown compound from the HPLC mobile phase.**

In order to identify the unknown compound Mass spectroscopy was performed by Mr B. Frost and (Rothmans International Services) and the compound identified by a comparison of the data obtained with information on a chemical database, which was performed by Mr H. Elmenhorst (Rothmans International Services: Chemical Research, Bremen). For the above analysis it was necessary to have a final extract in chloroform.

The fraction collected from the HPLC column contained a mixture of methanol and the phosphoric acid buffer. In order to extract the unknown compound from this solvent system into chloroform the pH was adjusted to pH10.8 with 20% (v/v) ammonium hydroxide. The alkaline sample was then transferred to a separating funnel and extracted with 3 X 40ml of chloroform. The chloroform extract was dried by the addition of approximately 2g of anhydrous sodium sulphate and the removal of the slurry by filtration under reduced pressure through a Whatman No.1 filter paper. The volume of the extract was then reduced by 50% by rotary evaporation before storage and transport on dry ice under a nitrogen atmosphere.

Before storage 5ml of the final extract was removed and rotary evaporated to dryness. The residue was resuspended in 0.5ml of HPLC grade methanol and prepared for HPLC analysis. 20µl of this sample were analysed using a Hewlett Packard 1090 liquid chromatogram (as in Section 2.5.3.1) so as to compare the retention time and the spectra of the purified unknown compound with that of the unknown peak in the original extract.

## **2.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.**

High performance liquid chromatography (HPLC) was used in the identification and quantification of several compounds. HPLC analysis provided an analytical system that was rapid, automated and allowed quantification of compounds from small sample volumes.

### **2.6.1 Preparation Of The Mobile Phase.**

All solvents were either of HPLC grade or the highest grade commercially available. Before use all mobile phases were filtered under reduced pressure through either 0.45 $\mu$ m cellulose acetate membranes (Schleicher and Schuell, Dassel, ~~Dassel~~, W. Germany) for aqueous solutions or through 0.45 $\mu$ m Nylon-66 membrane filters (Rainin Instrument Co. Inc., Woburn, MA.) for organic solvents. Immediately prior to use all mobile phases were degassed for 10min with a steady stream of helium (BOC) coming from a sparger. This was a precaution to prevent air bubbles developing within the HPLC system where the solvents were subjected to large pressure differences. While in use mobile phase reservoirs were sealed with Parafilm to reduce the build up of dissolved air and to prevent the entry of dust particles.

### **2.6.2 Preparation Of The Samples.**

Prior to HPLC analysis all samples were filtered using a Bioanalytical Systems Microfilter fitted with a 0.45 $\mu$ m Nylon-66 membrane filter (Rainin) by centrifugation at 1000xg for 5min. The filtrate was then transferred to a 2ml crimp top rubber sealed autosampler vial (Chromacol, London). Samples which were not analysed immediately were stored by placing the vials in a -40°C freezer.

### **2.6.3 The HPLC system.**

HPLC analysis was performed using a Gilson 302 liquid chromatograph fitted with: a Gilson 301 autosampler, a Gilson variable wavelength UV. detector and a Shimadzu chromatopac C-RIB data processor. Samples were separated at ambient temperature on a Nova-Pak C<sub>18</sub> 15cm X 3.9mm column (Millipore-Waters, Bedford, MA.). The column was eluted with an appropriate mobile phase and the UV detector set at a wavelength close to the optimal absorbance of the compounds of interest. Changes in absorbance against time were plotted by the data processor.

### **2.6.4 Collection Of Compounds Eluted From The HPLC Column.**

Having determined the retention times of the compounds of interest it was possible to programme an LKB 2211 Superrac fraction collector (LKB, Croydon) to collect the eluted fractions containing the compounds required. The fraction collector was linked to the Gilson Autosampler and connected to the outlet from the Gilson UV detector flow cell, a delay was included in the fraction collector programme to account for the time between detection of a compound at the flow cell and its arrival at the fraction collector. The Autosampler and the Gilson gradient manager system were programmed so that the sample was injected onto the HPLC column and simultaneously the data processor and the fraction collector activated.

## **2.7 THE EXTRACTION AND DETERMINATION OF QUINOLINIC ACID PHOSPHORIBOSYL TRANSFERASE ACTIVITY FROM TOBACCO PLANTS AND CULTURES.**

In order to determine the activity of the enzyme quinolinic acid phosphoribosyl transferase (QaPRT'ase) a crude cellular extraction was obtained in a MOPS based extraction buffer. After centrifugation a coarse fractionation was carried out and the protein fraction containing enzyme activity assayed in one of two ways. Enzyme activity was either determined spectrophotometrically or with the use of  $^{14}\text{C}$ -quinolinic acid. For the latter method it was necessary to synthesise radio-actively labelled quinolinic acid from universally labelled  $^{14}\text{C}$ -aniline and glycerol.

### **2.7.1 The Synthesis And Characterization Of $^{14}\text{C}$ -Quinolinic Acid.**

#### **2.7.1.1 The synthesis of $^{14}\text{C}$ -quinolinic acid.**

$^{14}\text{C}$ -Quinolinic acid was synthesised from universally labelled  $^{14}\text{C}$ -aniline (Amersham) and unlabelled glycerol according to the method of Nishizuka and Nakamura (1970) (Fig 2.7.1). All operations were carried out in a fume cupboard in a radioactive suite, the use of which was kindly provided by Drs. R. Baxter and T. Simpson (The Department of Chemistry, The University of Edinburgh).

Universally labelled  $^{14}\text{C}$ -aniline (250 $\mu\text{Ci}$  with a specific activity of 13.62 $\mu\text{Ci } \mu\text{mole}^{-1}$ ) (Amersham) was combined with unlabelled aniline (Sigma) to give a total weight of 93 mg. The aniline was placed in a 50ml round bottomed flask (Quickfit) and 218 $\mu\text{l}$  of glycerol (BDH) added. Also added to the reaction vessel were 21 mg of crushed ferrous sulphate (BDH) (which acted as a catalyst), 137 $\mu\text{l}$  of nitrobenzene (BDH) (which stopped the back reaction of aniline to nitrobenzene) and 137 $\mu\text{l}$  of concentrated sulphuric acid (BDH) (which caused the protonation of any quinoline formed thus stabilizing it). The flask

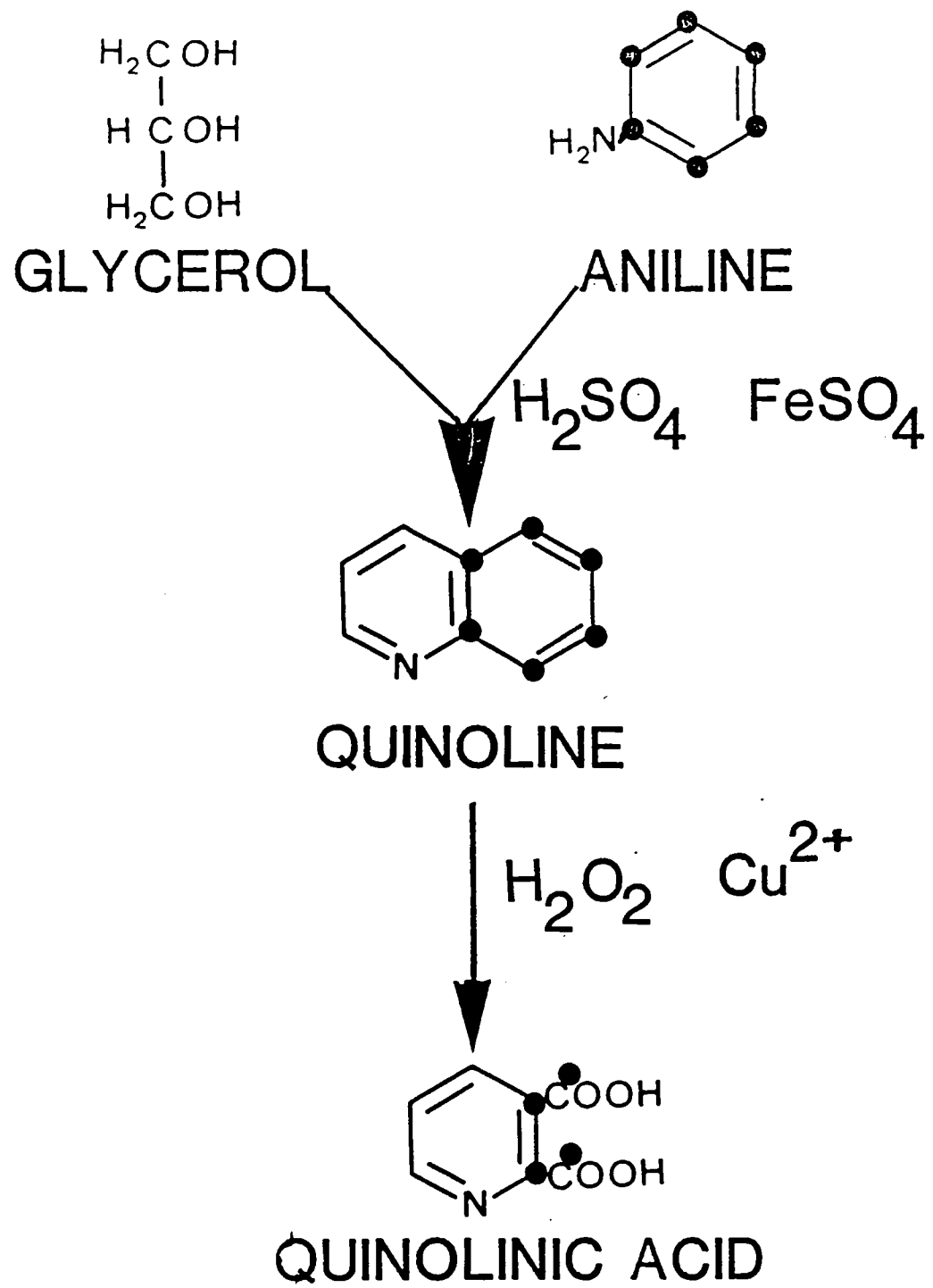


FIGURE 2.7.1

A schematic representation of the synthesis of  $^{14}\text{C}$ -quinolinic acid from universally labelled  $^{14}\text{C}$ -aniline and glycerol. See text for details of the synthesis.

● =  $^{14}\text{C}$ -carbon atoms.

was then heated at 140°C, using a heating block and a liquid paraffin bath, for 6 hours under air reflux. After 6 hours the vessel was cooled and antibumping granules (BDH) along with 20 ml of distilled water added. The air reflux tube was replaced by a right angle tube (Quickfit) which led to a water cooled condenser (Quickfit) thus allowing steam distillation. The flask was heated until the contents boiled and then maintained at boiling point. The first 3 ml of steam distillate collected were discarded since this contained the nitrobenzene added to the reaction vessel. The contents of the flask were then made alkaline with 2 ml of 40% (w/v) potassium hydroxide (BDH), making any quinoline formed steam volatile. The flask was again heated to boiling point and 10 ml of steam distillate collected. The creamy white distillate was acidified with 0.2 ml of glacial acetic acid (BDH) thus reprotonating the quinoline and ensuring that the right-hand ring broke during the subsequent oxidation, due to its being more electron dense. To the acidic distillate 200 mg of cupric acetate (BDH) were added and the solution heated to 60°C with constant stirring. Once at 60°C 2 ml of 30% (v/v) hydrogen peroxide (BDH) was added dropwise over 2 hours, thus oxidising the quinoline to the blue copper salt of quinolinic acid. The salt was collected on a Whatman glass fibre filter paper by filtration under reduced pressure and then washed with 5 ml of distilled water. The copper salt residue was resuspended in 10 ml of distilled water and heated to 60°C with constant stirring. To obtain free quinolinic acid hydrogen sulphide (BOC) was bubbled through the solution, at 60°C for 1 hour so as to form black copper sulphide, which precipitated out, and free quinolinic acid. The copper sulphide was removed by filtering through a 2 cm layer of cellite (BDH) contained between two layers of Whatman No.1 filter paper in a funnel. The quinolinic acid formed was contained in the filtrate which was freeze dried, after being frozen onto the inside of a 250 ml round bottom flask. The residue was resuspended in 1 ml of 50% (v/v) ethanol and the quinolinic acid crystallized by heating and adding ethanol drop wise until crystallization began. The solution was then cooled and left for 16 hours before the crystals of quinolinic acid were collected by filtration through a 45 µm cellulose acetate membrane filter (Rainin) under reduced pressure. The filter and crystals were then dried at 60°C for 4 hours and then the <sup>14</sup>C-quinolinic acid was stored at -20°C in an air tight container.

*The final yield of quinolinic acid was 38 mg.*

## **2.7.2 Determination Of The Purity And The Specific Activity Of The <sup>14</sup>C-Quinolinic Acid.**

Both the determination of the purity of the synthesised <sup>14</sup>C-quinolinic acid and the determination of its specific activity involved high performance liquid chromatography (HPLC) analysis of the synthesised <sup>14</sup>C-quinolinic acid.

### **2.7.2.1 Separation and quantification of quinolinic acid by high performance liquid chromatography.**

Quinolinic acid was analysed and quantified according to the HPLC method of Wagner & Wagner (1984). The preparation of extracts and mobile phases for HPLC and the HPLC system were as described in Section 2.6. The C<sub>18</sub> column was eluted isocratically, at ambient temperature, with a mobile phase of 200mM diammonium hydrogen phosphate and 10mM tetrabutylammonium hydrogen sulphate with the pH adjusted to pH5.1. The flow rate was 1ml min<sup>-1</sup> and detection at 254nm.

The quinolinic acid was quantified by measuring the height of the peak on the chromatogram, with a retention time corresponding to authentic quinolinic acid (Sigma), in conjunction with a calibration curve (Fig 2.7.2). The calibration curve was obtained by injecting known amounts of quinolinic acid onto the HPLC column and plotting the height of the resulting peak against the amount injected.

A known weight of <sup>14</sup>C-quinolinic acid was dissolved in a known volume of distilled water containing 1% (v/v) potassium hydroxide. The solution was then prepared for HPLC analysis as described in Section 2.6.2. 20µl of the solution was injected onto the HPLC column and the amount of quinolinic acid present in the solution determined as described above. The purity of the <sup>14</sup>C-quinolinic acid, Q<sub>p</sub> (%), was calculated as:



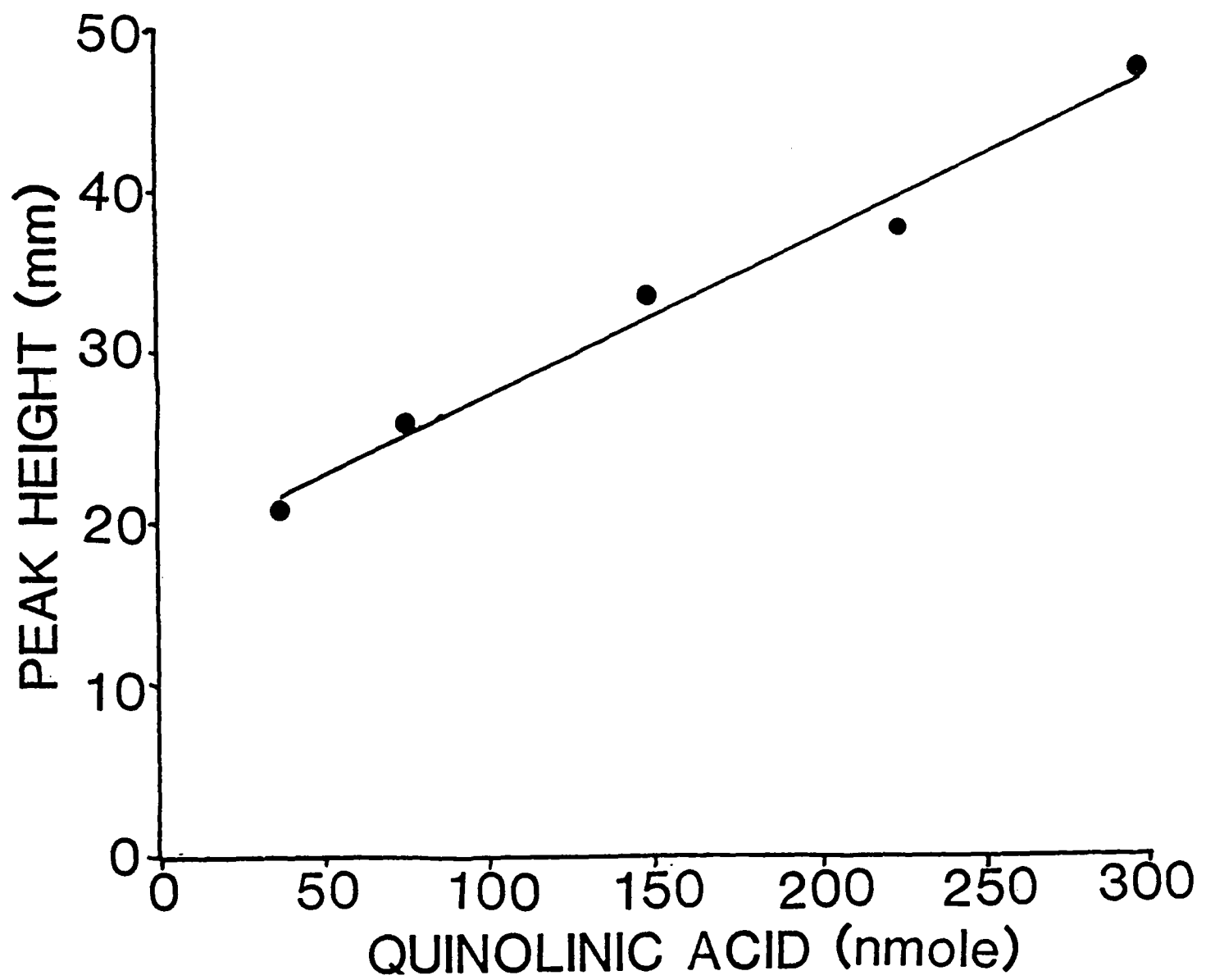


FIGURE 2.7.2

The HPLC calibration curve for quinolinic acid.

$$Q_p = \frac{Q_c}{Q_w} \cdot 100$$

where:  $Q_c$  = The weight (g) of quinolinic acid as determined by HPLC quantification.  
 $Q_w$  = The weight (g) of quinolinic acid added to the solution.

Using this method the purity of the  $^{14}\text{C}$ -quinolinic acid was determined as 30%. The impurities present were mainly insoluble, these accounted for 91% of the impurity. Since further purification of the  $^{14}\text{C}$ -quinolinic acid would have led to a decrease in the yield of  $^{14}\text{C}$ -quinolinic acid no further purification was carried out. The insoluble contaminant, which was found to be non-radioactive, was removed by filtration when the  $^{14}\text{C}$ -quinolinic acid was dissolved.

#### **2.7.2.2 Determination of the specific activity of the synthesised $^{14}\text{C}$ -quinolinic acid.**

The specific activity of the synthesised  $^{14}\text{C}$ -quinolinic acid was determined by collecting the HPLC elution fraction from a 20 $\mu\text{l}$  injection of a 10mg ml $^{-1}$  solution of the  $^{14}\text{C}$ -quinolinic acid. This was achieved using a LKB fraction collector as described in Section 2.6.4. The amount of quinolinic acid present in the solution was calculated and the activity of the  $^{14}\text{C}$ -quinolinic acid determined using the fraction collected. 0.1ml of the fraction was taken and placed in a scintillation vial along with 5ml of toluene/triton/PBD scintillation fluid, and liquid scintillation counted for 4min (Section 2.7.3). The specific activity was calculated using the HPLC quantification of the quinolinic acid, and the number of disintegrations per minute in the HPLC fraction containing the quinolinic acid. Since  $2.22 \times 10^6$  disintegrations per minute represent an activity of 1 $\mu\text{Ci}$  the activity of the quinolinic acid fraction,  $A_q$  ( $\mu\text{Ci}$ ), was given by:

$$A_q = \frac{A_f \cdot D_f}{2.22 \times 10^6}$$

where:  $A_f$  = The activity ( $\mu\text{Ci}$ ) in 0.1ml of the HPLC fraction.

$D_f$  = The dilution factor, given by:

$$D_f = \frac{V_f}{0.1}$$

where:  $V_f$  = The volume (ml) of the HPLC fraction.

The specific activity,  $S_q$  ( $\mu\text{Ci } \mu\text{mol}^{-1}$ ) was then calculated as:

$$S_q = \frac{A_q}{M_q}$$

where:  $M_q$  = The number of  $\mu\text{moles}$  of quinolinic acid giving the activity  $A_q$ , and given by:

$$M_q = \frac{Q_c}{M_r}$$

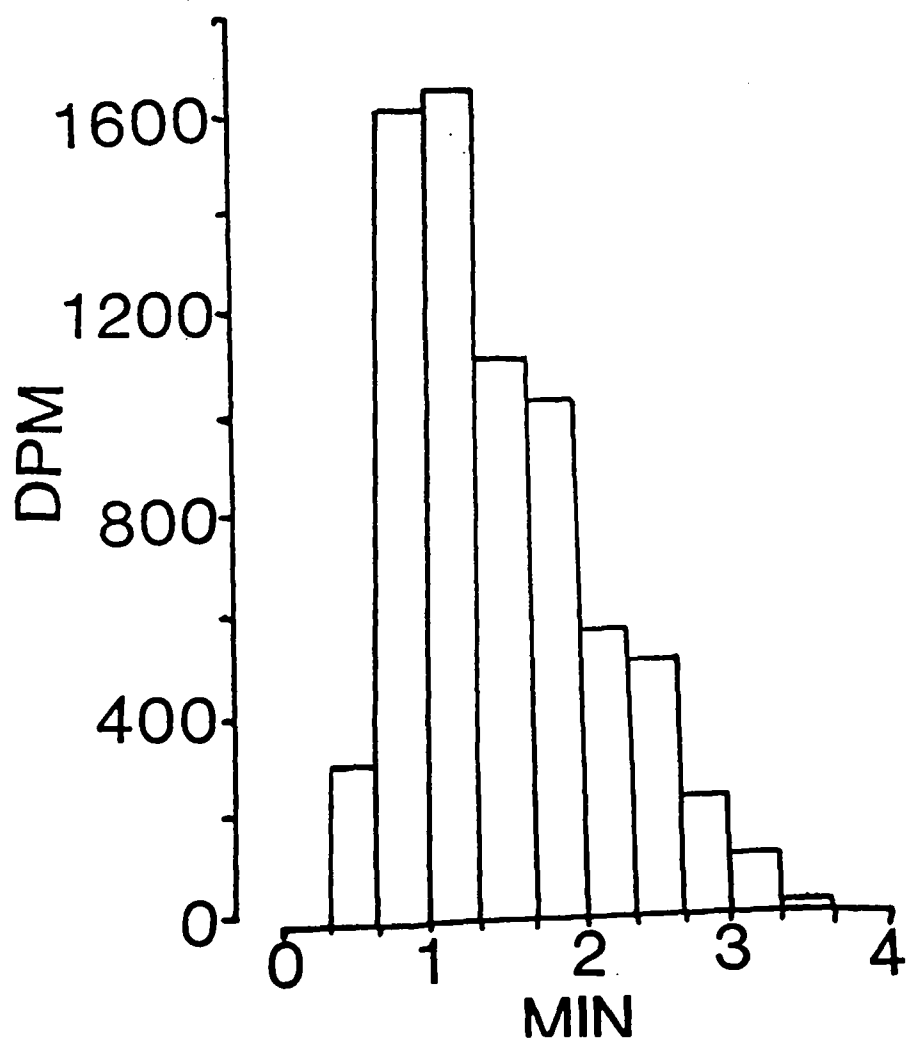
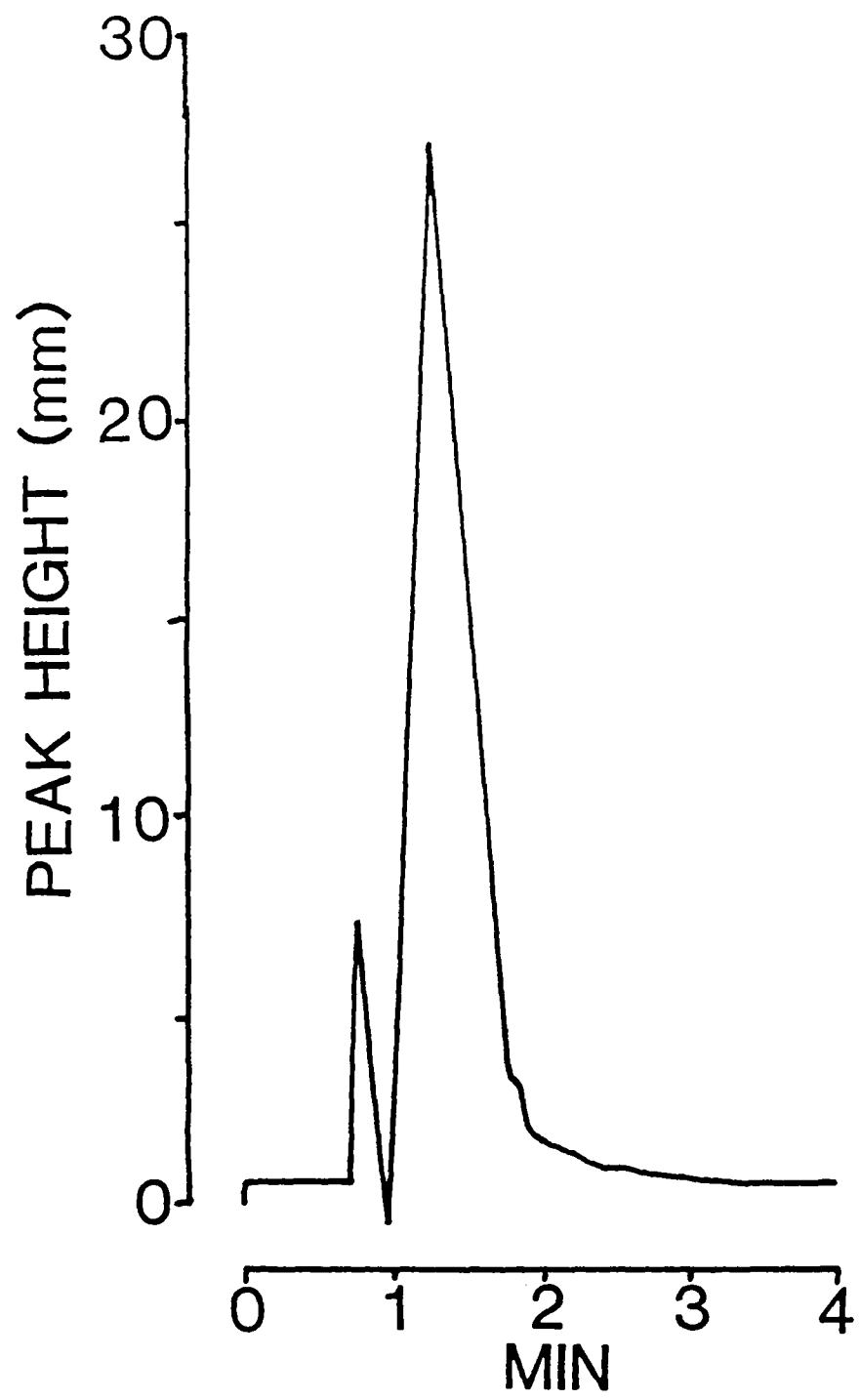
where:  $Q_c$  = The weight (g) of quinolinic acid as calculated from HPLC analysis (see Section 2.7.2.1).

$M_r$  = The molecular weight of quinolinic acid (167.1).

Using this technique the specific activity of the synthesised  $^{14}\text{C}$ -quinolinic acid was determined. A 1.2mM solution of  $^{14}\text{C}$ -quinolinic acid was made up and 20 $\mu\text{l}$  of this injected onto the HPLC column. Fractions of the eluate were collected every 20sec, as described in Section 2.6.4. The radioactivity in each of these fractions was then determined by liquid scintillation counting in triton PBD scintillant (see below). The HPLC chromatogram and the radioactivity in each fraction is shown in Fig 2.7.3. The radioactivity in a sample containing 4 $\mu\text{g}$  of  $^{14}\text{C}$ -quinolinic acid was also determined, since 4 $\mu\text{g}$  of  $^{14}\text{C}$ -quinolinic acid had been injected onto the HPLC column. The total amount of radioactivity in the HPLC fractions corresponding to quinolinic acid was 7,156dpm, while there were 7871dpm from a 4 $\mu\text{g}$  sample of  $^{14}\text{C}$ -quinolinic acid. Therefore 91% of the radioactivity injected onto the HPLC column was recovered. Using the above equations the specific activity of the synthesised  $^{14}\text{C}$ -quinolinic acid was calculated to be 0.13 $\mu\text{Ci } \mu\text{mole}^{-1}$ .

FIGURE 2.7.3

The HPLC chromatogram obtained with a 20 $\mu$ l injection of  $^{14}\text{C}$ -quinolinic acid, and the radioactivity in each of the 20sec fractions of the eluate collected. See text for details.



### 2.7.3 Liquid Scintillation Counting.

The radioactivity of the  $^{14}\text{C}$ -isotope present in quinolinic acid or subsequently in the enzymatic product was determined by liquid scintillation counting. Samples for scintillation counting were placed in scintillation vials together with 5ml of an appropriate scintillation fluid.

i. Toluene, PBD, Triton scintillation fluid.

This scintillation fluid was used for aqueous samples and contained triton to emulsify the water present. The fluid was composed of 500ml triton-X-100 (BDH) and 6.1g of butyl-PBD (BDH) per litre of toluene (BDH).

ii. PPO, POPOP scintillation fluid.

This scintillation fluid was used in counting solid samples. It was made up of 5g of PPO (BDH) and 0.3g of POPOP (BDH) per litre of toluene.

Samples were counted in an Intertechnique SC3000 scintillation counter for 4min ~~at an error of upto~~ 0.2%. The results obtained were as counts per minute (cpm) which were then converted to disintegrations per minute (dpm) by means of the calibration curve shown in Figure 2.7.4. The calibration curve was obtained by counting  $1\mu\text{Ci}$  of  $^{14}\text{C}$ -phenylalanine (Amersham) and then sequentially quenching with acetone. In this way it was possible to obtain different X-values which were plotted against the efficiency of counting (ie the percentage cpm after quenching of the unquenched cpm). Disintegrations per minute were then obtained from cpm by multiplying the cpm value by the efficiency determined from the X-value using the calibration curve.

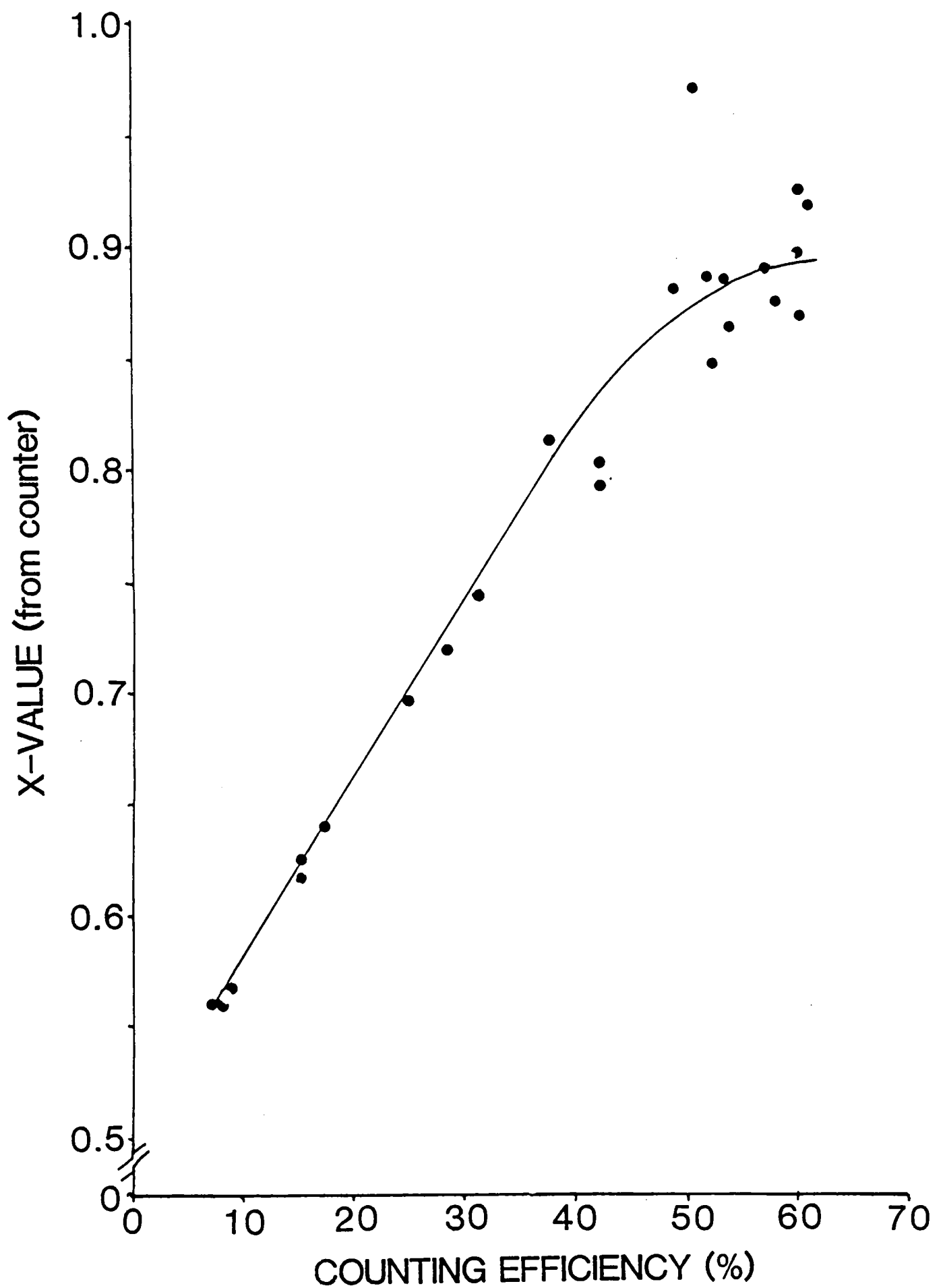


FIGURE 2.7.4

The radioactivity quenching curve used to convert cpm to dpm. The curve was prepared by sequentially quenching 1 $\mu$ Ci of  $^{14}\text{C}$ -phenylalanine with acetone. To convert cpm to dpm the counting efficiency (%) is obtained from the graph using the X-value. The counting efficiency is the percentage of the dpm's that are present in the radioactive sample.

## 2.7.4 The Extraction And Assay Of QaPRT'ase From Plants And Cultures Of *Nicotiana*

The extraction and assay of QaPRT'ase was based on the method of Wagner & Wagner (1984). However, the separation of the substrate (quinolinic acid) from the enzymatic product (nicotinic acid mononucleotide {NaMN}) was based on the methods of Mann & Byerrum (1974a) and Packman & Jakoby (1970).

### 2.7.4.1 The extraction of QaPRT'ase activity.

All of the steps in the extraction of the enzyme QaPRT'ase were carried out at 0-4°C. Plants were divided into roots and shoots, and after separation from their compost, roots were washed in distilled water and blotted dry. Cells were separated from their medium and in the case of suspension cultured cells lyophilized without freezing. Approximately 3g of tissue was suspended in 6ml of: 100mM 3-(N-morpholino) propane sulphonilic acid (MOPS-NaOH), 5mM magnesium chloride, 0.1mM ethylenediamine-tetra acetic acid (Na<sub>2</sub>-EDTA), 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 10mM DL-dithiothreitol (DTT) at pH7.4, all chemicals from Sigma, except magnesium chloride from BDH. The tissue was then ground in a pestle and mortar for 5min before being strained through a single layer of Miracloth. The filtrate was centrifuged for 20min at 27,000xg in 15ml glass Corex centrifuge tubes. After centrifugation 2.5ml of the supernatant was applied to a PD10 Sephadex column (Pharmacia) which was equilibrated with the extraction buffer. The column was eluted with the same buffer and 0.3ml fractions collected using an LKB fraction collector. The protein content of the fraction containing enzyme activity

(Fraction 14) was determined and the activity of the enzyme measured. When loading 2.5ml of supernatant onto the PD<sub>10</sub> column all proteins would elute immediately after the void volume and at least nine fractions should contain activity. With activity being detected in only two fractions it suggests that the method of cutting only 1cm strips around the R<sub>F</sub> value of the product, on paper chromatograms, was insufficient. (The R<sub>F</sub> values of compounds vary between samples and on each running of chromatograms.) This being the case the activity in other protein fractions may have been undetected.



#### 2.7.4.2 Determination of protein content.

The protein content of the enzyme extract was determined using the method of Bearden (1978). Dye reagent was made up by dissolving 100mg of coomasie Brilliant Blue (Sigma) in 50ml of ethanol and 100ml of orthophosphoric acid. This solution was stirred for 30min and then made up to 1l with distilled water. The solution was filtered through a Whatman No.1 filter paper and the filtrate stored, until use, in a dark bottle.

To 0.1ml of enzyme extract was added 3ml of dye reagent. This mixture was vortexed for 15sec and left to stand for 5min at room temperature. The optical density was then measured at 595nm against an extraction buffer and protein reagent blank, using a Pye Unicam SP8-100 spectrophotometer. The protein concentration was estimated using a calibration curve prepared using Bovin albumin serum (Sigma) (Fig 2.7.5).

#### 2.7.4.3 The assay for QaPRT'ase activity.

The <sup>enzymatic</sup> reaction was started by bringing 80 $\mu$ l of enzyme extract to a final volume of 300 $\mu$ l with assay buffer so that the final concentrations of the constituents were: 115mM potassium phosphate ( $K_2 HPO_4$  and  $KH_2PO_4$ ) at pH7, 0.2mM  $^{14}C$ -quinolinic acid (or unlabelled quinolinic acid), 0.4mM phosphoribosylpyrophosphate (Na-PRPP), 12.5mM magnesium chloride and 10mM DTT, all chemicals from Sigma except magnesium chloride (BDH) and  $^{14}C$ -quinolinic acid (synthesised personally). The reaction was allowed to proceed at 30°C for 5min before termination by boiling for 2min. The NaMN was then separated from other compounds in the reaction mixture, in particular from quinolinic acid.

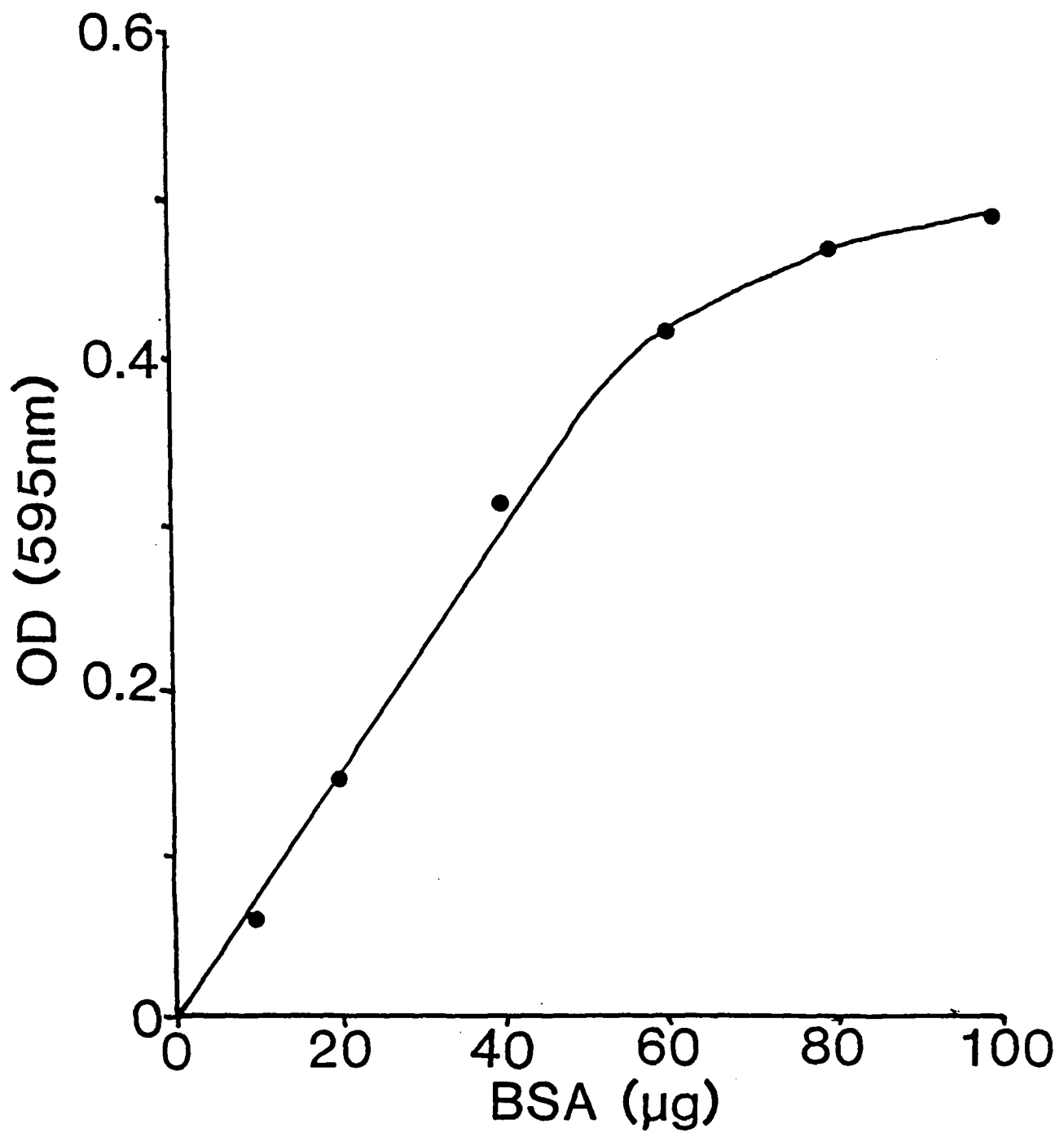


FIGURE 2.7.5

The calibration curve prepared with BSA for the estimation of the protein content of extracts.

#### 2.7.4.4 The separation of NaMN from quinolinic acid by descending paper chromatography.

The total reaction volume was loaded as a 2cm streak onto a sheet of Whatman 3MM chromatography paper (46cm X 57cm). The paper was *developed* with a mobile phase of butanol: acetic acid: water (4:1:2) (Mann & Byerrum 1974a) in a descending chromatography tank, equilibrated with the mobile phase, for 16hours. After this period the solvent front was marked and the paper dried. The NaMN was then detected by one of two methods.

i. The spectroscopic determination of the NaMN formed during the assay of QaPRT'ase.

The R<sub>f</sub> of NaMN was determined by *developing* a chromatogram onto which authentic NaMN had been loaded. After drying the chromatogram was cut into 1cm strips which were eluted with 1ml of distilled water. The strips and water were agitated for 16hours and then filtered using a Bioanalytical Systems Microfilter fitted with a 0.45µm Nylon-66 membrane filter (Rainin) which was centrifuged at 1,000xg for 10min. The filtrate was transferred to a 15ml plastic centrifuge tube (Corning) and 0.5ml of 7M sodium cyanide added (all subsequent operations were carried out in a fume cupboard). The solution was left to stand for 15min before the optical density of the cyanide adduct of NaMN was measured at 315nm (Packman & Jakoby 1970), against a water and cyanide blank. Using this method the R<sub>f</sub> of NaMN was found to be 0.12.

To quantify the amount of NaMN formed during the enzymatic reaction a 2cm strip was cut to include the R<sub>f</sub> of NaMN. The strip was eluted for 16hours and centrifuged as above. The filtrate was mixed with 0.5ml of 7M sodium cyanide and after 15min the optical density was measured at 315nm against the eluate of a strip from the chromatogram where a sample that had been boiled prior to the assay had been run. The amount of NaMN present was quantified using a calibration curve obtained using known amounts of authentic NaMN (Fig 2.7.6).

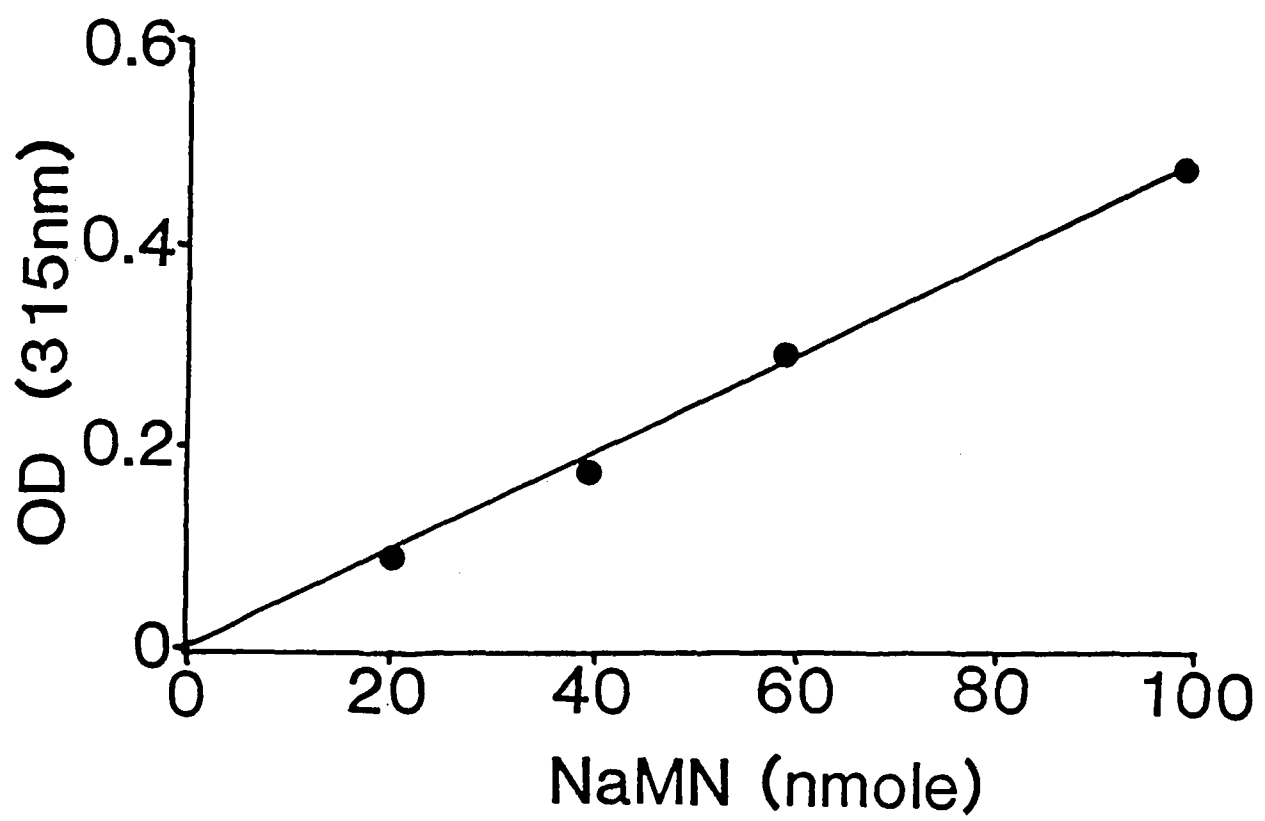


FIGURE 2.7.6

The calibration curve used for the quantification of NaMN.

ii. Determination of the activity incorporated into NaMN from  $^{14}\text{C}$ -quinolinic acid using liquid scintillation counting.

The Rf value of quinolinic acid was determined by cutting 1cm strips from a chromatogram on which a solution of  $^{14}\text{C}$ -quinolinic acid had been run. Each strip was liquid scintillation counted in 5ml of PPO/POPOP scintillant (Section 2.7.3). The Rf of quinolinic acid was found to be 0.38.

The amount of quinolinic acid and NaMN present after the enzymatic reaction was determined by cutting 1cm strips around the Rf values of each of the compounds. These strips were liquid scintillation counted in 5ml of PPO/POPOP scintillant (Section 2.7.3) and the results used to calculate the conversion of quinolinic acid to NaMN.

## **2.8 MATHEMATICAL ANALYSIS OF DATA.**

### **2.8.1 Calculation Of The Standard Error Of A Mean.**

In the majority of experiments three replicates per treatment were used and the mean of the values calculated. The standard error of the replicates was also calculated to determine the amount of variation within a treatment as well as giving an indication of differences between treatments. The standard error was calculated according to the method of Parker (1979).

### **2.8.2 Calculation Of Correlation Coefficients.**

In certain experiments the association between two variables was of interest. The association between two variables is commonly measured by the correlation between them. In this study the correlation between two variables was calculated using the statistical computing package "Minitab" (version 5.1.3 1985).

### **2.8.3 Calculation Of The Doubling Times.**

In order to calculate the doubling time of cells in culture the relative growth rate must firstly be determined. The relative growth rate in a given period of time was calculated according to Hunt (1981) and this value used in calculating the doubling time also using the method of Hunt (1981).

**CHAPTER 3**  
**RESULTS**

The aims of this project were firstly to determine whether light influenced alkaloid accumulation in culture and secondly to determine whether any effects of light were a direct result of photosynthetic activity or due to light regulation of an enzyme or enzymes.

In Part A of the results the development of apparatus and techniques, required for this investigation, are described. Apparatus was designed and fabricated which facilitated the detection of chlorophyll fluorescence from cultured cells (Section 3.1) thus enabling a detailed assessment of the photosynthetic competence of cells. This was an essential prerequisite to the selection processes and for the characterization of culture-lines. Having obtained a range of cultured material a method capable of extracting the four main tobacco alkaloids was required. The development of this extraction procedure is described in Section 3.2. Subsequently the apparatus and techniques were employed in pursuing the objectives of the project (Part B).

The alkaloid content of whole plants of *N.tabacum* is described in the first section of Part B (Section 3.3). Having defined the alkaloid content *in vivo*, material of *N.tabacum* was put into suspension culture and the effects of six basal media on alkaloid accumulation were investigated (Section 3.4). The growth and photosynthetic characteristics were also determined for several culture-lines in the chosen medium using different levels of carbon supplementation (Section 3.5). Once the suspension culture material had been characterized a series of experiments were carried out to determine whether light affected alkaloid accumulation in the suspended cells (Section 3.6). Following this the effect of light on the metabolism of nicotine added to the culture medium of suspended cells was investigated (Section 3.7). Having determined that light had an affect on alkaloid synthesis *in vitro* the activity of the enzyme quinolinic acid phosphoribosyl transferase was determined *in vivo* (Section 3.8) and *in vitro* <sup>h</sup>were the affect of light on it was determined (Section 3.9).



**PART A.**

**THE DEVELOPMENT OF TECHNIQUES REQUIRED FOR THE INVESTIGATION OF THE  
EFFECT OF LIGHT ON ALKALOID BIOSYNTHESIS.**

### **3.1 THE DEVELOPMENT OF A SYSTEM FOR THE DETECTION OF CHLOROPHYLL FLUORESCENCE FROM CULTURED CELLS.**

A system was required for screening cultured cells for their photosynthetic potential, using chlorophyll fluorescence, which would allow for the processing of several cultures using small samples of each. Such a system should also be capable of being used to estimate and detect the features of chlorophyll fluorescence.

#### **3.1.1 Fluorescence Profiles From Chloroplasts Isolated From Pea Seedling And Tobacco Leaves.**

Isolated pea chloroplasts have been used extensively in the development and interpretation of fluorescence systems and the signals detected from them. Since isolated pea chloroplasts provide defined material, with respect to chlorophyll fluorescence emission, they were used as a "standard" in the development of a fluorescence detector for cultured cells. Having determined the fluorescence profiles for pea chloroplasts the profiles for tobacco chloroplasts were also recorded to determine whether there were any species specific differences in the profiles. In both treatments a chloroplast suspension containing 100 $\mu$ g of chlorophyll was used for the fluorescence determinations at an oscilloscope sensitivity of 0.1V cm<sup>-1</sup>.

##### **3.1.1.1 Chlorophyll fluorescence profiles from isolated pea seedling chloroplasts.**

The fluorescence profiles from isolated pea chloroplasts were recorded at two time-bases as described in Section 2.3.3. When the fluorescence emission was resolved at a time-base of 10msec cm<sup>-1</sup> the level  $F_0$ , which denotes the "constant" fluorescence, could be determined. Also from this profile an estimation of the redox state of the primary electron acceptor could be made, by determining the area above the curve from  $F_0$  to the

maximum level of emission (these measurements were made using a Cambridge Instruments, Quantimet 970 Image Analyser). If the primary electron acceptor is fully oxidized it has maximum electron accepting ability and fluorescence rises gradually as the electron acceptor becomes more reduced, until an equilibrium is reached between reduction and reoxidation. However, if the primary electron acceptor is totally reduced no photochemical event is possible and fluorescence thus rises rapidly to a maximum.

When the fluorescence emission was resolved at a time-base of  $0.5\text{sec cm}^{-1}$  a complex transient was detected. This profile allowed an estimation to be made as to the functional state of the photosynthetic electron transport chain and associated photosynthetic events. The fluorescence level above  $F_0$ , termed "variable" fluorescence, rises rapidly in two phases to a peak. The first phase is a rapid rise in fluorescence emission which is then followed by a dip before rising again to reach a peak. From the peak the fluorescence yield declines, with slower kinetics, to a stationary level (Krause & Weis 1984), this distinctive fluorescence transient is termed the Kautsky effect (Butler 1977). For the screening of cells for photosynthetic competence it was not necessary to investigate the processes underlying the transients obtained (for reviews see Butler 1977, Govindjee *et al* 1967, Krause & Weis 1984, Papageorgiou 1975).

i. The high resolution fluorescence profile from isolated pea seedling chloroplasts.

When a pea seedling chloroplast suspension containing  $100\mu\text{g}$  of chlorophyll was illuminated after an incubation of 5min in the dark and the resulting fluorescence resolved at a time-base of  $10\text{msec cm}^{-1}$  the fluorescence profile shown in Fig 3.1.1 was obtained. On illumination there was an instantaneous rise in fluorescence to the value  $F_0$  which was followed by a more gradual increase in fluorescence until a maximum was reached. The gradual rise in fluorescence indicated that the primary electron acceptor was at least partially in an oxidised state and therefore capable of accepting electrons thus allowing a photochemical event and quenching fluorescence. The area above the curve from  $F_0$  to the maximum level of fluorescence was  $1100 \text{ mm}^2$ .

FIGURE 3.1.1

The fluorescence profile from a suspension of isolated pea chloroplasts containing 100 $\mu$ g of chlorophyll. The chloroplast suspension was incubated in the dark for 5min before illumination at an intensity of 300 $\mu$ E m<sup>-2</sup> sec<sup>-1</sup> by an LED light source. The fluorescence emission was resolved at a time-base of 10msec cm<sup>-1</sup> and a sensitivity of 0.1V cm<sup>-1</sup>.

F<sub>0</sub> = The "constant" fluorescence emission seen when photosynthetic tissues are illuminated.



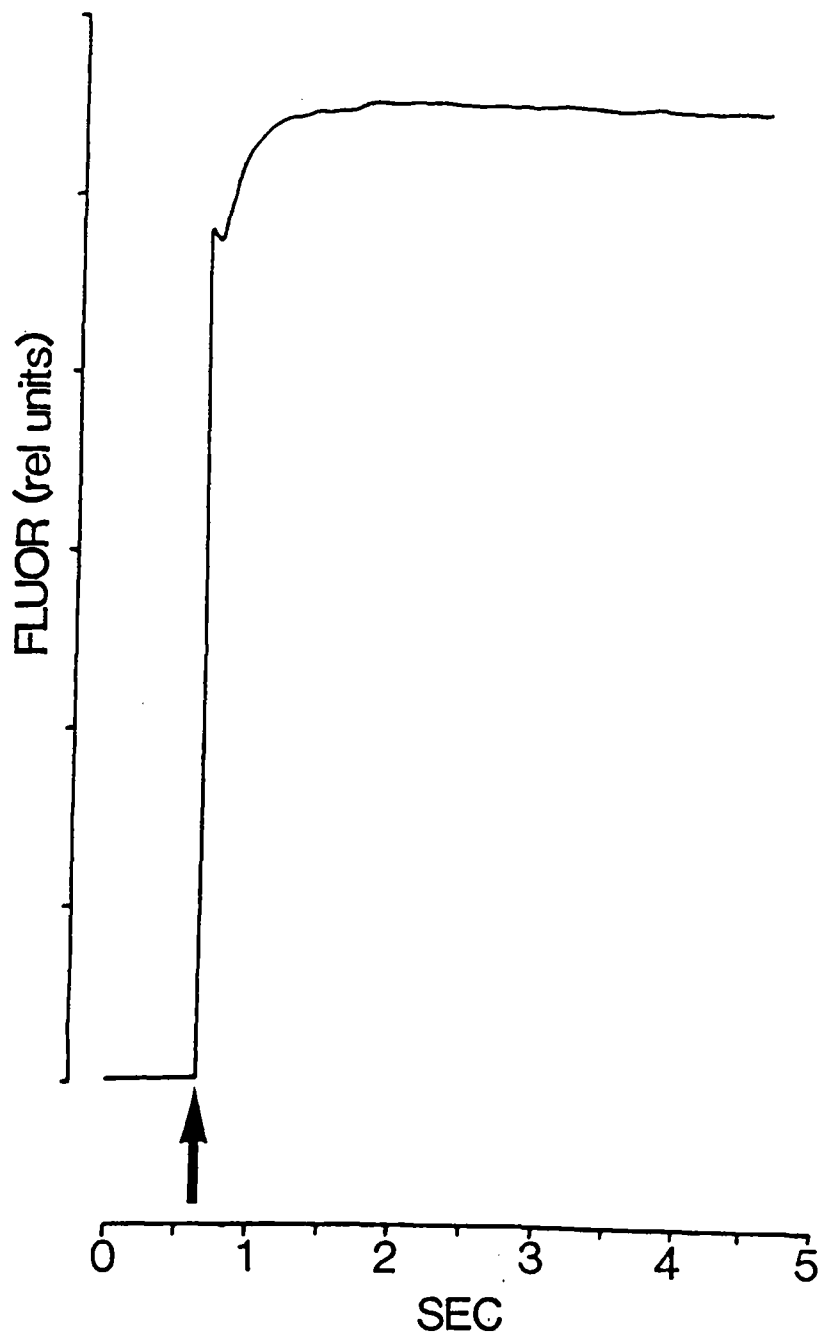
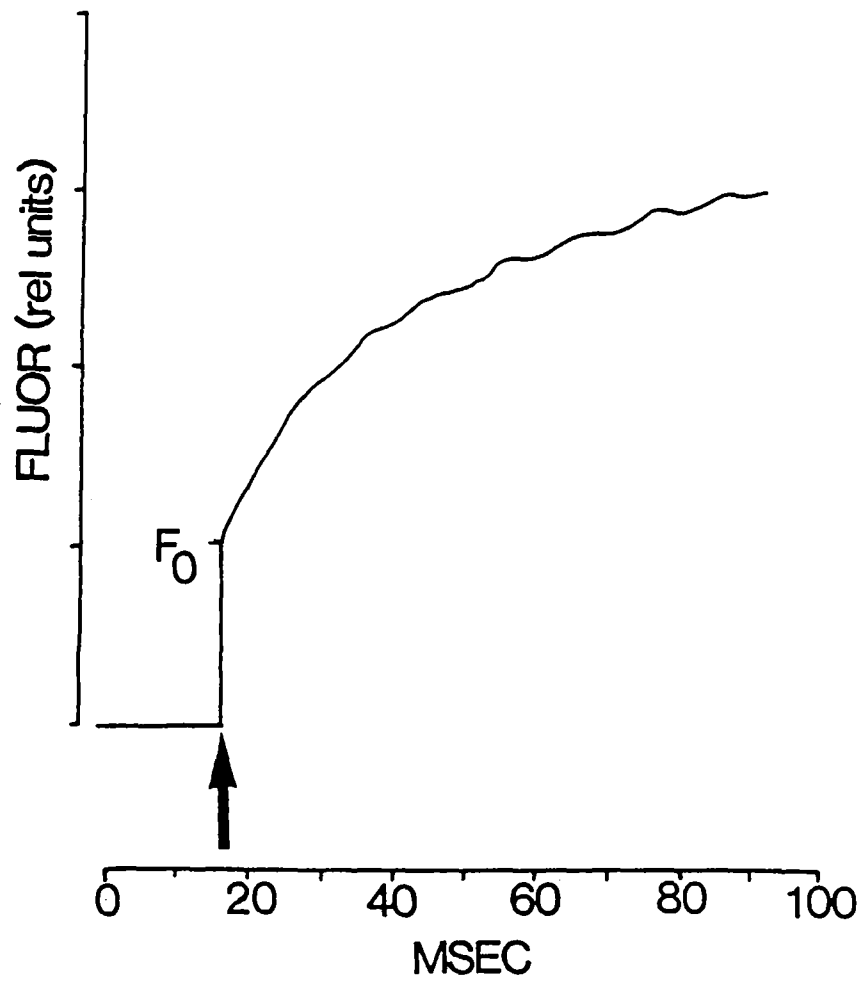
= The point of illumination.

FIGURE 3.1.2

The fluorescence profile obtained from isolated pea chloroplasts containing 100 $\mu$ g of chlorophyll. After a 5min incubation in the dark the chloroplasts were given a 2.5sec flash of light and then illuminated at an intensity of 300 $\mu$ E m<sup>-2</sup> sec<sup>-1</sup>. The fluorescence emission was resolved at a time-base of 0.5sec cm<sup>-1</sup> and a sensitivity of 0.1V cm<sup>-1</sup>.



= The point of illumination.



ii. The low resolution fluorescence profile from isolated pea seedling chloroplasts.

The fluorescence transient recorded after a pre-incubation of 5min in the dark followed by a 2.5sec flash of light before illumination at  $300\mu\text{E m}^{-2}\text{ sec}^{-1}$  and resolved at  $0.5\text{sec cm}^{-1}$  showed several distinct phases (Fig 3.1.2). On illumination there was a rapid rise to an initial maximum. The rate of increase decreased prior to the maximum and there was a decrease to a dip after the maximum. From the dip the fluorescence yield increased, with slower kinetics than the initial increase, to a new maximum. After reaching this maximum level of fluorescence there were slight fluctuations in emission with an overall downward trend.

#### **3.1.1.2 Fluorescence profiles from isolated tobacco leaf chloroplasts.**

Chloroplasts were isolated from the four most recently fully expanded leaves of twelve week old tobacco plants. The isolated chloroplasts were then treated in the same way as isolated chloroplasts from pea seedlings.

i. The high resolution fluorescence profile from isolated tobacco leaf chloroplasts.

The fluorescence profile obtained when a tobacco leaf chloroplast suspension containing  $100\mu\text{g}$  of chlorophyll was illuminated after an incubation of 5min in the dark is shown in Fig 3.1.3. On illumination fluorescence rose immediately to  $F_0$  and then increased gradually to a maximum. The area above the curve from  $F_0$  to the maximum was  $1439\text{ mm}^2$ .

FIGURE 3.1.3

The fluorescence profile from a chloroplast suspension, containing 100 $\mu$ g of chlorophyll, isolated from twelve week old tobacco leaves. The chloroplasts were incubated in the dark for 5min before illumination at an intensity of 300 $\mu$ E m<sup>-2</sup> sec<sup>-1</sup>. The resulting fluorescence was detected at a sensitivity of 0.1V cm<sup>-1</sup> and a time-base of 10msec cm<sup>-1</sup>.

F<sub>0</sub> = The "constant" fluorescence emission which occurs on illumination of photosynthetic tissue.



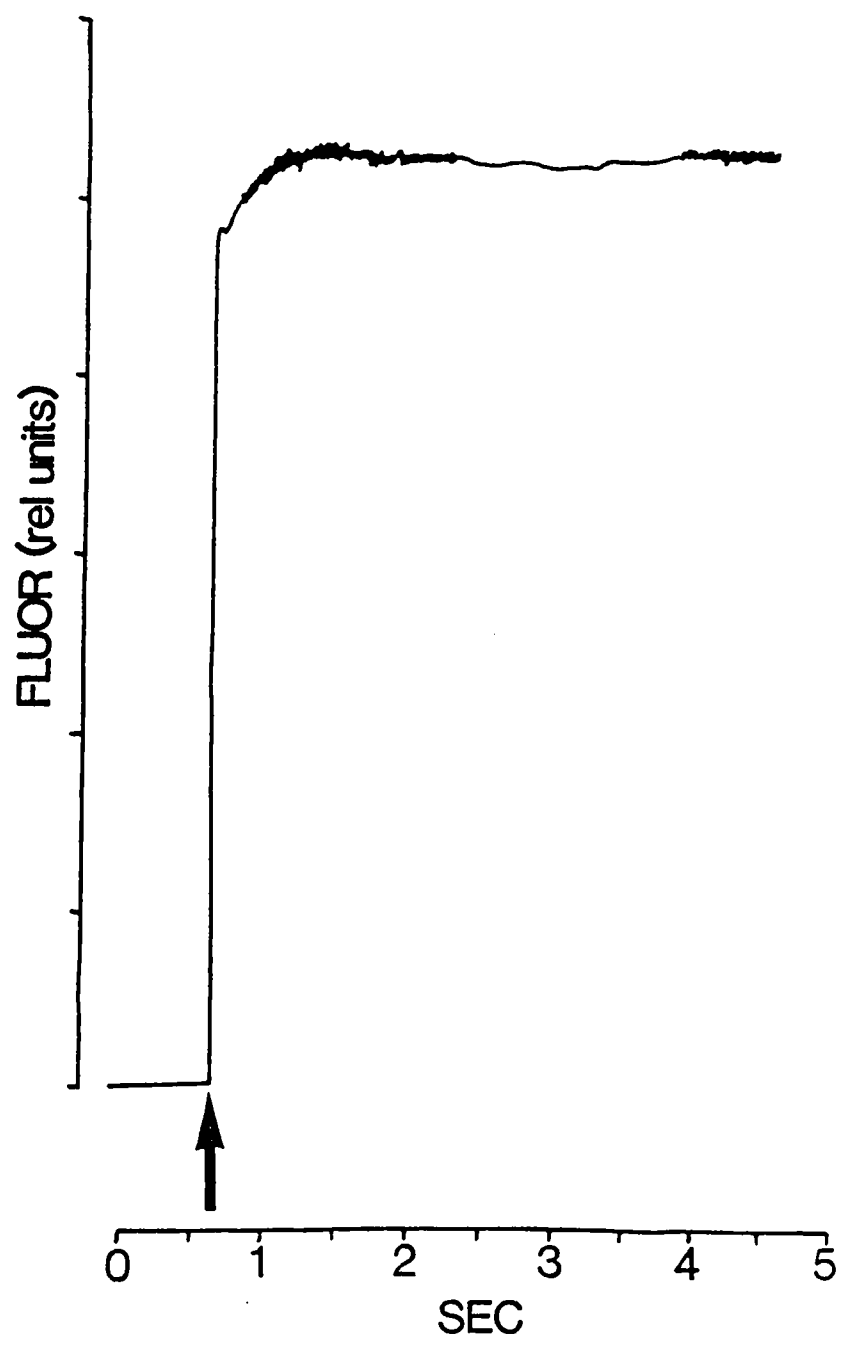
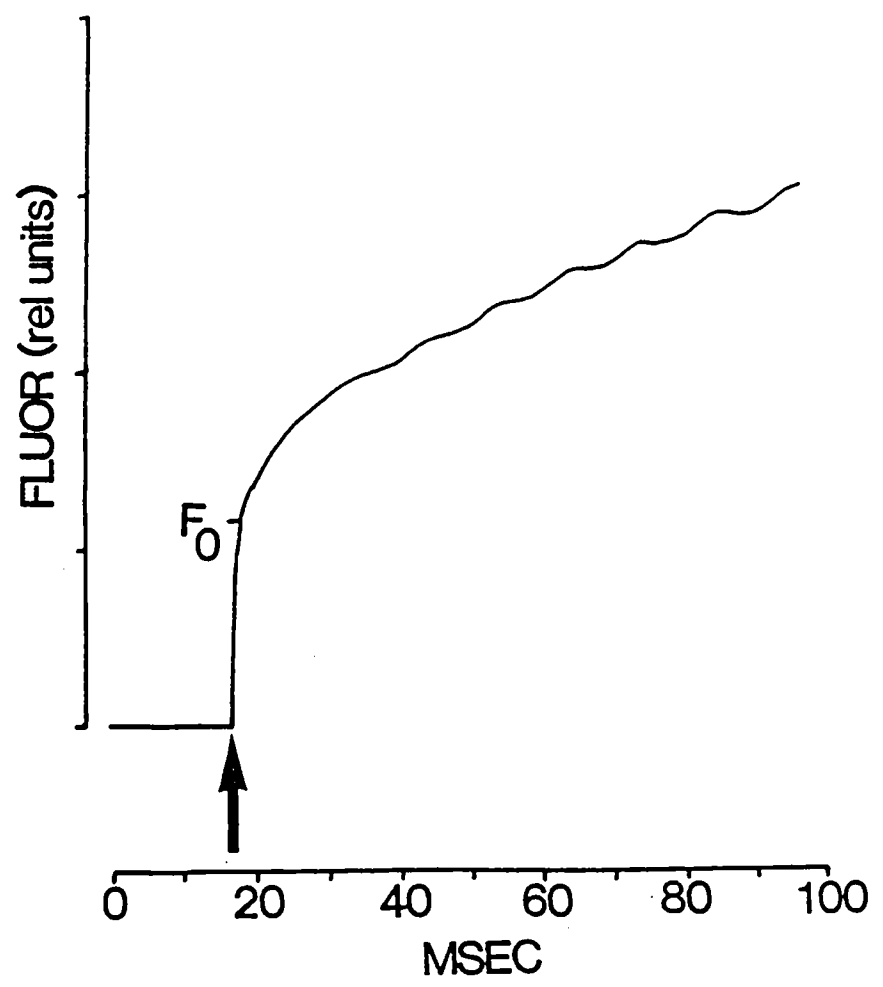
 = The point of illumination.

FIGURE 3.1.4

The fluorescence emission from chloroplasts isolated from 12 week old tobacco leaves, detected at a time-base of 0.5sec cm<sup>-1</sup>. The chloroplast suspension, containing 100 $\mu$ g of chlorophyll was incubated in the dark for 5min and then given a 2.5sec flash of light before the fluorescence profile was recorded at a sensitivity of 0.1V cm<sup>-1</sup>.

 = The point of illumination.





ii. The low resolution fluorescence profile from isolated tobacco leaf chloroplasts.

The fluorescence transient obtained at a time-base of  $0.5\text{sec cm}^{-1}$  with isolated tobacco chloroplasts, incubated for 5min in the dark followed by a 2.5sec flash of light, showed several distinct phases on illumination at  $300\mu\text{E m}^{-2}\text{ sec}^{-1}$  (Fig 3.1.4). There was an initial rise on illumination to a maximum after which the fluorescence yield decreased. Following the decrease there was a rise to a new maximum which was proceeded by a gradual decrease in fluorescence.

There were no gross differences between the fluorescence transients obtained from isolated pea seedling and tobacco leaf chloroplasts. When the fluorescence emission was resolved at  $10\text{msec cm}^{-1}$  it was found that the  $F_0$  value was lower in isolated tobacco chloroplasts and the value for the primary electron acceptor in its fully oxidised state was greater in isolated tobacco chloroplasts. At the slower time-base very similar fluorescence profiles were obtained for the chloroplasts from the two tissues. The Kautsky effect was more pronounced in the isolated pea chloroplasts but the kinetics were the same in the two treatments. Since the fluorescence transients obtained from isolated tobacco chloroplasts did not differ to any great extent from the "standard" transients obtained with isolated pea chloroplasts the profiles from isolated tobacco chloroplasts were used as the "standard" to which all future profiles were compared.

The extraction of chloroplasts required a large amount of tissue to obtain the necessary yields of chloroplasts therefore a system capable of detecting fluorescence from a small quantity of whole cells was developed. The development of this system was carried out using cells grown in suspension culture in MSS medium supplemented with 2% fructose and under constant illumination of  $80\text{--}90\mu\text{E m}^{-2}\text{ sec}^{-1}$ .

### 3.1.2 Modification Of The Detection System Used For Detecting Fluorescence From Isolated Chloroplasts.

Cell suspension cultures were sampled by aseptically removing 5, 10 or 15ml of media and cells. The cells were pelleted by centrifugation at 750xg for 5min and then resuspended in 3ml of MSS medium plus fructose. The cell suspension was placed in a 4ml fluorescence cuvette (Hughes and Hughes) and treated in the same manner as isolated chloroplasts (Section 2.4.3). On illumination of the cells there was no detectable fluorescence signal, even when the sensitivity of the oscilloscope was increased. It was found that the cells were settling out in the cuvette because the magnetic stirrer was not efficient enough to keep them suspended. In an attempt to keep the cells in suspension, cell aggregates were removed by sieving the sample through 400 $\mu$ m nylon mesh (Henry Simon Ltd.) prior to centrifugation. After resuspension, as previously, it was possible on illumination of the cells to detect a very weak fluorescence signal. However, the sensitivity of the system had been increased to such a level (50mV cm<sup>-1</sup>) that the fluorescence signal intermittently became masked by external electrical noise. So as to increase the level of fluorescence and thus allow for a decrease in the sensitivity the quantity of cells in the cuvette was increased but this had little if any effect.

For fluorescence measurements with isolated chloroplasts approximately 100 $\mu$ g of chlorophyll per cuvette was used. The chlorophyll content of the cultured cells was determined (Section 2.3.2.1) so as to establish the weight of cells needed per cuvette to give a chlorophyll content of 100 $\mu$ g. The chlorophyll concentration of the cells was between 10–20 $\mu$ g g<sup>-1</sup> fresh weight, therefore for a chlorophyll content in the cuvette of 100 $\mu$ g it was necessary to use between 5–10g fresh weight of cells. In order to obtain a fine suspension in the cuvette the sample was digested for 1hour prior to analysis in 10ml of 2% (w/v) macerozyme (Yakult Honsha Co. Ltd., Tokyo, Japan) before being centrifuged down at 750xg for 5min and resuspended in 3ml of MSS medium. However, the volume of cells was greater than 3ml, therefore a system that either did not require such a large amount of cells or did not rely on the cells being suspended in a cuvette was required.

### **3.1.3 Development Of A Chlorophyll Fluorescence Detection System In Which The Cells Were Stationary.**

Initially the LED light source and the photodiode, along with their control systems, were removed from their holder and secured in clamps on retort stands. Cells were removed from their bathing medium by filtration under reduced pressure and blotted dry with Whatman No. 3 filter paper. 2–5g fresh weight of cells were placed on a non-reflective matt black card, in a dark room, and the LED light source and the photodiode mounted above them. Using this set up it was possible to obtain a satisfactory fluorescence signal on illumination of the cells. Having detected a fluorescence signal the distances and angles for optimal focusing of the light source and the photodiode were determined and a holder designed around the specifications. The holder (Fig 2.3.1) was constructed from grey PVC to limit the amount of reflection, since high levels activated the photodiode due to "leakage" through the 740nm cut off interference filter.

It was not possible to use isolated chloroplasts to test this system, since there was no means of suspending them, therefore a tobacco leaf disc was used secured across the top of the sample well.

#### **3.1.3.1 Chlorophyll fluorescence profiles from a tobacco leaf disc.**

The fluorescence profiles at time-bases of 10msec  $\text{cm}^{-1}$  and 0.5sec  $\text{cm}^{-1}$  were determined for a disc of the most recently expanded leaf of a 12 week old tobacco plant. The leaf disc was secured across the top of the sample well in the fluorescence detector holder and treated in the same way as isolated chloroplasts (Section 2.4.3).

i. The high resolution fluorescence profile from a tobacco leaf disc.

On illumination at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$  and detection at an oscilloscope sensitivity of  $0.5\text{V cm}^{-1}$  the fluorescence profile seen in Fig 3.1.5 was obtained from a tobacco leaf disc, which had been incubated for 5min in the dark. There was an instantaneous rise in fluorescence to  $F_0$  and from  $F_0$  there was a gradual increase in fluorescence to a maximum and thereafter oscillations in the fluorescence yield with an overall decrease in fluorescence. This transient did not directly correspond to the transient obtained at  $10\text{msec cm}^{-1}$  with isolated tobacco chloroplasts. A possible reason for this was that the light intensity used to illuminate the leaf disc was too high and thus fluctuations in the photochemical use of captured photons and their re-emission as fluorescence were seen. To determine whether this was the case and if so what the optimal illuminating intensity for leaf discs was the fluorescence profiles from a tobacco leaf disc were recorded at a series of intensities. Light intensities between 20 and  $125\mu\text{E m}^{-2} \text{sec}^{-1}$ , as determined using a LI-185 Quantum/Radiometer/Photometer (Lambda Instruments) photometer, were used to illuminate the leaf disc, since detection was possible across this range at a sensitivity of  $0.2\text{V cm}^{-1}$ . The profiles obtained are shown in Fig 3.1.6 and from these it can be seen that light intensity did affect the fluorescence profiles as well as the yield and that the optimal intensity was  $75\mu\text{E m}^{-2} \text{sec}^{-1}$ , since it gave a good signal with a similar profile to that seen from isolated tobacco chloroplasts.

Using an intensity of  $75\mu\text{E m}^{-2}\text{sec}^{-1}$  to illuminate a leaf disc incubated in the dark for 5min an instantaneous increase to  $F_0$  was seen. From  $F_0$  there was a gradual rise to a maximum with the area above the curve from  $F_0$  to the maximum being  $451 \text{ mm}^2$ .

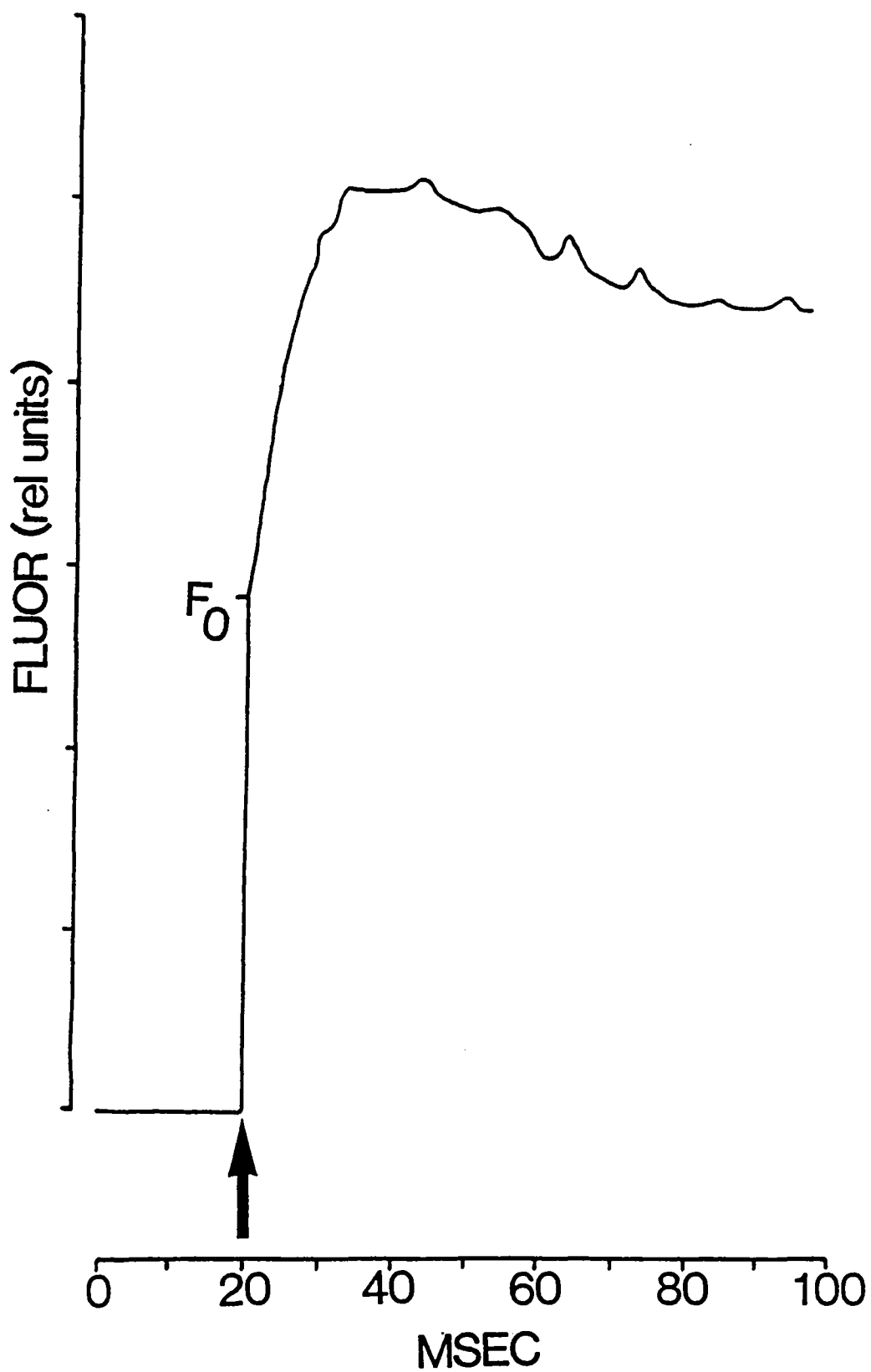


FIGURE 3.1.5

The fluorescence profile obtained from a twelve week old tobacco leaf disc. After a 5min incubation in the dark the disc was illuminated at an intensity of  $300\mu\text{E m}^{-2} \text{sec}^{-1}$  and the resulting fluorescence detected at a sensitivity of  $0.5\text{sec cm}^{-1}$  and a time-base of  $10\text{msec cm}^{-1}$ .

$F_0$  = The "constant" fluorescence which occurs on illumination of photosynthetic tissues.

↑ = The point of illumination.

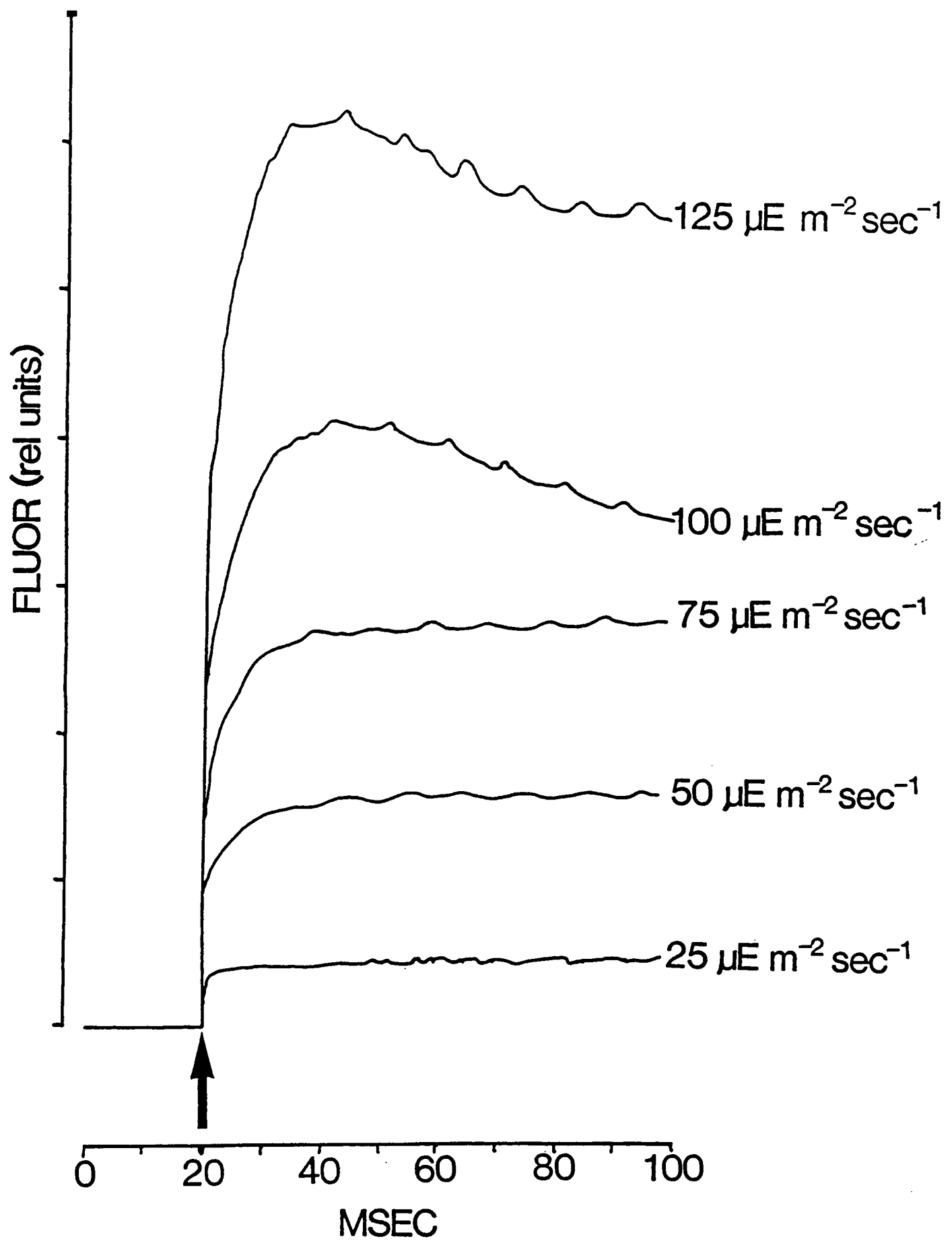


FIGURE 3.1.6

The effect of the intensity of the illuminating light on the fluorescence emission of 12 week old tobacco leaf discs. Before illumination at each intensity the disc was preincubated in the dark for 5min. The fluorescence emission was resolved at a time-base of 10msec  $\text{cm}^{-1}$  and a sensitivity of 0.2V  $\text{cm}^{-1}$ .

↑ = The point of illumination.

ii. The low resolution fluorescence profile from a tobacco leaf disc.

The fluorescence profile produced on illumination of a tobacco leaf disc at a time base of  $0.5\text{sec cm}^{-1}$  at  $300\mu\text{E m}^{-2}\text{ sec}^{-1}$  and a sensitivity of  $0.5\text{V cm}^{-1}$  (Fig 3.1.7) was different from the profile obtained with isolated tobacco leaf chloroplasts at this time-base. To determine whether the intensity of illumination was responsible for the differences fluorescence profiles were recorded at a range of illuminating intensities, as above, and at a sensitivity of  $0.2\text{V cm}^{-1}$ . From the profiles obtained (Fig 3.1.8) it can be seen that the intensity of illumination did have an effect although it did not account for all of the differences. Due to the fact that an intensity of  $75\mu\text{E m}^{-2}\text{ sec}^{-1}$  was optimal for fluorescence detection at a time-base of  $10\text{msec cm}^{-1}$  this intensity was also used for the lower resolution profiles.

On illumination of the leaf disc at  $75\mu\text{E m}^{-2}\text{ sec}^{-1}$  after 5min incubation in the dark followed by a 2.5sec flash of light there was a rapid rise in fluorescence to a maximum (Fig 3.1.9). After the maximum there was a decline in fluorescence yield before it increased again, more gradually, to a second maximum. Having reached the second maximum the fluorescence emission gradually decreased and reached a steady level. The kinetics and the profile of fluorescence emission over the first 0.5sec was directly comparable to the kinetics and profile obtained from isolated tobacco chloroplasts. The increased second maximum and the subsequent decrease seen in profiles obtained from tobacco leaf discs and not in isolated chloroplasts may be due to the fact that the chloroplasts *in situ* represent a fully functional system in contact with all other cellular processes. On the otherhand fluorescence profiles from isolated chloroplasts may give a slightly distorted view of photosynthetic events since the integrity of the whole leaf is lost.

Although there were differences between the fluorescence profiles obtained from isolated tobacco chloroplasts and tobacco leaf discs the main features of chlorophyll fluorescence profiles from functional photosynthetic tissue were detectable. The fluorescence detection system designed was therefore suitable for use in detecting chlorophyll fluorescence from cultured cells of *N.tabacum*.

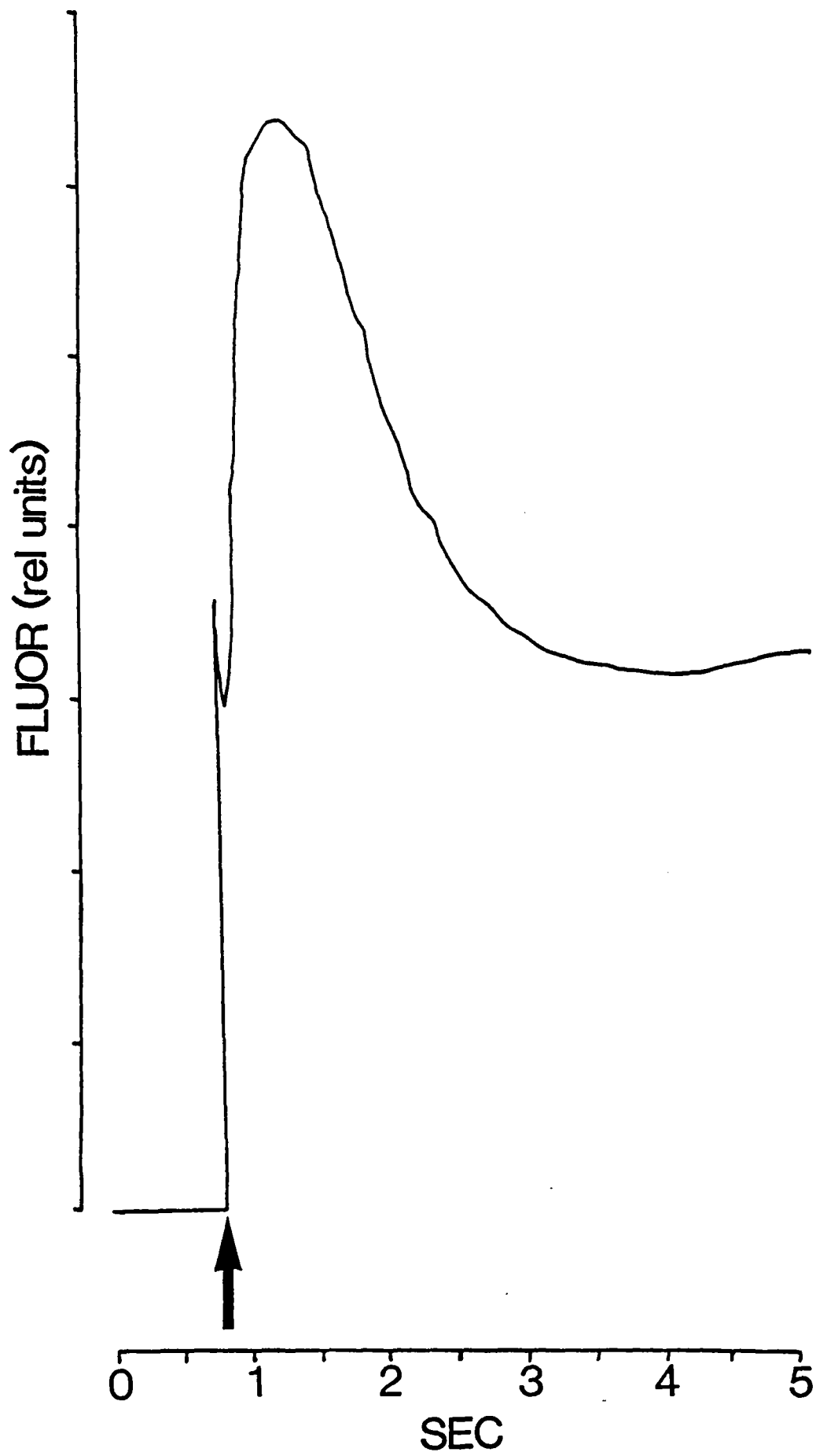


FIGURE 3.1.7

The fluorescence transient recorded when the fluorescence emission from an illuminated twelve week old tobacco leaf disc was resolved at a time-base of  $0.5\text{sec cm}^{-1}$ . After a 5min incubation in the dark the disc was flashed with light for 2.5sec and then illuminated at  $300\mu\text{E m}^{-2}\text{sec}^{-1}$  and the signal detected at a sensitivity of  $0.5\text{V cm}^{-1}$ .

↑ = The point of illumination.



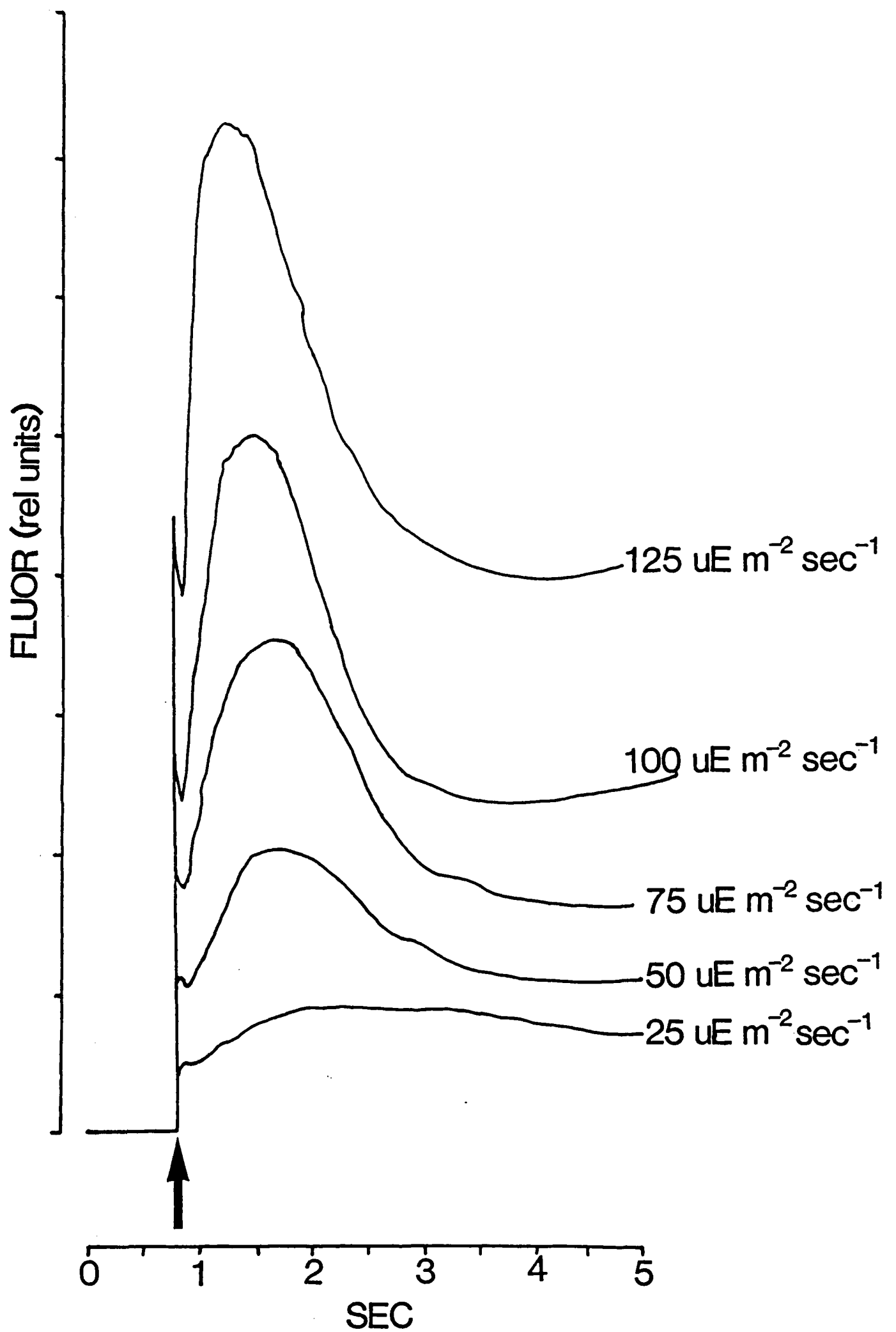


FIGURE 3.1.8

The effect of illuminating intensity on the fluorescence profiles of twelve week old tobacco leaf discs resolved at a time-base of  $0.5\text{sec cm}^{-1}$ . The leaf disc was illuminated at each intensity after a 5min dark incubation followed by a 2.5sec flash of light. Detection of the fluorescence emission was at a sensitivity of  $0.2\text{V cm}^{-1}$ .

↑ = The point of illumination.

FIGURE 3.1.9

The fluorescence profile of a twelve week old tobacco leaf disc resolved at a time-base of  $0.5\text{sec cm}^{-1}$ . After a 5min dark incubation the disc was flashed with light for 2.5sec before being illuminated at  $75\mu\text{E m}^{-2}\text{ sec}^{-1}$ . The fluorescence emission was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .



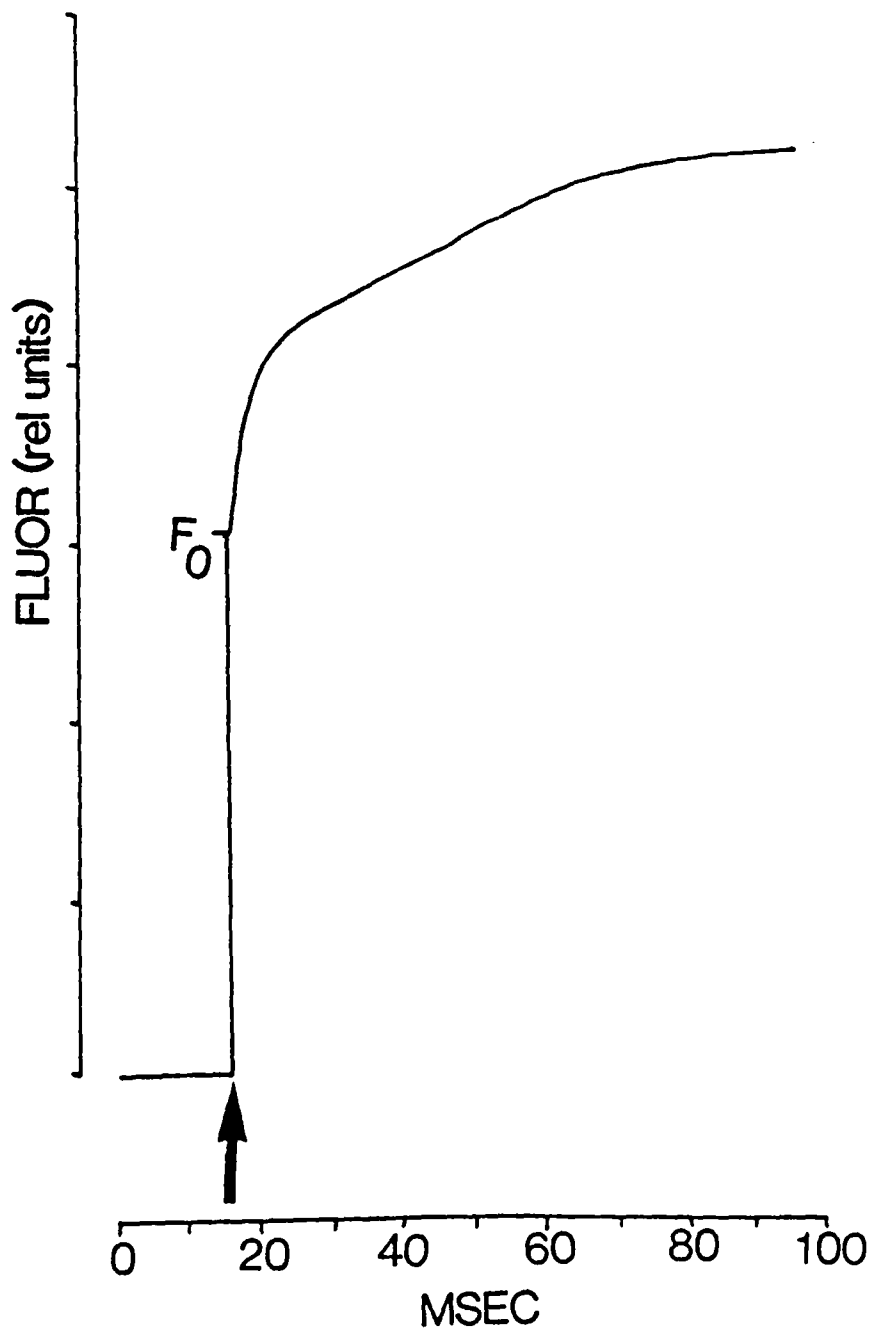
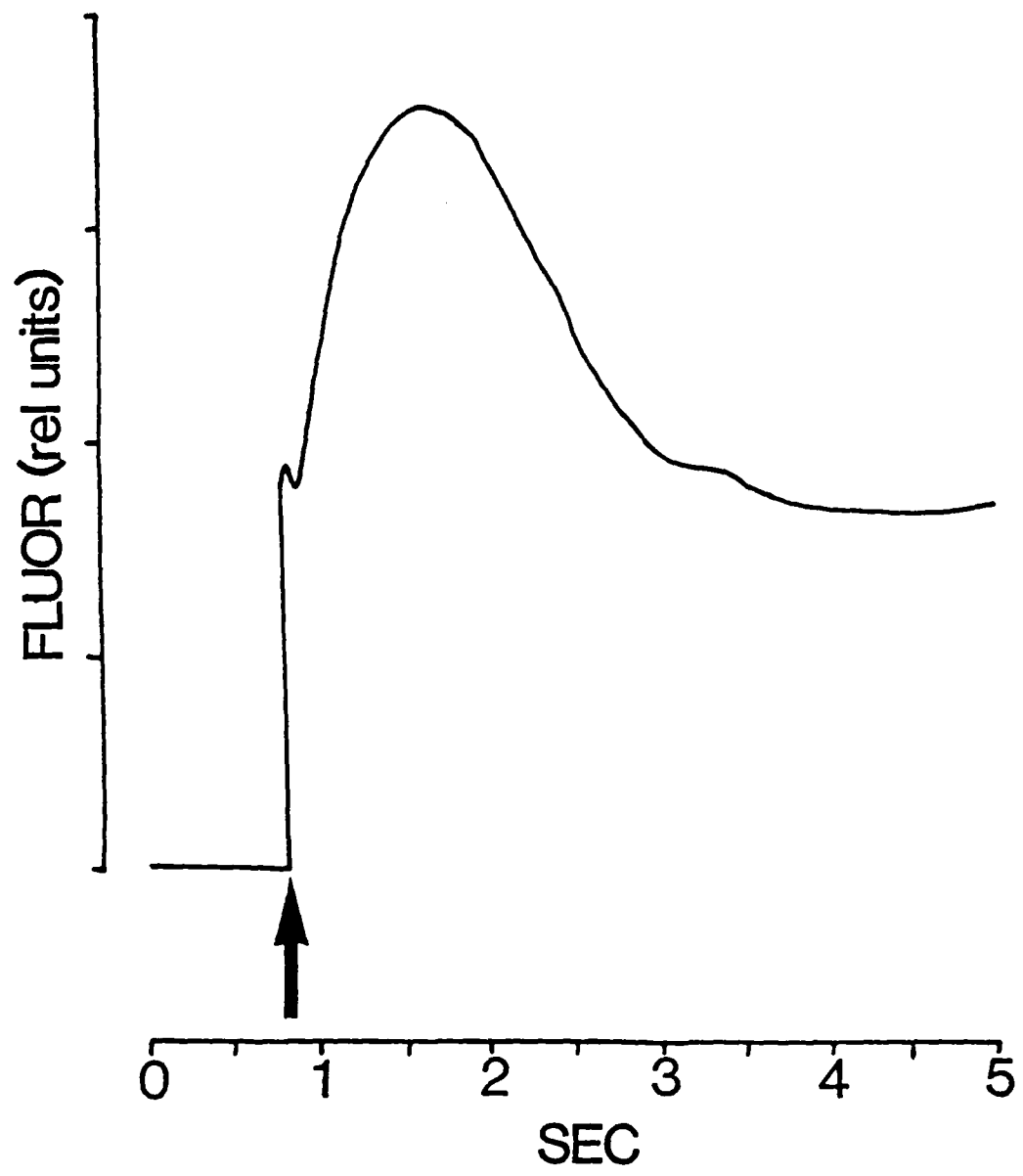
 = The point of illumination.

FIGURE 3.1.10

The fluorescence profile obtained from 2g of cells that had been grown in the light in MSS medium containing 1% fructose for 25 days. After a 5min incubation in the dark the cells were illuminated at an intensity of  $300\mu\text{E m}^{-2}\text{ sec}^{-1}$ . The fluorescence signal was resolved at a time-base of  $10\text{msec cm}^{-1}$  and a sensitivity of  $0.1\text{V cm}^{-1}$ .

$F_0$  = The "constant" fluorescence which occurs when photosynthetic tissue is illuminated.

 = The point of illumination.



### 3.1.3.2 Determination of the fluorescence signals obtained from chlorophyllous and non-chlorophyllous suspension cultured cells.

Although the optimal illuminating intensity for tobacco leaf discs was  $75\mu\text{E m}^{-2} \text{sec}^{-1}$  this was not used for cultured cells since the fluorescence signal generated was greatly reduced. So as to obtain a signal that was detectable at an oscilloscope sensitivity of  $0.2\text{V cm}^{-1}$ , which gave a clear profile with little if any electrical noise being detected, an illuminating intensity of  $300\mu\text{E m}^{-2} \text{sec}^{-1}$  was used.

The fluorescence profiles obtained with chlorophyllous cells did not have all of the characteristics of a fully functional photosynthetic electron transport chain (Fig 3.1.10 and 3.1.11). The profile detected at a time-base of  $10\text{msec cm}^{-1}$  rose instantaneously to  $F_0$  on illumination and then rose to a maximum (Fig 3.1.10). The area above the curve from  $F_0$  to the maximum being  $898 \text{ mm}^2$ . The profile obtained at the slower time-base rose rapidly on illumination to a maximum and then continued rising more gradually to a second maximum (Fig 3.1.11). After reaching the second maximum there was very little change in the fluorescence yield.

In order to determine the contribution of reflection of light by the cells to the signal detected a sample of non-chlorophyllous cells was treated in the same manner as chlorophyllous cells. A signal was detectable that accounted for no greater than 10% of the signal obtained with chlorophyllous cells, and had none of the characteristics of a chlorophyll a fluorescence profile (Fig 3.1.12).

The low non-fluorescence signal in conjunction with the fluorescence signals obtained from leaf discs and chlorophyllous cells showed that the system designed was suitable for the detection of chlorophyll fluorescence signals from whole cells. The sample well of the detector allowed for a sample in the region of 2g fresh weight to be used for the determinations.

FIGURE 3.1.11

The fluorescence transient recorded from tobacco cells cultured for 25 days in MSS medium containing 1% fructose and maintained in the light. After 5min in the dark the cells were flashed with light for 2.5sec and then illuminated at an intensity of  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . The fluorescence emission was detected at a sensitivity of  $0.1\text{V cm}^{-1}$  and a time-base of  $0.5\text{sec cm}^{-1}$ .



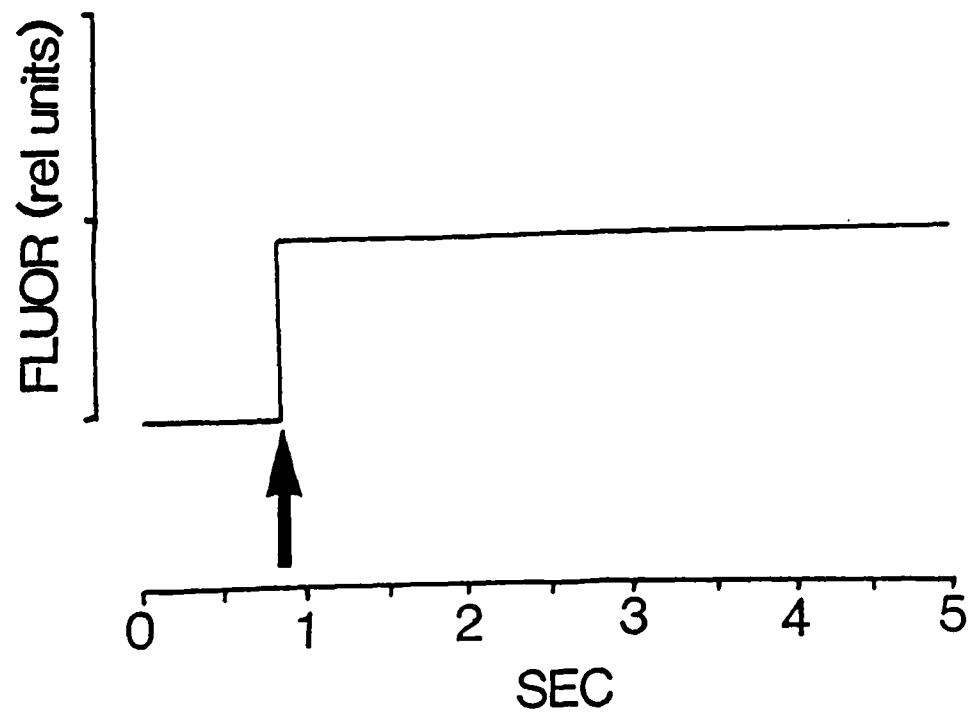
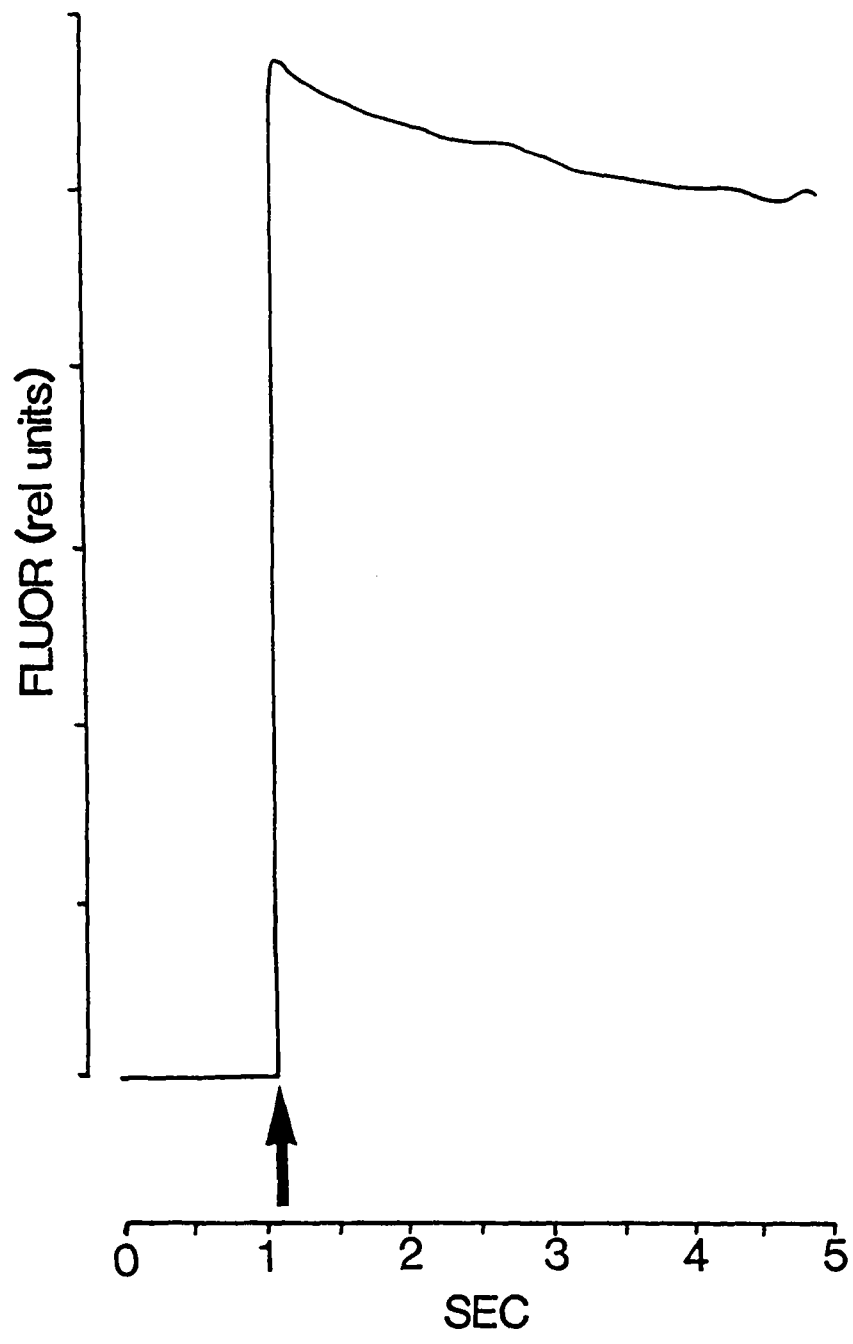
 = The point of illumination.

FIGURE 3.1.12

The signal produced on illumination of non-chlorophyllous cells. Cells grown in the dark for 25 days in MSS medium supplemented with 3% fructose were incubated in the dark for 5min before illumination at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . The resulting signal was resolved at a time-base of  $0.5\text{sec cm}^{-1}$  and a sensitivity of  $0.1\text{V cm}^{-1}$ .

 = The point of illumination.



Having designed a suitable system and established that it was suitable for the detection of chlorophyll fluorescence from cultured cells it was used in determining the fluorescence profiles of cells. The profiles obtained were then compared with those from photosynthetically active tissue thus enabling an estimation of the photosynthetic capacity of the cells. From the results obtained it was possible to decide on the most suitable cultures for transfer to media either promoting photosynthesis or to media inhibiting photosynthetic development (the data recorded at various sampling times are presented in Appendix 2). Since it was possible to aseptically remove a sample from a culture for analysis the remaining tissue could be used, after determination of the photosynthetic capacity, for inoculation of the desired medium.

## **3.2 DEVELOPMENT OF A PROCEDURE FOR THE EXTRACTION OF THE TOBACCO ALKALOIDS FROM CELL CULTURES OF *Nicotiana***

The development of a procedure for the extraction of the tobacco alkaloids from cultured cells of *N.tabacum* involved testing existing methods and modifying the most suitable of these. The procedure established by this process is described in Section 2.5.1.

### **3.2.1 The Selection And Modification Of A Procedure For The Extraction Of The Tobacco Alkaloids From Cultured Cells And Their Medium.**

An extraction procedure was required capable of extracting the four major pyridine alkaloids of tobacco (ie nicotine, nornicotine anabasine and anatabine) and yielding a final extract suitable for analysis by HPLC and TLC. Numerous procedures have been described in the literature for the extraction of the tobacco alkaloids, especially nicotine, from plants and/or from plant cell cultures (Burns & Collin 1977, Hamill *et al* 1986, Lockwood & Essa 1984, Piade & Hoffmann 1980, Saunders & Blume 1981, Severson *et al* 1981). From these procedures three were selected for their apparent ability to extract all four alkaloids simultaneously. Modified forms of these procedures were tested for their ability to extract known amounts of each of the alkaloids and to extract endogenous alkaloids from leaf material of *N.tabacum*. Two of the methods were rejected and the remaining one modified before the recovery of known amounts of exogenous alkaloids from cultured cells was determined.



### 3.2.1.1 A preliminary trial of methods for the extraction of tobacco alkaloids.

Modified versions of three procedures which had been previously used for the extraction of tobacco alkaloids from plant tissue and/or plant cell cultures were tested in this experiment. Solutions containing 500µg of each of the alkaloids or approximately 5g of leaf material from four month old *N.tabacum* plants were subjected to each of the selected procedures (i.-iii.) and the resulting extracts analysed by HPLC and TLC.

#### i. Sodium phosphate extraction (Saunders & Blume 1981).

The sample was mixed with 50ml of 25mM sodium phosphate (sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate, BDH) at pH7.8 and left in the dark for 16hours with constant agitation. After this period the mixture was filtered under reduced pressure through a Whatman No. 1 filter paper, and the resultant filtrate used as the final extract.

#### ii. Acidified methanol extraction (Saunders & Blume 1981).

The sample was mixed with 50ml of 96% methanol containing 0.1% (v/v) concentrated hydrochloric acid, then left in the dark for 16hours with constant agitation. After this period the mixture was filtered under reduced pressure through a Whatman No. 1 filter paper and the filtrate reduced to dryness by rotary evaporation under reduced pressure at 30°C. The residue was then resuspended in 1ml of methanol.

#### iii. Extraction in ammonium hydroxide and chloroform (Hamill *et al* 1986).

The sample was mixed with 15ml of 2% (v/v) conc ammonia/water before 15ml of chloroform was added. The mixture was left to stand for 5min after which it was filtered under reduced pressure through a Whatman No. 1 filter paper. The phases of the filtrate were separated using a 250ml separating funnel and the aqueous phase reextracted with 2 X 15ml of chloroform.

Dissolved water was removed from the chloroform extract by adding anhydrous sodium sulphate which was subsequently removed by filtration through a Whatman glass microfibre filter. The filtrate was rotary evaporated to dryness under reduced pressure at 30°C and the residue taken up in 1ml of methanol.

The extracts obtained using methods i.-iii. were prepared for TLC and HPLC analysis as described in Sections 2.6.2. The R<sub>f</sub> values (TLC) of alkaloids in the extracts were compared with the R<sub>f</sub> values of standards dissolved in methanol (Fig 3.2.1 and Table 3.2.1). HPLC was used to identify the alkaloids according to their coelution with authentic standards (Fig 3.2.2) and to quantify each alkaloid (as described in Section 2.5.3.1). The quantity of alkaloid recovered was compared with the known amount at the start of the extraction procedure and expressed as a percentage recovery (Table 3.2.2).

All three methods of extraction gave acceptable recoveries of each of the alkaloids, as determined by HPLC. However, alkaloids in extracts obtained using methods i. and ii. had R<sub>f</sub> values that were different from those of authentic standards, when analysed by TLC (Table 3.2.1). The extraction methods each extracted different amounts of each of the alkaloids from leaf material (Table 3.2.3). Assuming that at the start of the extractions each leaf sample had the same alkaloid content the most efficient extraction of nicotine was achieved with method i.. However, using this method nicotine was the sole alkaloid detectable by HPLC or TLC. Using extraction procedure ii. it was not possible to identify any of the alkaloids by HPLC and only nornicotine was detected by TLC. The third extraction method (iii) gave a final extract which enabled quantification of three of the four alkaloids, by HPLC, and these alkaloids had R<sub>f</sub> values similar to those of authentic standards when analysed by TLC.

From the results of the above experiment extraction methods i. and ii. were rejected since they did not extract all four of the alkaloids simultaneously. Therefore extraction procedure iii. was selected and modified so as to produce a clean final extract from plant material and cultured cells and medium.

## SOLVENT FRONT

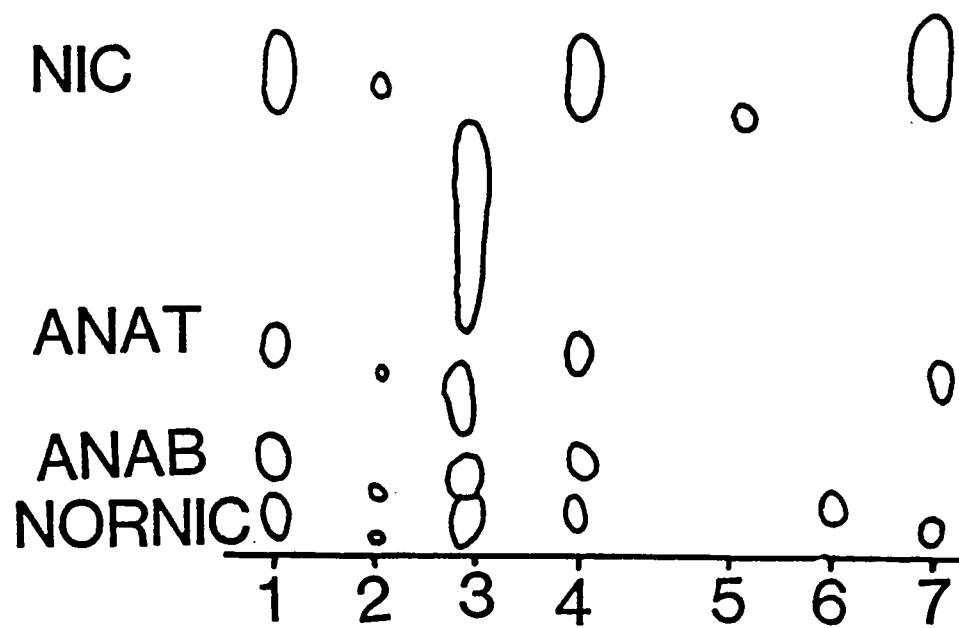


FIGURE 3.2.1

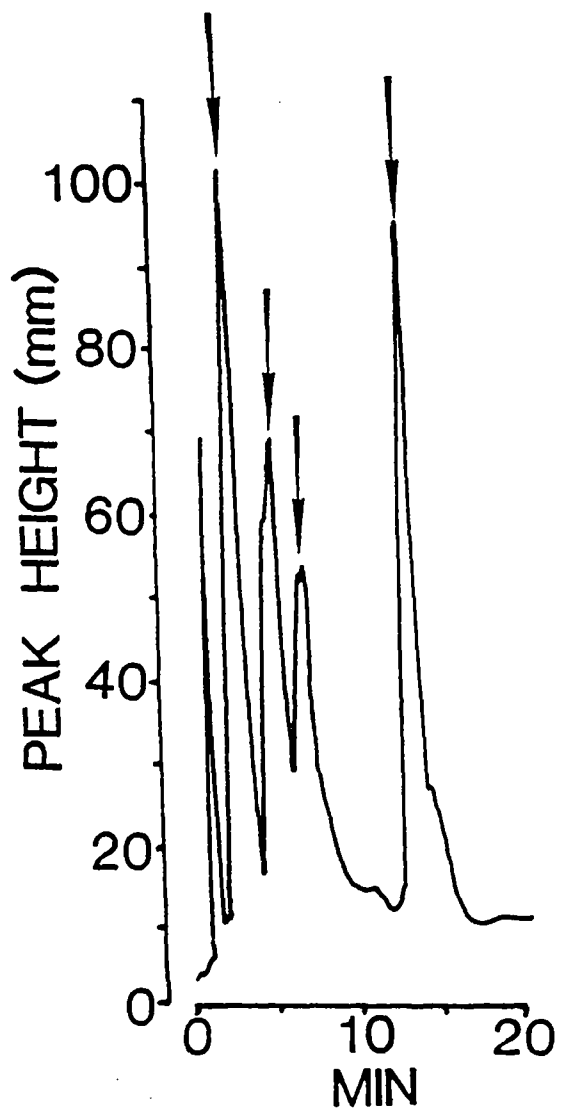
TLC separation of tobacco alkaloid extracts. 50  $\mu$ l of alkaloid standards (1), and extracts of standards using extraction methods: i. (2), ii. (3), iii. (4), and leaf extracts obtained using extraction methods: i. (5), ii. (6) and iii. (7). Silica TLC plates were *developed* with 96% (v/v) ethanol and chloroform (4:1), and the alkaloids detected by spraying the plate with iodoplatinate reagent.

### FIGURE 3.2.2

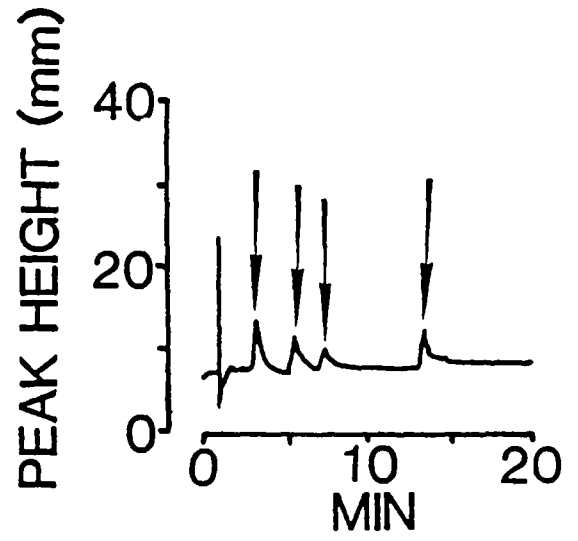
HPLC chromatograms showing the elution of alkaloid standards before and after extraction by one of three methods. In each case peaks with retention times corresponding to those of alkaloid standards are arrowed. A solution containing 500µg of each of the alkaloid standards was subjected to three extraction procedures. 20µl of each of the final extracts was then injected onto a C<sub>18</sub> reverse phase HPLC column for identification and quantification of the alkaloids extracted. The alkaloids were detected by their UV absorbance at 260nm as they were eluted from the column with a gradient of methanol against a weak phosphoric acid buffer.

- a. A chromatogram obtained with a solution containing 50µl of each of the alkaloid standards.
- b. The chromatogram obtained from extract obtained with extraction procedure i.
- c. The chromatogram obtained with the extract obtained using extraction procedure ii. The final extract was diluted two-fold before analysis.
- d. The chromatogram obtained with the extract from extraction procedure iii. The sample was diluted two-fold prior to analysis.

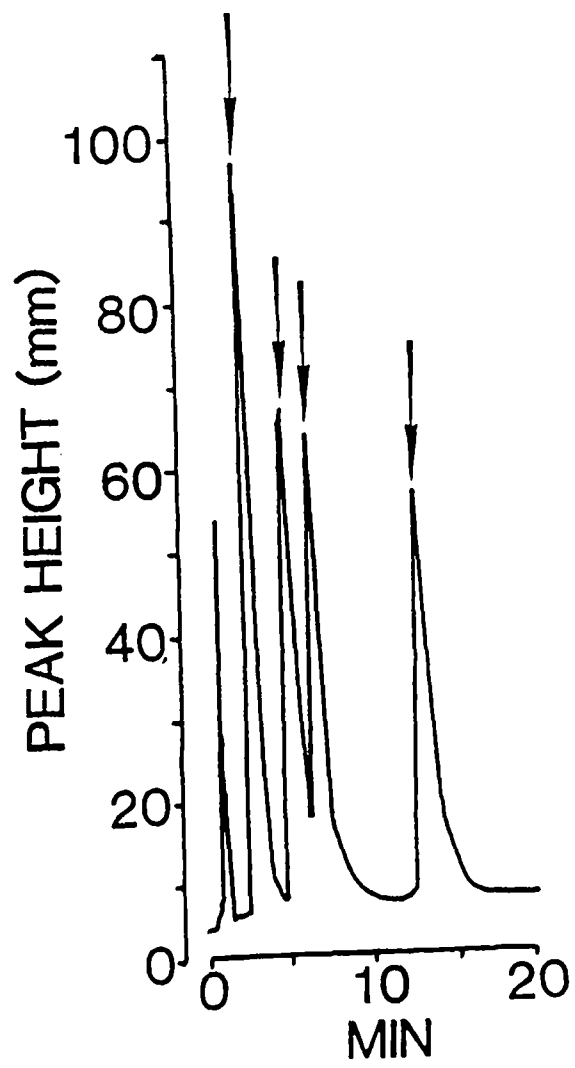
a



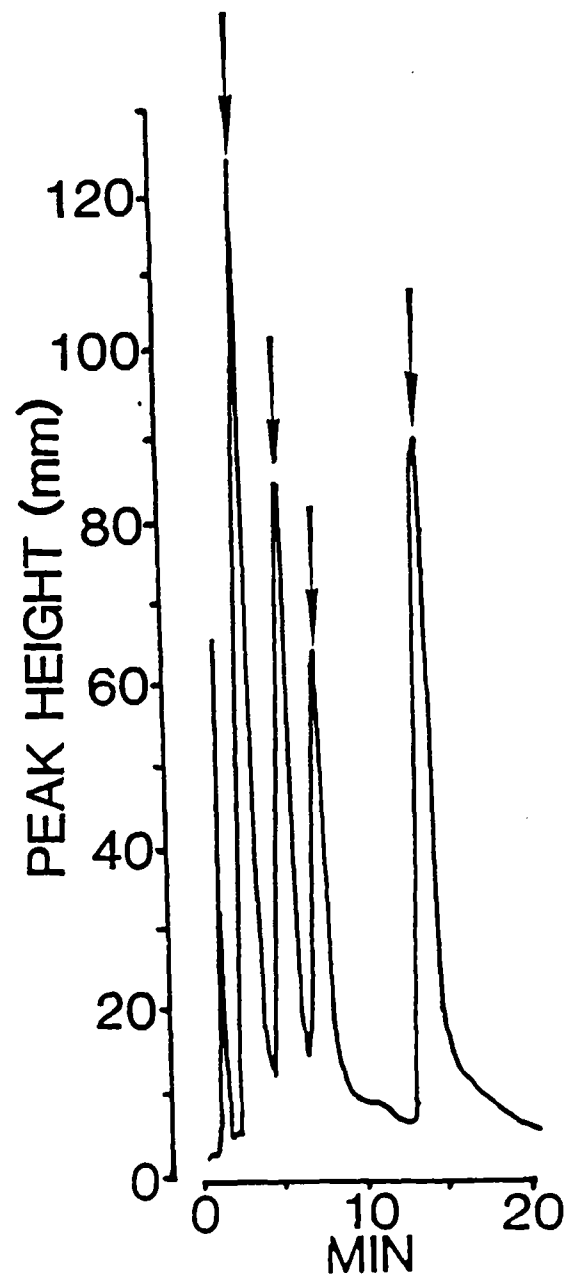
b



c



d



	Authentic standards	Rf VALUE		
		Extract i	Extract ii	Extract iii
Nornicotine	0.04	0.03	0.04	0.04
Anabasine	0.09	0.06	0.07	0.09
Anatabine	0.18	0.14	0.13	0.19
Nicotine	0.43	0.34	0.24	0.43

TABLE 3.2.1

The Rf values as determined by TLC of tobacco alkaloid standards before and after extraction by one of three methods. For each sample 50µl was loaded onto a Silica TLC plate and eluted with 96% (v/v) ethanol and chloroform (8:2). The alkaloids were detected by spraying the eluted plate with iodoplatinate reagent.

	PERCENTAGE RECOVERY		
	Extract i	Extract ii	Extract iii
Nornicotine	108	86	76
Anabasine	78	88	85
Anatabine	37	95	81
Nicotine	60	86	91

TABLE 3.2.2

The percentage recovery, as determined by HPLC, after extraction using the three extraction methods, described in the text, of 500µg of authentic standards of nornicotine, anabasine, anatabine and nicotine. Each value represents the mean of three replicates.

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	Extract i		Extract ii		Extract iii	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Nornicotine	ND		UQ		16.4	3.5
Anabasine	ND		ND		ND	
Anatabine	ND		ND		16.8	0.6
Nicotine	500	80	ND		150.6	19.8

TABLE 3.2.3

The alkaloid content of 15 week old tobacco leaves, as determined by HPLC, after extraction using the three methods described in the text. The two most fully expanded leaves from were pooled and extracted by each method and three plants were used to provide the replicates. The data from the extractions of the three replicates was used to calculate the means and standard errors.

ND = Not detectable.

UQ = Unquantifiable by HPLC but detectable by TLC.

### **3.2.1.2 Extraction of alkaloids from cultured cells using the chosen extraction procedure.**

Cultured cells suspended in MS medium supplemented with 3% sucrose and grown in the dark were harvested 20 days after inoculation into fresh medium. The cells were separated from the medium by filtration under reduced pressure through a Whatman No1 filter paper. Approximately 9-10g fresh weight of cells were then extracted according to method iii as described above.

When analysed by HPLC the extract was found to have nine ~~main~~ components (Fig 3.2.3). The UV spectra of peaks with retention times close to those of alkaloid standards were determined using a Hewlett Packard HPLC systems diode array facility (Fig 3.2.4). Only the peaks with retention times of 3.02, 5.76 and 11.82min were found to have spectra that corresponded to the spectra of alkaloid standards (Fig 3.2.5). Maximum UV absorbtion for all four of the major alkaloids was found to be at 258+/-4nm. By comparing the spectra Peak 1 (Fig 3.2.3) was confirmed as nornicotine while Peaks 2 and 3 were confirmed as anatabine and nicotine respectively. However, the UV spectrum of Peak 4 (Fig 3.2.4c) did not resemble the spectrum of known tobacco alkaloids. Since this contaminating substances had a retention time similar to nicotine quantification of the nicotine peak was impossible and therefore the extraction method required modification.

### **3.2.1.3 Modification of the chosen extraction method to remove contaminating substances.**

Cells grown in MS medium containing 3% sucrose and incubated in the dark harvested 20-24 days after inoculation into fresh medium were used in the experiments to refine the extraction procedure. In each case 9-10g fresh weight of tissue was extracted.

In order to clean up the extract it was necessary to remove as many non-alkaloid compounds as possible. Since the final extract was the result of

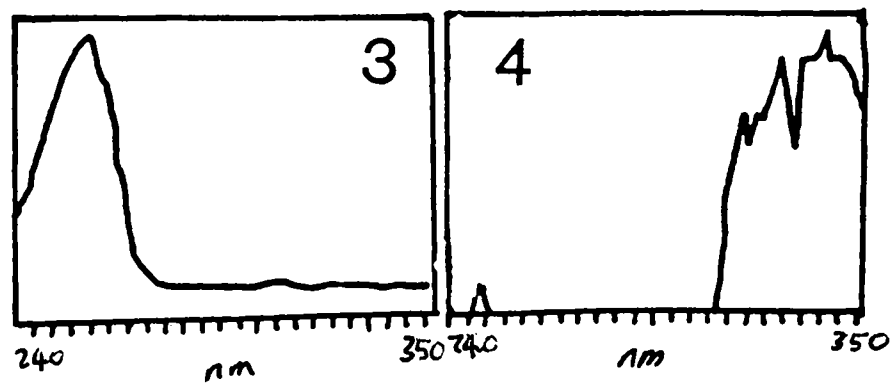
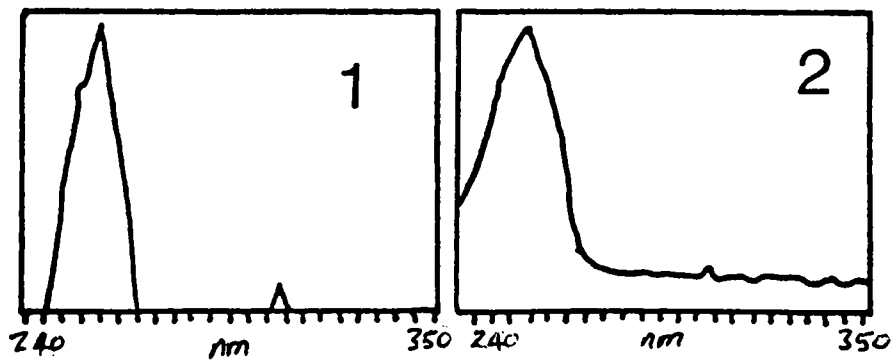
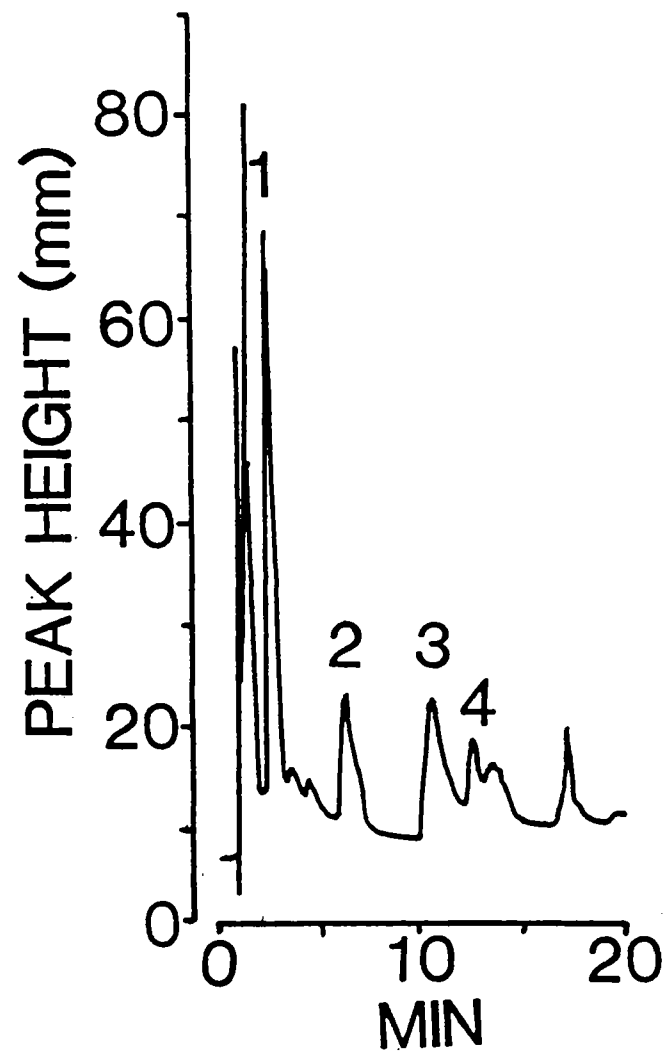


**FIGURE 3.2.3**

A chromatogram obtained with a 20 $\mu$ l injection onto the HPLC column of an extract from cultured cells. The peaks numbered are those with retention times similar to the retention times of alkaloid standards.

**FIGURE 3.2.4**

The UV spectra of peaks 1-4 in Fig 3.2.3 detected using a Hewlett Packard diode array analysis system.



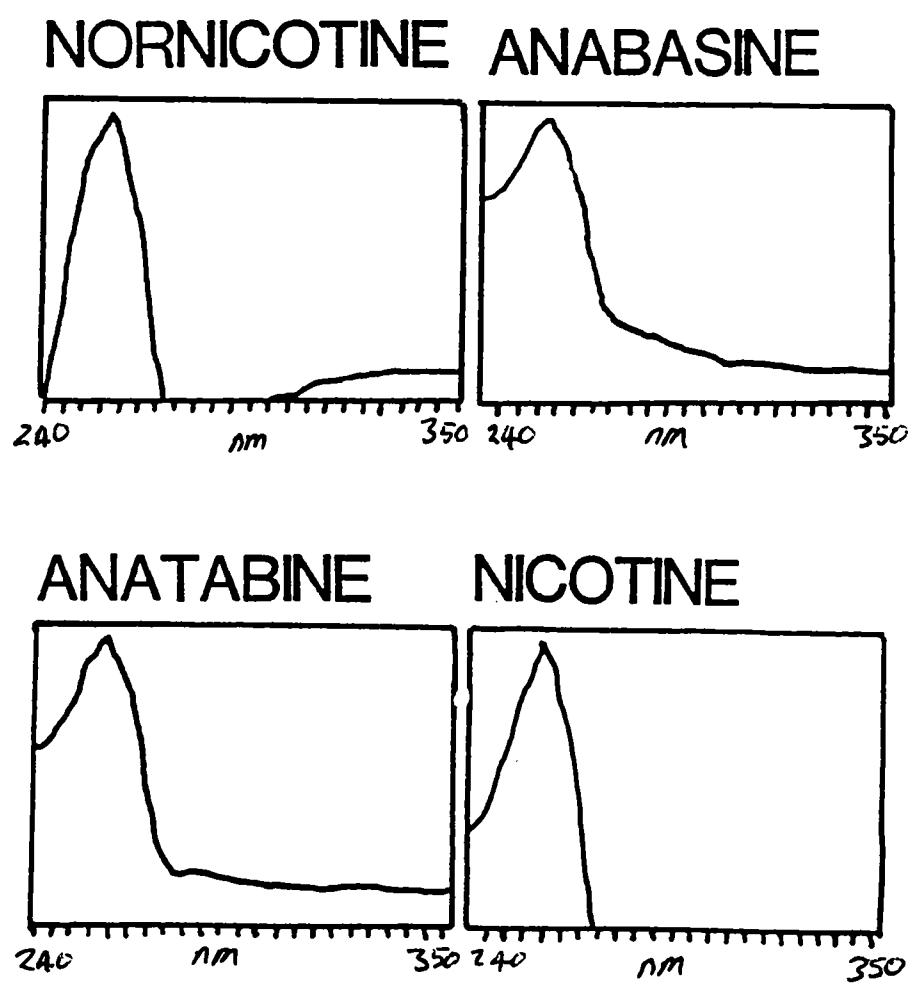


FIGURE 3.2.5

The UV spectra of tobacco alkaloid standards. The spectra were determined using the diode array detector facility on a Hewlett Packard HPLC system.

an extraction from an alkaline solution into chloroform the majority of polar compounds would have been discarded. In order to remove contaminating non-polar compounds an additional step was introduced into the extraction procedure. After extraction of the alkaloids into chloroform and rotary evaporation to dryness the residue was resuspended in 1ml of 0.3% (v/v) sulphuric acid. The alkaloids are soluble in the acidic aqueous phase because acidic conditions cause the protonation of the nitrogen *atom* of the alkaloids. Under these conditions the majority of non-polar compounds would not have resuspended due to their insolubility in the aqueous phase. This aqueous phase extract was then prepared for analysis by HPLC.

On analysis of the extract by HPLC three main peaks with retention times corresponding to those of alkaloids were detected (Fig 3.2.6). However, the base-line obtained with the sulphuric acid extract was extremely unstable and did not allow for accurate quantification of the peaks. However, a good base-line was achieved with extracts resuspended in methanol and therefore the alkaloids were extracted from the acidic aqueous phase back into an organic phase. This was achieved by resuspending the residue, after rotary evaporation to dryness, in 10ml of 0.3% sulphuric acid and transferring this to a separating funnel. The pH of the solution was adjusted to pH 10.8 with 20% (v/v) conc ammonia/water, causing the deprotonation of the nitrogen *atom* of the alkaloids and rendering them soluble in an organic phase. The alkaloids were thus extracted from the aqueous phase into 3 X 15ml of chloroform which was dried with anhydrous sodium sulphate before being reduced to dryness by rotary evaporation at 30°C. The residue from rotary evaporation was resuspended in 1ml of methanol and prepared for HPLC analysis.

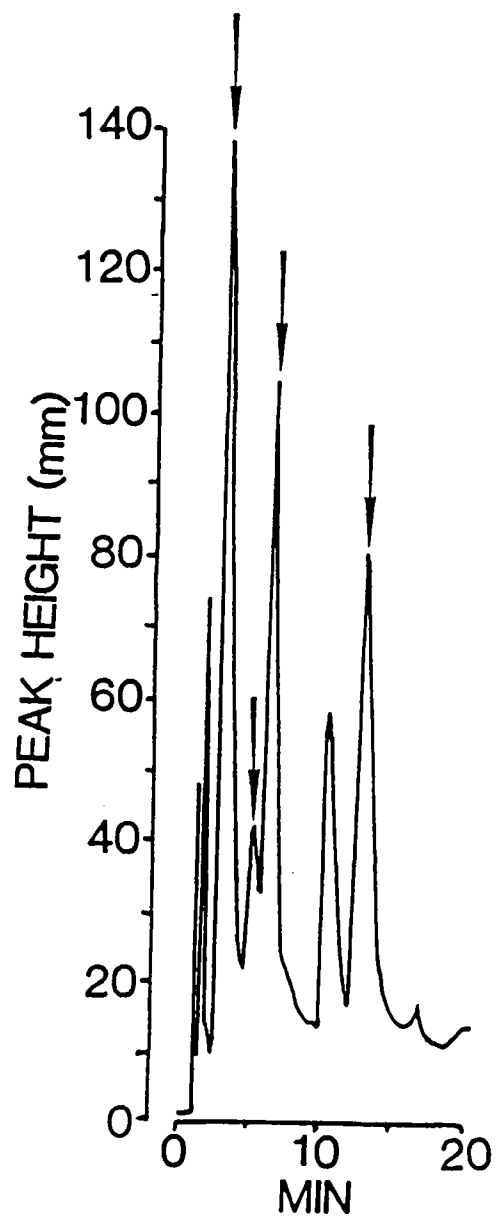
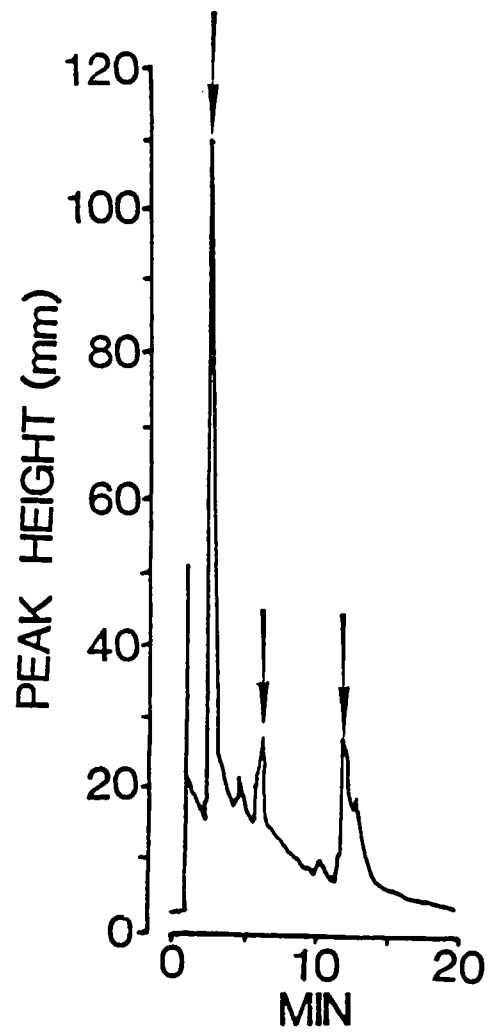
The extract produced by this method was suitable for HPLC analysis since the base-line was steady throughout the analysis and the peaks with retention times corresponding to alkaloids were clearly defined (Fig 3.2.7) allowing accurate quantification. The method was therefore modified to allow extraction of the alkaloids from the cells bathing medium.

**FIGURE 3.2.6**

The HPLC chromatogram obtained with a 20 $\mu$ l injection of a final extract of cultured cells in 0.03% (v/v) sulphuric acid. The alkaloid peaks are arrowed.

**FIGURE 3.2.7**

The chromatogram obtained with a 20 $\mu$ l injection of a final extract of cultured cells in chloroform after an extraction involving an acid clean up stage. Peaks with retention times corresponding to those of alkaloid standards are arrowed.



#### **3.2.1.4 Modification of the extraction procedure to allow extraction of the alkaloids from the culture medium.**

The pH of the culture medium varied between pH4 and pH6 during the culture cycle, thus alkaloids in the medium were likely to be protonated. If the medium, after separation from the cells, was made alkaline the alkaloids would become deprotonated and thus soluble in an organic phase. In this way the alkaloids could be extracted from the medium.

After separation of the cells from the medium the pH was adjusted to pH10.8 with 20% (v/v) conc ammonia/water. The alkaline medium was then extracted with 3 X 50ml of chloroform and the resulting chloroform extract treated in the same manner as the initial cellular chloroform extract (see above).

On analysis of cellular and medium extracts prepared using this extraction procedure five peaks (Fig 3.2.8 a,b) were detected by HPLC analysis. Four of these peaks (Peaks 1,2,3 and 5) had retention times corresponding to the alkaloids while the other peak (Peak 4) did not. The UV spectra obtained using the diode array facility on a Hewlett Packard chromatograph confirmed the identity of the alkaloid peaks but it was not possible to either confirm or disprove that the unknown peak was of alkaloid origin by its spectrum. When the extract was analysed by TLC the only iodoplatinate sensitive spots that were detectable were those with Rf values corresponding to the Rf values of authentic standards of the four main tobacco alkaloids. Thus it appeared that the unknown compound was of non-alkaloid origin. However, the unknown compound appeared to have very similar properties to those of alkaloids since it was extracted by an alkaloid specific procedure therefore it was necessary to determine the identity of the unknown compound.

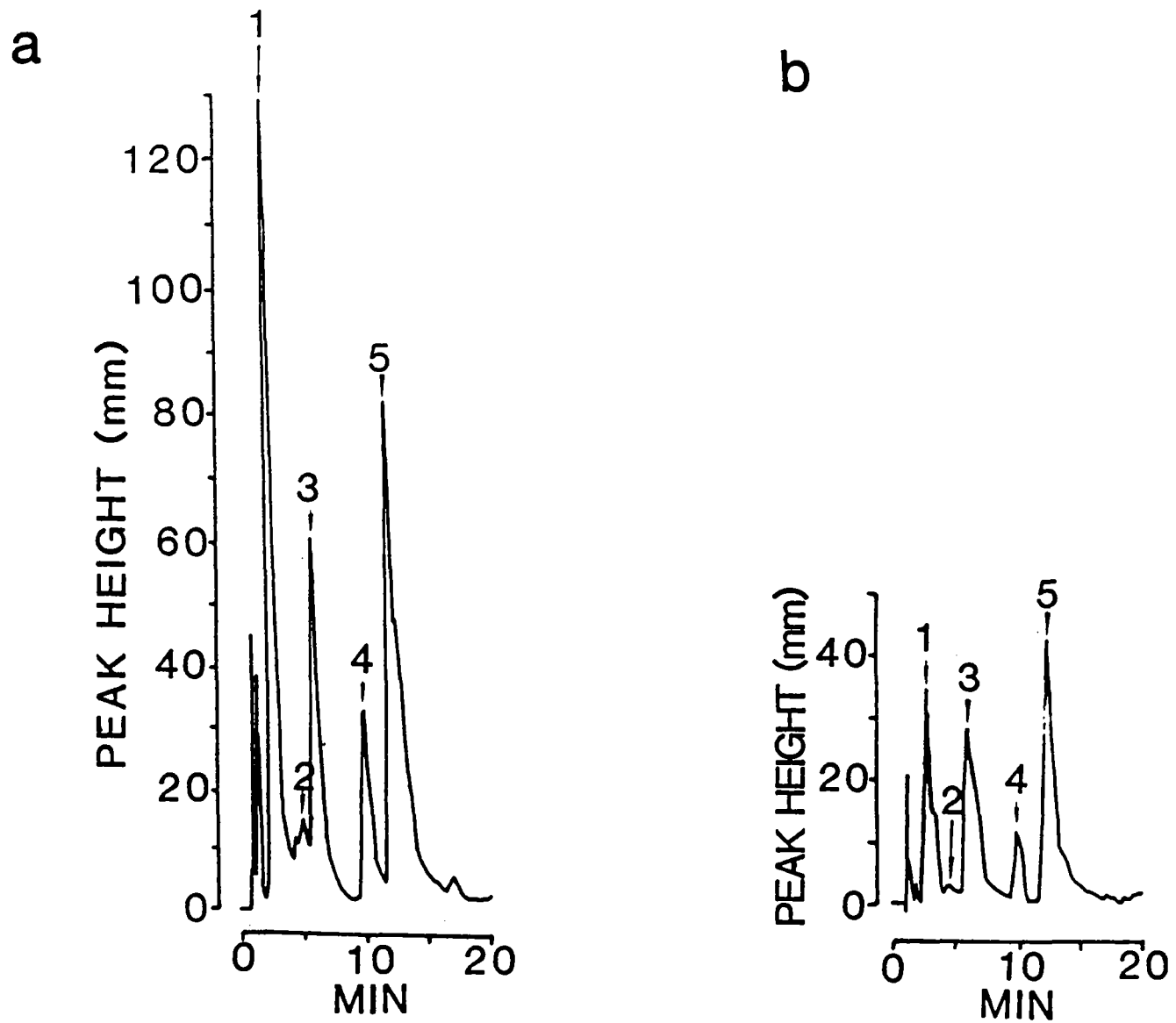


FIGURE 3.2.8

The HPLC chromatograms obtained from a 20 $\mu$ l injection of extracts of cells (a) and medium (b).

- 1 = Nornicotine.
- 2 = Anabasine.
- 3 = Anatabine.
- 5 = Nicotine.



### 3.2.2 Identification Of The Unknown Compound In Alkaloid Extracts.

In order to identify the unknown compound that was extracted along with the alkaloids the eluate, from the HPLC column, between 9 and 10min was collected (Section 2.6.4) so that a fraction containing the unknown compound was obtained. The unknown was then re-extracted from the eluate. This was achieved by adjusting the pH of the eluate to pH11 with 20% conc ammonia and extracting this alkaline solution with 3 X 50ml of chloroform. Any residual aqueous phase was removed from the chloroform extract with anhydrous sodium sulphate and after the removal of the sodium sulphate the filtrate was reduced to approximately 5ml by rotary evaporation. The sample was then stored under nitrogen at  $-40^{\circ}\text{C}$ . Analysis was carried out using GC-MS at Rothmans International Services, Basildon (by Mr B. Frost) and the compound identified by Rothmans International Services, Bremen (by Dr H. Elmenhorst) from the GC-MS data using their data base of GC-MS spectra (Appendix 3). The compound was found to be di-(2-ethyl-hexyl)-phthalate (the most common plasticizer used in tubings and other laboratory plasticware).

On analysis of the alkaloids in extracts by GC-MS (carried out by Rothmans International Services: Chemical Research, Bremen) it was found that a variety of polar compounds could be separated from nornicotine. The major contaminants of these extracts were different derivatives of phthalate the two main compounds being dimethylphthalate and diethylphthalate (Appendix 4). The compounds associated with nornicotine would appear to be complexed with it in some way rather than coeluting with it because chromatograms of extracts that did not contain nornicotine did not have a peak at the retention time of nornicotine.

From these results it was clear that alkaloid quantification needed to take into account the contaminating compounds.

### 3.2.2.1 Alkaloid quantification.

Alkaloid quantification was achieved by extracting a range of known amounts of each of the alkaloids added to cultured cells which did not contain alkaloids (as determined by TLC). The height of peaks on HPLC chromatograms with retention times corresponding to the alkaloids were plotted against the amount of the given alkaloid at the start of the extraction procedure. The calibration curves obtained were used in alkaloid quantification as described in Section 2.5.3.1. This assumed that the ~~proportion~~<sup>was</sup> of complexing of nornicotine with contaminating compounds  $\lambda$  constant. This appeared to be the case since the calibration curves were linear over the alkaloid concentrations employed.

The use of the peak height on the chromatogram, rather than peak area, may have reduced the accuracy of the quantification because the tailing of peaks would not be accounted for. However, there variations in the order of 40% between the peak areas of identical standards.\* Therefore the reduction in accuracy through using peak height was justifiable when the problems with using peak area were considered.

The methods of alkaloid extraction, identification and quantification, described above, were used to determine the alkaloid content of plant tissue and cultured material. Using the method of quantification described the complexing of non-alkaloid compounds with the alkaloids could be taken into account.

\* These variations being caused by the nature of the electronic integration system operating in the data processor.

**PART B.**

THE RESULTS OF INVESTIGATIONS INTO ALKALOID BIOSYNTHESIS IN TOBACCO

### 3.3 THE DISTRIBUTION OF ALKALOIDS IN PLANTS OF *NICOTIANA TABACUM*

Plants of *Nicotiana tabacum* Cv. Wisconsin-38 were grown under standard greenhouse conditions (Section 2.1.2) and were harvested 25 to 30 weeks after planting. Plants were divided up into leaves and roots and the alkaloid content of each tissue determined. Due to variations in the alkaloid content of leaves on an individual plant (Papenfus & Quin 1984) the sampling of leaves was standardised so that the two most fully expanded leaves from the plant were harvested for alkaloid determinations. Roots were separated from the compost and washed in distilled water prior to extraction.

The results show roots to have a significantly higher alkaloid content than leaves, having a total alkaloid content of  $473 \pm 139 \mu\text{g g}^{-1}$  fr wt compared with  $280 \pm 11 \mu\text{g g}^{-1}$  fr wt in leaves. On a percentage dry weight basis this equated as 0.3 % and 0.4 % for leaves and roots respectively. The composition of the total alkaloid content for each tissue is shown in Table 3.3.1. In the leaves nicotine accounted for 86% of the total alkaloid content and for 62% in the roots and on a whole plant basis for 71% of the total alkaloid content. In the roots the amount of each of the alkaloids was higher than the level in the leaves. Anabasine was not detectable in either the leaves or the roots. Nornicotine was not detectable in the leaves while in the roots there was no significant difference between the anatabine content and the nornicotine content,  $73 \pm 6.6 \mu\text{g g}^{-1}$  fr wt and  $106 \pm 58 \mu\text{g g}^{-1}$  respectively. The overall ratio of nornicotine to nicotine was 0.2.

Once the alkaloid content and distribution had been determined for whole plants an experiment was carried out to compare the alkaloid composition of cells grown *in vitro* with that in whole plants. Sterile seed was germinated under conditions of total asepsis and callus arising from the radicle excised and used to initiate cell cultures. After initial culture on MS medium cell suspensions were established on a range of basal media and the alkaloid content of each of the resulting cultures compared with the alkaloid content of the plant.

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	LEAVES		ROOTS		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	ND		106	58	106	58
ANABASINE	ND		ND		ND	
ANATABINE	40	3	73	6	113	10
NICOTINE	240	11	293	75	533	81
TOTAL	280	15	473	139	753	144

TABLE 3.3.1

The alkaloid content and distribution in 25 week old greenhouse grown plants of *N.tabacum*. Each replicate consisted of material from the two most fully expanded leaves or from the root system. The mean and standard errors were calculated from the data obtained from three determinations with each tissue.

### **3.4 THE EFFECT OF BASAL MEDIUM ON ALKALOID ACCUMULATION IN CELL SUSPENSION CULTURES OF *NICOTIANA TABACUM***

In the majority of previous studies on alkaloid accumulation in cell cultures of *N.tabacum*, MS medium, with either 2% or 3% sucrose, has been used as the basal medium. However, MSS medium supplemented with fructose has been shown to promote photosynthetic development (Dalton & Street 1977). A study was therefore undertaken to determine the effect of *each* basal medium on alkaloid accumulation. In addition to MS with sucrose and MSS containing fructose, the media shown in Table 2.2.1 were examined. The hormone concentrations employed with each medium were those most frequently reported in the literature, and ascorbic acid included since it increases alkaloid synthesis *in vitro*.

All treatments were inoculated with 1.5 to 2.0g wet weight of cells, from stock cultures grown for at least four culture cycles under the same conditions. Cultures were grown in the dark at 25+/-2°C on an orbital shaker for 23 days before harvest. For each treatment three replicates were harvested and the cells separated from their medium by filtration under reduced pressure. The medium was retained for alkaloid analysis and the fresh weight of the cells recorded before 10g (or the total biomass if less than 10g) was extracted as described in Section 2.5.1.1.

#### **3.4.1 Alkaloid Accumulation In Cultures Grown In MS Medium.**

In cell suspension cultures grown in MS medium, supplemented with: 3% sucrose,  $0.18 \times 10^{-6}$ M NAA,  $0.93 \times 10^{-6}$ M kinetin and  $28 \times 10^{-6}$ M ascorbic acid, alkaloids were only detectable in the cellular fraction. There was a total alkaloid content of  $17.5 \mu\text{g g}^{-1}$  fr wt, with nornicotine as the major alkaloid (Table 3.4.1 and 3.4.2). The ratio of nornicotine to nicotine, which accounted for 16 % of the total alkaloid content, was 3.5.

**TABLE 3.4.1**

Alkaloid accumulation by suspension cultures of *N.tabacum* grown in six different basal media. The media employed were: Murashige and Skoog (MS) medium, Murashige and Skoog Plant Salts (MSS and MSS<sup>1</sup>) medium, Gamborgs B<sub>5</sub> (B<sub>5</sub>) medium, Schenk and Hildebrandt (SH) medium and Whites (W) medium. The media were supplemented with 3% sucrose, with the exception of MSS<sup>1</sup> which contained 3% fructose. Cultures were grown in the dark for 23 days before the alkaloid contents were determined.

ND = Not detectable.

a. The alkaloid content of the cells.

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	BASAL MEDIA					
	MS	MSS	MSS <sup>1</sup>	SH	B <sub>5</sub>	W
NORNICOTINE	11	ND	17	10	26	0.24
ANABASINE	2	ND	ND	1	3	0.10
ANATABINE	ND	ND	14	ND	18	0.08
NICOTINE	3	ND	6	0.07	7	ND
TOTAL	17	ND	38	12	56	0.42

b. The alkaloid content in the media.

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	BASAL MEDIA					
	MS	MSS	MSS <sup>1</sup>	SH	B <sub>5</sub>	W
NORNICOTINE	ND	ND	1.8	1.1	10	ND
ANABASINE	ND	ND	ND	ND	ND	ND
ANATABINE	ND	ND	0.6	ND	6.9	ND
NICOTINE	ND	ND	0.4	ND	ND	ND
TOTAL	ND	ND	2.8	1.1	17	ND

c. The total alkaloid content.

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	BASAL MEDIA					
	MS	MSS	MSS <sup>1</sup>	SH	B <sub>5</sub>	W
NORNICOTINE	11	ND	19	11	37	0.24
ANABASINE	2	ND	ND	1.1	3.8	0.10
ANATABINE	ND	ND	14	ND	25	0.08
NICOTINE	3.2	ND	7.2	0.07	7.8	ND
TOTAL	17	ND	30	13	74	0.42



	ALKALOID CONTENT ( $\mu\text{g culture}^{-1}$ )					
	BASAL MEDIA					
	MS	MSS	MSS <sup>1</sup>	SH	B <sub>5</sub>	W
NORNICOTINE	218	ND	313	141	432	1.5
ANABASINE	54	ND	ND	14	45	0.1
ANATABINE	ND	ND	243	ND	298	0.1
NICOTINE	63	ND	118	8	91	ND
TOTAL	335	ND	674	163	867	2.1

TABLE 3.4.2

The alkaloid content on a culture basis of cultures grown in six different basal media for 23 days. Cell suspensions of *N.tabacum* were grown in the dark in: Murashige and Skoog (MS) medium, Murashige and Skoog Plant Salts (MSS and MSS<sup>1</sup>) medium, Gamborgs B<sub>5</sub> (B<sub>5</sub>) medium, Schenk and Hildebrandt (SH) medium and Whites (W) medium. The media were supplemented with 3% sucrose, with the exception of MSS<sup>1</sup> which *was supplemented with 3% fructose.*

ND = Not detectable.

### 3.4.2 Alkaloid Accumulation In Cultures Grown In MSS Medium Supplemented With Either 3% Sucrose or 3% Fructose.

The alkaloid content of cells grown in MSS medium was affected by the additions to the medium. The treatment supplemented with: 3% sucrose,  $0.81 \times 10^{-6}$ M NAA,  $0.93 \times 10^{-6}$ M kinetin and  $28 \times 10^{-6}$ M ascorbic acid had *no detectable alkaloids (Table 3.4.1 and 3.4.2)*

When MSS<sup>1</sup> medium was supplemented with: 3% fructose,  $1.62 \times 10^{-6}$ M NAA and  $1.33 \times 10^{-6}$ M BAP<sub>1</sub>, nornicotine and anatabine were found in the medium as well as in the cellular fraction (Table 3.4.1 and 3.4.2). Overall *nornicotine* was the most abundant alkaloid ( $19 \mu\text{g g}^{-1}$  fr wt) followed by *anatabine* ( $14 \mu\text{g g}^{-1}$  fr wt). The total alkaloid content was  $47 \mu\text{g g}^{-1}$  fr wt and 8.8% of this was accounted for by nicotine. The ratio of nornicotine to nicotine was 3.4.

### 3.4.3 Alkaloid Accumulation In Cultures Grown In B<sub>5</sub> Medium.

When cells were grown in B<sub>5</sub> medium supplemented with: 3% sucrose,  $0.81 \times 10^{-6}$ M NAA,  $0.93 \times 10^{-6}$ M kinetin and  $28 \times 10^{-6}$ M ascorbic acid an overall alkaloid content of  $74 \mu\text{g g}^{-1}$  fr wt was achieved. Of this 55 % was associated with the cellular fraction. Nicotine was not detected in ~~either the medium, or the cellular extracts and~~ In both fractions *nornicotine* was the most abundant alkaloid (Table 3.4.1 and 3.4.2).

#### 3.4.4 Alkaloid Accumulation In Cell Suspension Cultures Grown In SH Medium.

Cultures grown in SH medium, supplemented with: 3% sucrose,  $0.81 \times 10^{-6}$  M NAA,  $0.93 \times 10^{-6}$  M kinetin  $28 \times 10^{-6}$  M ascorbic acid and  $1.75 \text{ g l}^{-1}$  potassium nitrate, had an alkaloid content of  $13 \mu\text{g g}^{-1}$  fr wt. Nicotine, associated entirely with the cellular fraction, accounted for 6 % of the total alkaloid content while the ratio of nornicotine, the major alkaloid, to nicotine was 13. Both nornicotine and anatabine were released into the medium (Table 3.4.1 and 3.4.2), such that 11 % of the total alkaloid content was in the medium.

#### 3.4.5 Alkaloid Accumulation In Cells Grown In W Medium.

Whites medium supplemented with: 3% sucrose,  $0.81 \times 10^{-6}$  M NAA,  $0.93 \times 10^{-6}$  M kinetin and  $28 \times 10^{-6}$  M ascorbic acid caused a nearly complete inhibition <sup>of</sup> alkaloid accumulation (Table 3.4.1 and 3.4.2). The total alkaloid content was  $0.4 \mu\text{g g}^{-1}$  fr wt which was all from the cellular fraction. There was no nicotine detectable and nornicotine was the major alkaloid.

#### 3.4.6 *The Effect of basal medium on biomass yield of suspension cultures.*

In addition to drastically affecting alkaloid accumulation the basal medium employed also affected biomass production (Table 3.5.3). The fresh weights of the cultures, on day 24, ranged from 6.12 g (with B<sub>5</sub> medium) to 19.70 g (with MS medium). However, there was not a correlation between the final fresh weight and the alkaloid content of the cultures (the correlation coefficient being 0.25).

MSS medium, supplemented with 3% fructose  $1.62 \times 10^{-6}$  M NAA and  $1.33 \times 10^{-6}$  M BAP, did not inhibit growth and the cultures achieved an alkaloid content second only to the cultures grown in B<sub>5</sub> medium. The MSS medium was also known to promote photosynthetic development and was therefore favourable when compared with all of the other media tested. The alkaloid

BASAL MEDIA	FRESH WEIGHT (g)
MS	19.70
MSS	15.81
MSS <sup>1</sup>	16.23
SH	12.17
B <sub>5</sub>	11.61
W	6.12

TABLE 3.4.3

The effect of basal media on biomass production by *N.tabacum* cells in suspension culture. The fresh weights of cultures were determined after 23 days growth in the dark in: Murashige and Skoog (MS) medium, Murashige and Skoog Plant Salts (MSS and MSS<sup>1</sup>) medium, Schenk and Hildebrandt (SH) medium, Gamborgs B<sub>5</sub> (B<sub>5</sub>) medium and Whites (W) medium. Each medium, with the exception of MSS<sup>1</sup> which contained 3% fructose, was supplemented with 3% sucrose.

spectrum was of minor importance since the synthesis of the pyridine ring of the alkaloids was of interest, and this ring is common to all of the nicotine alkaloids. Therefore MSS medium, with varying fructose concentrations, was used in all subsequent experiments involving cell suspension cultures of *N.tabacum*.

### **3.5 CHARACTERIZATION OF THE GROWTH OF SUSPENSION CULTURES.**

#### **3.5.1 An analysis Of The Growth Of *N.tabacum* Cells In Suspension Culture And Of The Kinetics Of Sucrose Uptake During A Culture Cycle.**

The aim of this analysis was to determine the time after inoculation of fresh medium at which changes in the pattern of growth occurred, along with the kinetics of sucrose uptake by the cells during the culture period. This data was required for determining the optimum times for single cell cloning and for the screening of cells for photosynthetic potential.

Culture growth was measured by fresh weight, dry weight, and cell number in order to determine whether or not it was feasible to use fresh weight to describe culture growth in future experiments rather than cell number, which is regarded as a more accurate descriptor of culture growth. Using fresh weight as the parameter for measuring culture growth has the advantages of not requiring tissue to be sacrificed and being less time consuming.

At the start of the experiment 30 conical flasks (250ml) each containing 50ml of MS medium with 2% (v/v) sucrose were inoculated with 5.0–5.5g wet weight of cells from a stock suspension that was the result of pooling the contents of 8 culture flasks 14 days after inoculation. After inoculation the cultures were maintained under continuous illumination and standard culture conditions. At intervals of 1–2 days over a 14 day period 3 flasks were sampled and determinations of fresh weight, dry weight and cell

number per culture made. The medium from each flask was retained and stored at  $-40^{\circ}\text{C}$  until the end of the time course when the residual sucrose concentration was determined for each sample.

#### **3.5.1.1 The growth of cells in liquid culture as expressed by fresh weight, dry weight and cell number.**

Between days 0 and 1 there was no significant change in cell number or dry weight but there was a decrease in fresh weight (Fig 3.5.1). After the first day of culture, dry weight and cell number per culture increased linearly until the tenth day after inoculation, while fresh weight increased gradually from day 1 to day 4 before increasing more rapidly to a maximum at day 10. Subsequent to the tenth day after inoculation there were no significant changes in the values of any of the parameters being measured.

From the results of this experiment the start of the stationary phase was determined to be at day 12 after inoculation. Single cell cloning was therefore carried out on the eighth day of culture since the viability of single cells is greatest at the end of the exponential phase (Thomas & Davey 1975).

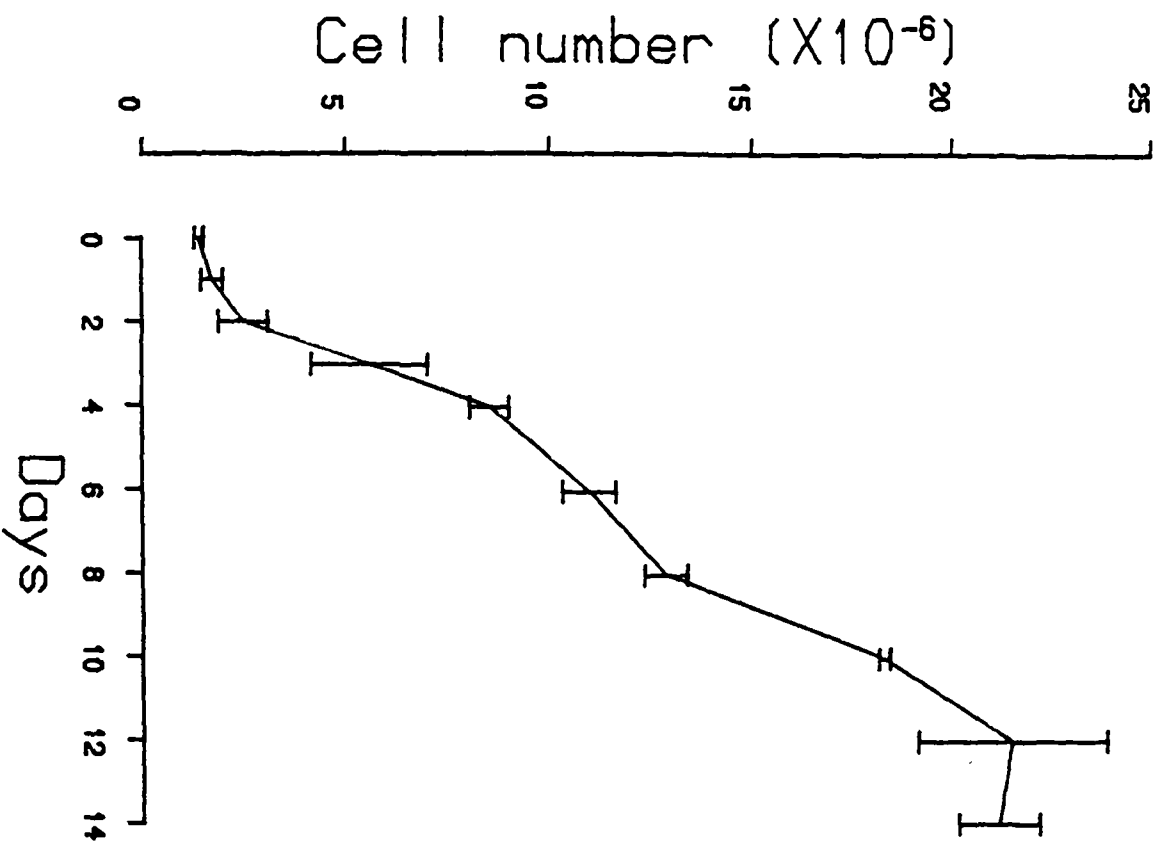
It can be seen (Fig 3.5.1) that although fresh weight did not mirror the cell number per culture exactly it did show changes in the pattern of growth as seen when using cell number as the descriptor of culture growth. Thus fresh weight was used to measure growth of cells in culture in all subsequent growth related studies.

#### **3.5.1.2 The uptake of sucrose by cells in suspension culture.**

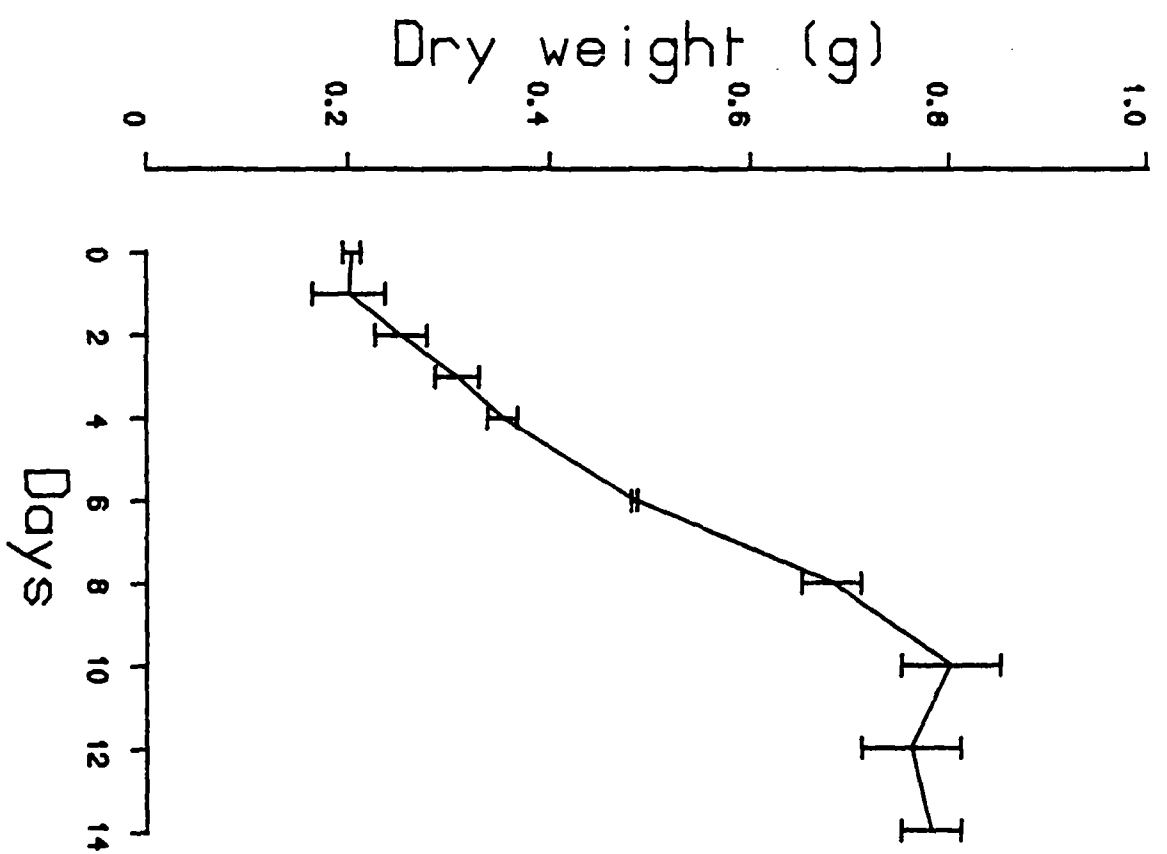
During the first 4 days after inoculation of the medium there was no significant sucrose uptake (Fig 3.5.2). However, by day 14 of the culture period 98 % of the sucrose in the medium had been taken up by the cells. Uptake was most rapid between days 4 and 10 when 89% of the sucrose present at inoculation was taken up by the cells.

FIGURE 3.5.1

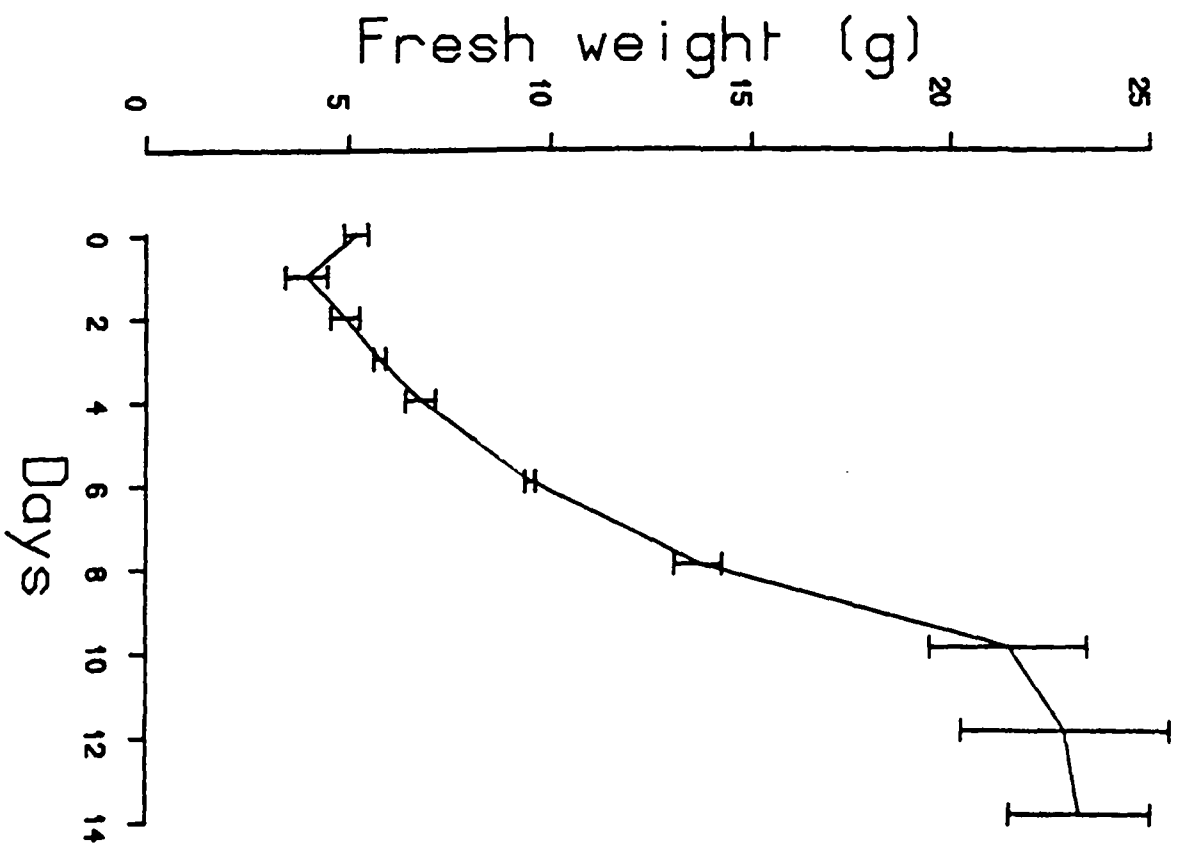
Changes in the growth of *N.tabacum* suspension cultures during a culture cycle, as measured by cell number (a), dry weight (b) and fresh weight (c). Cells were grown in MS medium supplemented with 2% sucrose,  $0.81 \times 10^{-6}$ M NAA and  $0.93 \times 10^{-6}$ M kinetin and cultured in the light. Each point represents the mean  $\pm$  the standard error of three replicates.



Cell number per culture.



Dry weight per culture



Fresh weight per culture



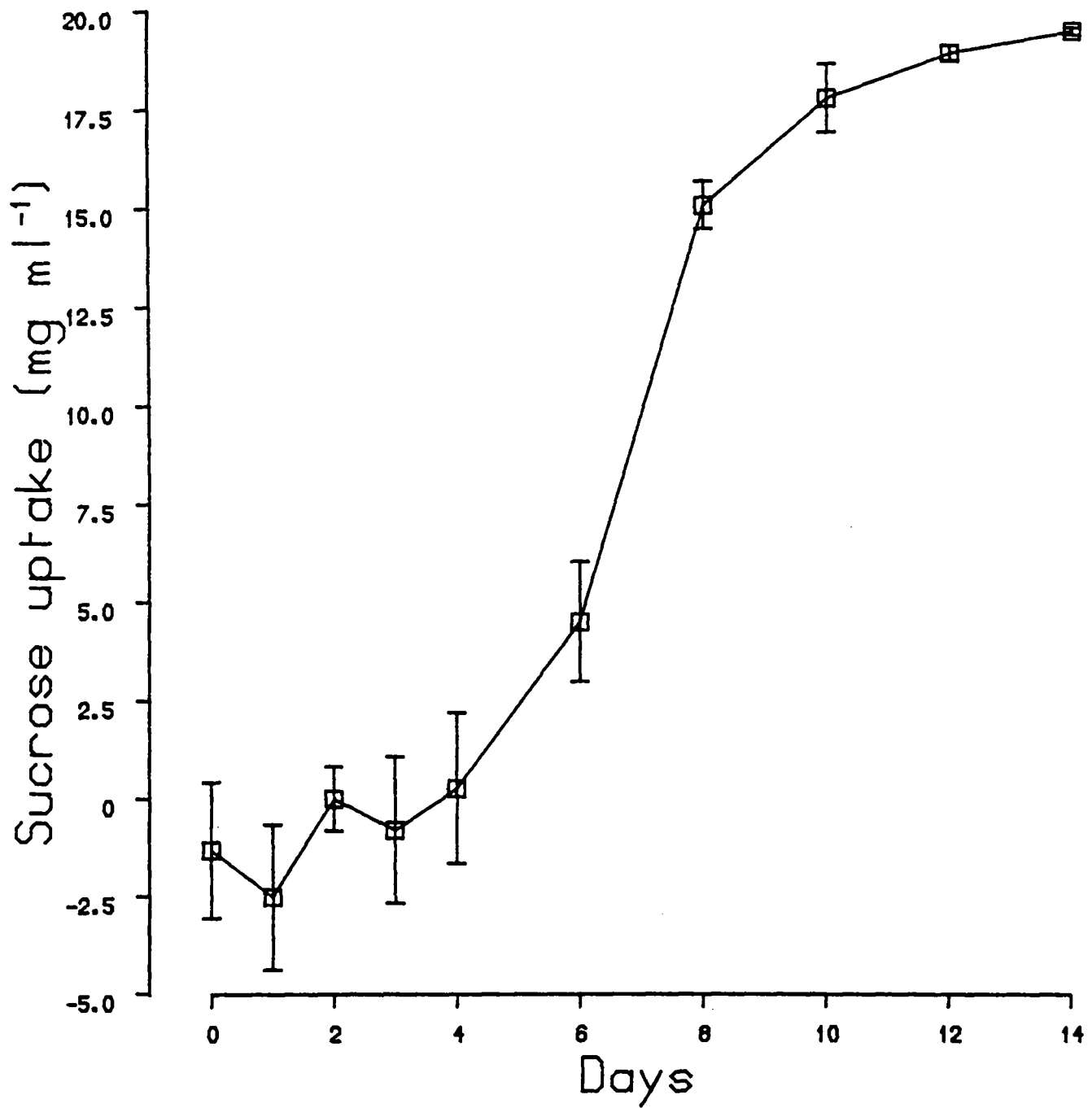


FIGURE 3.5.2

The uptake of sucrose from MS medium, initially containing 2% sucrose, by suspension cultured cells of *N. tabacum*. Cultures were grown in the light for a 14 day period with  $0.81 \times 10^{-6}$ M NAA and  $0.93 \times 10^{-6}$ M kinetin in the medium. Each point represents the mean +/- the standard error of three replicates.

The photosynthetic activity of cultured cells has been found to be greatest when cultures reach the stationary phase of the growth cycle at which time carbon in the medium is depleted (Bender *et al* 1985). From the results of the growth and sucrose analyses the twelfth day of the culture cycle was determined to be the optimal time for screening for photosynthetic activity.

### **3.5.2 An Analysis Of The Effects Of Fructose Concentration In The Medium On Growth, Photosynthesis, Respiration And Nutrient Uptake Of Suspension Cultured Cells Of *N.Tabacum***

The growth, photosynthetic and respiratory characteristics and uptake kinetics of several major nutrients were studied in two treatments. In one treatment MSS medium was supplemented with 3% fructose and the cultures grown in the dark, while in the other MSS medium was supplemented with 1% fructose and the cultures grown in the light. The aim was to determine the effect of fructose concentration in the medium on the growth of the cells, and in the treatment with a reduced carbon source to assess the importance of photosynthetic carbon fixation.

At the start of the experiment 48 conical flasks (250ml) each containing 50ml of MSS medium with 3% (w/v) fructose and 48 flasks containing MSS medium with 1% fructose were inoculated with 2.5–3.0g wet weight of cells. These were taken from a stock cell suspension, which was the result of pooling the contents <sup>of</sup> five culture flasks, for each treatment. The cultures used for the stock suspension were taken 20 days after the previous subculture and had been grown for the previous three culture cycles under the same conditions employed in the experiment. Those cultures in medium supplemented with 3% fructose were incubated in the dark while those in medium with 1% fructose were cultured under continuous illumination of 80–90  $\mu\text{E m}^{-2} \text{sec}^{-1}$ . Three flasks, per treatment, were harvested every two days for the first 18 days and every 3–5 days thereafter until the end of the experiment.

### **3.5.2.1 Growth, photosynthetic and respiratory characteristics of cultures supplemented with either 1% or 3% fructose.**

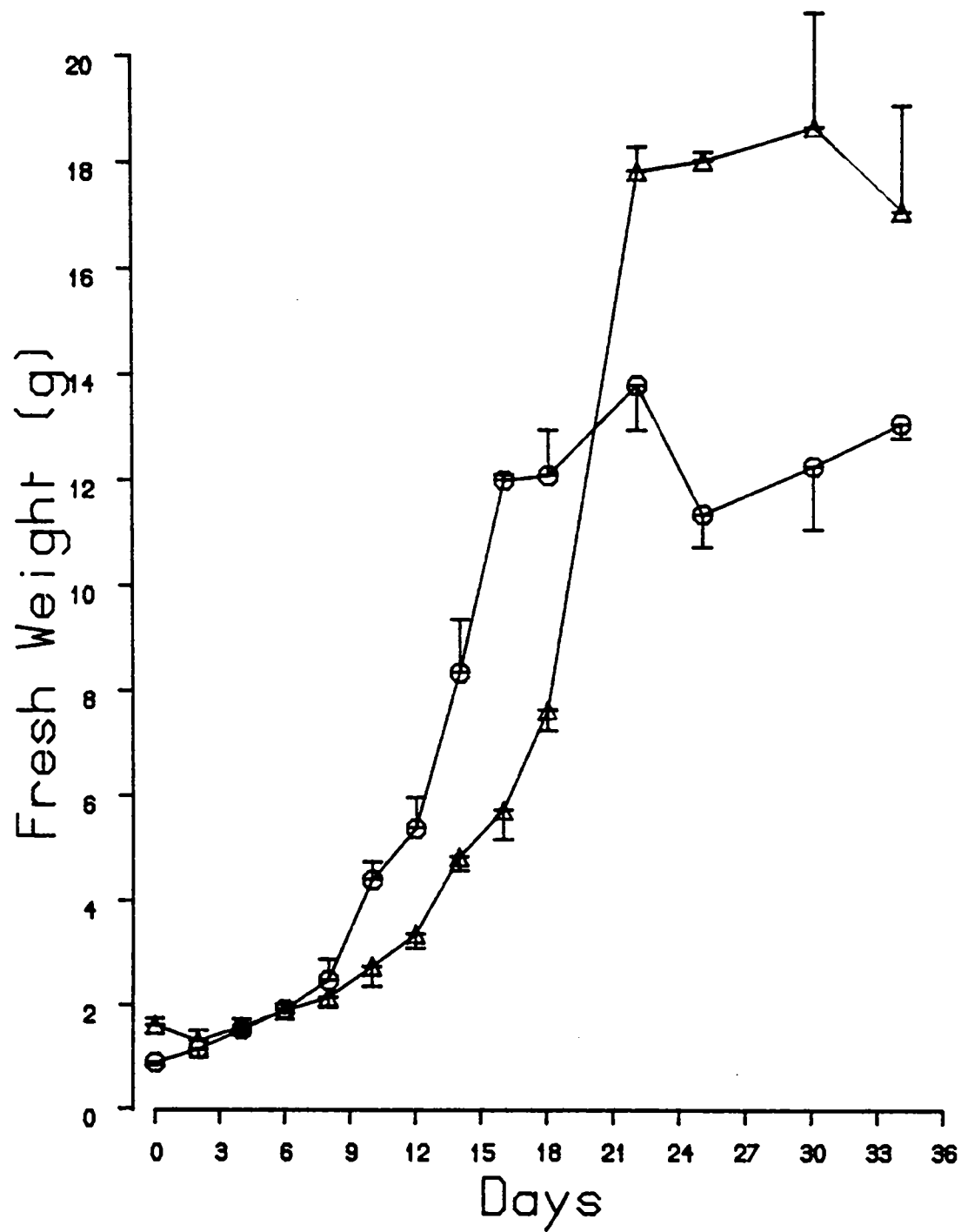
#### **i. The Growth Of Cells With Different Levels Of Fructose.**

The growth of the cultures was measured by following changes in the weight of cells separated from the medium by vacuum filtration (fresh weight). At inoculation there was no significant difference between the mean inoculation weight for each treatment (2.72  $\pm$  0.04 g and 2.63  $\pm$  0.04 g for 1% and 3% fructose treatments respectively). Between inoculation and day 8 there was no significant difference between the fresh weight of the two treatments (Fig 3.5.3). During this period, in the treatment with 3% fructose, there was a lag phase from day 0 to day 2, during which there was no increase in fresh weight. Logarithmic growth occurred between days 0 and 9 in the treatment with 1% fructose and between days 2 and 16 in the 3% fructose treatment (Fig 3.5.4). During the period of logarithmic increase in fresh weight the doubling times were 5.4 days and 6.5 days for the 1% and 3% fructose treatments respectively. In both treatments logarithmic growth was preceded by linear growth which continued until the stationary phase was reached. The maximum level of biomass was reached by day 22 in both culture-lines, with a significantly higher value for the 3% fructose treatment, and in neither treatment was there any significant difference between that time and the end of the experiment.

#### **ii. Respiratory And Photosynthetic Characteristics Of Cultures Grown In Medium Supplemented With Either 1% Or 3% Fructose.**

##### **a. Respiratory rates.**

Immediately after inoculation a significant difference was obtained between the respiration rates of the two treatments, with the rate in the 1% fructose treatment being much higher (Fig 3.5.5). Both treatments showed an increase in respiration rate in the early stages of the culture period, with a greater increase in the 3% fructose treatment. Maximum respiration rates of 77  $\pm$  8  $\mu$ mol O<sub>2</sub> min<sup>-1</sup> g<sup>-1</sup> and 104  $\pm$  15  $\mu$ mol O<sub>2</sub> min<sup>-1</sup> g<sup>-1</sup> on days



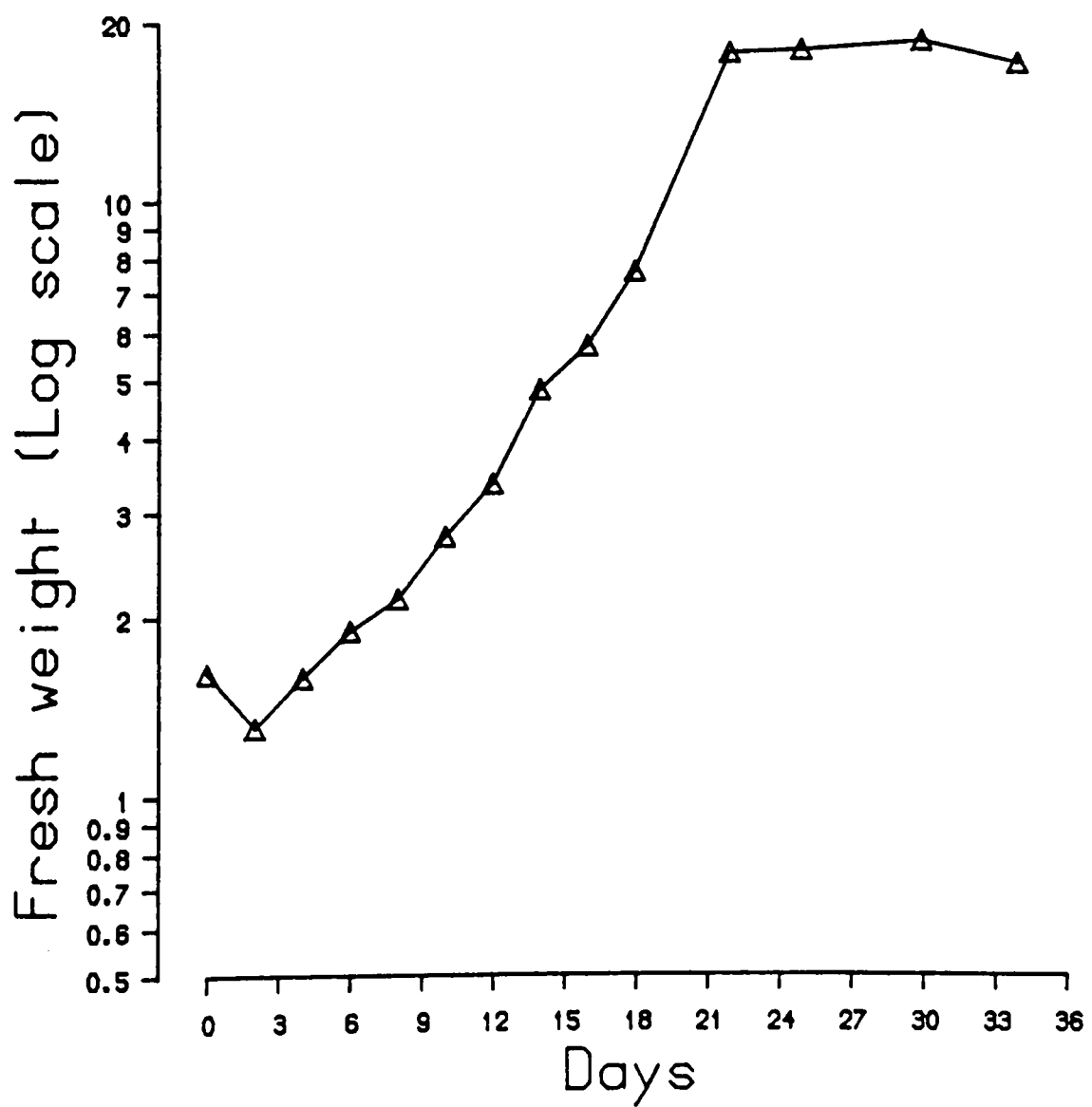
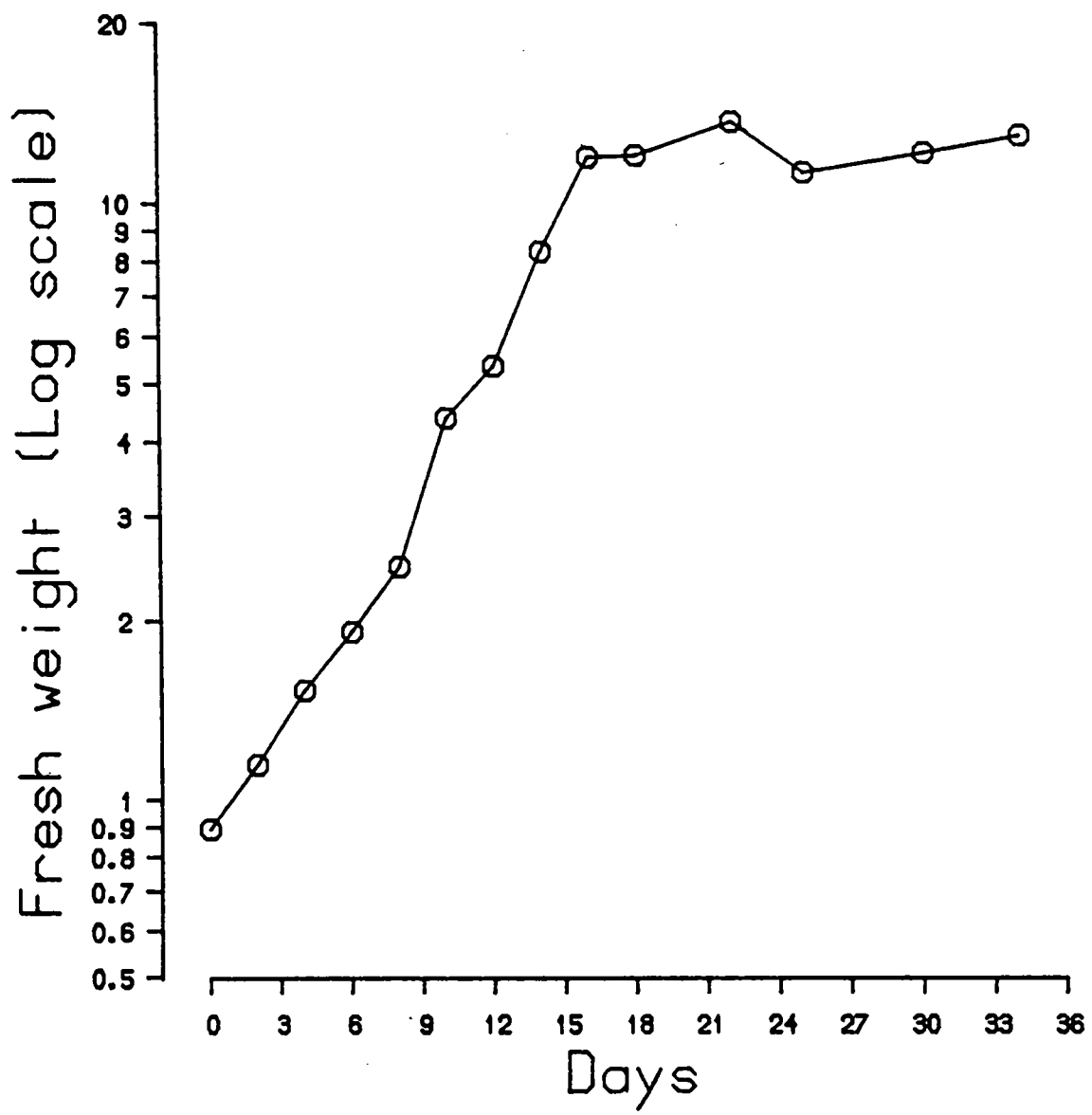
○ 1% Fructose Treatment.  
 △ 3% Fructose Treatment.

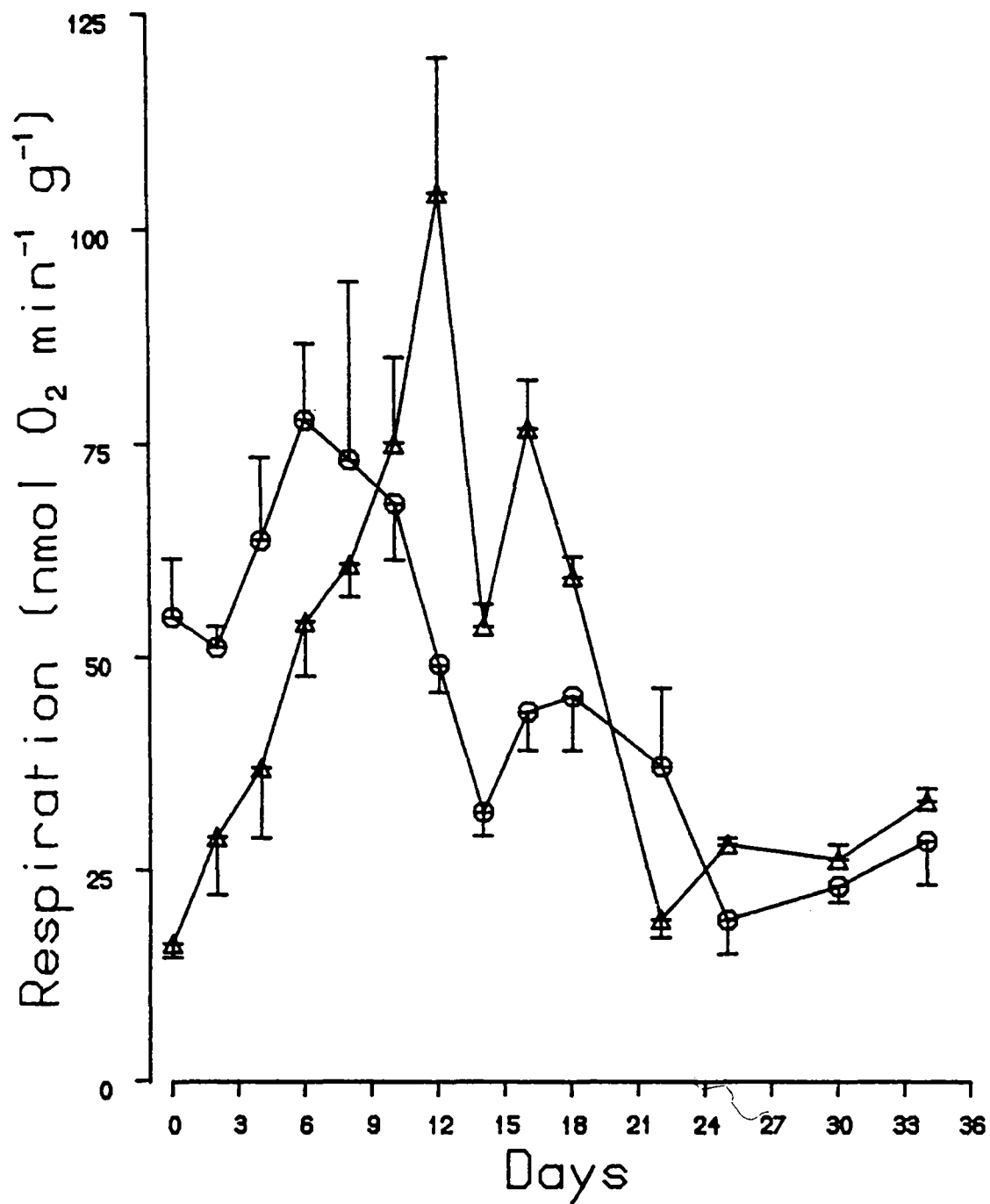
FIGURE 3.5.3

Changes in fresh weight during a culture cycle of cells of *N.tabacum* grown in suspension culture. MSS medium was supplemented with either 3% or 1% fructose. Each point represents the mean +/- the standard error of three replicates.

**FIGURE 3.5.4**

Plot of fresh weight on a log scale against time after inoculation for suspension cultured cells of *N.tabacum* grown in MSS medium supplemented with either 3% (a) or 1% (b) fructose.





○ 1% Fructose Treatment.  
 △ 3% Fructose Treatment.

FIGURE 3.5.5

Changes in the respiration rates of cells of *N. tabacum*, over a 36 day period, grown in medium supplemented with either 3% or 1% fructose. Respiration rates were measured by determining oxygen uptake by cells in the dark using an oxygen electrode. Each point represents the mean +/- the standard error of three replicates.

6 and 12 were attained by the 1% and 3% treatments respectively. Having reached the maximum there was an overall decline in respiration rates in both treatments until day 25. Between days 25 and 34 there were no significant differences within treatments and from day 30 to day 34 there was no significant difference between treatments.

b. Net and gross photosynthetic rates and photosynthetic competence in cultures supplemented with 1% fructose.

In cultures grown in the dark with 3% fructose in the medium there was no detectable photosynthetic rate throughout the experimental period. *However, cultures grown in the light with 1% fructose in the medium were photosynthetic.*

After inoculation there was no change in net photosynthesis until after day 2 (Fig 3.5.6). Between days 2 and 10 there was an overall increase in net photosynthesis giving a maximum at day 10. After day 10 there was a decrease until day 14 then the net photosynthetic rate increased before becoming constant between days 16 and 22. From day 22 to day 25 there was a decrease in net photosynthesis which was followed by a slight increase to day 30 after which there was no significant change.

At day 0 gross photosynthesis was substantially below compensation point and remained so, without any significant variation, until day 8 (Fig 3.5.6). Between days 8 and 10 the gross photosynthetic rate rose from its initial level to just above the compensation point. Subsequently there was no significant deviation from the compensation point between days 10 and 22 after which there was a significant increase until day 25. Between day 25 and the end of the time course the gross photosynthetic rate decreased to the compensation point.

Photosynthetic competence was constant for the first 2 days after inoculation then increased slightly, although not significantly above the value for day 0 (Table 3.5.1). Between days 8 and 10 photosynthetic competence increased to above 1 (the value above which there is a positive carbon balance). From day 10 to day 18 the value fluctuated around 1 and then



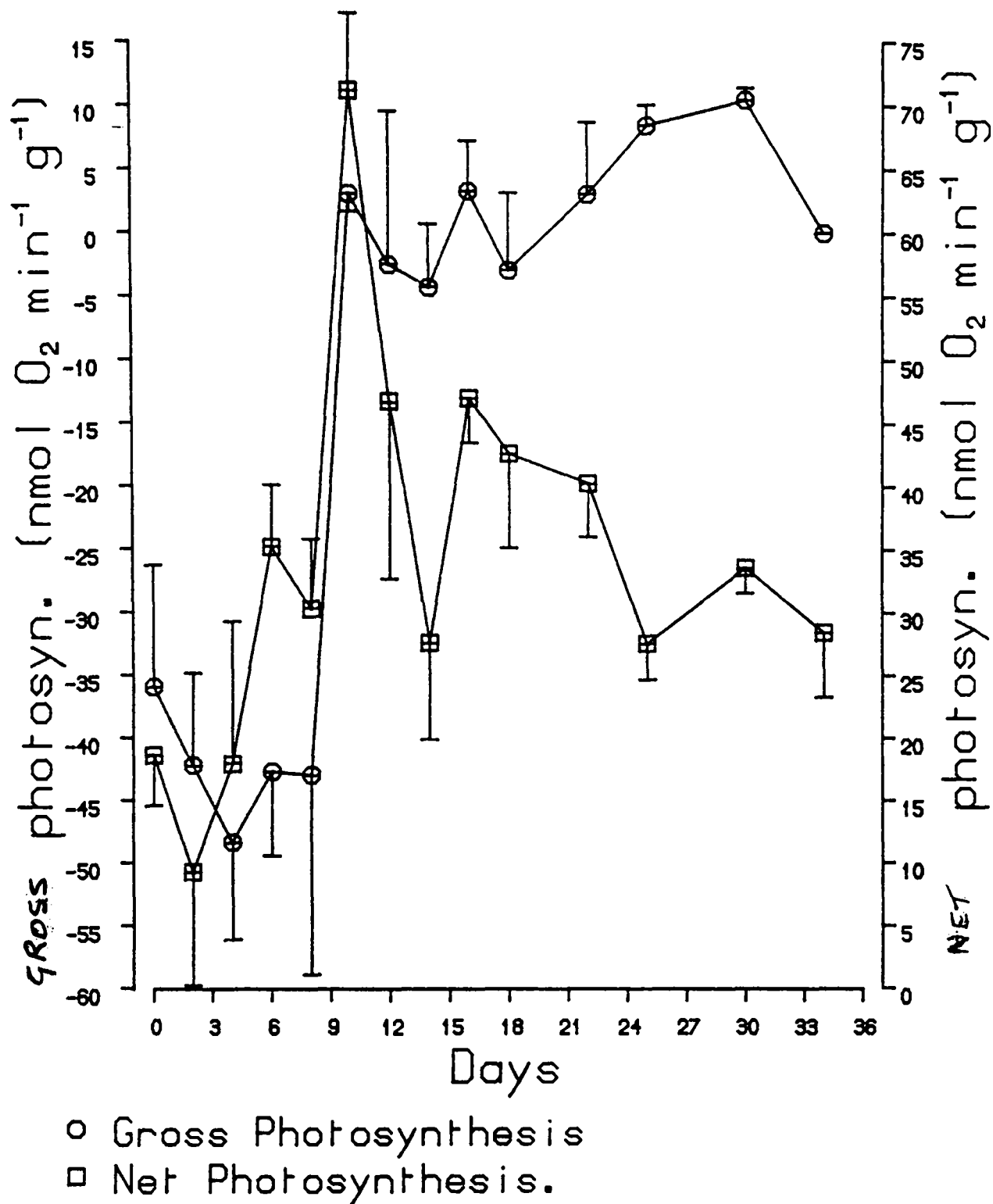


FIGURE 3.5.6

Changes in the rates of photosynthesis (net and gross rates) during a culture cycle of *N. tabacum* cells grown in suspension culture in MSS medium containing 1% fructose. Photosynthetic rates were measured by determining the amount of oxygen evolution from cells in the light using an oxygen electrode. Net photosynthetic rates were the actual rates of photosynthesis that were determined while gross photosynthetic rates took oxygen uptake, due to respiration, into account. Each point represents the mean  $\pm$  the standard error of three replicates.

DAYS AFTER INOCULATION	PHOTOSYNTHETIC COMPETENCE	
	$\bar{X}$	SE
0	0.37	0.11
2	0.16	0.17
4	0.28	0.13
6	0.45	0.04
8	0.45	0.05
10	1.05	0.03
12	0.92	0.26
14	0.82	0.17
16	1.10	0.09
18	0.96	0.15
22	1.30	0.29
25	1.59	0.23
30	1.47	0.06
34	1.00	-

TABLE 3.5.1

Changes in photosynthetic competence, over a 34 day period, of suspension cultured cells of *N.tabacum* in MSS medium supplemented with 1% fructose and maintained in the light. The photosynthetic competence was calculated as the ratio of the rate of respiration to gross photosynthesis. The mean and standard error at each sampling time were calculated from the results from three replicates.

increased to a maximum of  $1.59 \pm 0.23$  on day 25. Subsequent to day 25 there was an overall decline in photosynthetic competence until the end of the analysis.

c. Chlorophyll content of cultures over a 34 day period when grown with 1% fructose in the medium.

In the treatment with 3% fructose there was no detectable level of chlorophyll throughout the time course.

During the first 8 days of the growth cycle there were no significant changes in the chlorophyll content (Fig 3.5.7). There was a significant decrease in chlorophyll content between days 8 and 14, but the value at day 14 was not significantly different from the value at inoculation. From day 14 there was an increase in chlorophyll content until day 30 when a maximum of  $22.0 \pm 1.8$   $\mu\text{g chlorophyll g}^{-1}$  fr wt was attained. After day 30 there was a slight decrease in chlorophyll content until the end of the analysis.

The effect of fructose concentration in the medium and the effect of light on chlorophyll accumulation can be seen from Fig 3.5.8 (a b c). It can be seen that the culture grown in medium supplemented with 3% fructose and maintained in the dark was non-chlorophyllous and a dark brown in colour. The cells grown in the two treatments in the light both contained chlorophyll, with the treatment in medium containing 1% fructose being the greener of the two. The cultures grown in medium supplemented with 1% fructose became more aggregated than the cultures grown with 3% fructose in the medium.

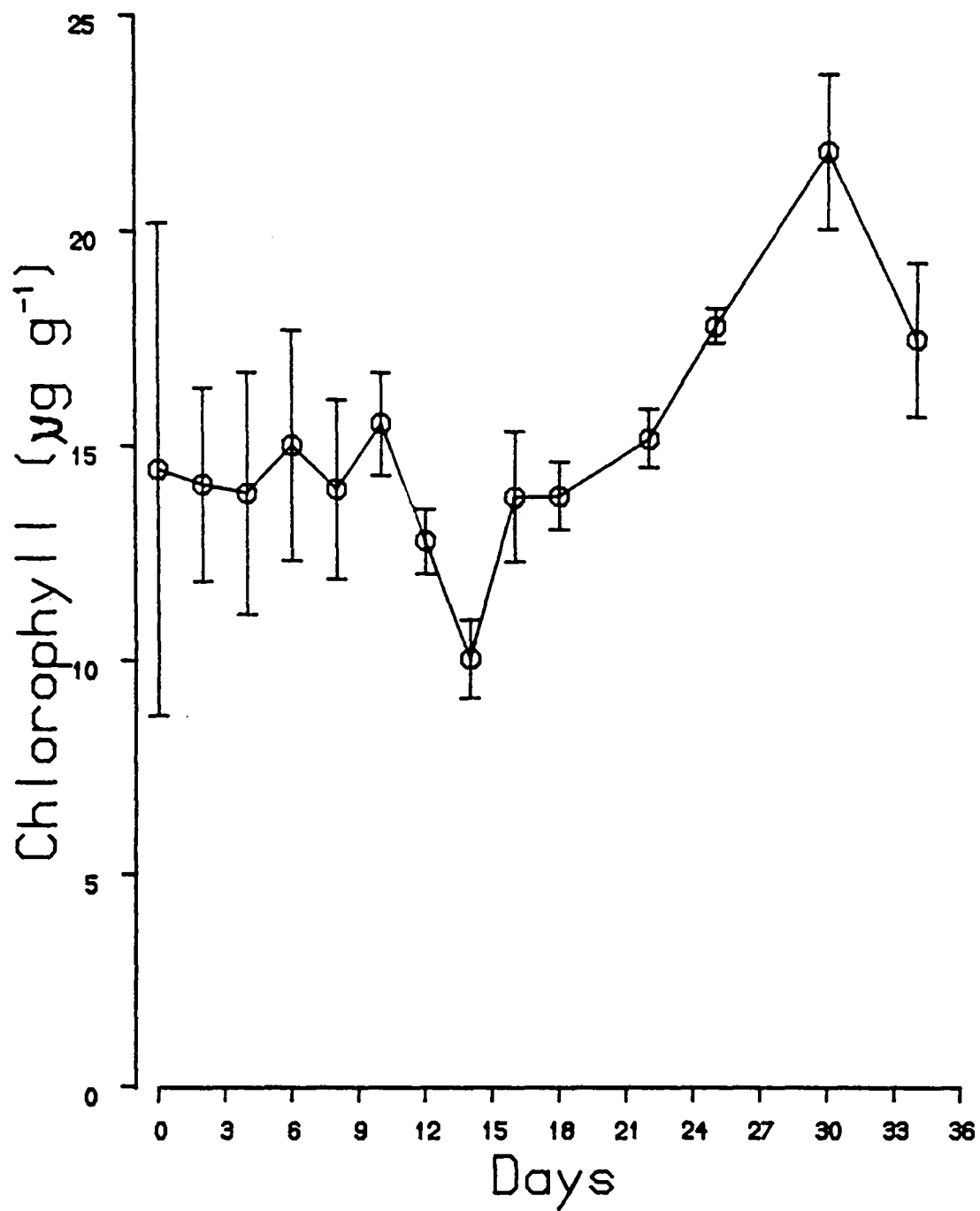


FIGURE 3.5.7

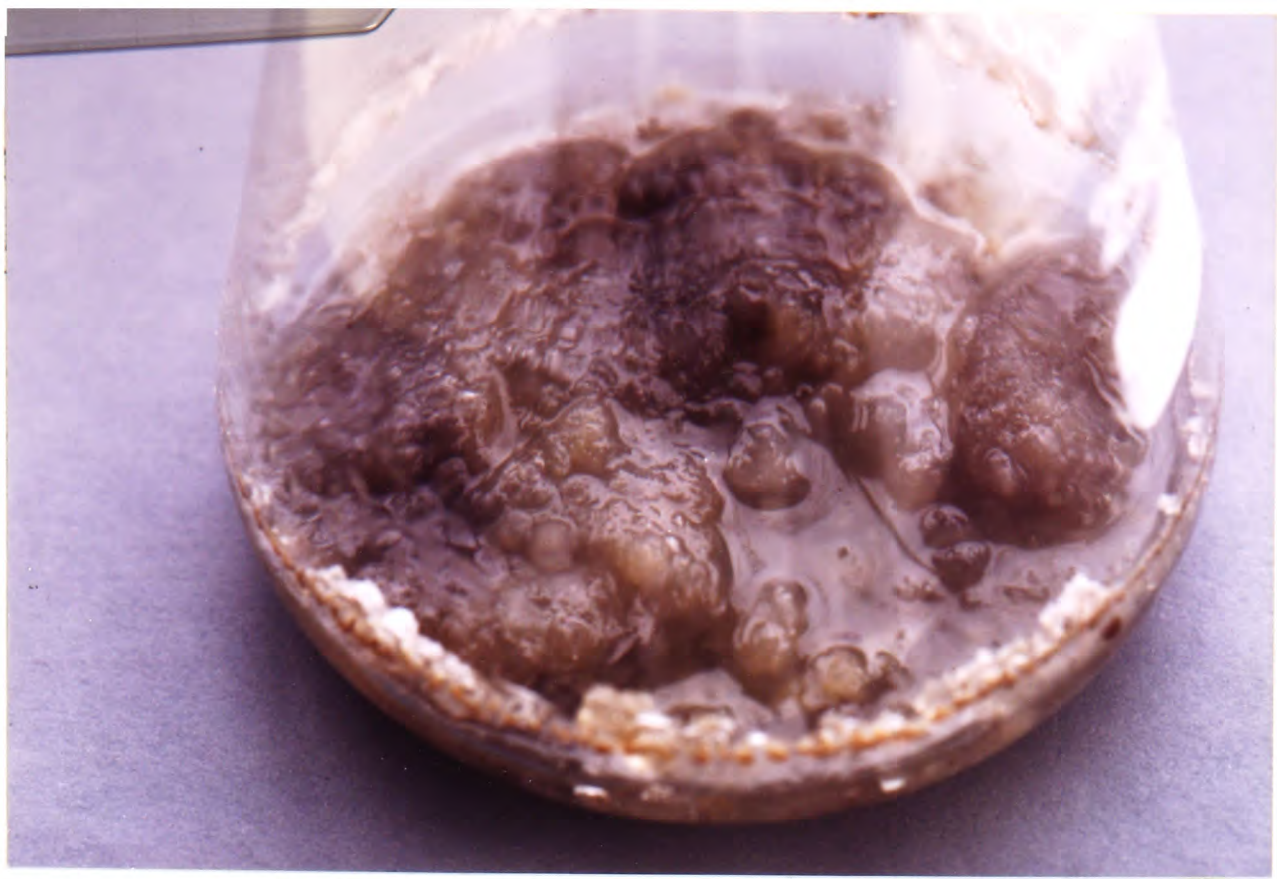
Changes in the chlorophyll content of cells of *N. tabacum* grown in MSS medium supplemented with 1% fructose during a culture cycle. Each point represents the mean +/- the standard error of three replicates.

**FIGURE 3.5.8**

The appearance of cultures 22 days after inoculation grown with different levels of fructose in their medium and with or without illumination.

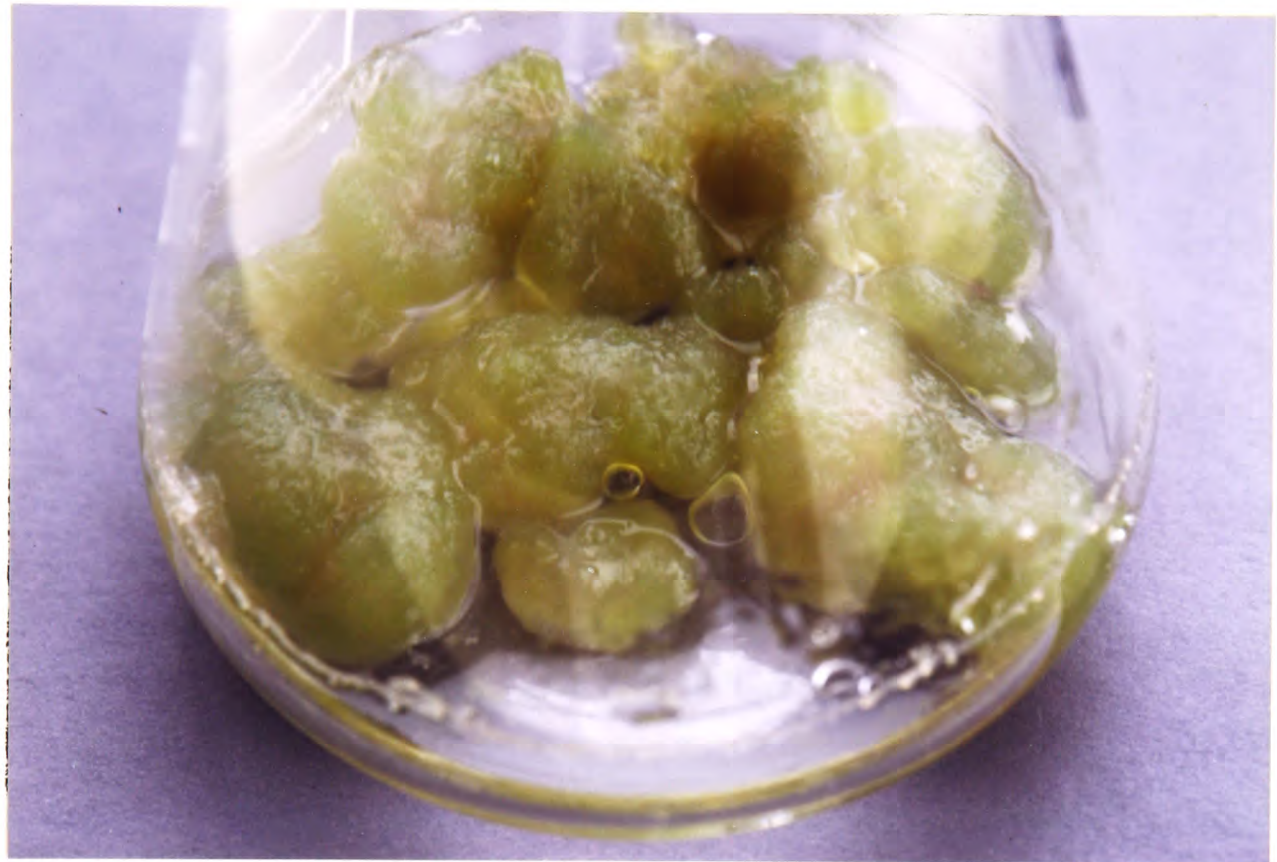
- a. Grown in the dark in MSS medium supplemented with 3% fructose.
- b. Grown in the light in MSS medium supplemented with 3% fructose.
- c. Grown in the light in MSS medium supplemented with 1% fructose.

a



1 cm

b



1 cm

c



1 cm

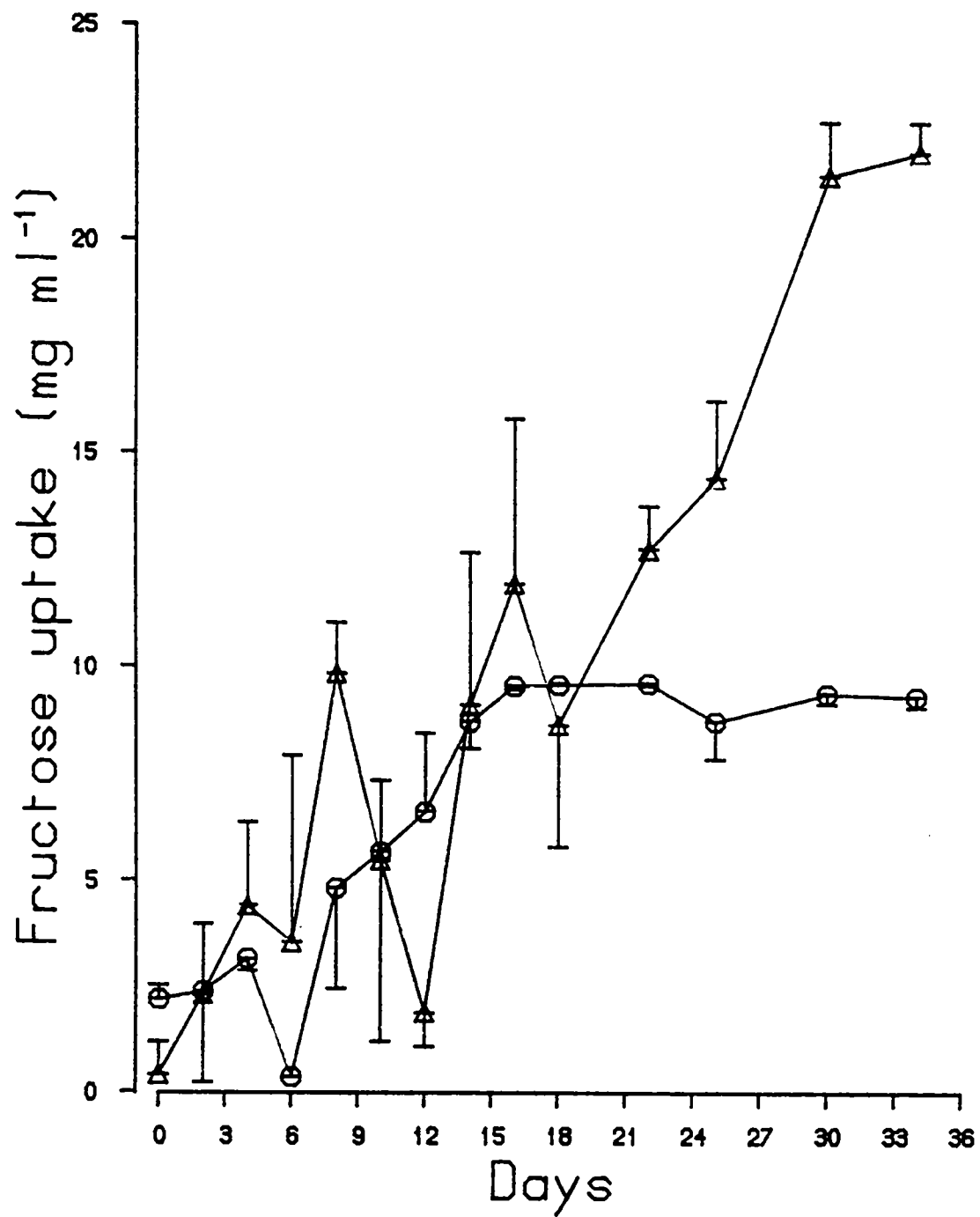


### **3.5.2.2 Nutrient uptake by cells supplemented with either 1% or 3% fructose.**

The uptake of four major nutrients (fructose, phosphate, ammonium and nitrate) by cells grown in MSS medium containing either 10,000 or 30,000  $\mu\text{g ml}^{-1}$  fructose (1% and 3% respectively) was followed throughout the experiment. In the medium at the start of the experiment there were 118.6  $\mu\text{g ml}^{-1}$  of phosphate (from potassium phosphate), 361  $\mu\text{g ml}^{-1}$  of ammonium (from ammonium nitrate), and 2,420  $\mu\text{g ml}^{-1}$  of nitrate (from ammonium nitrate and potassium nitrate). There were also small amounts of other nitrogen containing compounds in the medium at the start of the analysis but these have not been included in the calculations since they would not have made a significant contribution to the total nitrogen utilization figures obtained. The aim of the analysis was to establish whether or not one or more of the nutrients was limiting growth and also, to determine any changes which occurred in the utilization of nutrients between the two fructose treatments.

#### **i. Fructose Uptake By Cells From Medium Containing Either 1% Or 3% Fructose At The Start Of The Experiment.**

There was no significant uptake of fructose between day 0 and day 4 in either treatment, and this continued until day 6 in the treatment with 3% fructose (Fig 3.5.9). However, in medium supplemented with 1% fructose there was a significant uptake between inoculation and the time of the first sample. In the 1% fructose treatment there was an increase in fructose in the medium between days 4 and 6 which was followed by a period of uptake by the cells until day 16. Cultures supplemented with 3% fructose showed a significant uptake between days 6 and 8, followed by an increase in fructose in the medium to day 12. From day 12 to day 30 there was an overall uptake of fructose in the 3% fructose treatment such that on day 30 71% of the original 30,000  $\mu\text{g ml}^{-1}$  had been taken up by the cells (Table 3.5.2). In the 1% fructose treatment there was no significant fructose uptake after day 16 of the experiment whereas in the 3% fructose treatment uptake did not cease until day 30.



○ 1% Fructose Treatment.  
 △ 3% Fructose Treatment.

FIGURE 3.5.9

The uptake of fructose by cells of *N.tabacum* from MSS medium initially containing either 3% or 1% fructose. each point represents the mean +/- the standard error of three replicates.



a. Nutrient uptake by cells grown in MSS medium supplemented with 3% fructose and maintained in the dark.

Days after inoculation	Fructose	PERCENTAGE UPTAKE		
		Phosphate	Ammonium	Nitrate
0	1	22	63	-36
2	7	11	59	-18
4	14	39	65	-7
6	11	49	73	-18
8	32	55	73	32
10	18	59	63	0
12	6	89	77	38
14	30	100	70	0
16	40	100	77	42
18	28	100	78	51
22	42	100	84	65
25	48	100	84	100
30	71	100	91	100
34	73	100	93	100

b. Nutrient uptake by cells grown in MSS medium supplemented with 1% fructose and maintained in the light.

Days after inoculation	Fructose	PERCENTAGE UPTAKE		
		Phosphate	Ammonium	Nitrate
0	21	54	17	3
2	23	51	18	-21
4	31	61	20	0
6	3	65	23	-28
8	48	73	9	27
10	56	98	40	3
12	66	80	37	20
14	87	99	51	3
16	96	99	74	44
18	96	97	78	65
22	96	97	75	71
25	87	92	78	29
30	94	86	71	61
34	93	90	78	35

TABLE 3.5.2

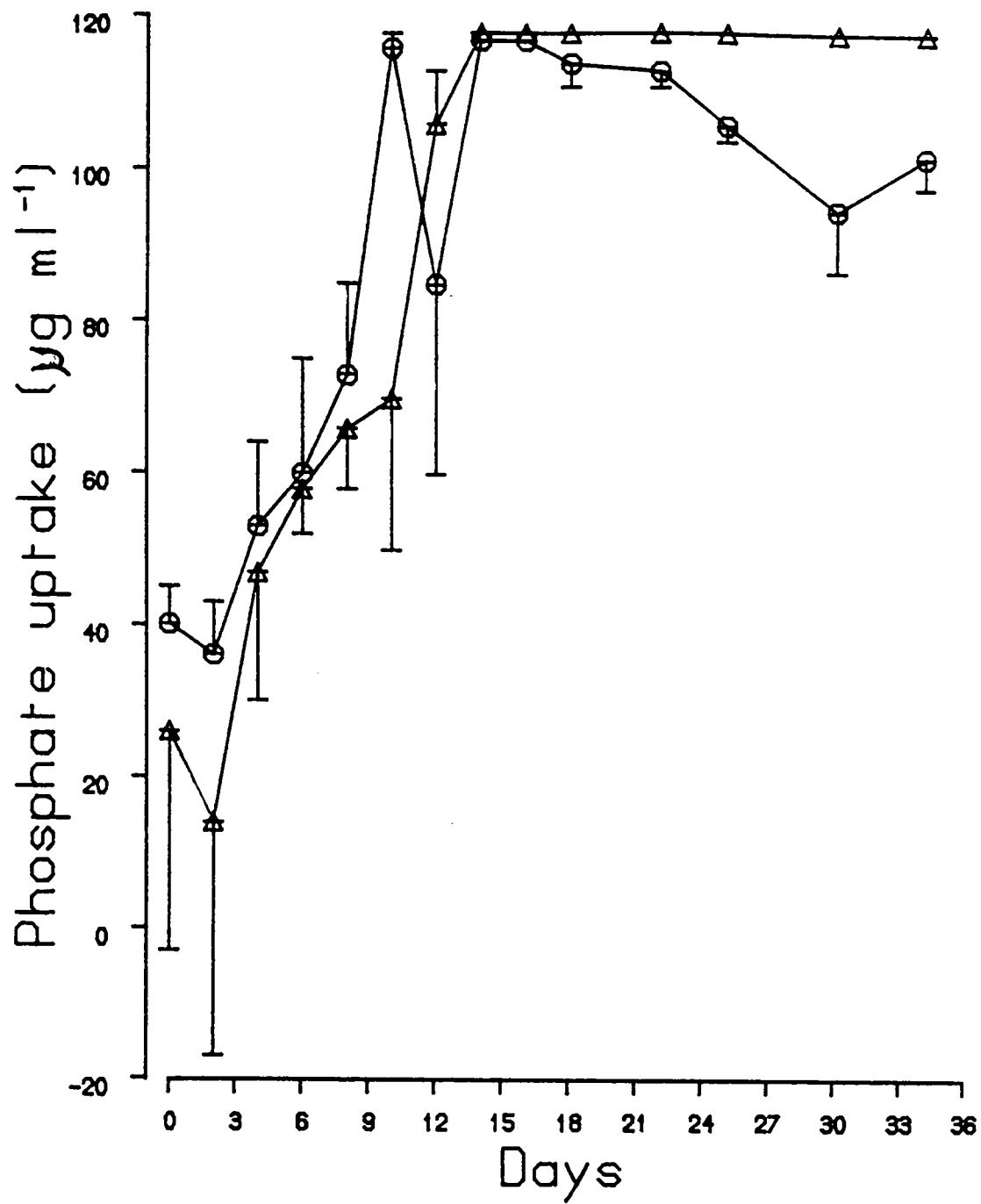
The percentage uptake over a 34 day period of four major nutrients from MSS medium supplemented with either 3% or 1% fructose by cell suspension cultures of *N.tabacum*.

ii. The Uptake Of Phosphate By Cultures Grown In Medium Supplemented With Either 1% Or 3% Fructose.

In both treatments there was a mean uptake of phosphate between inoculation and analysis on day 0, although this was only significant in the 1% fructose treatment (Fig 3.5.10 and Table 3.5.2). Between day 0 and day 4 there was no significant uptake in either treatment and from inoculation until day 14 (with the exception of day 10) there was no significant difference between treatments. From day 4 to day 10 there was significant uptake of phosphate such that by day 10 there had been 98 % and 59 % uptake in 1% and 3% fructose treatments respectively. After day 10 there was a release of phosphate from the cells in the 1% fructose treatment until day 12 which was followed by further uptake to day 14. In both treatments phosphate uptake was complete on day 14 after the start of the experiment and remained so in the 3% fructose treatment. However, in the 1% fructose treatment there was a release of phosphate into the medium from day 16 until day 30 after which there was no change.

iii. Ammonium Uptake By Cells Grown in Medium Supplemented With Either 1% Or 3% Fructose.

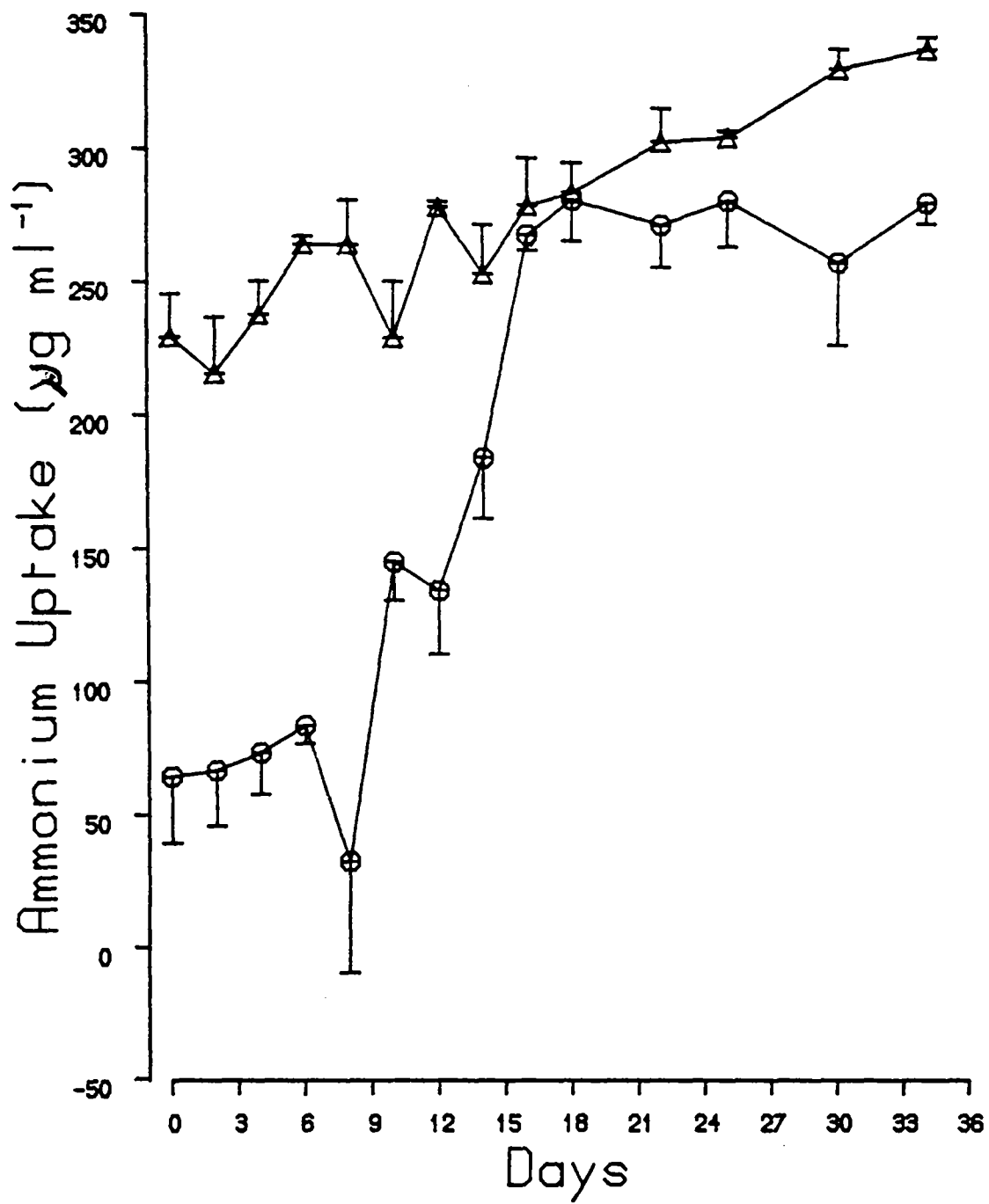
In both treatments there was a significant uptake of ammonium between inoculation and the time of the first sample. In the 1% fructose treatment this uptake was 17 % while in the 3% fructose treatment the initial uptake was 63 % (Table 3.5.2). Between days 0 and 4 there was no further significant change in either treatment, and this continued until day 8 in the 1% fructose treatment (Fig 3.5.11). In the treatment with 3% fructose there was a significant uptake of ammonium between days 4 and 6 after which there was a period of 8 days when there was no significant uptake. From day 14 to day 34 there was a significant ammonium uptake in the 3% fructose treatment so that at the end of the time course 93 % of the ammonium present at inoculation had been taken up by the cells. In the treatment supplemented with 1%



○ 1% Fructose Treatment  
 △ 3% Fructose Treatment

FIGURE 3.5.10

The uptake of phosphate by suspension cultured cells of *N. tabacum* cultured in MSS medium supplemented with either 3% or 1% fructose. Each point represents the mean +/- the standard error of three replicates.



○ 1% Fructose Treatment.  
 △ 3% Fructose Treatment.

FIGURE 3.5.11

Ammonium uptake from MSS medium supplemented with either 3% or 1% fructose by suspension cultured cells of *N.tabacum* each point represents the mean +/- the standard error of three replicates.

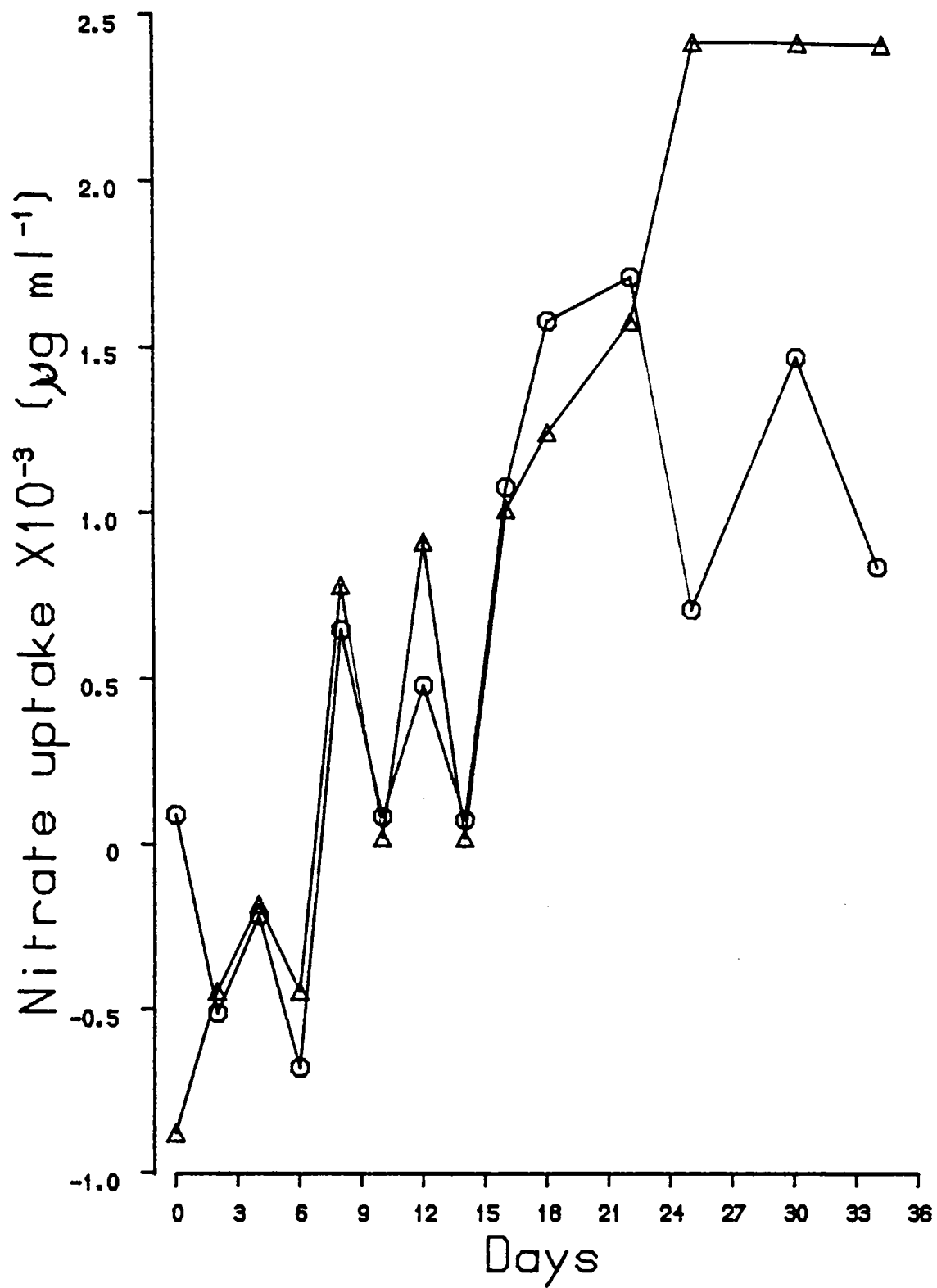
fructose there was significant ammonium uptake between days 8 and 10 and again from day 12 to day 18. There was no significant uptake of ammonium in the latter treatment between day 18 and the end of the analysis when 78 % of the original ammonium had been removed from the medium by the cells.

iv. Nitrate Uptake By Cells Grown In Medium Supplemented With Either 1% Or 3% Fructose.

Due to the contamination of some of the samples it was not possible to analyse 3 replicates for each sampling time, therefore the points on the graph (Fig 3.5.12) represent either the mean of 2 replicates or the value for 1 sample. For this reason the graphs obtained may show greater fluctuations in values at successive sampling times.

In the 3% fructose treatment there was an overall uptake of nitrate between days 0 and 25 (Fig 3.5.12). By day 25 there had been 100% uptake of the available nitrate (Table 3.5.2) in the 3% fructose treatment and this remained the case until the end of the analysis. There was also an overall uptake in the 1% fructose treatment between days 0 and 22 such that on day 22 there had been a 71 % uptake. From day 22 to day 34 there was a release of nitrate into the medium from cells grown in medium with 1% fructose.

From the results of this experiment it was concluded that the culture-line grown with 3% fructose in the medium was heterotrophic and that the culture-line with 1% fructose in the medium was mixotrophic. The changes in the chlorophyll fluorescence emissions of the mixotrophic and a heterotrophic culture grown in the light over a culture cycle were examined. This experiment was carried out to determine the amount of influence the fructose concentration in the medium had on photosynthetic development.



○ 1% Fructose Treatment  
 △ 3% Fructose Treatment

FIGURE 3.5.12

The uptake of nitrate over a 36 days by cells of *N. tabacum* grown in MSS medium containing either 3% or 1% fructose.

### **3.5.3 Changes In The Chlorophyll Fluorescence Emissions Of Two Culture-lines Grown In The Light For 31 Days.**

In this experiment changes in the photosynthetic apparatus, as estimated by chlorophyll fluorescence, were examined over a 31 day period in two suspension culture-lines. One of the lines was mixotrophic in its mode of growth while the other was heterotrophic but grown in the light, rather than the dark. At the start of the experiment 20 flasks (250ml) each containing 50ml of MSS medium with 3% fructose and 20 flasks containing MSS medium with 1% fructose were inoculated with 1.5–2.0g wet weight of cells. These were taken from a stock cell suspension, which was the result of pooling the contents of five culture flasks per treatment 27 days after the previous subculture, having been grown for five subcultures under the conditions employed during the experiment. The cultures in both treatments were incubated under constant illumination of  $80\text{--}90\mu\text{E m}^{-2}\text{ sec}^{-1}$ . Initially cultures were harvested once every five days and then every seven days after the tenth day of the growth cycle, with one flask and three flasks per treatment being taken at alternate sampling times.

#### **3.5.3.1 Changes in the chlorophyll fluorescence profiles of two suspension culture-lines grown in MSS medium containing either 1% or 3% fructose over a 31 day period.**

In the previous experiment chlorophyll content was found to change during the culture cycle therefore it was important to know whether there were corresponding changes in fluorescence profiles from the cells during the culture cycle. In order to investigate this two culture-lines were sampled over a 31 day period.

i. Changes over a 31 day period in the photosynthetic ability of cells cultured in MSS medium containing 3% fructose.

At inoculation the cells had the fluorescence profile seen in Fig 3.5.13a detected at a time-base of  $0.5\text{sec cm}^{-1}$ . When illuminated, after 5min in the dark followed by a 2.5sec flash of light, there was an instantaneous rise in fluorescence before a decline which in turn was followed by a gradual rise to a maximum. By day 5 of the time-course only a very weak fluorescence signal was detectable (Fig 3.5.13b). On illumination there was rapid increase in fluorescence the rate of which declined until a maximum was reached. The fluorescence signal detected on the seventeenth day of the experiment (Fig 3.5.13c) had a decline in emission after the initial rapid rise and then a secondary more gradual increase to a maximum. The kinetics of the changes in the fluorescence emission were different to those at day 0 and the signal was weaker than on day 0. On day 24 the fluorescence profile recorded (Fig 3.5.13d) had very similar kinetics and a similar profile to the profile recorded on day 0 yet by day 31 the fluorescence signal had lost the dip after the initial rise (Fig 3.5.13e). The fluorescence emission rose rapidly on illumination before the rate of increase in fluorescence declined until a maximum was achieved.

During the experiment there were changes in the size of the primary electron acceptor pool as well as in the fluorescence profiles detected. During the growth cycle the area above the fluorescence curve from  $F_0$  to the maximum, at a time-base of  $10\text{msec cm}^{-1}$  (which indicates the size of the primary electron acceptor pool) decreased before increasing again (Table 3.5.3). At the start of the experiment the value was similar to the value of cells at the same stage in the culture cycle as were the cells used as the inoculum. The area decreased until it was negligible 17 days after inoculation after which it increased to a final value which was approximately twice that at inoculation.



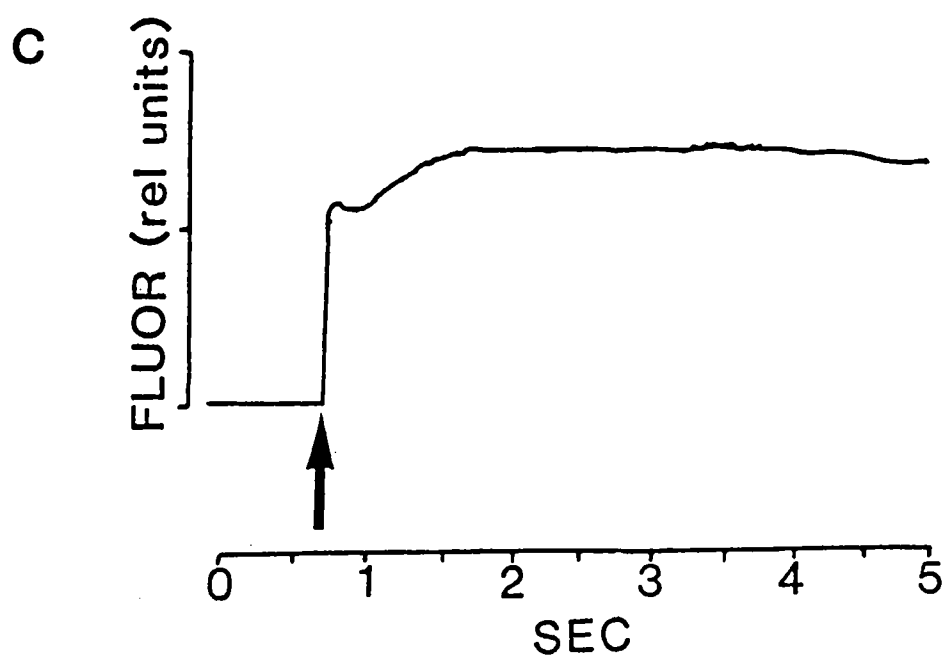
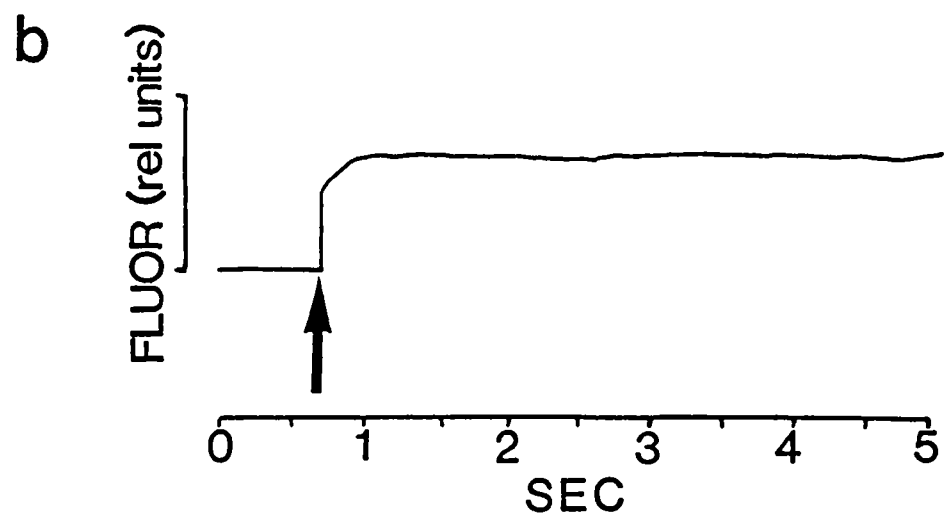
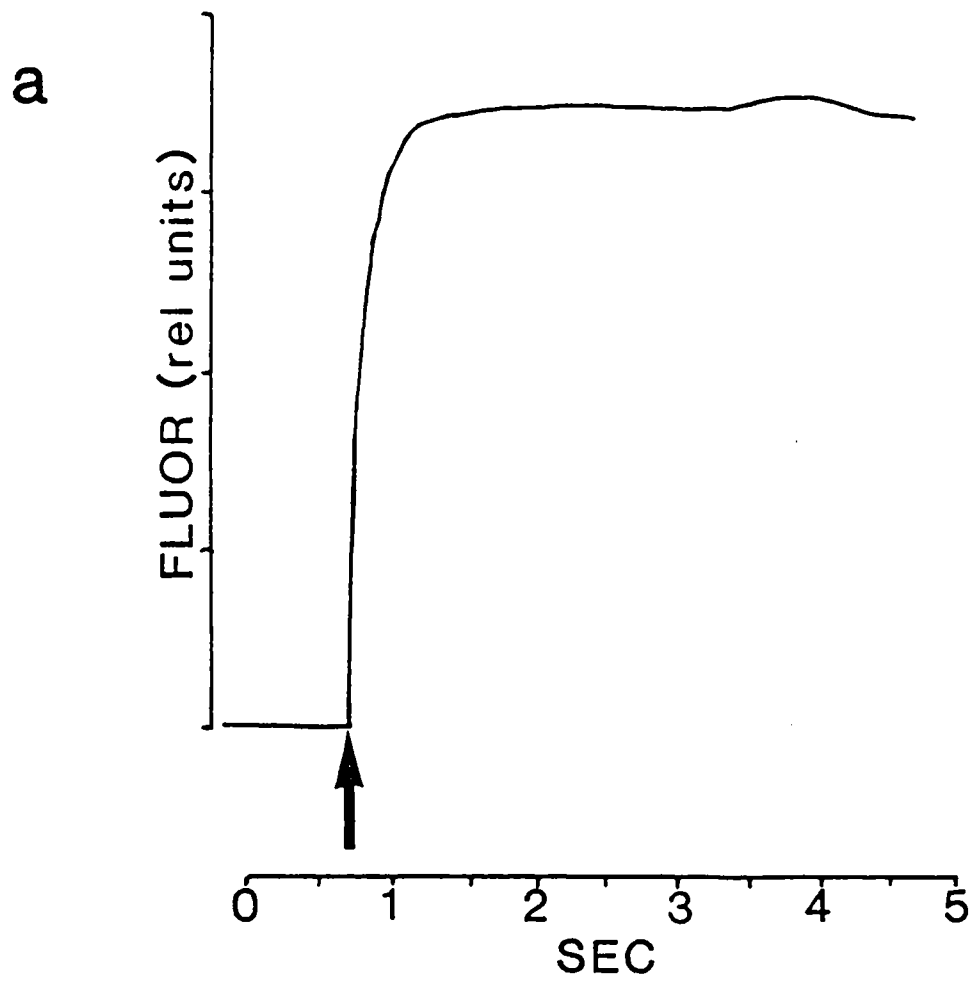
**FIGURE 3.5.13**

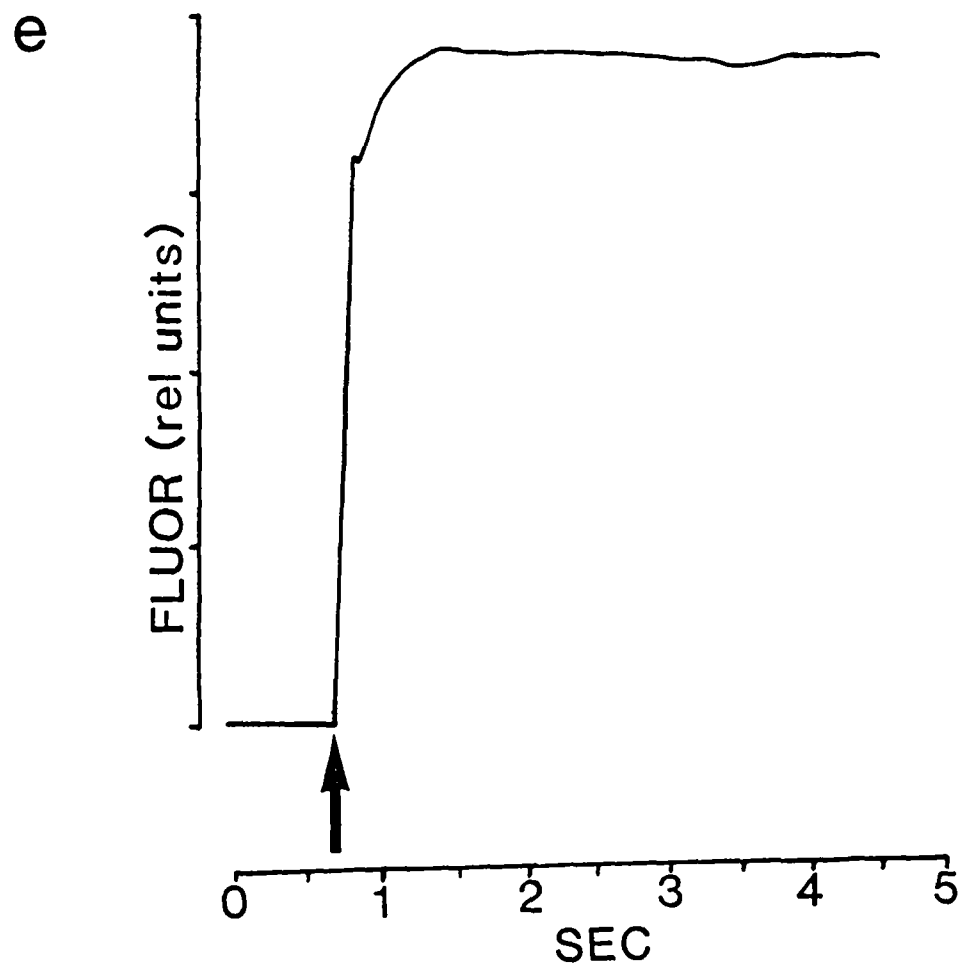
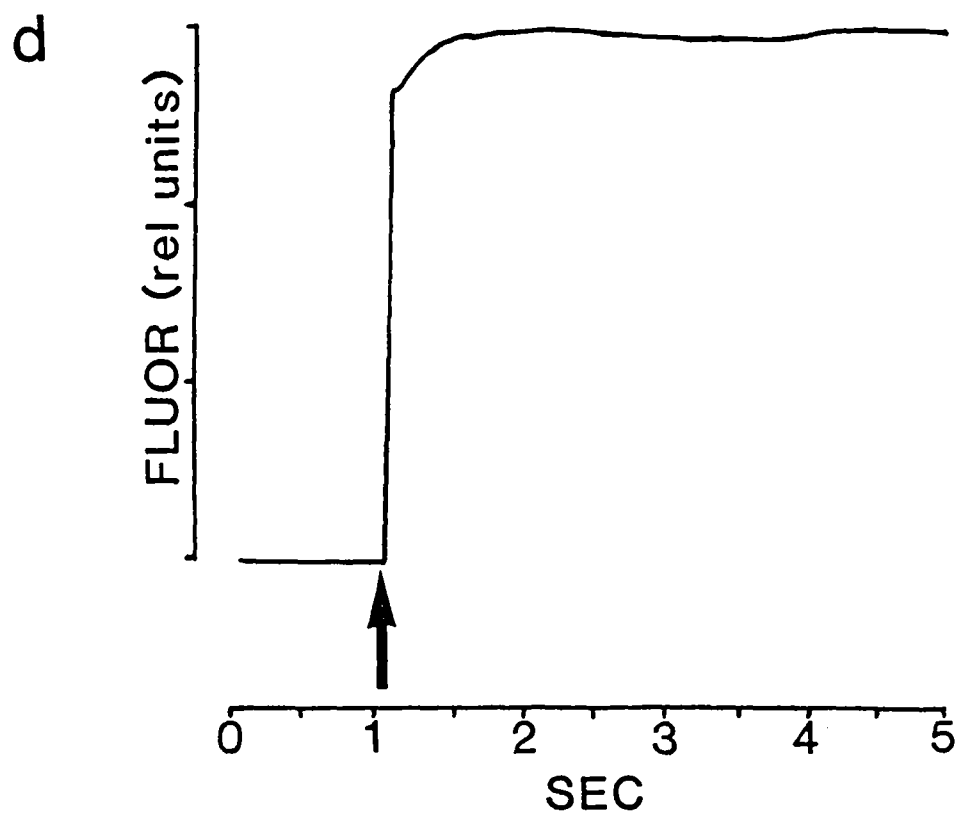
Changes in the chlorophyll fluorescence emissions from cells of *N.tabacum* cultured in MSS medium containing 3% fructose, over a 31 day period. Cells were separated from their medium and blotted dry before being incubated in the dark for 5min. The cells were then given a 2.5sec flash of light and immediately afterwards illuminated at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . The fluorescence emitted was resolved at a time-base of  $0.5\text{sec cm}^{-1}$ .



= Activation of the light source.

- a. The chlorophyll fluorescence profile of the cells used as the inoculum. The signal was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .
- b. The chlorophyll fluorescence profile of cells 5 days after inoculation. The signal was detected at a sensitivity of  $0.1\text{mV cm}^{-1}$ .
- c. The chlorophyll fluorescence emission from cells after 17 days of the culture cycle. The signal was detected at a sensitivity of  $0.1\text{V cm}^{-1}$ .
- d. The chlorophyll fluorescence of cells 24 days after subculture. The signal was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .
- e. The chlorophyll fluorescence profile from cells after 31 days growth in fresh medium. The signal was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .





DAYS AFTER INOCULATION	AREA ABOVE CURVE (mm <sup>2</sup> )	
	3% FRUCTOSE	1% FRUCTOSE
0	465	476
5	334	34
17	139	76
24	350	161
31	800	725

TABLE 3.5.3

Changes in the size of the photosynthetic primary electron acceptor pool, as measured by the area above the fluorescence curve from  $F_0$  to the maximum, over a 31 day period. At each sampling time the fluorescence profile at a time-base of 10msec  $\text{cm}^{-1}$  was recorded for a sample of approximately 2g of cells after incubation in the dark for the previous 5min.

ii. Changes in the fluorescence characteristics over a 31 day period of cells grown in MSS medium containing 1% fructose.


At inoculation the fluorescence profile detected at a time-base of  $0.5\text{sec cm}^{-1}$  rose instantaneously on illumination (Fig 3.5.14a). After this initial rapid increase there was a drop in fluorescence followed by a second more gradual rise until a maximum was reached. There was an overall decrease, after the maximum was reached, during which there was a slight increase followed by a corresponding decrease. After 5 days of the experiment the fluorescence signal was greatly reduced and consisted of an initial rapid rise which became more gradual until a maximum was reached (Fig 3.5.14b). By day 17 the fluorescence signal had increased and the transient recorded was similar in shape to that recorded on day 0 although the kinetics were slightly different (Fig 3.5.14c). The fluorescence profile recorded on day 24 (Fig 3.5.14d) was nearly identical to that at the start of the experiment. By day 31 the fluorescence profile recorded showed several phases (Fig 3.5.14e). There was an instantaneous rise in fluorescence on illumination of the cells which was followed by a decrease in fluorescence. After the decline in fluorescence there was a second slower increase in fluorescence to a maximum. From the maximum the fluorescence signal declined before leveling off. During this period of steadier emission there was an increase followed by a corresponding decrease in fluorescence.

While the fluorescence profiles were changing the size of the primary electron acceptor pool was also changing. The area above the fluorescence curve at inoculation was very similar to that of cultures grown with 3% fructose in the medium (Table 3.5.3). After inoculation the area decreased until it reached a minimum 17 days after inoculation. After this there was an increase until a maximum was reached on the last day of the experiment.

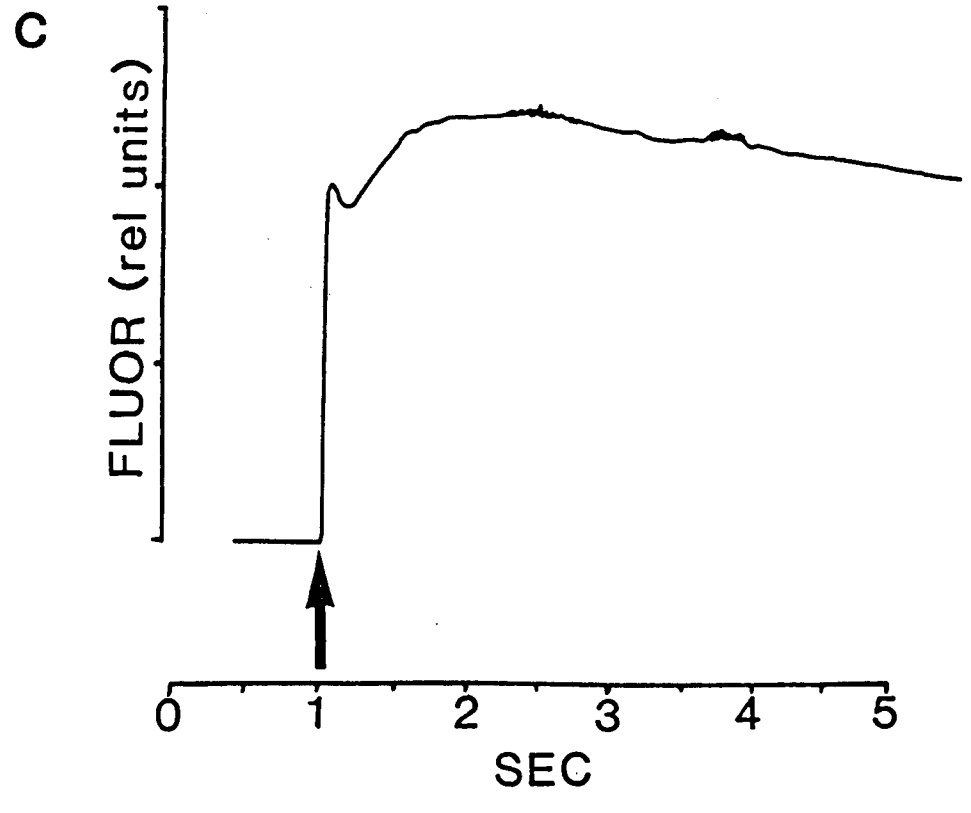
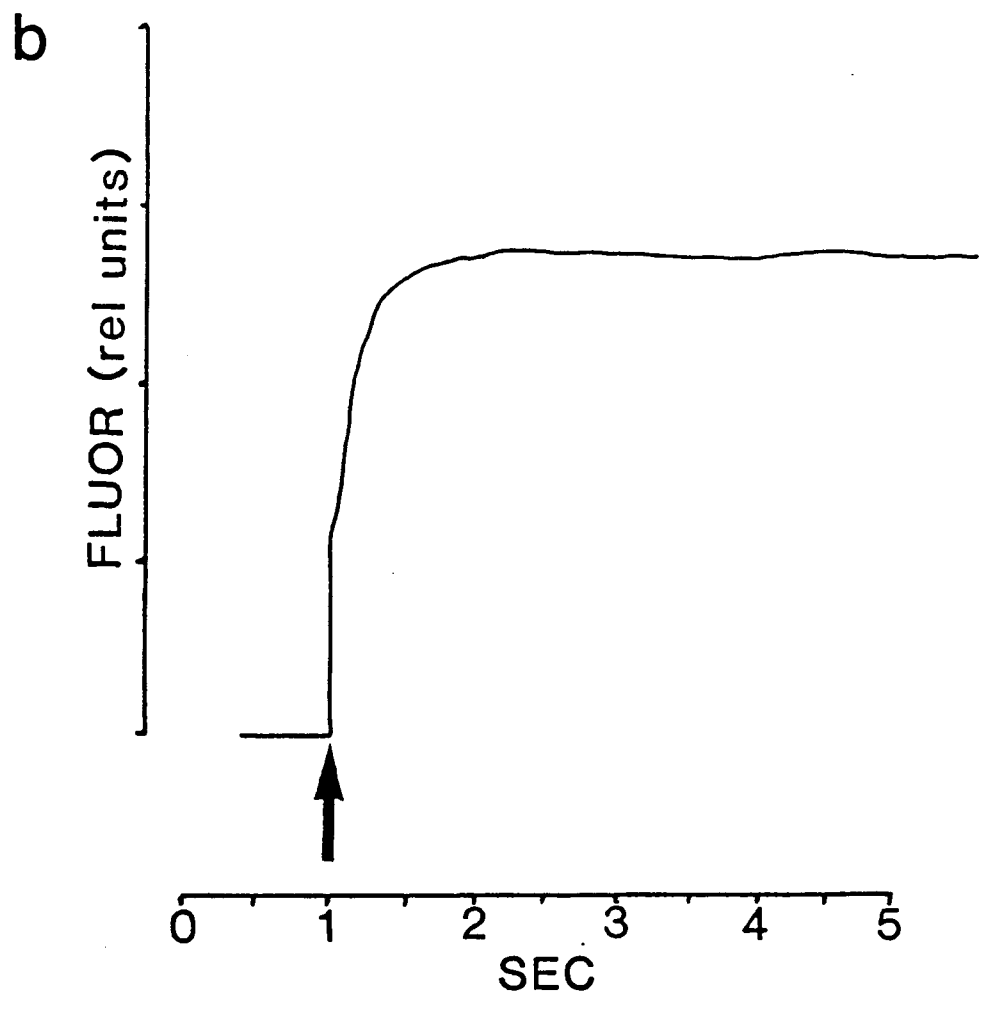
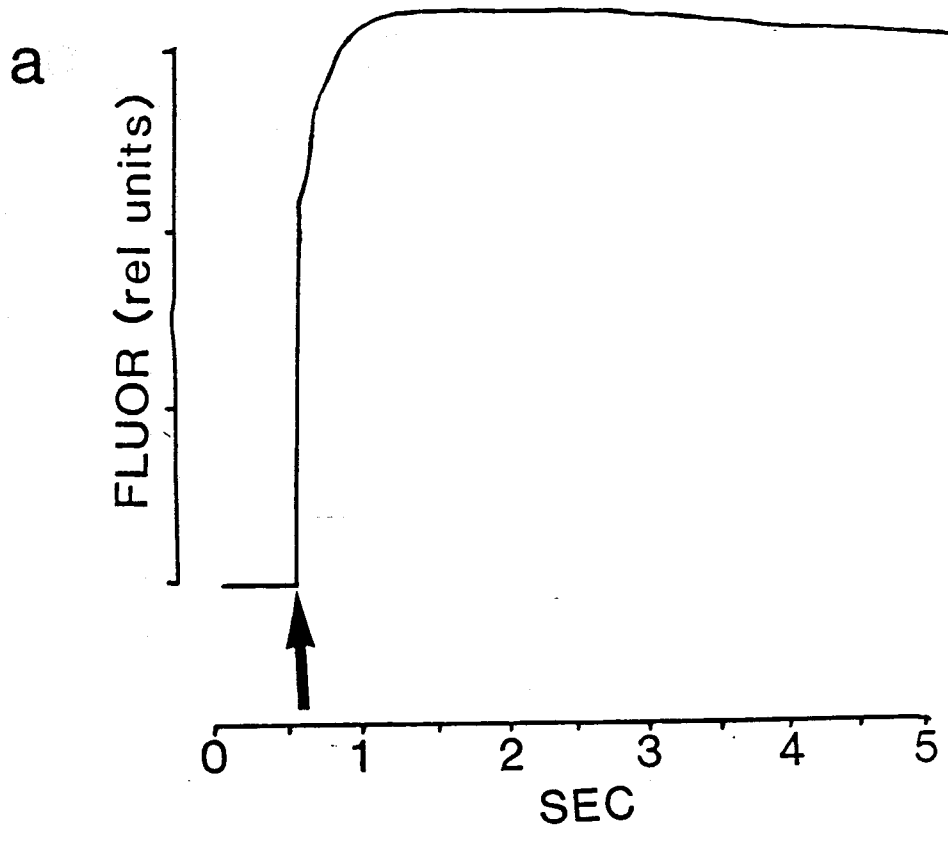
From these results it appeared that there was very little difference in the size of the primary electron acceptor pool between the two culture-lines and that during the growth cycle it changed in the way in both treatments. However, the fluorescence profiles recorded at a time-base of  $0.5\text{sec cm}^{-1}$  showed that the mixotrophic cells had a greater capacity for

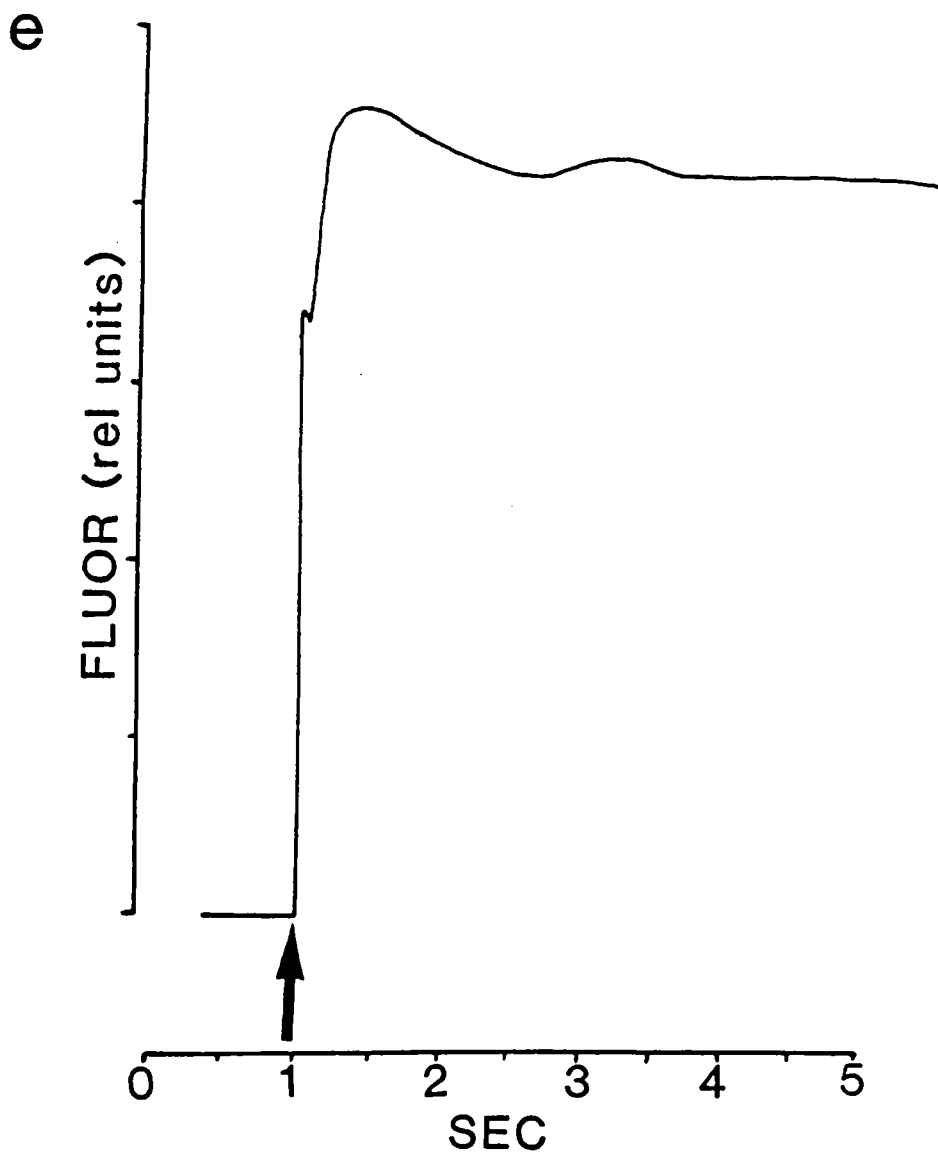
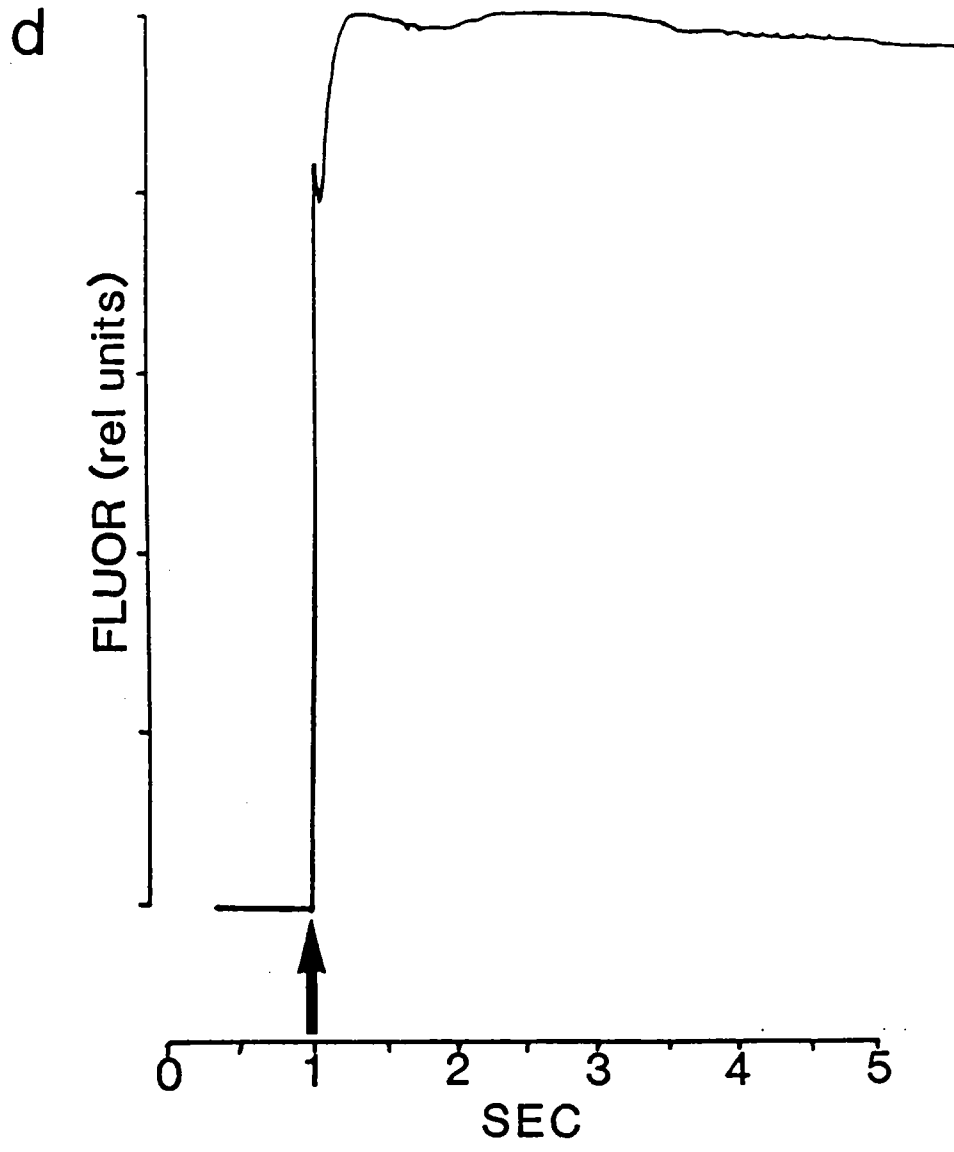
#### FIGURE 3.5.14

The chlorophyll fluorescence emissions from cells cultured in MSS medium containing 1% fructose during a culture cycle. Cells were separated from their medium and blotted dry. They were then incubated in the dark for 5min before being given a 2.5sec flash of light followed by illumination at  $300\mu\text{E m}^{-1} \text{sec}^{-1}$  during which the fluorescence emission was recorded. The fluorescence signal was resolved at a time-base of  $0.5\text{sec cm}^{-1}$ .

 = Activation of the light source.

- a. The chlorophyll fluorescence profile of the cells used as the inoculum. The signal was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .
- b. The chlorophyll fluorescence profile of cells 5 days after subculture. The signal was detected at a sensitivity of  $1\text{mV cm}^{-1}$ .
- c. The chlorophyll fluorescence emission from cells 17days after subculture. The signal was detected at a sensitivity of  $0.1\text{V cm}^{-1}$ .
- d. The chlorophyll fluorescence profile of cells 24 days after the start of the culture cycle. The signal was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .
- e. The chlorophyll fluorescence emission from cells 31 days after subculture. The signal was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .







photosynthetic carbon fixation than did the cultures grown with 3% fructose in the medium. Photosynthesis did not appear to contribute to the growth of the latter cultures. Therefore it was concluded that the culture-line grown with 3% fructose in the medium and cultured in the light was photoheterotrophic in its mode of nutrition.

The chloroplasts were extracted from the cells of 31 day old mixotrophic cultures and the fluorescence emissions from the isolated chloroplasts recorded. The profiles obtained allowed a comparison of the fluorescence characteristics of isolated chloroplasts from leaves and cultured cells.

### **3.5.3.2 The fluorescence profile of chloroplasts isolated from cells grown in MSS medium supplemented with 1% fructose for 31 days.**

After 31 days in culture the fluorescence profiles of cells grown in MSS medium containing 1% fructose were similar to those of tobacco leaf discs. In order to confirm the photosynthetic capacity of the cells chloroplasts were isolated from them and the fluorescence profile of the isolated chloroplasts determined. This enabled a comparison to be made between chloroplasts isolated from cells and those isolated from leaves.

Cells that had been grown for 31 days in MSS medium containing 1% fructose were harvested and the medium removed by filtration under reduced pressure. The contents of five culture vessels were pooled and 50g fresh weight subjected to the chloroplast isolation procedure (Section 2.4.1). The yield of chloroplasts was very low and it was only possible to add a volume of chloroplasts containing 50 $\mu$ g of chlorophyll to the fluorescence cuvette.

The fluorescence signal detected at a time-base of 0.5sec cm<sup>-1</sup> on illumination of the chloroplasts after 5min in the dark followed by a 2.5sec flash of light can be seen in Fig 3.5.15. There was an instantaneous increase in fluorescence which was followed by a decrease. After the decrease in fluorescence emission there was an increase to a maximum and thereafter the

signal was relatively constant.

FIGURE 3.5.15

The chlorophyll fluorescence profile from chloroplasts isolated from cells grown for 31 days in MSS medium containing 1% fructose. The chloroplasts were incubated in the dark for 5min and then given a 2.5sec flash of light before being illuminated at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . The fluorescence signal was resolved at a time-base of  $0.5\text{sec cm}^{-1}$  and a sensitivity of  $20\text{mV cm}^{-1}$ .



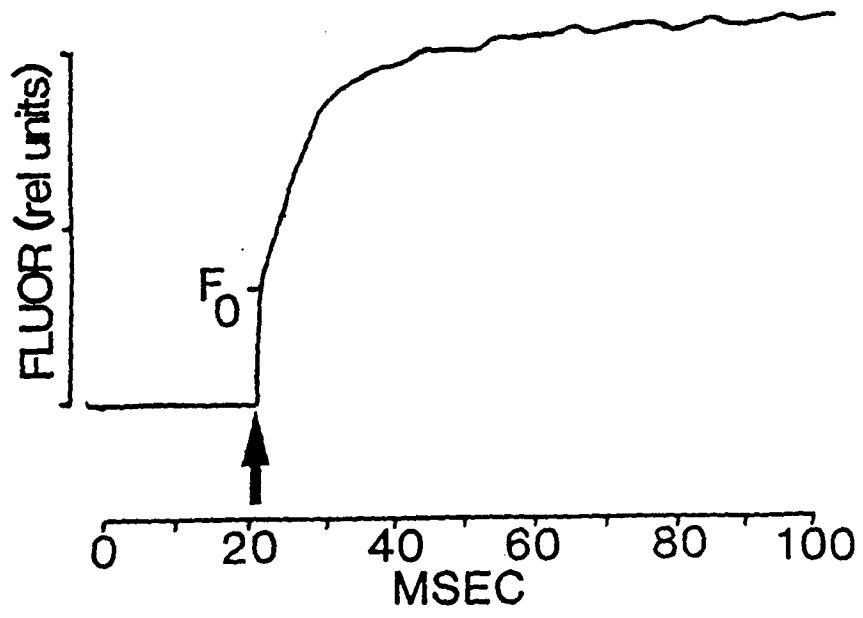
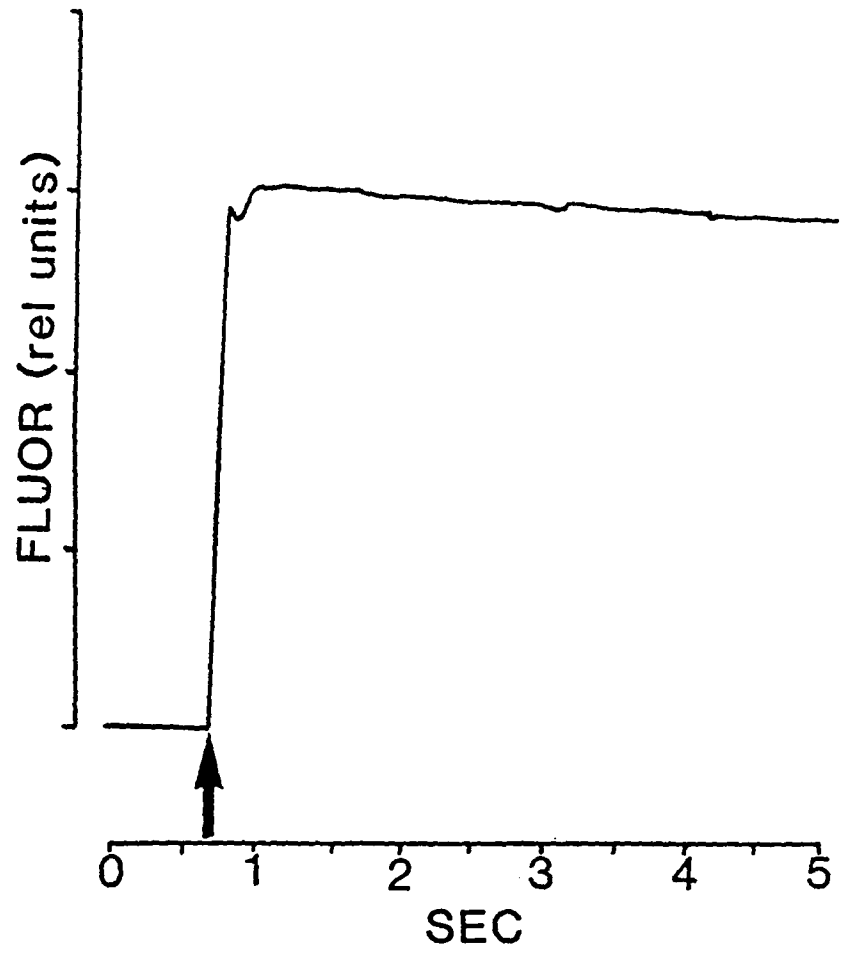
 = Activation of the light source.

FIGURE 3.5.16

The chlorophyll fluorescence profile from chloroplasts isolated from cells grown for 31 days in MSS medium supplemented with 1% fructose. The chloroplast were incubated in the dark for 5min before illumination at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . The fluorescence signal was resolved at a time-base of  $10\text{msec cm}^{-1}$  and a sensitivity of  $20\text{mV cm}^{-1}$ .

$F_0$  = The "constant" fluorescence seen in chlorophyllous tissues.

 = Activation of the light source.



When the fluorescence signal was recorded at a time-base of  $10\text{msec cm}^{-1}$  after a 5min incubation in the dark there was an instant rise in fluorescence to  $F_0$  followed by a gradual increase to a maximum (Fig 3.5.16). The size of the primary electron acceptor pool, as determined by the area above the curve from  $F_0$  to the maximum, was  $89\text{g}^{-1}\text{mm}^2$ .

### **3.5.4 Characterization Of Cells In The "Flat-bed" Culture System.**

The growth and photosynthetic characteristics of cells grown in the "flat-bed" system were determined 54 days after inoculation. However, it was possible to determine the nutrient uptake from the medium throughout the culture cycle by taking samples from the exhaust medium reservoir.

#### **3.5.4.1 The growth and photosynthetic characteristics of cells in the "flat-bed" system.**

Cells were harvested from the "flat-bed" culture system after 54 days of culture without any fructose in the medium. Over this period there was an increase in fresh weight of 14.86g. The culture consisted of several distinct layers. The uppermost layer was highly chlorophyllous, except for areas where cell proliferation was occurring which were friable in texture and white in colour. Underneath this there was a layer of less chlorophyllous cells followed by a layer of cells, that had grown through the substratum, that was white in colour and had a very hard texture. Due to the presence of different cell layers in the cultures samples were taken by cutting a vertical slice of cells so as to include cells from each layer therefore the values obtained for the parameters measured represent an average for the culture.

The chlorophyll content of the culture was found to be  $42\ \mu\text{g g}^{-1}$  fr wt and the net photosynthetic rate determined as  $57\ \text{nmol O}_2\ \text{g}^{-1}\ \text{min}^{-1}$ . The respiration rate was  $53\ \text{nmol O}_2\ \text{g}^{-1}\ \text{min}^{-1}$  which meant that the gross photosynthetic rate was  $110\ \text{nmol O}_2\ \text{g}^{-1}\ \text{min}^{-1}$  and that a value of 1.08 was

calculated for photosynthetic competence. The fluorescence profile obtained from a vertical section through the culture at a time-base of  $0.5\text{sec cm}^{-1}$  showed an instant increase in fluorescence on illumination followed by a dip before increasing to a maximum (Fig 3.5.17). When the fluorescence emission was analysed at a time-base of  $10\text{msec cm}^{-1}$  the primary electron acceptor pool size was found to be  $818 \text{ } \mu\text{m}^2$ .

#### **3.5.4.2 Nutrient uptake by cells in the "flat-bed" system.**

Throughout the culture cycle there were only small fluctuations in the uptake of nutrients from the medium (Table 3.5.4). Ammonium and nitrate were taken up at a similar rate, 10 and 11% uptake respectively. Phosphate was taken up to a far greater degree with approximately 50% being removed from the medium.

The results from the above experiments show that there are distinct differences between the nutritional requirements of the culture-lines investigated. The characteristics of the cells in the treatment grown with 3% fructose in the dark were those of a heterotrophic culture, where photosynthesis plays no part in carbon assimilation. Cells grown with 3% fructose in the medium and cultured in the light were photoheterotrophic, in that they appeared to have growth characteristics similar to heterotrophic cells but were chlorophyllous. The cells in the treatment with 1% fructose and grown in the light had the characteristics of mixotrophic culture, where photosynthesis contributes to some degree to the carbon balance of the cells. In the mixotrophic cultures the dependence on photosynthesis for the provision of organic constituents increased as the experiment progressed, in particular after the fructose in the medium was depleted. Finally cells from the "flat-bed" culture system grew without fructose added to the medium and were therefore almost entirely dependent on photosynthesis for the provision of organic constituents, such cultures were thus regarded as photosynthetic. The above cultures were used to investigate the effects of light/photosynthesis on alkaloid accumulation in cell suspension cultures.

FIGURE 3.5.17

The chlorophyll fluorescence profile of cells grown in the "flat-bed" culture system. Cells were blotted dry and incubated in the dark for 5min. The cells were then given a 2.5sec flash of light before illumination at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . The fluorescence signal was resolved at a time-base of  $0.5\text{sec cm}^{-1}$  and a sensitivity of  $0.5\text{sec cm}^{-1}$ .



= Activation of the light source.

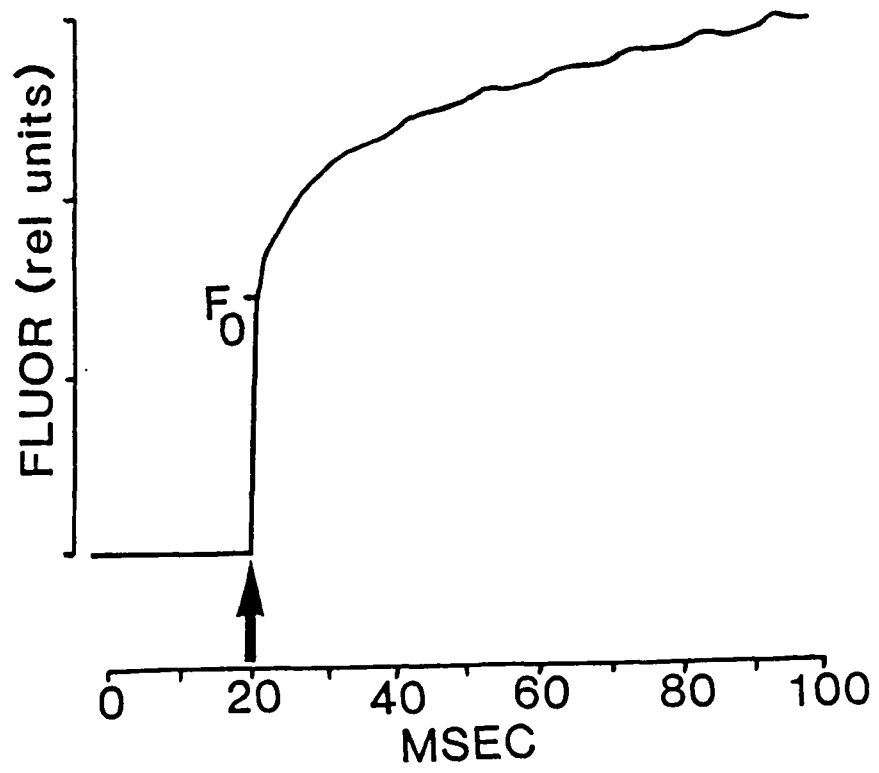
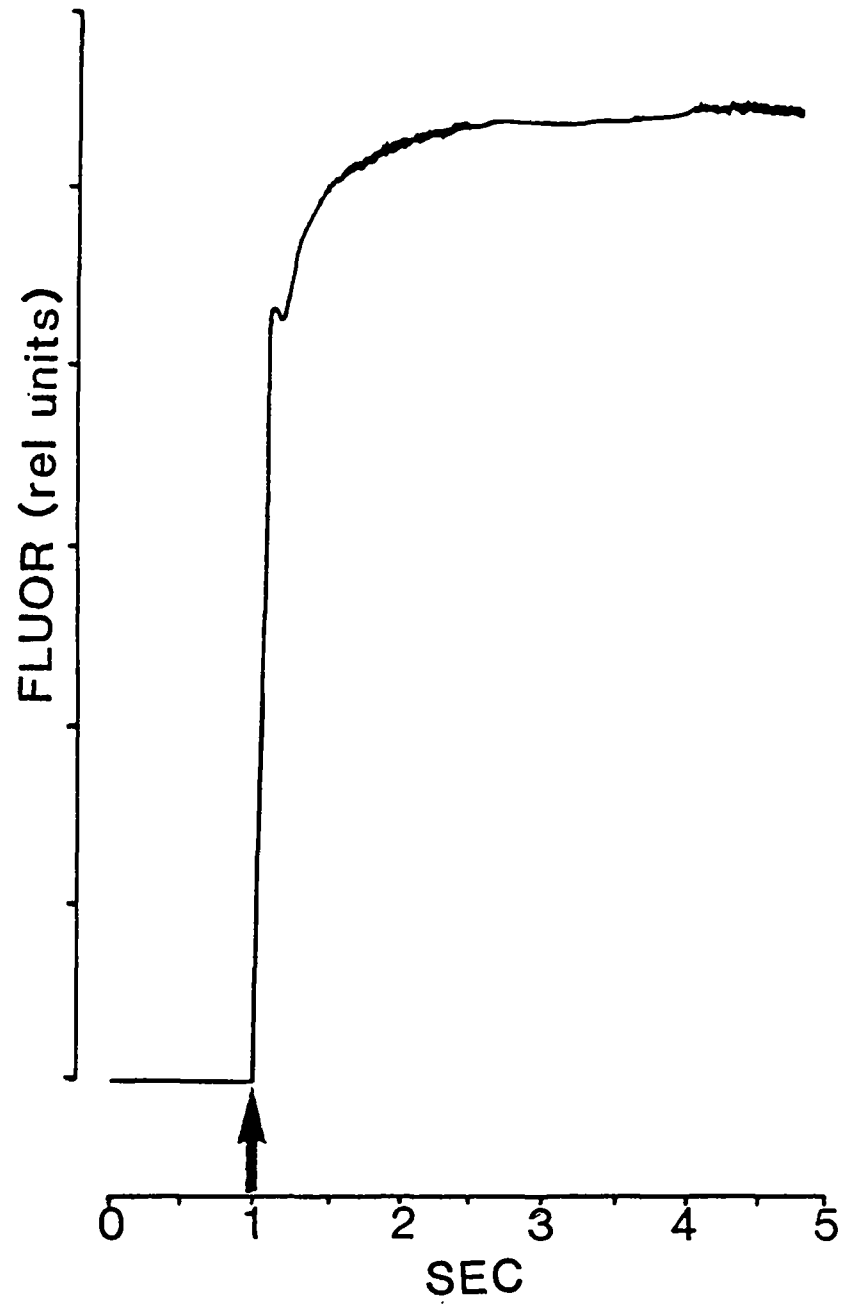
FIGURE 3.5.18

The fluorescence profile from cells cultured in the "flat-bed" culture system. Cells were blotted dry and then incubated in the dark for 5min. The cells were then illuminated at  $300\mu\text{E m}^{-1} \text{sec}^{-1}$ . The fluorescence signal was resolved at a time-base of  $10\text{msec cm}^{-1}$  and at a sensitivity of  $0.5\text{V cm}^{-1}$ .

$F_0$  = The level of "constant" fluorescence seen in chlorophyllous tissues.



= Activation of the light source.





Days after inoculation	PERCENTAGE UPTAKE		
	Phosphate	Ammonium	Nitrate
7	20	6	8
14	23	15	0
21	13	9	4
28	37	10	3
35	30	7	8
42	41	12	10
49	24	8	9
54	52	11	10

TABLE 3.5.4

The uptake of nutrients from the medium passing through the "flat-bed" culture system. The carbohydrate free MSS medium in the culture chamber was changed once every six hours and samples were taken at weekly intervals from the exhaust reservoir.

### **3.6 THE EFFECT OF LIGHT ON ALKALOID ACCUMULATION IN CULTURES OF *NICOTIANA* SPECIES.**

Light has been reported to either suppress (Ohta & Yatazawa 1978a, Pearson 1978) or stimulate (Tabata *et al* 1971) nicotine accumulation in cell cultures of *N.tabacum*. An experiment was therefore carried out to determine whether light either inhibited or stimulated alkaloid synthesis in suspension cultures of *N.tabacum* and to determine whether or not photosynthetic activity influenced alkaloid accumulation. The possible effect of light on alkaloid synthesis was investigated in a range of cultures of *Nicotiana* species with differing degrees of differentiation. This enabled information to be gathered about the effect of light on the synthesis of alkaloids other than nicotine, as well as giving information regarding any correlation between the level of culture differentiation and alkaloid accumulation. To look at the relationship between photosynthetic activity and alkaloid accumulation a range of cell suspension cultures of *N.tabacum* were used. At one extreme cultures were totally dependent on fructose in the medium for a source of organic constituents (heterotrophic) and at the other extreme photosynthesis was the major source of organic constituents (photosynthetic), while between these two extremes there were a series of transitional cultures (photoheterotrophic and mixotrophic). Thus it was possible to determine the alkaloid content of a series of cultures with increasing photosynthetic activity.

#### **3.6.1 Alkaloid Accumulation In Cell Cultures Of *N.tabacum*.**

Cell suspension cultures of *N.tabacum* Cv. Wisconsin-38 grown in MSS medium at one of two fructose concentrations were examined. In order to investigate the effect of illumination on alkaloid accumulation cultures with 3% fructose in the medium were grown either in the light (photoheterotrophic) or in the dark (heterotrophic). For the investigation into the effect of increasing photosynthetic activity on alkaloid content a treatment containing 1% fructose in the medium (mixotrophic) and a treatment in carbohydrate free medium (photosynthetic) were examined along with the above heterotrophic and photoheterotrophic cultures.

At the start of the experiment flasks containing 50ml of MSS medium containing the appropriate concentration of fructose along with  $1.62 \times 10^{-6}$ M NAA and  $1.33 \times 10^{-6}$ M BAP were inoculated with 1.5–2.0g wet weight of cells. The cells were taken from stock cultures produced by pooling the contents of four culture vessels grown in the manner employed in the experiment for the previous six subcultures. Cultures were harvested 25 days after inoculation and the cells separated from the medium by filtration under reduced pressure. The fresh weights were recorded before extraction of alkaloids from both the cells and the medium.

### **3.6.1.1 Alkaloid accumulation in heterotrophic suspension cultures of *N.tabacum***

Cell suspension cultures of *N.tabacum* Cv. Wisconsin-38 grown in the dark in MSS medium supplemented with 3% fructose (heterotrophic) had an alkaloid content of  $84 \pm 40 \mu\text{g g}^{-1}$  fr wt (Table 3.6.1a), which equated as  $1,185 \pm 577 \mu\text{g culture}^{-1}$  (Table 3.6.1b). Approximately 30% of the total alkaloid content was associated with the medium fraction. In neither fraction was there a significant difference between the concentrations of any of the three alkaloids detected (anabasine was not detected in any of the replicates) therefore the ratio of nornicotine to nicotine was one and nicotine accounted for approximately one third of the total alkaloid content.

### **3.6.1.2 Alkaloid accumulation in photoheterotrophic, mixotrophic and photosynthetic culture-lines of *N.tabacum***

Mixotrophic and photoheterotrophic cell suspension cultures of *N.tabacum* Cv. Wisconsin-38 grown in MSS medium in the light had very low alkaloid contents (Table 3.6.2a,b). In both treatments alkaloids were only detectable, in trace amounts, in one replicate. In the photoheterotrophic treatment one replicate had a nornicotine content of  $10 \mu\text{g culture}^{-1}$

a. Alkaloid content ( $\mu\text{g g}^{-1}$  fr wt).

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	23	9.5	10	2.0	33	12
ANABASINE	ND		ND		ND	
ANATABINE	13	5.4	6.6	2.1	20	8
NICOTINE	21	11	8.9	5.4	29	19
TOTAL	58	26	25	9.6	84	40

b. Alkaloid content ( $\text{culture}^{-1}$ ).

	ALKALOID CONTENT ( $\text{mg culture}^{-1}$ )	
	$\bar{X}$	SE
NORNICOTINE	481	193
ANABASINE	ND	
ANATABINE	281	265
NICOTINE	421	265
TOTAL	1185	557

TABLE 3.6.1

Alkaloid accumulation in heterotrophic cell suspension cultures of *N.tabacum* grown in the dark in MSS medium for a 25 day period. The mean and the standard error were calculated from the results from three replicates.

ND = Not detectable.

a. Alkaloid accumulation in photoheterotrophic cultures of *N.tabacum*

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	ND		0.28	0.28	0.28	0.28
ANABASINE	ND		ND		ND	
ANATABINE	ND		ND		ND	
NICOTINE	ND		0.30	0.30	0.30	0.30
TOTAL	ND		0.58	0.58	0.58	0.58

b. Alkaloid accumulation in mixotrophic cultures of *N.tabacum*

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	1.32	1.08	ND		1.32	1.08
ANABASINE	ND		ND		ND	
ANATABINE	ND		ND		ND	
NICOTINE	ND		ND		ND	
TOTAL	1.32	1.08	ND		1.32	1.08

TABLE 3.6.2

The alkaloid content of photoheterotrophic and mixotrophic cultures of *N.tabacum* grown in the light for 25 days. The mean and standard error were calculated from three determinations per treatment.

ND = Not detectable.

associated with the cellular fraction. In the mixotrophic treatment one replicate had a nornicotine content of  $5.2 \mu\text{g culture}^{-1}$  and a nicotine content of  $5.6 \mu\text{g culture}^{-1}$ , both alkaloids being only detected in the medium. There were no alkaloids detectable in either the cellular extracts or medium extracts of cultures that grew photosynthetically.

There was no significant difference between the final fresh weights of the heterotrophic and photoheterotrophic culture-lines (Table 3.6.3). However, there was approximately a 50% reduction in growth between the photoheterotrophic and the mixotrophic cultures.

Light had no significant effect on the growth of the cultures, whereas a reduction in carbon added to the medium decreased growth. However, light had an almost complete inhibitory effect on alkaloid accumulation, even in cultures with a very low dependency on photosynthesis for the provision of organic constituents (mixotrophic cultures) and a complete inhibitory effect on alkaloid synthesis in photosynthetic cultures, which were grown at a higher light intensity. However, it was unlikely that photosynthesis, by means such as substrate limitation, played a primary role in determining the level of alkaloid accumulation because photosynthetic activity in the photoheterotrophic culture-line was minimal. Further experiments were ~~therefore~~ carried out employing cultures other than cell suspension cultures of *N.tabacum* to determine whether light had a similar inhibitory effect on the synthesis of alkaloids other than nicotine and to discover if the level of differentiation of cultures influenced the effect of light.

### 3.6.2 Alkaloid Accumulation In Callus Cultures Of *N.glauca*

The effect of light on the accumulation of anabasine in callus cultures of *N.glauca* was investigated. This experiment was carried out since any regulatory effect of light on the synthesis of the pyridine moiety<sup>e</sup> of the tobacco alkaloids would influence the synthesis of all of the alkaloids, not just nicotine and nornicotine. Using a species of *Nicotiana* that predominantly accumulates anabasine in the whole plant and in callus culture allowed such an investigation.

	FRESH WEIGHT (g)	
	$\bar{X}$	SE
Heterotrophic	17.66	0.52
Photoheterotrophic	14.84	3.40
Mixotrophic	7.44	1.37

TABLE 3.6.3

The effect of illumination and carbon supplementation on biomass accumulation in suspension cultures of *N.tabacum* grown in MSS medium for 25 days. The fresh weights of three cultures per treatment were determined and from these the mean and standard errors for each treatment were calculated.

Callus cultures of *N.glauca* were grown in Petri dishes containing MS medium supplemented with 3% sucrose,  $0.81 \times 10^{-6}$ M NAA,  $0.93 \times 10^{-6}$ M kinetin,  $28 \times 10^{-6}$ M ascorbic acid and solidified with 0.8% (w/v) agar. At the start of the experiment 2–2.5g of callus, grown for at least the previous five culture cycles under the conditions employed in the experiment, were transferred to fresh medium.

### **3.6.2.1 The effect of illumination on anabasine accumulation by callus cultures of *N.glauca***

For one treatment the callus cultures were placed in the dark while for the second treatment they were placed under constant illumination of  $80\text{--}90 \mu\text{E m}^{-2}\text{sec}^{-1}$ . Both treatments were grown at  $25^{\circ}\text{C}$  for 30 days at which point the fresh weight of callus was determined before the alkaloids were extracted.

There was no significant difference between the final fresh weights of the two treatments, these being  $7.8 \pm 0.1$  g and  $7.4 \pm 0.1$  g for the dark and light treatments respectively. The callus grown in the dark accumulated both anabasine and nornicotine (Table 3.6.4). Anabasine was the major alkaloid accounting for 88% of the total alkaloid content with the cultures having a content of  $177 \pm 43$   $\mu\text{g}$ . There were no alkaloids detectable in any of the three replicates for the callus cultures grown in the light.

Light totally inhibited alkaloid synthesis in callus cultures of *N. glauca*, and had no significant affect on the final biomass yield. Due to the fact that light totally inhibited alkaloid synthesis in these cultures they were used in an experiment to determine the kinetics of alkaloid accumulation.



	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)			
	DARK		LIGHT	
	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	6.6	5.1	ND	
ANABASINE	156	27	ND	
ANATABINE	ND		ND	
NICOTINE	14	11	ND	
TOTAL	177	43	ND	

TABLE 3.6.4

The alkaloid content of callus cultures of *N.glauca* grown on MS medium supplemented with 3% sucrose and cultured either in the light or in the dark for 30 days. Three replicates were used to calculate the mean and standard error for each treatment.

### 3.6.2.2 Changes in fresh weight and anabasine content of callus cultures of *N.glauca* after transfer from the light into the dark or from the dark into the light.

Callus cultures of *N.glauca* grown under identical conditions except for illumination accumulated alkaloids in the dark and did not in the light. By transferring cultures from the non-producing (illuminated) environment to the environment more suited to alkaloid accumulation (dark) a determination of the onset of anabasine accumulation was made. Also by moving cultures from the dark into the light the kinetics of the inhibition of alkaloid synthesis could be determined. For this experiment four treatments were employed: callus that had been grown in the light was left in the light (treatment LL) or transferred to the dark (treatment LD) and callus grown in the dark was retained in the dark (treatment DD) or transferred to the light (treatment DL). For each treatment the medium was as previously used for *N.glauca* callus. At the start of the experiment 50 petri dishes per treatment were inoculated with 1.0–1.5g of callus from appropriate stock cultures, previously grown either in the light or in the dark. Cultures were grown with the required light regime at 25°C and three replicates harvested at 3 day intervals for a 33 day period. At each sampling time the fresh weights were determined and the tissue pooled for alkaloid extraction.

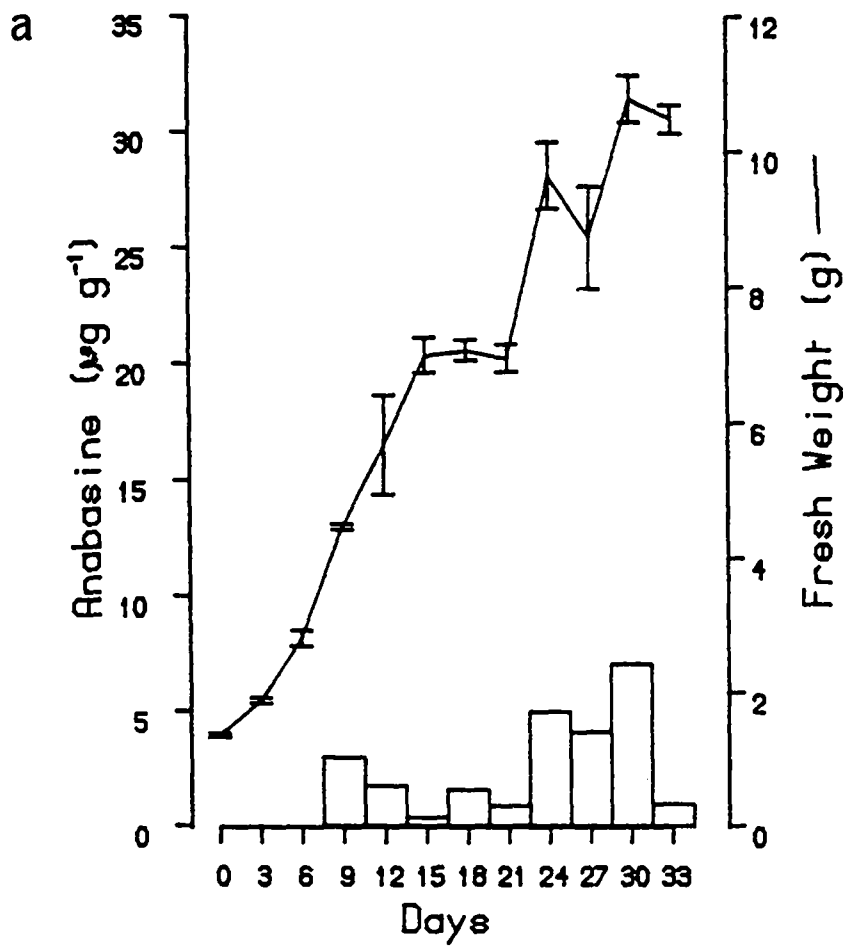
#### i. The LL treatment.

There was no detectable lag phase before fresh weight began to increase (Fig 3.6.1a). The increase continued until day 15 after which there was an overall increase until day 30. Between day 30 and the final reading on day 33 there was no significant change in fresh weight. Anabasine was not detectable for the first 9 days of the experiment. After the ninth day the amount present fluctuated between 0.8 and 7.0  $\mu\text{g g}^{-1}$  fr wt (Fig 3.6.1a) or on a per culture basis between 2.1 and 76  $\mu\text{g}$  (Fig 3.6.2a).

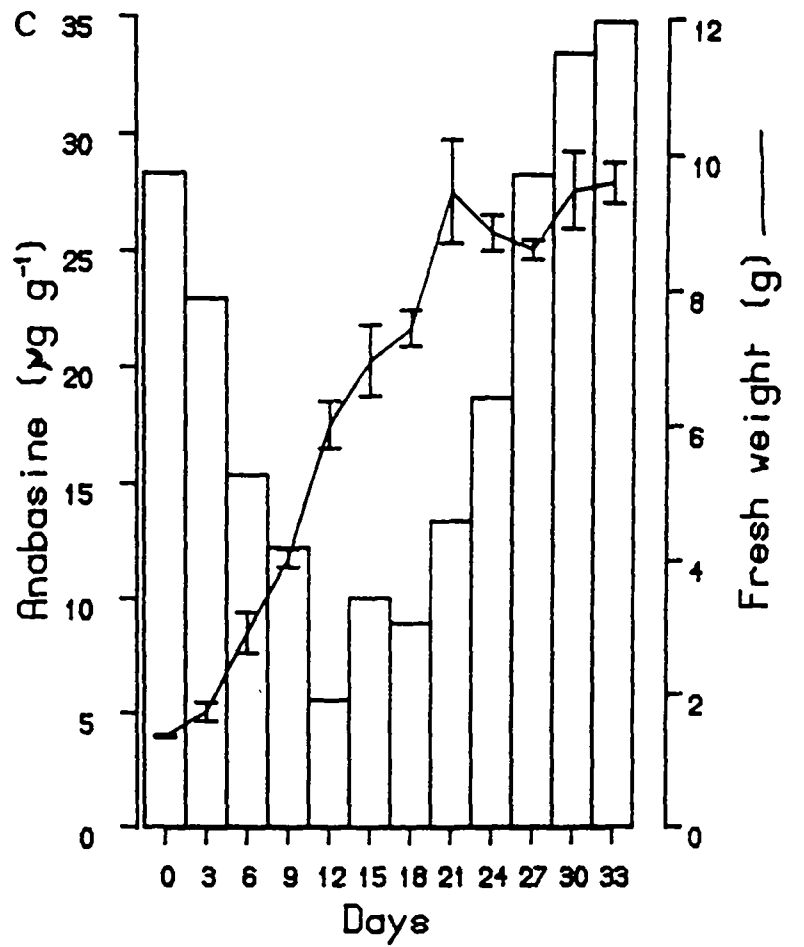
Anabasine had not previously been detected in cultures grown in the light. However, during this experiment roots arose from the callus and their presence may account for the alkaloid accumulation seen. The

FIGURE 3.6.1

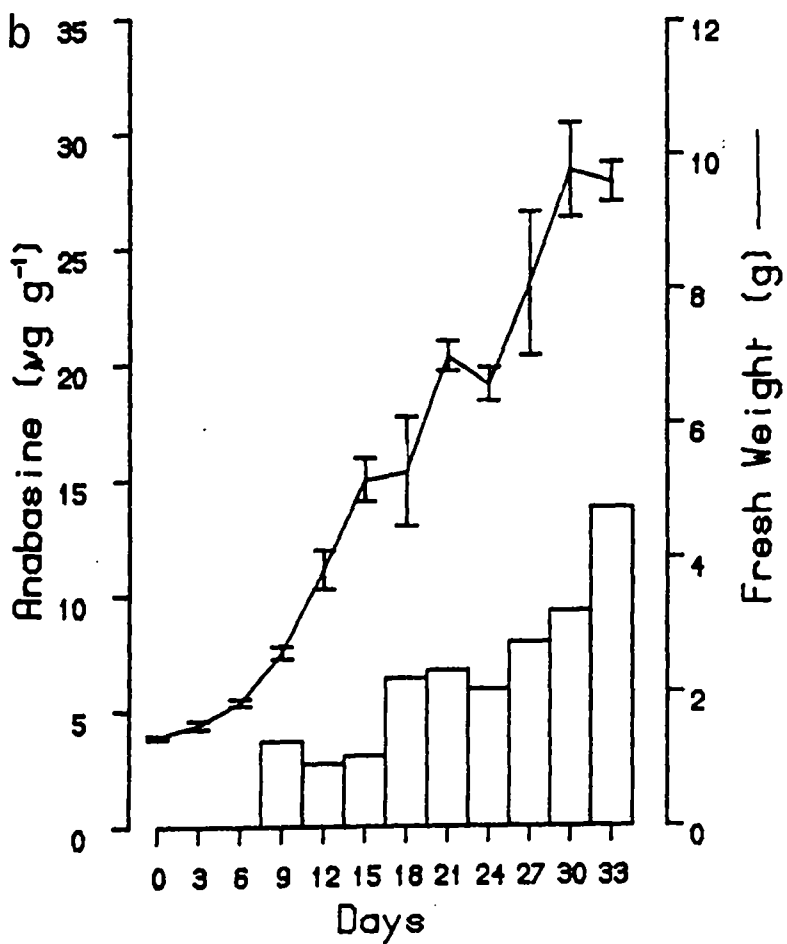
Changes in fresh weight and anabasine content, over 33 days, in callus cultures of *N.glauca* that had previously been grown under different illuminating conditions. Cultures grown in the light (LL treatment) were transferred into the dark (LD treatment) while cultures grown in the dark (DD) treatment were transferred into the light (DL) treatment. All cultures were grown on solid MS medium supplemented with 3% sucrose. The points for fresh weight represent the mean  $\pm$  the standard error of three replicates.



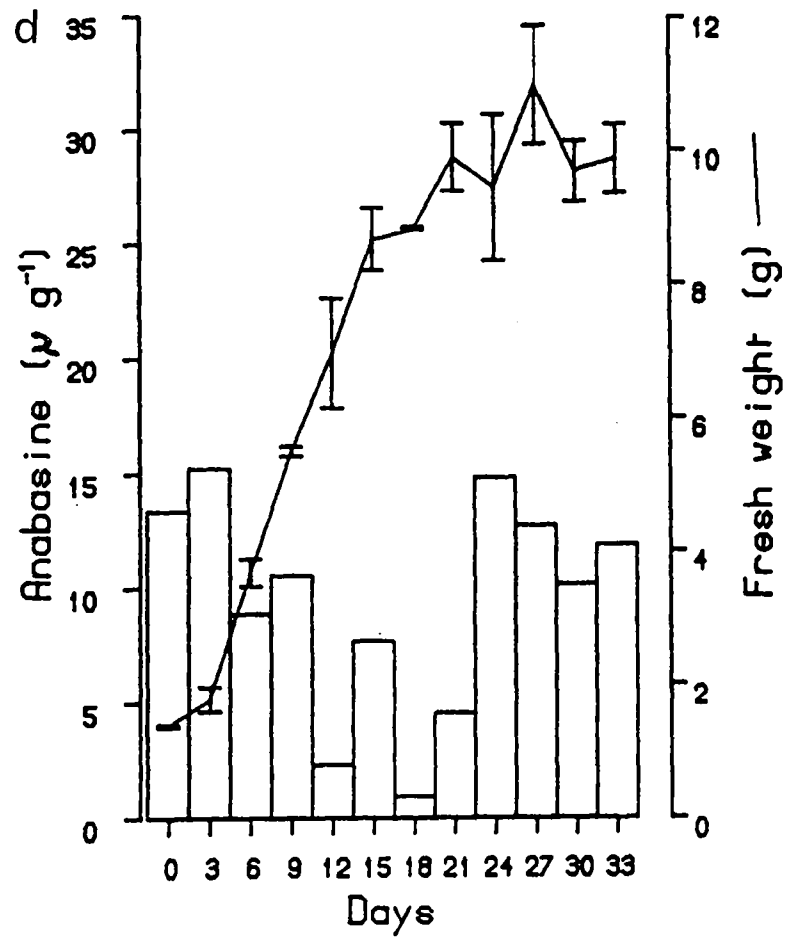
Light-light treatment



Dark-dark treatment



Light-dark treatment



Dark-light treatment

**FIGURE 3.6.2**

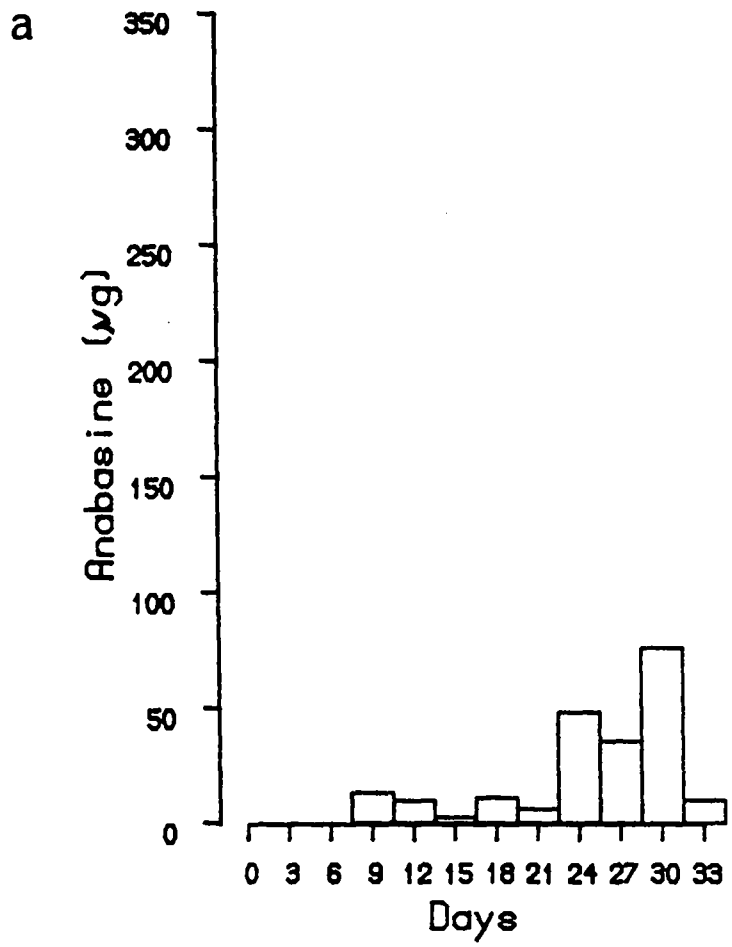
**The anabasine content on a per culture basis over a 33 day culture cycle.**

**a = Cultures continually grown in the light (LL treatment).**

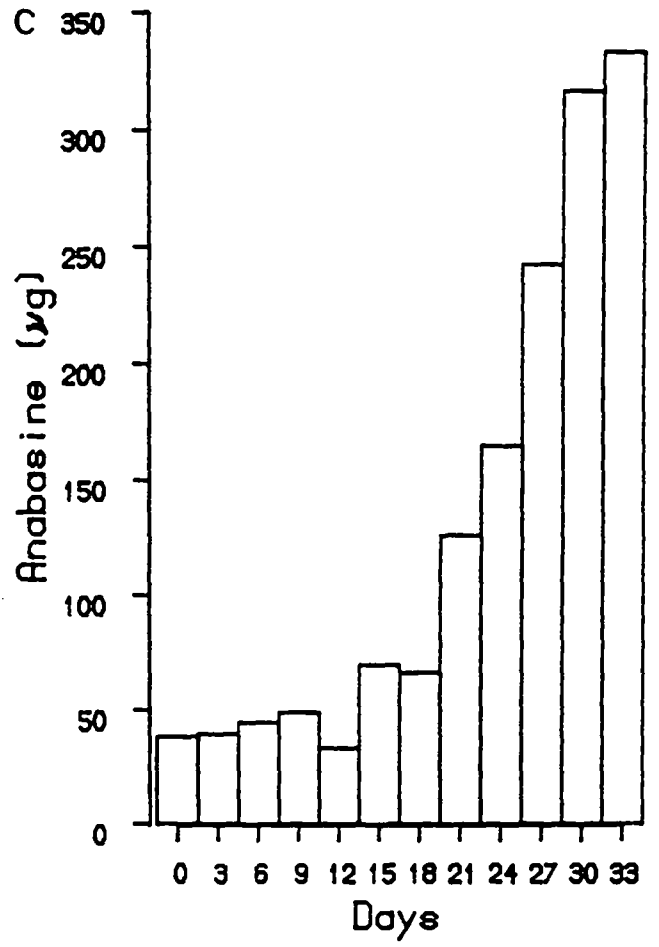
**b = Cultures grown in the light prior to the experiment and transferred into the dark at the start of the experiment (LD treatment).**

**c = Cultures continually grown in the dark (DD treatment).**

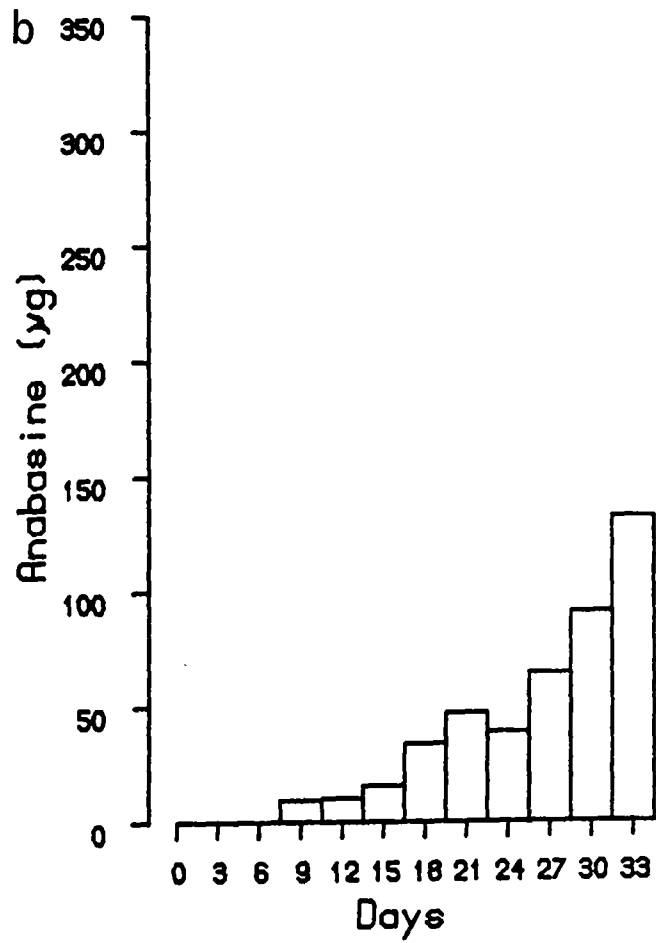
**d = Cultures grown in the dark prior to the experiment and transferred into the light at the start of the experiment (DL treatment).**



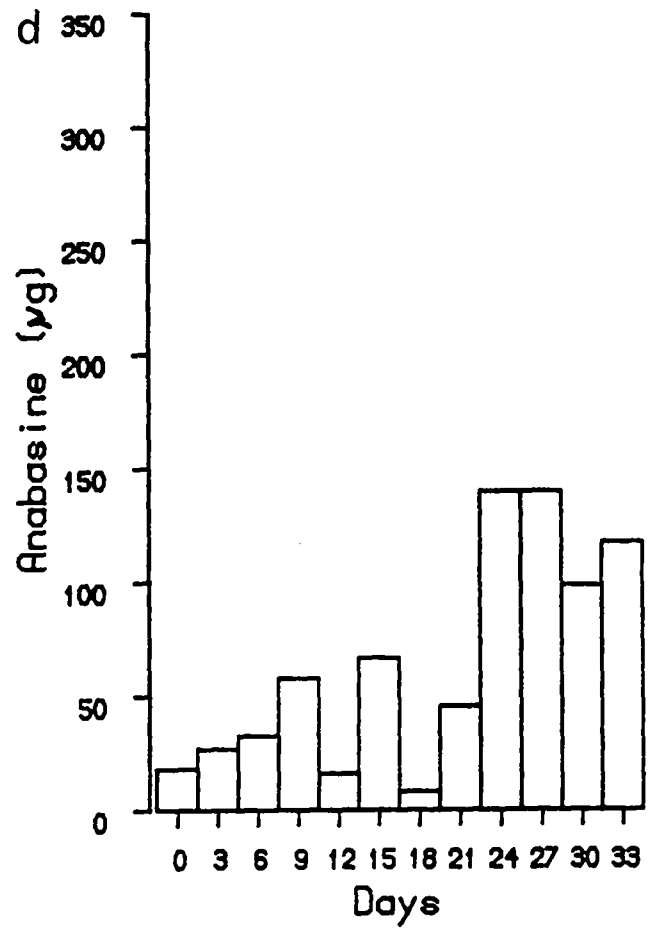
Light-light treatment



Dark-dark treatment



Light-dark treatment



Dark-light treatment

fluctuations in anabasine content between sampling time may be due to the fact that root differentiation did not occur to the same degree in the cultures taken at each sampling time.

ii. The LD treatment.

The fresh weight increased gradually between days 0 and 9 (Fig 3.6.1b) and then continued to increase at a more rapid rate until day 30. Anabasine was detected for the first time on day 9 and then increased until the end of the experiment (Fig 3.6.1b and 3.6.2b). At the end of the investigation cultures had an anabasine content of  $132 \mu\text{g}$  which equated as  $13 \mu\text{g g}^{-1}$  fr wt.

iii. The DD treatment.

The fresh weight increased between days 0 and 21 (Fig 3.6.1c) after which there was no significant change. There was a very little change in the anabasine content of the culture between days 0 and 18 (Fig 3.6.2c) with the value being near to  $50 \mu\text{g culture}^{-1}$ . However, expressed on a fresh weight basis the anabasine content was seen to decrease between days 0 and 12 before increasing to a maximum of  $34 \mu\text{g g}^{-1}$  fr wt at the end of the experiment (Fig 3.6.1c). The decrease coincided with the period of maximum growth with the subsequent increase occurring towards the end of the growth phase and into the stationary phase. This was also the case when the results were expressed on a per culture basis. The anabasine content increased from day 18 to day 33 when there was a content of  $335 \mu\text{g culture}^{-1}$ .

iv. The DL treatment.

The fresh weight increased from the time of inoculation until day 21 of the experiment after which there was no significant change (Fig 3.6.1d). The anabasine content at inoculation was the same as for the DD treatment at

inoculation, since the same material was used as the inoculum (Fig 3.6.1d and 3.6.2d). There was an overall decrease in anabasine content between days 0 and 18 and then there was an increase up to a maximum of  $14 \mu\text{g g}^{-1}$  fr wt on day 24. There was very little change in alkaloid content between day 24 and the end of the experiment.

There were small differences between the means of the final fresh weights of the four treatments (10.49g, 9.58g, 9.60g and 9.87g for the LL, LD, DD and DL treatments respectively). There were also differences in the growth patterns between treatments. The LL and DD had similar growth patterns but the LD treatment took a longer period of time to achieve its final fresh weight while the DL treatment had the highest initial rate of growth before growing more slowly during the later stages of the growth cycle.

The highest alkaloid content was recorded in the DD treatment, where accumulation of anabasine occurred chiefly between days 18 and 33. In the DL treatment the alkaloid content decreased until day 18 and then increased until day 24 but the final alkaloid content was lower than seen in the DD treatment. In the LD treatment anabasine was first detected on day 9 and increased thereafter to a value approximately a third of that for the DD treatment. In the LL treatment small amounts of anabasine were detectable between days 9 and 33. This may have been due to root differentiation in some of the sample cultures from this treatment, which may also have played a role in alkaloid synthesis in the DL treatment. The effects of light on the chlorophyll content and morphology of *N.glauca* callus cultures can be seen in Fig 3.6.3a,b,c,d. After 28 days of the culture cycle the callus of the DD treatment was friable and non-chlorophyllous. Callus transferred from the dark into the light (DL treatment) had become chlorophyllous, was compact and contained differentiated roots. The LL treatment was compact chlorophyllous and had several differentiated roots. The LD treatment was chlorophyllous and compact in the area of the tissue used as the inoculum but friable and non-chlorophyllous in areas where growth had occurred.

So as to determine whether differentiated roots in culture are capable of alkaloid synthesis in the light an experiment was conducted using root cultures of *N.rustica*



**FIGURE 3.6.3**

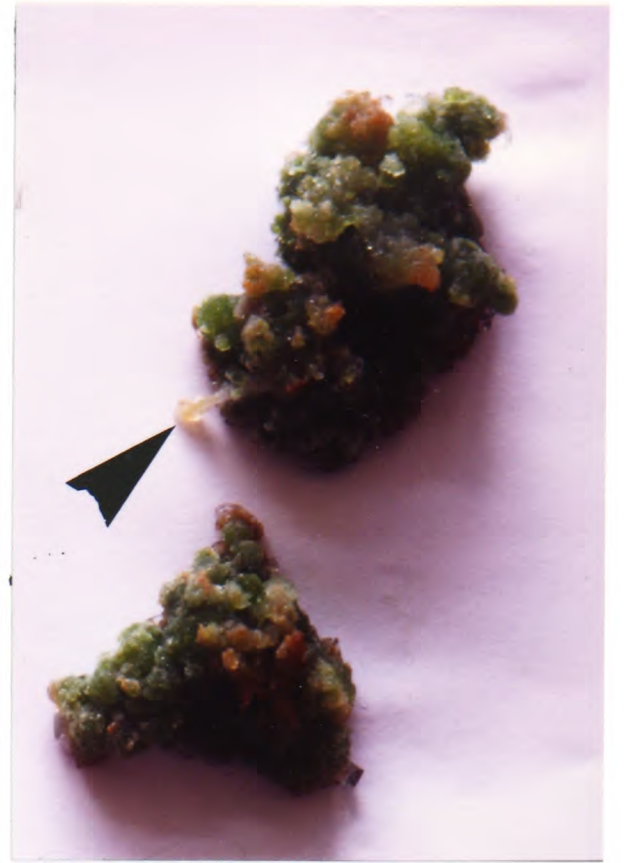
The appearance of callus cultures of *N.glauca* 28 days after subculture.

- a. Callus grown in the light (LL treatment). Differentiated roots are arrowed.
- b. Callus grown in the light (DL treatment). Differentiated roots are arrowed.
- c. Callus grown in the dark (DD treatment).
- d. Callus grown in the dark (LD treatment).

a



b



c



d



1 cm

### 3.6.3 The Growth And Alkaloid Accumulation Of Roots Of *N.rustica* In Liquid Culture.

For the presence of differentiated roots in callus cultures of *N.glauca* to have accounted for the ~~alkaloids~~ detected, the roots would have had to have been capable of alkaloid synthesis in the light. Roots were thus grown in either the light or the dark in 50ml of W medium containing 3% sucrose. At the start of the experiment flasks containing the medium were inoculated with two 3cm sections of cultured root, showing recent branching, that had been grown in the conditions employed throughout the experiment. After 32 days growth the cultures were harvested and the roots separated from the medium by filtration under reduced pressure. The fresh weight of the roots was determined before they and the medium were subjected to the alkaloid extraction procedure.

#### 3.6.3.1 Alkaloid accumulation in root cultures grown in the dark.

Root cultures of *N.rustica* grown in liquid culture in the dark had an alkaloid content of 0.25% on a fresh weight basis. The cells had a total alkaloid content of  $2,000\mu\text{g g}^{-1}$  fr wt while that of the *medium* was  $460\mu\text{g g}^{-1}$  fr wt (Table 3.6.5a), which was equivalent to a mean alkaloid content of  $1,078\mu\text{g culture}^{-1}$  (Table 3.6.5b). The major alkaloid was nicotine which accounted for approximately 79% of the total alkaloid content, and the ratio of nornicotine to nicotine was 0.17. Nearly 20% of the total alkaloid present was located in the medium, with each of the four major alkaloids being detectable.

#### 3.6.3.2 Alkaloid accumulation in root cultures grown in the light.

Nicotine was the predominant alkaloid in root cultures grown in the light accounting for 81% of the total alkaloid content (Table 3.6.6a). The alkaloid content was 0.1% on a fresh weight basis, with an alkaloid content on a culture basis of  $278 \pm 110 \mu\text{g}$  (Table 3.6.6b). Nearly 50% of the total

a. Alkaloid content ( $\mu\text{g g}^{-1}$  fr wt).

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	298	85	12	10	266	108
ANABASINE	46	13	4	2	51	15
ANATABINE	103	33	17	3	121	35
NICOTINE	1597	630	435	192	2067	804
TOTAL					2506	931

b. Alkaloid content ( $\text{culture}^{-1}$ ).

	ALKALOID CONTENT ( $\mu\text{g culture}^{-1}$ )	
	$\bar{X}$	SE
NORNICOTINE	144	33
ANABASINE	23	6.5
ANATABINE	54	8
NICOTINE	855	95
TOTAL	1078	122

TABLE 3.6.5

Alkaloid accumulation in root cultures of *N.rustica* grown in the dark in W medium for a 32 day period. The mean and the standard error were calculated from the results from three replicates.

ND = Not detectable.

a. Alkaloid content ( $\mu\text{g g}^{-1}$  fr wt).

	ALKALOID CONTENT ( $\mu\text{g g}^{-1}$ fr wt.)					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	70	48	13	7.0	83	55
ANABASINE	7	5	1.4	0.6	8.8	6
ANATABINE	32	10	10	5.6	42	12
NICOTINE	544	63	455	196	999	162
TOTAL					1134	114

b. Alkaloid content ( $\mu\text{g culture}^{-1}$ ).

	ALKALOID CONTENT ( $\mu\text{g culture}^{-1}$ )	
	$\bar{X}$	SE
NORNICOTINE	36	24
ANABASINE	3.7	2
ANATABINE	12	6
NICOTINE	225	70
TOTAL	278	110

TABLE 3.6.6

Alkaloid accumulation in root cultures of *N.rustica* grown in the light in W medium for a 32 day period. The mean and the standard error were calculated from the results from three replicates.

ND = Not detectable.

alkaloid content was located in the medium, where all four main alkaloids were detectable. The overall ratio of nornicotine to nicotine was 0.16

There was a significant difference between the alkaloid contents of the two treatments as calculated on a per gram fresh weight basis. This difference was accentuated when the alkaloid content was expressed on a per culture basis, since less biomass accumulated in the cultures grown in the light (Table 3.6.7). Root cultures grown in the dark attained a final biomass of 0.50+/-0.09g while similar cultures in the light attained 0.27+/-0.12g. There were no significant differences in the pattern of alkaloid accumulation between the two treatments, with both having ratios of nornicotine to nicotine of approximately 0.17 and in both nicotine accounting for nearly 80% of the total alkaloid content. However, cultures in the light had significantly higher proportion of their alkaloid content associated with the medium than did cultures in the dark.

Light did not cause a total inhibition of alkaloid synthesis in root cultures. The primary effect of light was to suppress growth which translated as a lower alkaloid yield per culture. Since light had a less pronounced effect on alkaloid accumulation in differentiated roots, than on callus or suspension cultures, an experiment using shoot cultures was conducted to determine whether or not they accumulated alkaloids when placed in the dark.

#### **3.6.4 Alkaloid Accumulation In Shoot Cultures Of *N.tabacum***

It has been reported (Dawson & Solt 1959) that shoots account for a small percentage of total alkaloid synthesis in the whole plant. This being the case shoots ~~must~~ possess the biosynthetic pathways required for alkaloid biosynthesis. An experiment was carried out to determine whether shoot cultures accumulated alkaloids when they were grown in the dark.

At the start of the experiment 8oz glass jars containing 50ml of MS medium, supplemented with 2% sucrose and  $5 \times 10^{-6}$ M BAP, adjusted to pH 5.6 and solidified with 1.2% agar were inoculated. The inoculum consisted of

	FRESH WEIGHT (g)	
	$\bar{X}$	SE
Dark	0.50	0.09
Light	0.26	0.11

TABLE 3.6.7

The effect of illumination on the biomass yield of *N.rustica* roots cultured for a 32 day period in W medium. The mean and standard error were calculated from the fresh weights of three replicates per treatment.

four 2–3cm sections of leaf and/or stem from a 30 day old shoot culture. The top of the jars were covered with a 4cm Petri dish and sealed with a double layer of parafilm. Cultures were either grown under constant illumination of  $80\text{--}90\mu\text{E m}^{-2}\text{ sec}^{-1}$ , or in the dark, at  $25^{\circ}\text{C}$ . Thirty days after inoculation the shoots were removed from the medium and the fresh weight recorded before determination of the alkaloid content.

#### **3.6.4.1 Alkaloid accumulation in shoot cultures of *N.tabacum* grown in the light.**

Shoot cultures of *N.tabacum* grown in the light for 30 days had a total alkaloid content of  $14 \pm 14 \mu\text{g g}^{-1}$  which on a culture basis was  $7 \pm 7 \mu\text{g culture}^{-1}$ . In only one of the three replicates were there any alkaloids detectable. Nicotine and nornicotine were present in equal amounts and anatabine was also detectable. There were no alkaloids detectable in the medium.

#### **3.6.4.2 Alkaloid accumulation in shoot cultures of *N.tabacum* grown in the dark.**

After 30 days culture in the dark tobacco shoot cultures were found to contain anatabine and nicotine. Neither nornicotine nor anabasine were detectable in these cultures but in all three replicates anatabine and nicotine were detectable. There was no significant difference between the anatabine and the nicotine contents these being  $24 \pm 19 \mu\text{g culture}^{-1}$  and  $32 \pm 17 \mu\text{g culture}^{-1}$  respectively. There were no alkaloids detectable in the medium.

In both treatments the alkaloid content was very low, and there was no significant difference between the alkaloid contents of the treatments. However there was a significant difference in the biomass accumulation with the cultures grown in the light accumulating significantly less biomass than those grown in the dark. This experiment showed that shoots possess the



metabolic pathways necessary for tobacco alkaloid synthesis and that synthesis does occur at very low levels irrespective of the degree of illumination.

This series of experiments showed that in undifferentiated cultures of *N.tabacum* and *N.glauca* light causes an almost total suppression of alkaloid biosynthesis, regardless of the photosynthetic state of the cultures. However, the effect of light on alkaloid biosynthesis was not so clear cut when differentiated cultures of *N.rustica* and *N.tabacum* were investigated. In root cultures of *N.rustica* light caused a reduction in alkaloid biosynthesis and in biomass yield while in shoot cultures of *N.tabacum* light caused a significant reduction in biomass yield but had no significant effect on alkaloid content.

To determine whether the inhibitory effect of light on alkaloid accumulation was due to its enhancing alkaloid metabolism to non-alkaloid compounds an experiment in which nicotine was added to the culture medium at the start of the experiment was carried out.

### **3.7 THE FATE OF NICOTINE ADDED TO SUSPENSION CULTURES OF *N.TABACUM***

In cell suspension cultures of *N.tabacum* alkaloid accumulation was not observed in cultures grown under constant illumination. It was therefore necessary to determine whether the alkaloids, and in particular nicotine, were metabolised under illuminated conditions. This was achieved by adding a known amount of nicotine to the culture medium at the start of the growth cycle and determining the alkaloid content after 14 days growth in either the light or the dark.

A known amount of nicotine was added to each culture vessel prior to autoclaving. After autoclaving the medium from three vessels was extracted and no significant difference was found between the nicotine concentrations before and after autoclaving. Having determined that autoclaving did not affect the nicotine concentration sterile medium was inoculated with 1.5–2.0g wet weight of cells.

#### **3.7.1 The Fate Of Nicotine Added To Heterotrophic, Photoheterotrophic And Mixotrophic Suspension Culture-lines Of *N.tabacum***

The fate of nicotine added to the culture medium was investigated using the culture-lines in which light was found to inhibit alkaloid accumulation and the heterotrophic culture-line was used as a control. Three culture-lines were used: heterotrophic (grown with 3% fructose in the medium in the dark), photoheterotrophic (cultured in the light in medium containing 3% fructose) and mixotrophic (grown with 1% fructose in the medium and maintained in the light).

At the start of the experiment 250ml flasks containing 50ml of MSS medium supplemented with either 3% or 1% fructose and having a final nicotine concentration of 1.25mM, were inoculated with cells from the appropriate culture-lines. After 14 days growth cultures were harvested and the

cells separated from the medium by filtration under reduced pressure. The fresh weight of the cells was recorded before the cells and medium were subjected to the alkaloid extraction.

### 3.7.1.1 The alkaloid content of heterotrophic cells.

i. The alkaloid content of cultures grown without nicotine added to the medium.

Suspension cultures of *N.tabacum* cultured in MSS medium supplemented with 3% fructose and grown in the light had an alkaloid content of 0.6 +/-0.2 mg 14 days after subculture. Nicotine and nornicotine were the predominant alkaloids in both the cellular and the medium extracts (Table 3.7.1), with 31% of the total alkaloid content released into the medium. The ratio of nornicotine to nicotine was 1.1 and nicotine accounted for 34% of the total alkaloid content.

ii. The alkaloid content of cells grown in medium containing 1.25mM nicotine.

Cultures of *N.tabacum* cultured in the dark in MSS medium containing 1.25mM nicotine had a total alkaloid content of 9.9 +/-0.9 mg. Cells grown in the same environment without added nicotine had an alkaloid content of 0.6 +/-0.2 mg. Therefore in the treatment with added nicotine there was an extra 9.2 mg of alkaloid present at the end of the experiment. At the start of the experiment 10mg (1.25mM) of nicotine were added to the medium. There was no significant difference between the alkaloid content of the cultures at harvest and the amount of nicotine added to the medium at the start of the experiment. However, at the end of the experiment only 0.4 +/-0.1 mg of the original 10mg of nicotine were detectable (Table 3.7.2), while there was a large increase in the nornicotine content compared with the control. Nornicotine accounted for 95% of the total alkaloid content and 32% of the alkaloid content was detected in the medium. Anabasine and anatabine were not detectable in either the cellular or the medium extracts.

	ALKALOID CONTENT (mg culture <sup>-1</sup> )					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	0.1	0.08	0.08	0.02	0.2	0.08
ANABASINE	ND		ND		ND	
ANATABINE	0.1	0.04	0.05	0.01	0.1	0.05
NICOTINE	0.1	0.09	0.06	0.03	0.2	0.13
TOTAL	0.4	0.20	0.19	0.26	0.6	0.26

TABLE 3.7.1

The alkaloid content of cultures grown for 14 days in the dark in MSS medium supplemented with 3% fructose. Each value is the mean +/- the standard error of three replicates.

ND = Not detectable.

	ALKALOID CONTENT (mg culture <sup>-1</sup> )					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	6.40	0.50	3.01	0.32	9.41	0.83
ANABASINE	ND		ND		ND	
ANATABINE	ND		ND		ND	
NICOTINE	0.35	0.12	0.14	0.05	0.49	0.17
TOTAL	6.74	0.62	3.14	0.37	9.90	0.99

TABLE 3.7.2

The fate of 62.5 μmoles of nicotine added to cell suspensions of *N.tabacum*. Cells were grown in the dark for 14 days in MSS medium supplemented with 3% fructose and 62.5 μmole nicotine. Each value represents the mean +/- the standard error of three replicates.

ND = Not detectable.

### **3.7.1.2 The alkaloid content of the photoheterotrophic and mixotrophic culture-lines.**

Neither the photoheterotrophic nor the mixotrophic cultures accumulated any alkaloids when there was no nicotine added to the medium. Therefore after 14 days growth with 1.25mM nicotine added to the medium the alkaloid content should be due to the presence of the nicotine in the medium.

i. The alkaloid content of photoheterotrophic cultures grown in medium containing 1.25mM nicotine.

Photoheterotrophic suspension cultures grown in MSS medium containing 1.25mM nicotine had a total alkaloid content of 8.6 +/-0.6 mg per culture (Table 3.7.3). At the start of the experiment 10mg of nicotine were present in the medium therefore 86 % of the original alkaloid content remained at the end of the experiment. Nornicotine accounted for 95% of the total alkaloid content with 51% of the alkaloid content being released into the medium. Anabasine and anatabine were not detectable in either extract.

ii. The alkaloid content of mixotrophic cultures grown in medium containing 1.25mM nicotine.

In mixotrophic cultures grown in MSS medium containing 1.25mM nicotine there was no significant difference between the alkaloid content at the beginning and at the end of the experiment (10mg and 9.8 +/-0.3 mg respectively) (Table 3.7.4). Nornicotine was the predominant alkaloid accounting for 61% of the total alkaloid content and 32% of the alkaloid content was found in the medium.

In only the photoheterotrophic culture-line there was a significant difference between the alkaloid content at the start of the experiment and that at the end of the experiment. However, the reduction in alkaloid content was not great enough to account for the lack of alkaloid accumulation in cultures grown in the light. Although the total alkaloid content of cultures was only affected to a small degree by light the nicotine added to the cultures had been

	ALKALOID CONTENT (mg culture <sup>-1</sup> )					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	3.97	0.40	4.27	0.84	8.23	0.58
ANABASINE	ND		ND		ND	
ANATABINE	ND		ND		ND	
NICOTINE	0.29	0.05	0.14	0.04	0.43	0.08
TOTAL	4.26	0.46	4.40	0.88	8.66	0.67

TABLE 3.7.3

The fate of 62.5  $\mu$ mole nicotine added to photoheterotrophic cultures of *N.tabacum*. The cultures were grown in the light for a 14 day period. The mean and standard error were calculated from three replicates.

ND = Not detectable.

	ALKALOID CONTENT (mg culture <sup>-1</sup> )					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	1.43	0.28	4.62	0.30	6.06	0.27
ANABASINE	ND		ND		ND	
ANATABINE	ND		ND		ND	
NICOTINE	0.66	0.18	3.16	0.25	3.82	0.15
TOTAL	2.09	0.46	7.77	0.56	9.88	0.42

TABLE 3.7.4

The fate of nicotine added to mixotrophic cell suspensions of *N.tabacum*. Each value represents either the mean or the standard error of three replicates.

ND = Not detectable.

transformed into nornicotine.

The transformation of nicotine to nornicotine occurs by a demethylation (Leete 1977b) and in order to account for the difference in molecular weights of the two alkaloids percentage transformations were calculated from the number of moles of each of the alkaloids present in the cultures. The untreated heterotrophic cultures had an alkaloid content of 1.7 $\mu$ moles of nornicotine while the treated cultures contained 63.5 $\mu$ moles. Therefore 61.8 $\mu$ moles of the nornicotine in the treated cultures derived from the nicotine added to the medium (Table 3.7.5a). At the end of the experiment the cultures with nicotine added to their medium contained 1.7 $\mu$ moles more of nicotine than cultures to which no nicotine had been added. There was a difference of 63.5 $\mu$ moles between the total alkaloid content of the treated and untreated cultures thus 101 % (which fell within the error limits for a 100% recovery) of the 62.5 $\mu$ moles of nicotine added were recovered. The percentage conversion of nicotine to nornicotine was 99 % (Table 3.7.5a). In the case of the photoheterotrophic culture-line there were 55.5 $\mu$ moles of nornicotine and 2.65 $\mu$ moles of nicotine present in the treated cells and no alkaloids were detectable in the untreated cells (Table 3.7.5b). The recovery of the added nicotine was therefore 93 % and a percentage conversion of nicotine to nornicotine of 84 %. For the mixotrophic culture-line the nornicotine and nicotine contents were 40.9 $\mu$ moles and 23.5 $\mu$ moles respectively (Table 3.7.5c). There was a total alkaloid content of 64.4 $\mu$ moles which was equivalent to a 103% recovery of the added nicotine. The percentage conversion of nicotine to nornicotine in these cultures was 65 %. There were no significant differences between the final fresh weights of cultures with and without added nicotine (Table 3.7.6).

The results of this experiment showed that light only caused a minor decrease in the alkaloid content of one of the cultures while in the other two culture-lines there was a more than 100% recovery of the added alkaloid. However, in both cases there was no significant difference between the recovery measured and 100% recovery. However, in all of the cultures there was a transformation of nicotine to nornicotine and this transformation was more efficient in cultures with decreased photosynthetic activity.

a. The percentage conversion of 62.5µmole nicotine to nornicotine by heterotrophic cultures.

	ALKALOID CONTENT (µmoles)	
	NORNICOTINE	NICOTINE
Control	1.7	1.3
62.5µmole Nicotine	63.5	3.0
Difference	61.8	1.7

Percentage conversion = 99 %

b. The percentage conversion of 62.5µmole nicotine to nornicotine by photoheterotrophic cultures.

	ALKALOID CONTENT (µmoles)	
	NORNICOTINE	NICOTINE
Control	ND	ND
62.5µmole Nicotine	55.5	2.65
Difference	55.5	2.65

Percentage conversion = 89 %

c. The percentage conversion of 62.5µmole nicotine to nornicotine by mixotrophic cultures.

	ALKALOID CONTENT (µmoles)	
	NORNICOTINE	NICOTINE
Control	ND	ND
62.5µmole Nicotine	40.9	23.5
Difference	40.9	23.5

Percentage conversion = 65 %

TABLE 3.7.5

The percentage conversion of 62.5µmole nicotine in MSS medium by suspension cultures of *N.tabacum* with different degrees of dependence on photosynthesis.

ND = Not detectable.



	FRESH WEIGHT (g)			
	CONTROL		62.5 $\mu$ mole NICOTINE	
	$\bar{X}$	SE	$\bar{X}$	SE
Heterotrophic	9.55	0.71	12.97	3.44
Photoheterotrophic	15.57	2.83	20.65	1.88
Mixotrophic	8.31	0.90	7.67	0.44

TABLE 3.7.6

The fresh weights of heterotrophic, photoheterotrophic and mixotrophic cultures grown with nicotine added to the culture medium. Cells were grown for 14 days in MSS medium, supplemented with either 3% or 1% fructose, containing 62.5 $\mu$ mole nicotine. One treatment with medium containing 3% fructose (heterotrophic) was grown in the dark while the other two treatments were cultured in the light (photoheterotrophic and mixotrophic). Alkaloid determinations were made on three replicates per treatment after 14 days growth, and each value represents the mean or the standard error of these replicates.

A further experiment was carried out using cultures grown in B<sub>5</sub> medium, since cells in this medium had been found to accumulate higher levels of nornicotine than cells grown heterotrophically in MSS medium (Section 3.4). Nicotine at a concentration of 2.5mM was added and growth allowed to proceed for 30 days.

### **3.7.2 The Biotransformation Of Nicotine To Nornicotine By Suspension Cultures Of *N.tabacum* Grown In B<sub>5</sub> Medium.**

At the start of the experiment 50ml of B<sub>5</sub> medium supplemented with 3% sucrose,  $0.81 \times 10^{-6}$ M NAA,  $0.93 \times 10^{-6}$ M kinetin,  $28 \times 10^{-6}$ M ascorbic acid and a final nicotine concentration of 2.5mM was inoculated with 1.5-2g wet weight of cells. Cultures were grown in the dark for 30 days at 25°C. At harvest the cells were separated from the medium and their fresh weight recorded before extraction of the alkaloids from both the cells and the medium.

#### **3.7.2.1 Alkaloid accumulation in cell suspension cultures of *N.tabacum* grown in B<sub>5</sub> medium.**

In cell suspension cultures of *N.tabacum* grown in B<sub>5</sub> medium nornicotine was the predominant alkaloid, with a total culture content of  $9.2 \pm 1.6$  mg (Table 3.7.7). Nornicotine accounted for 84% of the total alkaloid content of which 25% was found in the medium. Anabasine and nicotine were detectable in the cellular fraction ( $0.5 \pm 0.1$  mg and  $0.6 \pm 0.1$  mg respectively) and in trace amounts in the medium.

TABLE 3.7.7

The alkaloid content of *N.tabacum* cells grown in B<sub>5</sub> medium in the dark for 30 days. The mean and standard error were calculated from the results of alkaloid determinations from three replicates per treatment.

ND = Not detectable.

TABLE 3.7.8

The alkaloid content of cells grown, for 30 days in the dark, in B<sub>5</sub> medium containing 125µmoles of nicotine. Three replicates per treatment were analysed and the alkaloid contents found used to calculate the mean and standard error for each treatment.

ND = Not detectable.

TABLE 3.7.9

The percentage conversion of nicotine to nornicotine by suspension cultures of *N.tabacum* grown in B<sub>5</sub> medium. Cells were grown in the dark for 30 days in medium containing 125µmoles of nicotine.

ND = Not detectable.

	ALKALOID CONTENT (mg culture <sup>-1</sup> )					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	5.6	0.07	1.8	0.7	7.5	0.8
ANABASINE	0.5	0.1	0.17	0.1	0.58	0.2
ANATABINE	ND		ND		ND	
NICOTINE	0.6	0.1	0.1	0.1	1.1	0.5
TOTAL	6.8	0.3	2.1	1	9.2	1.6

	ALKALOID CONTENT (mg culture <sup>-1</sup> )					
	CELLS		MEDIUM		TOTAL	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
NORNICOTINE	16	1.2	10	0.7	26	1.9
ANABASINE	ND		ND		ND	
ANATABINE	ND		ND		ND	
NICOTINE	ND		ND		ND	
TOTAL	16	1.2	10	0.7	26	1.9

	ALKALOID CONTENT ( $\mu$ moles)	
	NORNICOTINE	NICOTINE
Control	50.1	6.8
125 $\mu$ mole Nicotine	181.0	ND
Difference	130.9	-6.8

Percentage conversion = 105%

### 3.7.2.2 The alkaloid content of cultures grown in B<sub>5</sub> medium containing 2.5mM nicotine.

After 30 days growth none of the nicotine added to the culture medium was detectable (Table 3.7.8). The nornicotine content showed a significant increase, above the control, in both the cells and the medium. No other alkaloids were detectable using HPLC analysis but analysis by TLC showed traces of nicotine and anabasine in both the cellular and the medium extracts. Nicotine was demethylated to nornicotine. This conversion was expressed as a percentage by subtracting the mean nornicotine content of the control cultures from the mean nornicotine content of cells grown in the presence of nicotine. The difference between the means was then expressed as a percentage of a 100% conversion. The control cultures had a mean nornicotine content of 50  $\mu$ moles (Table 3.7.9) while cultures treated with 2.5mM nicotine had a nornicotine content of 181  $\mu$ moles. The addition of nicotine to the medium of the cells therefore caused an increase in nornicotine content of 130  $\mu$ moles. A 100% conversion of the nicotine in the medium would have caused an increase in nornicotine content of 125  $\mu$ moles, thus a conversion of 105% had occurred. Although this value was greater than 100% the additional nornicotine was accounted for by variations between replicates rather than by a stimulation of alkaloid synthesis.

The alkaloid extracts from these cultures were analysed further using a more modified HPLC analysis and detection system and by GC-MS (carried out by Rothmans International Services: Chemical Research, Bremen). The results of this analysis showed the presence of large amounts of nornicotine as well as small quantities of the other major alkaloids and trace amounts of derivatives of the major alkaloids (Table 3.7.10 and Appendix 4).

This experiment showed that cell cultures of *N.tabacum* are capable of transforming nicotine to nornicotine at a high efficiency. It was also found that at the concentration of nicotine added to the cultures did not affect the growth of the cells (Table 3.7.11). Although nornicotine accounted for over 90% of the total alkaloid content, a range of tobacco alkaloids and their derivatives could be detected in trace amounts using modified GC-MS techniques.

CELLS	MEDIUM
Anabasine	Anabasine
Anatabine	Anatabine
-	Myosmine
N-acetylnornicotine	N-acetylnornicotine
N'-formylnornicotine	N'-formylnornicotine
-	N-nitrosonornicotine
Nicotine	Nicotine
Nornicotine	Nornicotine
Unknown <sup>1</sup>	-
-	Unknown

TABLE 3.7.10

Alkaloids and their derivatives detected in cellular and medium extracts of cultures grown in B<sub>5</sub> medium containing 125µmole nicotine. After routine HPLC and TLC analysis the samples were analysed further, using HPLC and GC-MS techniques, by Rothmans International Services: Chemical research, Bremen.

<sup>1</sup> = Unknown alkaloid derived from anabasine.

	FRESH WEIGHT (g)	
	$\bar{X}$	SE
Control	10.64	0.55
125µmole Nicotine	9.71	1.03

TABLE 3.7.11

The effect of nicotine added to the culture medium on biomass accumulation. Cells were grown in the dark for 30 days in B<sub>5</sub> medium containing 125µmole nicotine. Three replicates per treatment were harvested and the values obtained used to calculate the mean and standard error for each treatment.

After 14 days growth in the light in medium containing nicotine at least 80% of the *added* alkaloid at the start of the experiment could be accounted for. This indicates that photochemical degradation of the alkaloids does not account for the lack of alkaloid accumulation in cultures maintained in the light. Therefore it appears that light causes the metabolism of cells to be altered such that alkaloid synthesis may occur in the dark but not in the light. The activity of an enzyme that has previously been found to be associated with the synthesis of nicotine, and that is involved in the synthesis of the pyridine ring which is present in all of the alkaloids, was therefore determined. Initially the enzyme activity was measured in extracts from roots and shoots of *N.tabacum* plants in order to confirm previous reports of its activity.

### **3.8 THE ACTIVITY OF QUINOLINIC ACID PHOSPHORIBOSYL TRANSFERASE (QaPRT'ase) IN THE ROOTS AND SHOOTS OF *N.TABACUM* PLANTS.**

The results of the preceding experiments show that light inhibits alkaloid synthesis in unorganized cultures and that the lack of alkaloid accumulation is not due to the photochemical degradation of the alkaloids. In plants nicotine has been reported to be synthesised in the root system (Dawson 1942a,b) and the activity of the enzyme quinolinic acid phosphoribosyl transferase (QaPRT'ase) has been found to increase when plants are "topped" (a process that increases nicotine yield {Papenfus & Quin 1984}) (Saunders & Bush 1979). The activity of the enzyme has also been reported to be higher in the roots of plants that accumulate pyridine alkaloids than it is in the leaves of the same plants and higher than in plants that do not synthesise the alkaloids (Mann & Byerrum 1974a, Wagner & Wagner 1984, Wagner *et al* 1986a). The activity of the enzyme was therefore determined in plants of *N.tabacum* to investigate whether there were differences in activity between extracts from roots and shoots and between extracts from plants at different stages of development.

#### **3.8.1 Characterization Of The Methods Used In The Assay Of QaPRT'ase.**

##### **3.8.1.1 The separation and detection of the enzymatic product.**

There are two methods by which the enzyme QaPRT'ase can be assayed. The first of these is to determine the radioactivity of CO<sub>2</sub> liberated by the enzymatic reaction when <sup>14</sup>C-quinolinic acid is used as the substrate for the reaction. The second method is to quantify the enzymatic product, nicotinic acid mononucleotide (NaMN), after the assay has been terminated. The second of these two methods is more sensitive and was therefore chosen for use in this investigation.



NaMN can be detected in one of two ways, either by using  $^{14}\text{C}$ -quinolinic acid as the substrate for the reaction and determining the activity incorporated into NaMN or by using unlabelled quinolinic acid and using a spectroscopic method to quantify the NaMN. Both of these methods were used in this study since the specific activity of the  $^{14}\text{C}$ -quinolinic acid was low the spectroscopic method was used for confirmation of results. The presence of compounds in the extract that interfered with the spectroscopic quantification of NaMN necessitated the separation of NaMN from as many of these compounds as possible. The radioactive determination of enzyme activity required essential that the NaMN formed was completely separated from the  $^{14}\text{C}$ -quinolinic acid. Previously separation was achieved using HPLC (as described in Section 2.7.2.1) (Wagner & Wagner 1985). However, this technique did not allow complete separation of the  $^{14}\text{C}$ -quinolinic acid and NaMN (Fig 3.8.1). To get complete separation the paper chromatography system described in Section 2.7.4.4 was employed. Using this method the substrate could be separated from the product by up to 12cm (Fig 3.8.2). After ~~development~~ of paper chromatograms 1cm strips were cut so as to include the area corresponding to the Rf of the substrate (0.38) and the product (0.12). These strips were then either eluted with 1ml of distilled water for spectroscopic determination or scintillation counted in PPO/POPOP scintillant to determine the activity present in both the substrate and the product.

### **3.8.1.2 Determination of the protein elution profile of PD<sub>10</sub> columns and determination of which protein fraction contained QaPRT'ase activity**

After centrifugation of a root extract at 27,000xg for 20min <sup>(see section 2.7.4.1)</sup> 2.5ml of the supernatant was applied to a PD<sub>10</sub> column. The column was equilibrated and eluted with the extraction buffer and 0.3ml fractions were collected using an LKB fraction collector. The protein content of each fraction was determined using the Bradford protein assay method. The extract applied to the PD<sub>10</sub> column had a protein content of 1.35mg and the elution of this protein can be seen in Fig 3.8.3. The total amount of protein present in the fractions was 1.3 mg, therefore 98% of the protein applied to the column could be accounted for. The fractions containing protein were then assayed for QaPRT'ase activity.

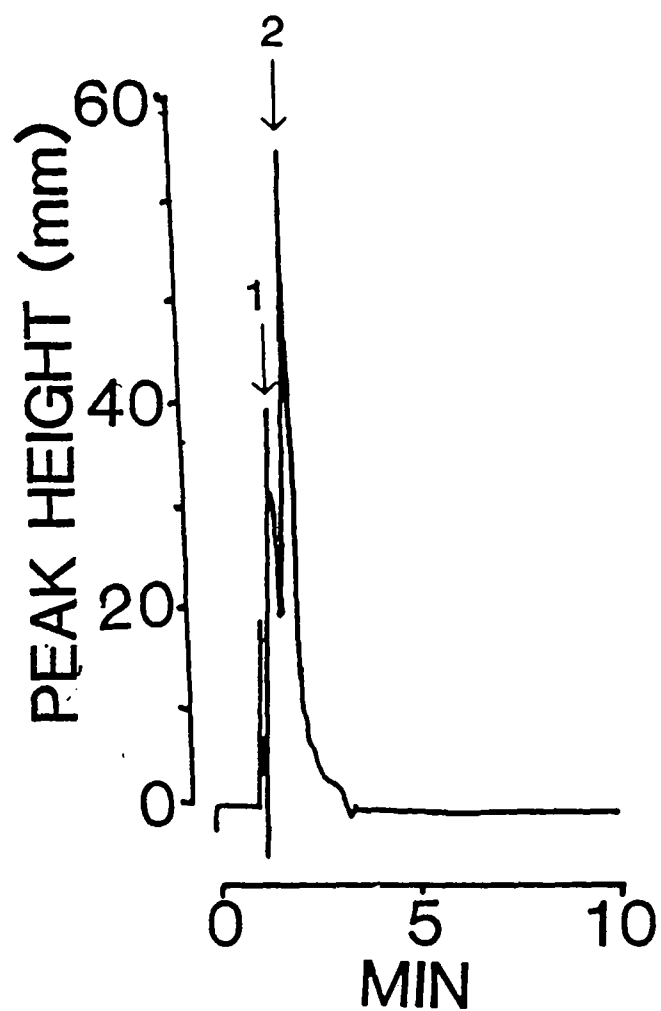


FIGURE 3.8.1

The separation of nicotinic acid mononucleotide (1) and quinolinic acid (2) by HPLC. A chromatogram from an injection of 20 $\mu$ l of a solution containing quinolinic acid at a concentration of 300 $\mu$ M and NaMN at a concentration of 150 $\mu$ M. The compounds were separated on a C<sub>18</sub> reverse phase column eluted isocratically with 200mM di-ammonium hydrogen orthophosphate and 10mM tetrabutylammonium hydrogen sulphate at a flow rate of 1ml min<sup>-1</sup>. The compounds were detected by their UV absorbance at 254nm.

# SOLVENT FRONT

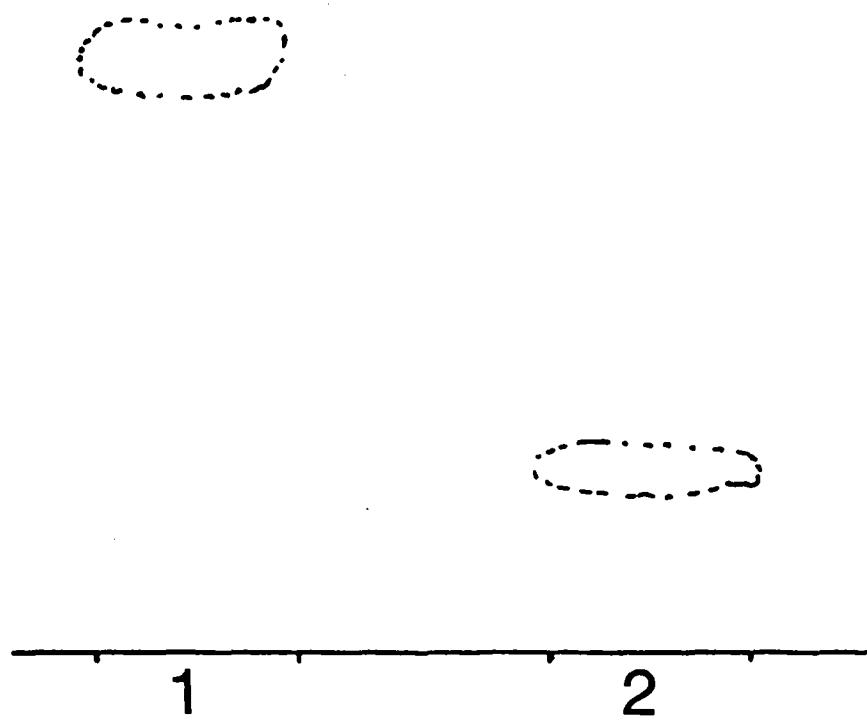


FIGURE 3.8.2

The *migration* of quinolinic acid (1) and NaMN (2) on a paper chromatogram *developed* for 16 hours with butanol: acetic acid: water (4:1:2) and *stained* with 0.2% fluorescein in ethanol.

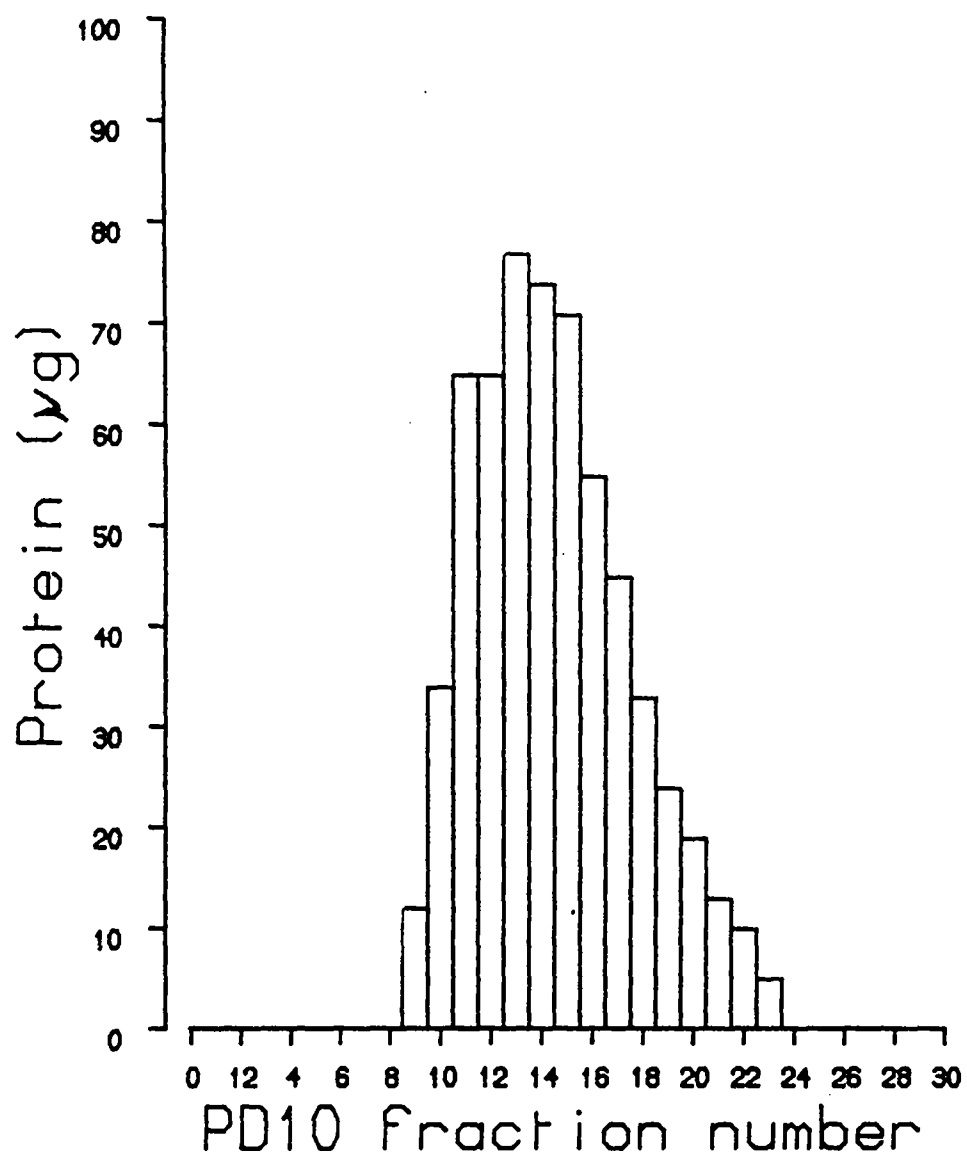


FIGURE 3.8.3

The elution of protein from a PD10 column. 2.5ml of the supernatant from a 27,000xg centrifugation was applied to the column and eluted. 0.3ml fractions were collected and 0.1ml of each fraction assayed for protein using the Bradford protein assay.

For the QaPRT'ase assay 80 $\mu$ l of each fraction were assayed with 220 $\mu$ l of the assay buffer. The reaction was terminated after 10min by boiling for 2min and the contents of each assay vessel was loaded onto a paper chromatogram as 2cm streaks. The chromatogram was *developed* and the chromatogram cut into 1cm strips which were then placed in PPO//POPOP scintillant and the activity on each strip counted. activity was found at the R<sub>f</sub> corresponding to NaMN in protein fractions 13 and 14 (Fig 3.8.4), with a higher activity in Fraction 14. Therefore protein Fraction 14 was used in all subsequent experiments in which QaPRT'ase was assayed.

### 3.8.2 Determination Of QaPRT'ase Activity In Roots And Leaves Of Plants Of *N.tabacum*

Roots were harvested from plants just prior to flowering. The extract obtained was used to determine the kinetics of the conversion of quinolinic acid to NaMN by QaPRT'ase. 80 $\mu$ l of extract were assayed in 220 $\mu$ l of assay buffer for 2.5, 5, 10, 15, 20, 60 and 90min. The contents of each assay vessel were then loaded onto paper chromatograms, as 2cm strips, and *developed*. After *development* 1cm strips were cut around the R<sub>f</sub> values of the substrate and the product and the amount of NaMN formed quantified. The conversion of quinolinic acid to NaMN was linear for 5min and then the rate of conversion reduced until a maximum conversion was reached at 10min (Fig 3.8.5). After longer periods of incubation, of the enzyme with the substrate, a decrease in the amount of product formed was seen. This decrease was observed irrespective of the method used to quantify the NaMN formed. From this time-course it appeared that the initial rate of reaction was linear for the first 5min and therefore this was used as the assay time in all subsequent determinations of QaPRT'ase activity.

The protein extracted and the specific activity of QaPRT'ase in extracts from leaves and roots of *N.tabacum* plants are shown in Table 3.8.1. Plants were harvested either 12 weeks after germination or at the time of initiation of the floral bud. There was no detectable QaPRT'ase activity in *leaf* extracts from plants of either age. However, QaPRT'ase activity was higher in extracts from roots of older plants, with specific activities of 0.3  $\pm$  0.1 nmoles  $\text{min}^{-1}$   $\text{mg protein}^{-1}$  and

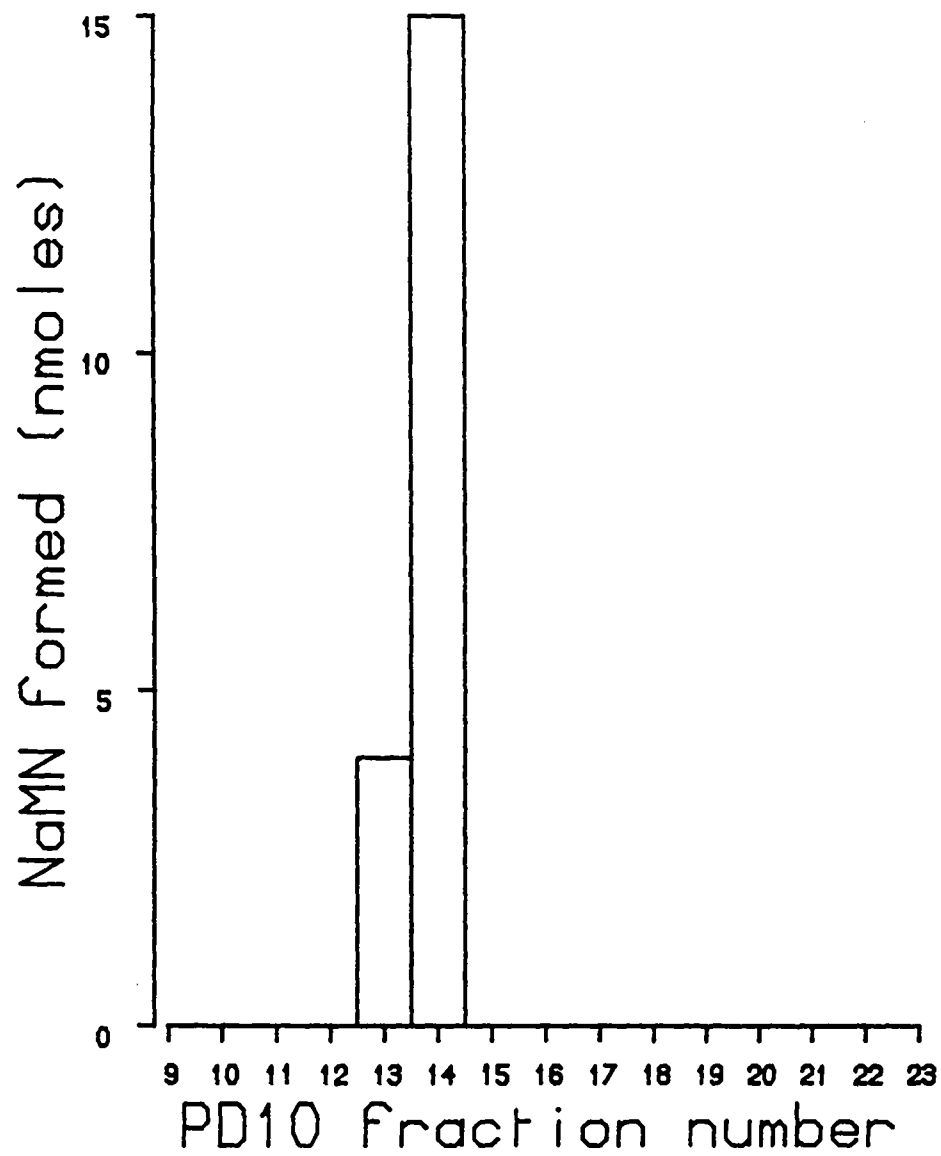


FIGURE 3.8.4

Determination of which protein fraction contains QaPRT'ase activity. 80 $\mu$ l of each, 0.3ml, protein fraction was assayed with 220 $\mu$ l of assay buffer for 5min. The product and the substrate were separated using paper chromatography and the product quantified by determining the amount of radio activity incorporated into it from  $^{14}$ C-quinolinic acid.

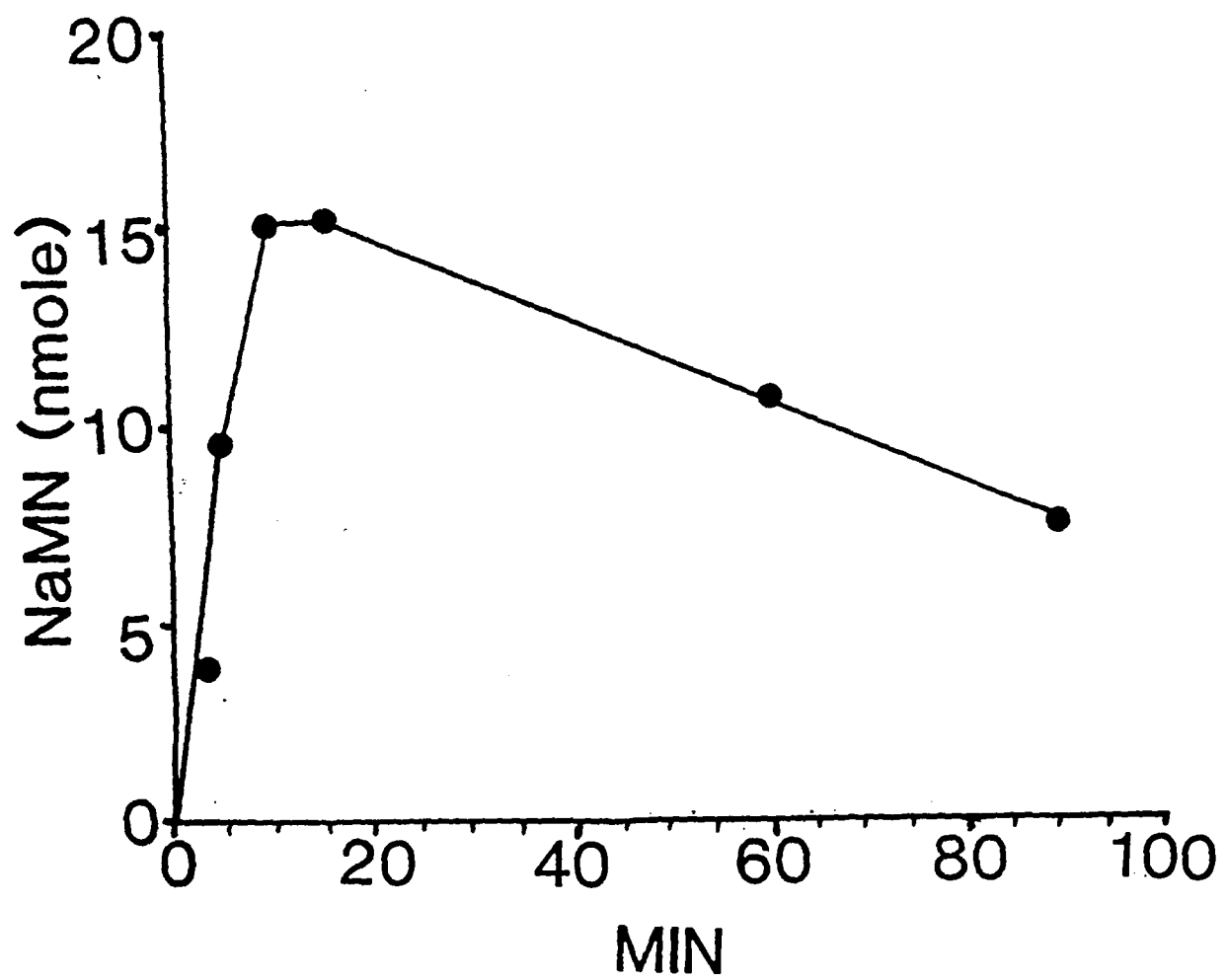


FIGURE 3.8.5

The kinetics of the conversion of quinolinic acid to NaMN by the enzyme QaPRT'ase.

	PROTEIN ( $\mu\text{g assay}^{-1}$ )		NaMN FORMED ( $\text{nmoles min}^{-1}$ )		SPECIFIC ACTIVITY ( $\text{nmoles min}^{-1} \text{mg}^{-1}$ )	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
LEAF <sup>1</sup>	104	11	ND		ND	
ROOT <sup>1</sup>	76	11	0.03	0.01	0.37	0.12
LEAF <sup>2</sup>	69	9	ND		ND	
ROOT <sup>2</sup>	84	6	1.47	0.09	17.62	1.66

TABLE 3.8.1

The protein content and the activity of QaPRT'ase in leaf and root extracts from *N.tabacum* plants 12 weeks and 38 weeks after germination.

<sup>1</sup> = plants 12 weeks after germination.

<sup>2</sup> = plants 38 weeks after germination.



17.6 ± 1.6 nmoles min<sup>-1</sup> mg<sup>-1</sup> for extracts from young and old roots respectively.

Having found that QaPRT'ase activity was higher in the roots than in the leaves, where activity was undetectable, the activity of the enzyme in cell cultures grown in the light or the dark was determined.

### **3.9 THE ACTIVITY OF QaPRT'ase IN CULTURES GROWN EITHER IN THE LIGHT OR IN THE DARK.**

It was found that light inhibits the synthesis of alkaloids in the cultures used in this investigation. It was also found that the activity of the enzyme QaPRT'ase was higher in the roots than in the leaves of plants, and previous work has suggested a role for this enzyme in the regulation of alkaloid synthesis (Mann & Byerrum 1974a,b, Saunders & Bush 1979, Wagner *et al* 1986). Therefore it was of interest to determine whether light reduced the activity of QaPRT'ase as well as alkaloid synthesis.

#### **3.9.1 QaPRT'ase Activity In Heterotrophic, Photoheterotrophic And Mixotrophic Suspension Cultures Of *N.tabacum***

Suspension cultures of *N.tabacum* were grown in MSS medium containing 1% or 3% fructose. One culture-line with 3% fructose was grown in the dark (heterotrophic) while another culture-line with 3% fructose and a culture-line with 1% fructose were grown in the light (photoheterotrophic and mixotrophic respectively). Cultures were harvested 23 days after subculture and the cells separated from their medium. The cells were then lyophilised, without freezing for 24 hours before extraction of QaPRT'ase. After centrifugation at 27,000xg the supernatant was black and the PD<sub>10</sub> fraction in which the enzyme eluted was also black. After incubating 80 µl of the extract with 220 µl of assay buffer and separation of the substrate from the product by paper chromatography it was not possible to detect any product using either the radioactive or the spectroscopic method.

### 3.9.2 QaPRT'ase Activity In *N.glauca* Callus Cultured In The Light Or The Dark.

Callus cultures of *N.glauca* were grown on solid MS medium containing 3% sucrose in either the light or the dark for 36 days. At harvest the callus was separated from the medium and QaPRT'ase extracted. 80µl of extract were assayed for 5min with 220µl of assay buffer. The specific activity of the enzyme was reduced by 99% in the extract from cells grown in the light when compared with cells grown in the dark (Table 3.9.1). There was no significant difference in the protein content in the assay vessel between these two treatments, 62.0 ± 16.0 and 71.0 ± 17.0 µg in the light and dark assays respectively. In the dark extracts there was a large variation in the QaPRT'ase activity between replicates with a maximum specific activity of 22.0 nmoles min<sup>-1</sup> mg<sup>-1</sup> and a minimum activity of 6.9 nmoles min<sup>-1</sup> mg<sup>-1</sup>.

From these results it appears that the activity of QaPRT'ase could be involved in the regulation of tobacco alkaloid biosynthesis and that the activity of QaPRT'ase may be regulated by light.

	PROTEIN ( $\mu\text{g assay}^{-1}$ )		NaMN FORMED ( $\text{nmoles min}^{-1}$ )		SPECIFIC ACTIVITY ( $\text{nmoles min}^{-1} \text{mg}^{-1}$ )	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
LIGHT	62	16	0	0	0	0
DARK	71	17	1.0	0.6	12	5

TABLE 3.9.1

The protein content and specific QaPRT'ase activity of enzyme extracts from *N.glauca* callus cultures after 36 days in either the light or the dark.

## SUMMARY.

1. A method for the detection of chlorophyll fluorescence from cultured cells has been developed to aid in the selection of photosynthetic cultures.
2. A method has been developed that enables the four main tobacco alkaloids to be extracted simultaneously in a final extract that is suitable for HPLC analysis.
3. Culture-lines that have an increasing dependence on photosynthesis for the provision of organic constituents have been established. The growth and nutritional characteristics of each of these culture-lines were determined.
4. Using cultures of *N.tabacum* with differing nutritional requirements it was found that light inhibited alkaloid accumulation and that this inhibition was independent of photosynthetic activity.
5. The synthesis of anabasine by callus cultures of *N.glauca* was also inhibited when the cultures were grown in the light. Analysis of the alkaloid content of these cultures during a culture cycle showed that alkaloid synthesis occurred during the stationary phase. On transfer of cultures from the light to the dark or *vice versa* it was found that the alkaloid content did not increase, or decrease, to the level of the two controls.
6. Using differentiated root cultures the effect of light on alkaloid synthesis was reduced and in shoot cultures there was very little alkaloid accumulation regardless of illumination.
7. By adding nicotine to the culture medium of cultures it was found that photochemical degradation does not account for the lack of alkaloid accumulation in cultures grown in the light. However, the majority of the nicotine added to the cultures was transformed into nornicotine.
8. The activity of the enzyme QaPRT'ase was undetectable in leaves but was detectable in roots of *N.tabacum* plants. The activity of the enzyme was higher in plants that were near to flowering.

9. The activity of the enzyme QaPRT'ase was drastically reduced by light in callus cultures of *N.tabacum*. In cultures grown in the dark there was a large variation in the activity of the enzyme.

**CHAPTER 4**  
**DISCUSSION**

All the *Nicotiana* species investigated accumulate one or more of the four main tobacco alkaloids (anabasine, anatabine, nicotine and nornicotine), the total amount and ratio of the individual alkaloids present in a plant depending on the species (Saitoh *et al* 1985). *Nicotiana* species grown *in vitro* as disorganized cultures (ie callus and suspension cultures) generally only accumulate small amounts of the alkaloids (Robins *et al* 1987), although levels comparable to those of the parent plant have been achieved (Ohta *et al* 1978a). Maximum alkaloid yields in so called undifferentiated cultures of *N.tabacum* have been achieved when cultures were maintained in the dark (Ohta *et al* 1978a). On illumination of cultures previously grown in the dark changes in the amount of alkaloid synthesis has been observed (Ohta & Yatazawa 1978b, Pearson 1978, Tabata *et al* 1971). Therefore the possibility exists that light plays a role in regulating alkaloid synthesis *in vitro*.

In many cases the transfer of cultures from the dark into the light induces chloroplast development (Dalton & Street 1976, de Klerk-Kiebert *et al* 1982). The presence of chloroplasts indicates that cellular differentiation is occurring in such a way as to promote photosynthetic carbon fixation. In several cases culture conditions have been manipulated, over a period, to produce cultures capable of sustained growth in the absence of any organic constituents in the medium (photoautotrophic cultures) (for review see Dalton & Peel 1983). The development of a functional photosynthetic system in cultured cells may create other metabolic differences between chlorophyllous and non-chlorophyllous cells (Dalton & Peel 1983, Dobberstein & Staba 1966, Yamada *et al* 1981).

Accordingly the effects of light on alkaloid synthesis in photosynthetic and non-photosynthetic cells were investigated. This was achieved using cultures of *N.tabacum* which obtained their organic constituents either from fructose added to the medium (heterotrophic cultures and photoheterotrophic cultures), or from carbon added to the medium and photosynthesis (mixotrophic cultures), or by photosynthesis <sup>alone</sup> (photosynthetic cultures). with different degrees of dependence on photosynthesis, and thus under different light conditions, for the provision of organic constituents. The results of this investigation along with the effects of light on alkaloid synthesis in two other *Nicotiana* species are now discussed.

## THE EXPERIMENTAL SYSTEM.

Three species of *Nicotiana* were used to investigate the effects of illumination on growth and alkaloid synthesis. Root cultures of *N.rustica*, grown in W medium, were selected because of their high alkaloid content ~~in this species~~ (Saitoh *et al* 1985) and the ability of cultured roots to accumulate alkaloids at levels similar to those in the whole plant (Hashimoto *et al* 1986, Endo & Yamada 1985, Tabata *et al* 1972). Thus root cultures of *N.rustica* provided an *in vitro* system with the potential for high levels of alkaloid synthesis, in particular nicotine. The second species chosen was *N.glauca*, which was maintained as callus on MS medium. This species was selected because anabasine, rather than nicotine, is the major accumulating alkaloid in the plant (Saitoh *et al* 1985). Using this species it was possible to investigate the effect of illumination on an alkaloid other than nicotine. The third species studied was *N.tabacum* which was selected because of its economic importance and the large amount of research previously undertaken with the plant. The cultivar of *N.tabacum* used was Wisconsin-38, since it had been used extensively in previous studies and is also of economic value. For these reasons *N.tabacum* was studied in greatest detail paying particular attention to the effects of illumination, in cells with varying degrees of photosynthetic activity, on growth and alkaloid synthesis in cultures.

### Selection Of A Basal Medium Suitable For The Growth Of *N.tabacum* Cells In Suspension Culture.

Here two criteria were used to judge the suitability of the basal medium and growth regulators:

1. That cells grown in the dark accumulated detectable and quantifiable alkaloid levels.
2. That the photosynthetic development of cells grown in the light was not inhibited by the medium.

Previous investigations of alkaloid synthesis by cell cultures of *N.tabacum* have employed several different basal media and a variety of growth



regulators, supplemented with sucrose as a carbon source (Feth *et al* 1986, Furuya *et al* 1966, Lockwood & Essa 1984, Shio & Ohta 1973, Tabata *et al* 1971, Takahashi & Yamada 1973). In each case detectable levels of nicotine and, to a lesser degree, the other alkaloids, were reported.

Cultures of spinach (*Spinacia oleracea*) (Dalton 1980) and tobacco (Kaul & Sabharwal 1971) capable of high levels of photosynthesis have been maintained on MSS medium, while photosynthetic cultures of periwinkle (*Catharanthus roseus*) have been grown on B<sub>5</sub> medium (Tyler *et al* 1986). It has also been found that the use of fructose as a carbon source promotes the photosynthetic development of cultures, whereas sucrose suppresses such development (Dalton 1977 & Street, Edelman & Hanson 1971). Due to the reported instability of IAA in the light (Yamada & Sato 1978), NAA was used as an auxin, since the effect of NAA on alkaloid synthesis has been shown to be similar to that of IAA (Pearson 1978). BAP was used as the cytokinin since it has been found to be more suitable than kinetin for the photosynthetic development of tobacco cell cultures (Yamada & Sato 1978).

In view of the wide range of media that have been used in previous investigations it was necessary to determine which medium fulfilled the criteria described above. Five basal media (MS, MSS, SH, W and B<sub>5</sub>) were supplemented with sucrose (which has been used as a carbon source in other studies of alkaloid synthesis in culture), NAA and kinetin. In a sixth treatment MSS medium was supplemented with fructose, NAA and BAP since these additions were favourable for photosynthetic development. After one culture cycle in the dark, cultures in the two media previously reported to promote the development of photosynthetic cultures (MSS and B<sub>5</sub>) were found to have the highest alkaloid contents. However, tobacco cultures grew poorly in B<sub>5</sub> medium in the light with a reduced percentage of fructose in the medium (which were prerequisites for obtaining highly photosynthetic cultures), therefore MSS medium was chosen as the medium for the growth of *N.tabacum* suspension cultures.

## **Selection Of Culture-lines Of *N.tabacum* With Different Nutritional Requirements.**

For the experiments conducted in this thesis suspension cultures of *N.tabacum* were established which could be placed in one of four nutritional classes:

1. Heterotrophic cultures: in which cells were totally dependent on carbon added to the medium for the provision of organic constituents (grown in the dark).
2. Photoheterotrophic cultures: in which cells were totally dependent on carbon added to the medium for the provision of organic constituents (grown in the light).
3. Mixotrophic cultures: in which cells were partially dependent on carbon added to the medium and partially dependent on photosynthesis for the provision of organic constituents (grown in the light).
4. Photosynthetic cultures: in which cells were capable of sustained growth in the presence of light and CO<sub>2</sub>, in a defined carbohydrate free medium, containing mineral nutrients and organic growth factors (eg vitamins and growth substances) (grown in the light).(Dalton & Peel 1983).

Cell cultures consist of a heterogeneous population of cells (Dougall 1987, Yeoman & Forche 1980)). On exposure of cell cultures to light some of the cells accumulate chlorophyll (Bender *et al* 1985) and through cloning (Bergmann 1977) selection according to chlorophyll content highly chlorophyllous cultures have been obtained (Dalton & Street 1976, Davey *et al* 1971). In this investigation cloning and selection were performed and calli either grown in the light or the dark according to their chlorophyll content. After initially selecting calli according to their chlorophyll content further screening was carried out using the chlorophyll fluorescence characteristics of the cells, rather than chlorophyll content alone. Chlorophyll fluorescence as an aid in selecting for photosynthetic cultures has the advantage of allowing an estimation of the functional state of the photosynthetic electron transport chain (Butler 1977). This is particularly useful since it has been found that the chlorophyll content of cultured cells is not a useful index of photosynthetic capacity (Seeni & Gnanam 1981). By taking a sample of cells from each

culture-line during the late exponential or early stationary phase of growth, when photosynthetic activity is likely to be maximal (Bender *et al*/ 1985), it was possible from the fluorescence profiles recorded to select cultures with high photosynthetic potential. The most photosynthetically able cells were then transferred to medium with a lower percentage of fructose, which has been shown to promote photosynthetic development (Dalton 1980). This process was repeated up to a point where any further reduction in carbon added to the medium caused substantial cell death. However, by plating cell suspensions on solid medium with no added carbon source and placing these under a high light intensity (see Berlyn and Zelitch 1975) several aggregates capable of survival in a sugar free medium were obtained. These aggregates were then transferred to a "flat-bed" culture system (Lindsey & Yeoman 1983a) in which the composition of the medium could be maintained at a relatively constant level and the gaseous atmosphere surrounding the cells could be adjusted. The gaseous atmosphere was adjusted to give an overall mixture of 2% CO<sub>2</sub>, 10% O<sub>2</sub> and 88% N<sub>2</sub>. Increased levels of CO<sub>2</sub> were required for photosynthetic cultures because of the high resistance to the entry of CO<sub>2</sub>. (Berlyn & Zelitch 1975). A lower oxygen concentration, than in air, promotes photosynthetic pigment synthesis (Dalton & Street 1976), and together with a high CO<sub>2</sub> concentration lowers the level of oxygen consumption (respiration) (Yamada *et al*/ 1981) and photorespiration (Berlyn & Zelitch 1975).

Using this selection procedure culture-lines that were either: heterotrophic, photoheterotrophic, mixotrophic or photosynthetic were established. Heterotrophic cultures were grown in the dark with 3% fructose in the medium. Photoheterotrophic cultures were grown in the light in the same medium as the heterotrophic cultures. Mixotrophic cultures were grown under the same light intensity as photoheterotrophic cultures but with a reduced level of fructose (1%). Photosynthetic cultures were grown at an increased light intensity in medium that contained no carbohydrate but did contain small amounts of organic compounds.. The development of the photosynthetic apparatus during this selection process is now discussed.

## **Photosynthetic Development In Culture-lines of *N.tabacum***

From the data recorded at each screening, and from the characterization of cultures at the end of the selection process, it was possible to follow the photosynthetic development of culture-lines of *N.tabacum*. Measurements, for screening purposes, were made in the late exponential or early stationary phase of the culture cycle when photosynthetic activity was maximal. Thus the developmental sequence recorded was obtained from values obtained for the maximal state of photosynthetic development during a given culture cycle, rather than from average values from that culture cycle. However, it was not possible to determine the growth phases of cultures in the "flat-bed" system and so these values were taken as the averages for the growth cycle. Also measurements of photosynthesis were made at a higher light intensity than that at which the cells were grown, therefore the photosynthetic rates recorded were an overestimation of the rates in the culture vessel.

It has been reported that within three days of transferring tobacco cultures from the dark into the light chloroplasts are recognizable (Nato *et al* 1981). After five to six days the average number of chloroplast genome copies per cell increases. This copy number per cell of a mixotrophic culture was found to be higher than in photosynthetic cultures and the chloroplast genome copy number per cell of the latter corresponded to the copy number per cell in leaves (Cannon *et al* 1985). Some of the cells in cultures which had been transferred into the light must therefore have rapidly attained the potential for photosynthetic activity. However, as discussed above constant screening and selection of cultures is required to obtain photosynthetic (photoautotrophic) cultures. Therefore cultural conditions or some other factor(s) would appear to suppress the expression of this potential for photosynthetic growth, such that selection of cells through a series of defined environmental conditions is required to obtain full expression of the photosynthetic activity. By examining the photosynthetic characteristics of cultures in these environments gross changes that occur during the development of the photosynthetic apparatus could be followed.

The chlorophyll content of culture-lines increased throughout the

selection process. However, increases in photosynthetic competence and the appearance of chlorophyll fluorescence profiles characteristic of photosynthetic tissue did not mirror the increases in chlorophyll content. In barley the fluorescence induction kinetics changed in parallel with other photosynthetic processes measured during the greening of etiolated leaves in the light (Buschmann 1981), thus the accumulation of chlorophyll and the development of the photosynthetic electron transport chain in cultures of *N.tabacum* is uncoupled. However, the primary electron acceptor pool (the amount of the primary electron acceptor of photosynthesis present) appeared to reach a maximum level early in the developmental sequence. After several screening and selection cycles, a mixotrophic culture-line with a chlorophyll content of  $22.0 \pm 1.8 \mu\text{g g}^{-1}$  fr wt was achieved. The photosynthetic competence of this culture-line was  $1.59 \pm 0.23$  while the chlorophyll fluorescence profile had some of the characteristics of profiles from photosynthetic material. The photosynthetic competence was positive therefore the cells were capable of splitting water, to give hydrogen ions and oxygen. This was supported by the decrease in the rate of chlorophyll fluorescence between  $F_0$  and the dip in fluorescence emission (Papageorgiou 1975). It was also concluded from the fluorescence profile that photosystems I and II were linked to one and other (this association being essential for complete photosynthetic electron transport), the onset of which gave rise to the quenching of fluorescence after the initial peak (Papageorgiou 1975). The rise in fluorescence, after quenching, to the maximum level, which was not as great as the rise seen in leaf discs, is attributable to a transient block in electron transport in photosystem I (Munday & Govindjee 1969). In cultured cells the lack of organization of the thylakoids, within the chloroplast, (Laetsch & Stetler 1965) may account for the decreased yield of fluorescence from the dip in fluorescence emission to the maximum, since thylakoid damage in leaves leads to a decrease in variable fluorescence (Krause & Weis 1984). Although cells from this culture-line were capable of photosynthetic electron transport, as determined from the chlorophyll fluorescence profile, they did not appear to be capable of  $\text{CO}_2$  fixation. This was detected because the processes involved in the fixation of  $\text{CO}_2$  in leaves lead to a quenching of fluorescence through both photochemical and non-photochemical events (Sivak & Walker 1986) and this decrease in fluorescence was greatly reduced in mixotrophic cells.

With continued screening and selection and growth of the cells in a "flat-bed" culture system a photosynthetic culture-line with a chlorophyll content of  $48.8\mu\text{g g}^{-1}$  fr wt was obtained. This is comparable with the chlorophyll content ( $42.8\mu\text{g g}^{-1}$  fr wt) reported for photoautotrophic cultures of tobacco (Berlyn & Zelitch 1975) but only about half of that reported, in the same species, by Yamada and Sato (1978). The photosynthetic competence of the cells had increased to 2.08 but the chlorophyll fluorescence profile obtained from a vertical section through the culture showed very little change from the profiles from mixotrophic cultures. However, the photosynthetic cultures must have been capable of  $\text{CO}_2$  fixation since their medium contained no carbohydrate thus making  $\text{CO}_2$  fixation a prerequisite for growth. This apparent discrepancy between the fluorescence profile and the growth regime, of the cells may be due to several factors: The surface layer of the cells was highly chlorophyllous whereas in the suspension cultured mixotrophic cells the chlorophyll content was more uniformly distributed within the culture. Another factor that needs to be considered is that the growth of the photosynthetic cultures occurred at the surface of the culture which, in conjunction with the sampling time (which was not necessarily at the time of optimal photosynthetic development), may have affected the fluorescence profile. The growth phase of the culture is important because granal differentiation in chloroplasts has been found to be incomplete in non-stationary phase cultures, with differentiation increasing during the stationary phase (Laetsch & Stetler 1965). The degree of differentiation of the grana and their orientation with respect to one another has an effect on the fluorescence emissions of photosynthetic tissues (Papageorgiou 1975). Chloroplast ultrastructure may determine, to some extent the fluorescence profiles recorded, as has been found in carrot cultures (Kumar *et al* 1983). The differences between the fluorescence profiles of cultures <sup>of</sup> cells and leaf discs may also be associated with the lower level of organization in the cultured tissue. This lack of organization may affect the movements of photosynthetic metabolites between cells and thus disrupt photosynthetic  $\text{CO}_2$  fixation which in turn affect the fluorescence emissions.

Changes in fluorescence profiles during the culture cycle of mixotrophic cells were similar to those seen during the selection process through which this culture-line was established. The subculturing of these cells therefore leads to changes in the photosynthetic capacity of the cells through the culture cycle, as has previously been reported (Nato *et al* 1985), and as

discussed in more detail in the next section. The growth and nutritional requirements of the established culture-lines, during a culture cycle are now discussed.

## **THE GROWTH AND NUTRITIONAL REQUIREMENTS OF CULTURES OF *N.TABACUM* WITH DIFFERENT DEGREES OF DEPENDENCE ON PHOTOSYNTHESIS FOR GROWTH.**

Plant cell cultures at different stages of photosynthetic development offer a system in which the effect of the nutritional status of cells, as well as the effect of light on those cells, on secondary metabolism can be investigated. Before the accumulation of alkaloids in heterotrophic, photoheterotrophic, mixotrophic and photosynthetic is discussed the growth and nutritional requirements of these cultures are considered.

The nutrient medium of cultured suspended cells was not replenished during the culture cycle, therefore as the cycle progressed the availability of nutrients from the medium decreased. The depletion of nutrients leads to one or more nutrients becoming limiting with such limitation having an effect on the metabolic activity of the cells (Dalton & Peel 1983). Nutrient effects were kept to a minimum in the "flat-bed" culture system, in which photosynthetic cells were grown, because the nutrient medium was replenished at frequent intervals. Due to this difference in the availability of nutrients between culture-lines, and the effect on the metabolism of the cells, the photosynthetic culture-line is discussed separately after the heterotrophic and mixotrophic culture-lines have been considered.

### **The Growth And Nutrition Of Heterotrophic And Mixotrophic Suspension Cultures Of *N.tabacum*.**

In heterotrophic and mixotrophic cultures carbohydrate, in the form of fructose, was added to the medium at inoculation at concentrations of 30g

$l^{-1}$  (heterotrophic) and  $10g\ l^{-1}$  (mixotrophic). After inoculation the heterotrophic cultures showed a lag phase of 2 days, similar to that seen in tobacco cultures grown in 3% sucrose in the light (Nato *et al* 1977). The lag phase was not seen in the mixotrophic cultures, as was previously the case with cultures grown in 2% sucrose (Filner 1965). The mixotrophic cultures had a doubling time of 5.4 days and exponential growth lasted for 8 days, while the exponential phase in the heterotrophic cultures proceeded for 14 days during which the doubling time was 6.5 days. In both of these culture-lines the doubling time and the duration of the exponential phase were longer than previously reported. Exponential phases of 6 days (Nato *et al* 1977) and 8 to 10 days (Filner 1965) with corresponding doubling times of 2.5 and 2 days have been reported for suspension cultures of tobacco. However, in this study carbohydrate was supplied as fructose, rather than sucrose, and this may influence the growth of the cells as was found when fructose, instead of sucrose, was used as the carbon source for spinach cultures (Dalton & Street 1977). Also the size of the initial inoculum and the state of the cells used for the inoculum influence the final biomass and the nature of the growth curve (King & Street 1977).

The termination of growth of the mixotrophic cultures coincided with the, almost, complete depletion of fructose from the medium. At this point there was no significant difference in the uptake of fructose by the heterotrophic and mixotrophic cultures. However, the higher initial fructose content of the medium bathing the heterotrophic cells meant that appreciable amounts were still available in the medium and it was therefore not constraining growth. The greater final biomass achieved by the heterotrophic cultures than by the mixotrophic cultures was due to the extended exponential and linear growth phases, as has been found with heterotrophic and mixotrophic cultures of potato (La Rosa *et al* 1984). In the heterotrophic cultures the onset of the stationary phase of growth was not accompanied by complete uptake of fructose from the medium.

In both culture-lines phosphate was completely removed from the medium during the period of exponential growth, although its uptake was slower than previously reported (Knobloch *et al* 1981). However, this difference in the rate of phosphate uptake may be due to differences in the growth regulators supplied, since it has been found that auxin concentration<sup>s</sup>, in



particular, affect the rate of phosphate uptake (Pinoi *et al* 1985). The residual concentration of phosphate is not related to the rate of growth (Dalton 1983) because it is a "conserved" nutrient (Nyholm 1976). Therefore phosphate would not have been limiting growth in either culture-line at the time of its complete uptake from the medium. In mixotrophic cultures phosphate was detected in the medium after its complete uptake and its appearance corresponded with a decrease in fresh weight. It appeared that on cell death, in the mixotrophic cultures, phosphate was released into the medium. This indicated that the phosphate accumulated by the cells had not been completely utilized and therefore it was not likely to limit growth. In the heterotrophic culture-line no decrease in fresh weight was seen and phosphate was absent from the medium. It has been found that the intracellular phosphate levels in tobacco cells are depleted 15 days after phosphate was completely removed from the medium (Knobloch *et al* 1981). This being the case it is possible that phosphate was limiting growth in the heterotrophic cultures.

The limiting nutrient would appear to differ between the two culture-lines such that phosphate limited growth in the heterotrophic cultures while in the mixotrophic cultures fructose was limiting. This is in agreement with changes in the limiting nutrient reported during the transition from heterotrophic to mixotrophic growth of suspension cultures of *Ocimum basilicum* (Dalton 1983). However, it must be remembered that cells are not only taking up nutrients from their medium but are also releasing compounds into the medium. Some of these compounds may be deleterious to growth and therefore could play a role in determining the onset of the stationary phase (Murashige 1986).

In both culture-lines nitrate was taken up by the cells to a greater degree than ammonium. Ammonium is usually utilized preferentially to nitrate (Dougall 1980) since its uptake is normally passive. The passive nature of ammonium uptake could explain the rapid initial uptake of this nutrient observed in both culture-lines. However, ammonium uptake causes a decrease in the pH of the culture medium (Sheat *et al* 1959). The decrease in medium pH causes the protonation of ammonium making it unavailable to the cells because in its protonated form it cannot cross the cell membrane. In cell suspension cultures of *Ipomoea* nitrate uptake was found to increase as the pH of the

medium decreased while ammonium uptake decreased, such that at pH's below 5.6 nitrate uptake was preferential to ammonium uptake (Martin & Rose 1976). In the present investigation heterotrophic cultures only took up small amounts of ammonium after the initial few hours. In the mixotrophic cultures ammonium uptake continued, after the initial uptake, until the cultures entered the stationary phase of the growth cycle. Nitrate uptake continued in both culture-lines until the stationary phase was reached. The increased uptake of both ammonium and nitrate by heterotrophic cultures was probably due to the increased yield of biomass in this culture-line. The difference between the two types of culture in ammonium uptake indicates that differences in nitrogen metabolism exist between them. Whether the differences in ammonium uptake were due to differences in cellular metabolism or to differences in the pH of the medium of both was unclear.

#### **Oxygen uptake (respiration) And Photosynthetic Changes During A Culture Cycle Of Heterotrophic And Mixotrophic Cultures Of *N.tabacum***

In both heterotrophic and mixotrophic cultures the rate of respiration increased, after inoculation, to a maximum on days six and 12 for the mixotrophic and heterotrophic culture-lines respectively. This increase may have been a response to a combination of: trauma, dilution and increased metabolic activity prior to cell growth. It is likely that respiration increases, at least in part, as a consequence of increased metabolic activity prior to cell division since respiration rates peaked just before maximal rates of increase in fresh weight. When cells were grown in MS medium supplemented with 3% fructose an increase in cell number was seen before fresh weight increased. This is in agreement with the possibility that increases in respiration were associated with the onset of cell division (Yeoman *et al* 1965, Yeoman 1970). Increases in respiration prior to cell division have previously been reported in tobacco suspension cultures (Nato *et al* 1977) and are thought to be associated with auxin induced growth (Leonova *et al* 1985) and/or a high rate of carbohydrate oxidation via the glycolytic pathway (Fowler 1971). Respiration rates declined before decreases in fresh weight were observed. However, the increase in dry weight of cultures grown in MS medium supplemented with 3% sucrose declined before the increase in fresh weight was complete. This

indicates that at later stages of the linear growth phase increases in fresh weight were due to cell expansion rather than cell division, as has been found in suspension cultures of *Acer pseudoplatanus* (King & Street 1977). Therefore metabolic activity due to cell division would have declined and in turn may have led to a decrease in respiration rates.

In the heterotrophic culture-line chlorophyll, and consequently photosynthetic activity, was not detectable throughout the growth cycle. In the mixotrophic culture-line the chlorophyll content was relatively constant until it increased during the stationary phase. This result was in agreement with previous findings (Laetsch & Stetler 1965, Lindsey 1982). The apparent lack of chlorophyll accumulation during the period of cell growth was probably due to a combination of inter-related factors: modifications of chloroplast structure due to the subculturing process (Nato *et al* 1977), dilution of the chlorophyll content per unit weight caused by unbalanced growth (La Rosa *et al* 1984), carbohydrate suppression of chlorophyll synthesis (Edelman & Hanson 1971, Pamplin & Chapman 1975) and increased organization of cultures during the stationary phase (Lindsey 1982). Since the chlorophyll content increased during the stationary phase (which was itself brought about by fructose limitation) when cell division had ceased and culture organization was maximal it was not possible to elucidate which of these factors was instrumental in bringing about the increased chlorophyll content.

During the exponential phase of growth the gross photosynthetic rate gradually increased and then reached a maximum during the linear growth phase before starting to decline. However, the photosynthetic competence of the cells did not follow the same pattern. During the increase in gross photosynthesis, the rate of respiration was high and as a result the value for photosynthetic competence was below 1. Towards the end of the exponential phase the rate of respiration decreased while the rate of gross photosynthesis continued to increase and therefore the photosynthetic competence increased reaching a value of approximately 1 in the linear growth phase. A value of nearly 2 has previously been reported for the photosynthetic competence during the exponential phase (Nato *et al* 1985). However, in the same study it was found that light intensity not only affected the value of photosynthetic competence but also affected the times during the growth cycle at which

changes in rates of respiration took place. In the present study a lower light intensity was used and this may account for the difference in the value for photosynthetic competence during the exponential phase. A maximum photosynthetic competence of approximately 1.5 was reached during the stationary phase, after the depletion of fructose from the medium and as chlorophyll content increased. The photosynthetic competence then decreased to 1 at the end of the growth cycle. These results would appear to be very similar to those of Nato *et al* (1985), when the difference in light intensity is taken into account.

The chlorophyll fluorescence profiles show that the cells became more characteristic of photosynthetically active tissue at the same time as the photosynthetic competence became maximal. During the early stages of the culture cycle chlorophyll fluorescence emissions indicated that photosystems I and II were not associated with one another and therefore photosynthetic electron transport was incomplete. This has been reported during the development of the photosynthetic system in carrot cells (Kumar *et al* 1983) and is probably due to a loss of chloroplast integrity. Changes in chloroplast structure brought about by such factors as starch deposition, as a result of carbohydrate in the medium, have previously been found in mixotrophic tobacco suspension cultures (Nato *et al* 1977). Towards the end of the culture cycle the fluorescence profiles indicated that, although the photosynthetic competence had decreased, the utilization of the light energy captured in fixing CO<sub>2</sub> had increased.

These results support the model of growth and nutritional state during a growth cycle proposed for mixotrophic cultures by Bender *et al* (1985). After inoculation cultures utilized carbohydrate in the medium as an energy and carbon source (heterotrophic growth). As fructose was removed from the medium photosynthetic activity increased and began to contribute to the carbon balance of the cells (mixotrophic growth). Finally, when the carbon in the medium was depleted, photosynthetic carbon fixation was capable of sustaining the metabolic activity of the cells, although no increase in fresh weight was observed during this period. The prolonged stationary phase in mixotrophic cultures, due to the availability of carbon through photosynthetic CO<sub>2</sub> fixation, showed the potential of these cells for photosynthetic growth.

## **Growth, Photosynthesis And Nutrient Uptake In Photosynthetic Cultures.**

After 54 days growth in the "flat-bed" culture system cultures that were supplied with medium minus carbohydrate showed an increase in fresh weight of 14.86g. Assuming that growth had been constant throughout this period the doubling time can be calculated as 13.5 days. At the time of harvest the respiration rate was similar to that for mixotrophic cells during the initial days in suspension culture. The chlorophyll content at this time was nearly double that in the mixotrophic culture-line and was similar to that of photoautotrophic suspension cultures established by Berlyn and Zelitch (1975). The gross rate of photosynthesis was more than double the maximum rate seen in mixotrophic cultures and the photosynthetic competence had increased to over 2. Chlorophyll fluorescence emissions showed that photosynthetic electron transport was taking place, although the level of fluorescence quenching, caused by the process of CO<sub>2</sub> fixation, was not as great as seen in leaf discs.

Analysis of medium that had passed through the culture system showed that there was a small uptake of ammonium and nitrate while there was a much greater uptake of phosphate. Like ammonium, phosphate normally enters the cell passively and, due to its being a "conserved" nutrient, the high uptake does not correlate with a high cellular requirement for this nutrient. The growth and survival of the cells in the "flat-bed" system employing carbohydrate free medium confirmed that tobacco cell cultures are capable of photosynthetic growth.

The different modes of growth and nutrition in three culture-lines of *N.tabacum* and changes in growth, nutrient uptake and photosynthetic activity throughout a culture cycle have been considered. In the next section the effect of light on the synthesis and accumulation of alkaloids in these cultures is discussed.

## THE EFFECT OF LIGHT ON ALKALOID ACCUMULATION IN CULTURES OF *NICOTIANA*

In many cases light has been reported to stimulate the formation of secondary products in plant cell cultures (for review see Seibert & Kadkade 1980). In general the cultures studied were not dependent on photosynthesis for the supply of carbon and the increased synthesis was probably due to the presence of chloroplasts (Dalton & Peel 1983). In tobacco cell cultures there have been reports of light both enhancing nicotine synthesis (Tabata *et al* 1971) and causing a suppression of synthesis of nicotine (Ohta & Yatazawa 1978, Pearson 1978). The effects of light on alkaloid accumulation in cultures of *N.tabacum* with different modes of nutrition are discussed first. The effect of light on anabasine accumulation in callus cultures is then considered. Finally changes in alkaloid synthesis due to light in root cultures of *N.rustica* and shoot cultures of *N.tabacum* are discussed.

### The Effect Of Light And Mode Of Nutrition On Alkaloid Accumulation In Cultures Of *N.tabacum*.

Ohta and Yatazawa (1978) found that three weeks after transferring callus of *N.tabacum* from the dark into the light nicotine synthesis was greatly reduced. However, callus cultures grown with a 12 hour photoperiod (Tabata *et al* 1971) showed increases in nicotine accumulation. In this investigation the effect of light and mode of nutrition on alkaloid synthesis in cultures of *N.tabacum* was investigated to determine whether light suppressed or enhanced alkaloid synthesis and to determine whether any effects of light were photomorphogenic or photosynthetic in nature.

On determination of the alkaloid contents of heterotrophic, photoheterotrophic, mixotrophic and photosynthetic cultures, the heterotrophic cultures were found to have the highest alkaloid content. The alkaloids detectable in the roots and leaves of plants, grown from the same seed as was used to initiate the cultures, were also detectable in the cells and to a lesser degree in the medium of heterotrophic cultures. However, there was an 88% reduction in alkaloid content, on a per gram basis, between the plant and the

cultures. Also nicotine was not the major alkaloid in the cultures whereas it was in the plant. The alkaloid contents of plants grown under green-house conditions were different from the alkaloid contents previously reported for *N.tabacum*. The total alkaloid content of plants used was approximately half that previously reported and the proportion of this in the roots was greater than in the shoots which was contrary to previous findings (Saitoh *et al* 1985). The overall ratio of nornicotine to nicotine, which is determined by a single dominant gene (Griffith *et al* 1955), was 0.2 compared to a published value of 0.03 (Saitoh *et al* 1985). Cultures grown on several media, under production conditions, were all found to have a higher nornicotine, rather than nicotine, content. In the heterotrophic culture-line the ratio of nornicotine to nicotine was approximately 1, with anatabine being the other alkaloid detected. This accumulation of nornicotine at the expense of nicotine, nornicotine is formed by the demethylation of nicotine (Leete 1977b, Stepka & Dewey 1961), in cultures of *N.tabacum* Cv. Wisconsin-38 has previously been found (Tiburcio *et al* 1985). The increase in the demethylation of nicotine to nornicotine in cultures of this cultivar could be due to a mutation of the gene involved, which in the field occurs at a high frequency (Wernsman & Matzinger (1970). The difference in the proportions of each alkaloid between the plant and cultures was not important since the effect of light on any or possibly all of the alkaloids was of interest.

The alkaloid content of the photoheterotrophic and the mixotrophic cultures was greatly reduced, when compared to that of the heterotrophic cultures, and alkaloids were undetected in the photosynthetic cultures. There was a greater than 99% reduction in the alkaloid content of the cultures grown in the light. In both of the culture-lines grown in the light nicotine and/or nornicotine were the only alkaloids detected. Ohta & Yatazawa (1978a) found a 80% decrease in nicotine accumulation after callus had been illuminated for three weeks at a light intensity of approximately  $75\mu\text{E m}^{-2} \text{sec}^{-1}$ , which was slightly lower than the intensity,  $80-90\mu\text{E m}^{-2} \text{sec}^{-1}$ , used in this investigation. In this study the cultures had been maintained in a defined environment for at least three months prior to analysis, rather than being transferred into the light at the start of the experiment, and this probably accounts for the higher degree of inhibition of alkaloid synthesis. This inhibition of alkaloid accumulation *in vitro* by light is similar to the effect that light and dark treatments have been found to have on the nicotine content of tobacco seedlings *in vivo* (Mantell &

Smith 1983). The high level of inhibition found when illumination was the only cultural factor changed (ie. the difference in alkaloid content between the heterotrophic and the photoheterotrophic cultures) suggests that photosynthesis was not directly involved in suppressing alkaloid synthesis. This was supported by the fact that there was no further inhibition of alkaloid synthesis in the mixotrophic cultures, which were grown at the same light intensity but which were more photosynthetically active. The complete inhibition of alkaloid synthesis in the photosynthetic cultures could be due to an increase in light intensity or to increased photosynthetic activity. Although it was not possible to distinguish between these two possibilities it is probable, when considered with the results from the other culture-lines, that the increased light intensity is a determining factor. These results do not agree with the increase in nicotine accumulation under conditions of illumination previously reported (Tabata *et al* 1971). In this case the cultures were photoheterotrophic since the cells were capable of growth in the dark as well as in the light. A 12 hour photoperiod was used and this may account for the synthesis, since it has been found that the suppression of nicotine synthesis by light is reversible (Ohta & Yatazawa 1978a), and there was also a high level of organogenesis, which is often associated with increased secondary metabolism (Lindsey & Yeoman 1983b). There was no significant difference in the fresh weight accumulated by the heterotrophic and the photoheterotrophic cultures, while the mixotrophic culture had a lower final biomass. Since there was no difference in the final fresh weights of the heterotrophic and the photoheterotrophic cultures it was not possible to attribute the differences in alkaloid content to an increased biomass in one or other of the treatments. It is also unlikely that changes in the alkaloid content on illumination of the cells were caused by differences in nutrient uptake or utilization. It has been found that phosphate limitation increases alkaloid content (Pinol *et al* 1985). Although phosphate appeared to become limiting in the heterotrophic but not in the mixotrophic cultures this would not explain the decrease in alkaloid synthesis in cultures grown in the light since it is unlikely that phosphate was limiting in the photoheterotrophic cultures which also had a decreased alkaloid content.

It would appear from the results of the current study and those of previous studies (Ohta & Yatazawa 1978a, Pearson 1978) that the suppression of nicotine synthesis is photomorphogenic in nature. If this is the case the degree of suppression of synthesis may be affected by the illuminating



wavelength. In such a study Ohta and Yatazawa (1978a) found that there was no difference in the degree of suppression between red and blue light but that alkaloid synthesis was greater at these wavelengths than in white light. However, the intensities of red and blue light used varied from each other and both were much lower than the intensity of white light used. Therefore it is not possible to draw any conclusions as to the effect of illuminating wavelength on alkaloid synthesis. In the current study the synthesis of anatabine, as well as that of nicotine and nor nicotine, appeared to be suppressed. This was of interest because if light causes the suppression of synthesis of all of the alkaloids it is probable that the point of regulation is associated with the side of the pathway that synthesises the pyridine ring which is common to all of the alkaloids (Leete 1980). To investigate the effect of light on the alkaloid anabasine callus cultures of *N.glauca* were used and this is now discussed.

#### **The Effect Of Light On The Accumulation Of Anabasine By Callus Cultures Of *N.glauca***

Callus cultures of *N.glauca* showed a greater than three-fold increase in anabasine when grown in the dark, rather than in the light. The maximum anabasine content of the cultures was approximately 3.5% of that found in plants (Saitoh *et al* 1985). When the anabasine content of cultures grown in the dark was expressed on a per culture basis it was found that there was a small increase in the alkaloid content during the period of growth, as measured by fresh weight, while the anabasine content increased during the stationary phase. On a per gram fresh weight basis the anabasine content decreased during the period of growth and then increased to a level, similar to that of the initial inoculum, during the exponential phase. There are no previous reports of the kinetics of anabasine accumulation in callus cultures of *N.glauca*. However, the stationary phase of culture is often associated with increased secondary metabolite production, and this is particularly the case in *Solanaceous* species (Lindsey & Yeoman 1985). The accumulation of anabasine in root cultures of *N.glauca* follows the same pattern with maximum rates of increase occurring towards the end of linear growth and during the stationary phase (Solt *et al* 1960). Conversely, nicotine has been found to accumulate, especially in root cultures, during the linear and exponential phases of growth

(Hamill *et al* 1986, Solt *et al* 1960, Tabata *et al* 1978, Tiburcio *et al* 1985). The difference in the kinetics of the accumulation of these two alkaloids may be attributed to different sites of synthesis, such that nicotine is synthesised in young meristematic tissue and anabasine is synthesised in more mature tissues (Solt *et al* 1960).

The kinetics of anabasine accumulation were studied in callus cultures that were transferred from their normal illumination regime one (ie. callus grown in the light was placed in the dark and *vice versa*). On transfer of callus from the light into the dark an increase in anabasine content from zero at the time of transfer to 40% of the content of callus growing in the dark was observed. This increase in anabasine content occurred at a relatively constant rate on a per gram fresh weight basis throughout the culture cycle. The anabasine content of cultures transferred from the dark into the light decreased, on a per gram fresh weight basis, during the exponential phase of growth and then showed an increase during the later stages of the growth cycle. At the end of the time-course the anabasine content of these cultures was very similar to that of the cultures transferred from the light into the dark and was higher than the anabasine content of cultures continually grown in the light. The increase in the anabasine content, on a per culture basis, was detected before the corresponding increase occurred in cultures grown continuously in the dark. This was probably due to the fact that the initial growth of cultures in the light was more rapid than cultures grown in the dark, although the final fresh weights were not significantly different in any of the treatments. The stimulation of growth of cultures by light is a phenomenon that has been reported in the past (Bergmann & Balza 1966, Seibert *et al* 1975). The accumulation of anabasine in cultures grown continuously in the light and those transferred from the dark into the light was complicated by the formation of roots in these cultures. It was not possible to determine whether or not the formation of roots in these cultures was correlated with the anabasine content but the appearance of roots in cultures grown in the light may account for the fluctuations in alkaloid content observed in cultures in the light.

The presence of differentiated organs, roots in cultures of *N.glauca* grown in the light and shoots in cultures of *N.tabacum* grown with a 12 hour photoperiod (Tabata *et al* 1971), may account for the levels of alkaloid

synthesis seen in these cultures. The effect of light on root and shoot cultures is now discussed.

### **The Effect Of Illumination On Alkaloid Accumulation In Root Cultures Of *N.rustica* And Shoot Cultures Of *N.tabacum*.**

Root cultures of *N.rustica* grown in liquid medium had an alkaloid content greater than that reported for *N.rustica* plants. The alkaloid content on a per gram fresh weight basis was approximately one and half times greater than in the plant although the ratio of nornicotine to nicotine was different (0.16 in cultures compared with 0.02 in the plant) (Saitoh *et al* 1985). Cultures maintained in the dark had a higher alkaloid content than cultures grown in the light. Illumination had no effect on the proportions of each alkaloid present but light did appear to stimulate the release of alkaloids from the roots into the medium. However, the most pronounced effect of light was the inhibition of growth. Cultures grown in the light accumulated approximately 50% less biomass than the corresponding cultures in the dark. Therefore when alkaloid contents were expressed on a per culture basis there appeared to be a large difference between the two treatments. It has been found that there is a complex relationship between light intensity, spectral quality and root growth (for review see Seibert & Kadkade 1980). Root growth from cultured segments of *Helianthus tuberosus* was found to be maximal at low light intensities with a 12 hour photoperiod but at light intensities slightly higher than used in the current study a 50% decrease in root growth was observed (Gautheret 1969) and light has been found to inhibit *Brassica* root growth *in vivo* (Ball 1969). The decreased growth in cultures grown in the light may account for the decreased alkaloid yield seen in these cultures since nicotine synthesis has been proposed to be associated with young meristematic root tissue (Pinol *et al* 1985, Solt 1957). Root cultures grown in the light accumulate less biomass and it follows that they have less young meristematic tissue and therefore have a reduced capacity for nicotine synthesis.

Shoot cultures of *N.tabacum* had the same alkaloid content irrespective of illumination. However, the shoot cultures grown in the dark had previously been cultured in the light and were only in the dark for the duration

of the experiment. In both cases the alkaloid content was very low and may have been the result of synthesis in small amounts of callus present and subsequent accumulation in the shoots. In a previous study the shoot was found to account for between 1 to 3% of total alkaloid synthesis (Dawson & Solt 1959) but excised shoots and associated callus are unable to synthesise nicotine (Dawson 1946). It was not possible in the current investigation to determine whether the alkaloid content was the result of synthesis in the shoots or due to the presence of small amounts in the callus. However, the material used was of shoot origin and had been maintained as shoot cultures for at least six months prior to analysis. From these results and those previously published it appears that shoots possess the potential for alkaloid synthesis but that this potential is not realized.

The evidence available indicates that light causes a photomorphogenic suppression of alkaloid synthesis in unorganized cultures. This suppression of alkaloid synthesis is more pronounced in cultures that have been cultured in the light for prolonged periods and is complete in cultures grown at high light intensities. On transfer of cultures from the dark into the light alkaloid synthesis continues at a reduced level. Transfer from the light into the dark stimulates alkaloid synthesis, although one culture cycle is not sufficient to attain the same level of alkaloid synthesis as is seen in cultures continuously grown in the dark. In the case of root cultures light does not suppress alkaloid synthesis directly but does so by decreasing the rate of growth. In shoot cultures light had no effect on the alkaloid content although it did reduce growth. Therefore it appears that increased differentiation causes the effect of light to be modified.

It is possible that alkaloid synthesis is occurring in cultures in the light and in shoot cultures but that synthesised alkaloids are degraded in chlorophyllous tissues in culture. This possibility is considered in the next section.

## THE FATE OF NICOTINE ADDED TO SUSPENSION CULTURES OF *N.TABACUM*.

In the photoheterotrophic culture-line there was a 15% decrease in the alkaloid content during a 14 day period but in the heterotrophic and mixotrophic cultures the alkaloid content was the same as at the start of the experiment. The decrease in alkaloid content in the photoheterotrophic cultures could be due to several factors: metabolism of the nicotine to non-alkaloid compounds such as protein (Tso & Jeffrey 1961), binding of nicotine to the cell wall and/or cell membrane (Peters *et al* 1974), and binding of nicotine to phenolics (Kurkdjian 1982). It was not possible to determine the fate of the nicotine unaccounted for and further studies using radio-labelled nicotine are required in order to clarify the situation. In all of the culture-lines of *N.tabacum* studied (heterotrophic, photoheterotrophic and mixotrophic) a large proportion of the total alkaloid content was associated with the cellular fraction. It has been proposed that nicotine, in its uncharged form, enters the cell by diffusion (Kurkdjian 1982). Nicotine taken up by cells has been found to become associated with the cell wall and within the chloroplasts, of green cultures, (Kurkdjian 1982) rather than in the vacuole, which has been found to accumulate nicotine in the plant (Saunders 1979). In the present study the localisation of nicotine within the cell was not investigated although it is possible that it was associated with the cell wall since the cells were not washed prior to extraction.

In all of the culture-lines tested the added nicotine was transformed to nornicotine. The degree of transformation was greater than 50% in all cases and increased with decreasing photosynthetic activity, such that the highest level of transformation was in the heterotrophic cultures. There was no significant difference between the weights of the heterotrophic and the photoheterotrophic cultures while the mixotrophic cultures had significantly lower final fresh weights. The smaller amount of biomass in the mixotrophic culture may account for the reduced level of transformation seen in this culture. However, there was a higher percentage conversion of nicotine to nornicotine in the heterotrophic than in the photoheterotrophic cultures where there was no significant difference in biomass. The difference between these two treatments is unlikely to<sup>be</sup> due to photosynthetic activity since this was negligible in the photoheterotrophic cultures. The differences in the percentage

transformation may be due to the presence, in the photoheterotrophic and mixotrophic cultures, of chloroplasts which have been found to accumulate nicotine (Kurkdjian 1982). The association of nicotine with the chloroplasts would also explain the lower level of transformation in the more chlorophyllous mixotrophic cultures. The presence of chlorophyll did not aid in the photodegradation of nicotine as has been reported (Doumery & Chouteau 1975). Also the results did not support the suggestion that the conversion of nicotine to nornicotine occurs in green tissues in the plant (Stepka & Dewey 1961), although differences in the localisation of nicotine between the plant and cultured cells may account for this.

In a previous study the only alkaloid detectable after the addition of nicotine to the culture medium was nicotine. No conversion of nicotine to nornicotine was found even when a culture initiated from a high nicotine to nornicotine converting plant strain was used (Saunders *et al* 1981). The transformation of nicotine to nornicotine by these culture-lines may be due to the fact that under culture conditions favouring alkaloid synthesis a high level of nornicotine synthesis occurs, as has previously been found with this cultivar (Tiburcio *et al* 1985).

A further experiment was conducted using cells of *N.tabacum* grown as cell suspensions in B<sub>5</sub> medium. A higher concentration of nicotine was added to the medium at inoculation and the culture period extended to 30 days. After this period all of the nicotine had been transformed into nornicotine and although the percentage transformation was greater than 100% this was accounted for by the variation between replicates. On more detailed analysis of the alkaloid extracts from these cultures a wide range of alkaloids at low concentrations were detected. All of the four main tobacco alkaloids were detectable along with derivatives of nornicotine, and two unidentifiable alkaloids. These derivatives of nornicotine along with many other derivatives of each of the four main alkaloids have been found in the plant (Enzell & Wahlberg 1980) but have not been reported in cultured material. Since other extracts were not analysed in such detail it is unclear whether these compounds occurred as a result of adding nicotine to the culture medium or whether they were present in all producing cultures.

The addition of nicotine to the culture medium at relatively low concentrations (1.25mM and 2.5mM) did not inhibit growth. Nicotine has been found to have no effect on growth at concentrations up to 10mM (Robins *et al* 1987) but at a concentration of 20mM growth over a 28 day period was reduced by 50% (Saunders *et al* 1981). It has also been found that nicotine added to cultures triggers the differentiation of roots (Peters *et al* 1974) while the formation of roots enhances alkaloid yield. The results presented in this thesis do not support this suggestion since no differentiation of cultures was observed when nicotine was added nor was there a stimulation of alkaloid production when nicotine was added.

These results show that the degradation of alkaloids by chlorophyllous tissues does not account for the decrease in alkaloid synthesis in cultures grown in the light. Therefore the suppression of alkaloid synthesis by light appears to be due to a change in the biosynthetic pathway leading to the alkaloids. The possible site of such a metabolic change and the effect of light on the enzyme catalyzing the reaction at that point is now discussed.

#### **A POSSIBLE SITE FOR THE REGULATION OF ALKALOID BIOSYNTHESIS IN TOBACCO.**

The synthesis of all of the four main alkaloids in unorganized cell cultures has been found to be inhibited by light. In the plant the alkaloids have been found to be chiefly synthesised in the root (Dawson 1942a,b, Kisasi & Tamaki 1966, Solt *et al* 1960) which indicates that in the plant non-photosynthetic tissues have a greater capacity for alkaloid synthesis. From these results it is probable that a change in metabolic activities occurs on the "greening" of plant cells. By examining changes in the activities of enzymes and fluxes in the pool sizes of metabolites involved in alkaloid synthesis, in producing and non-producing tissues, it may be possible to identify the site where regulation of alkaloid biosynthesis occurs.

Light inhibits the synthesis of all of the alkaloids by cultured cells to a similar degree. Since nicotinic acid is a common precursor to all of the alkaloids it is possible that light is affecting the availability of nicotinic acid for alkaloid biosynthesis. Nicotinic acid itself is an intermediate in the pyridine nucleotide cycle (PNC) (Gholson 1966, Ryrie & Scott 1969) (Fig 4.1) which is involved in the synthesis and recycling of NAD (White 1982). Quinolinic acid is a universal intermediate in the biosynthesis of the pyridine ring of pyridine nucleotides (Hadwinger *et al* 1963) and is the immediate precursor of nicotinic acid mononucleotide (NaMN) (Gholson *et al* 1964, Mann & Byerrum 1974a,b) which is the first compound in the PNC in the synthetic pathway to NAD (Waller & Dermer 1981). Therefore changes in the activity of the enzyme quinolinic acid phosphoribosyltransferase (QaPRT'ase), which converts quinolinic acid into NaMN, will have an effect on the availability of nicotinic acid for alkaloid synthesis.

The activity of QaPRT'ase has been found to be higher in plant species that accumulate pyridine alkaloids (Mann & Byerrum 1974a). Within plants that produce pyridine alkaloids the activity is higher in the roots (Wagner & Wagner 1984), which are the site of alkaloid synthesis. Saunders and Bush (1979) found that the activity of QaPRT'ase<sup>1965</sup> correlated with the nicotine content of plants and that when plants were "topped" (a process that stimulates nicotine synthesis {Papenfus & Quin 1984}) the activity of the enzyme increased prior to an increase in nicotine. When callus cultures of *N.tabacum* have been transferred from a medium promoting growth to one favouring alkaloid synthesis very little change in the growth pattern of the cells was seen but the activity of QaPRT'ase increased by up to ten-fold (Wagner *et al* 1986a). From these results it appears that the activity of the enzyme QaPRT'ase is an important factor in determining the level of pyridine alkaloid synthesis in a tissue. The activity of this enzyme in plants and cultures of *Nicotiana* and the effect of light on its activity in callus and suspension cultures is now discussed.



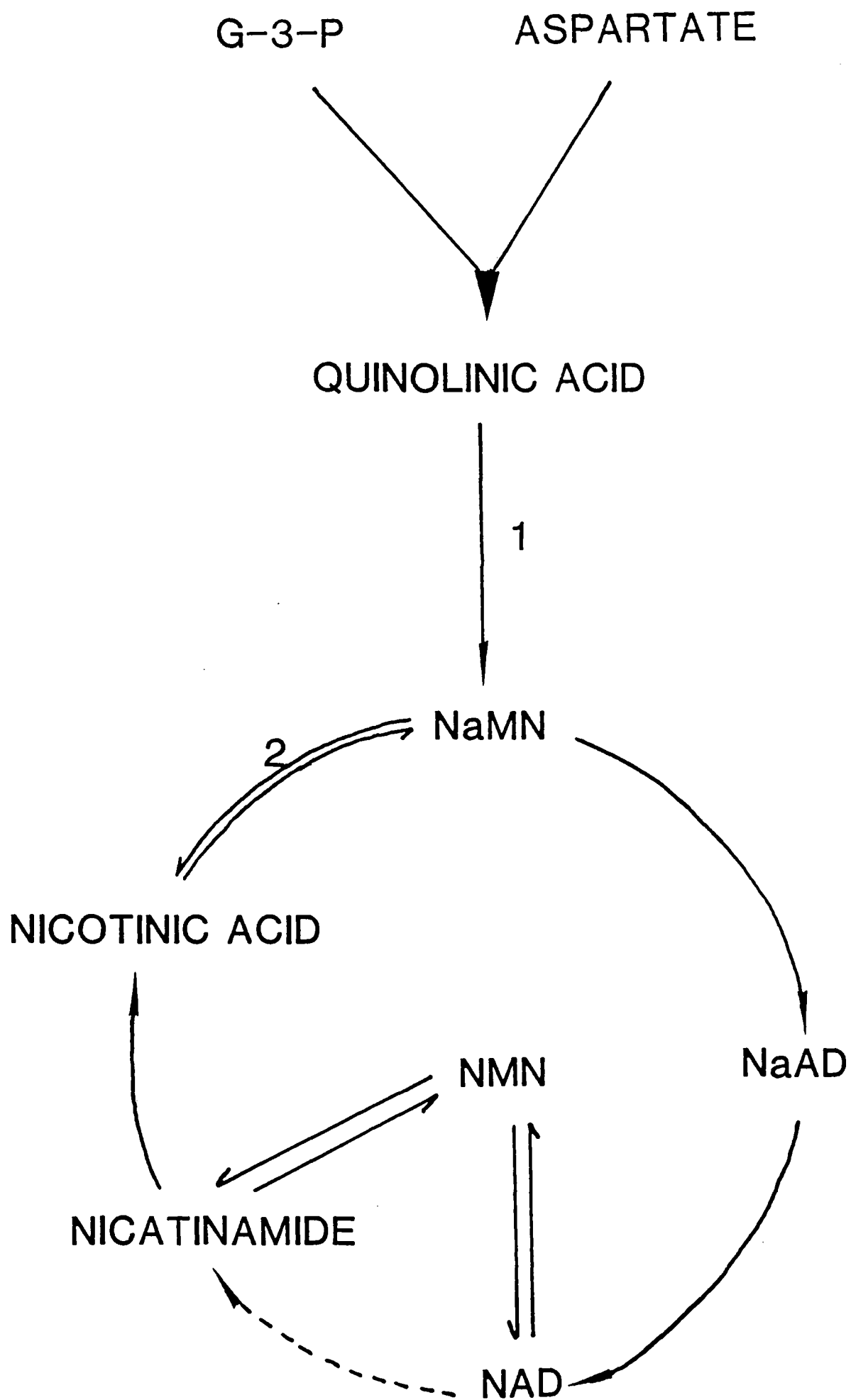


FIGURE 4.1

A schematic representation of the pathway from G-3-P and aspartate to nicotinic acid, via the pyridine nucleotide cycle.

G-3-P = Glyceraldehyde 3-phosphate.  
 NaMN = Nicotinic acid mononucleotide.  
 NaAD = Nicotinic acid adenine dinucleotide.  
 NAD = Nicotinamide adenine dinucleotide.  
 NMN = Nicotinamide mononucleotide.

1 = Quinolinic acid phosphoribosyl transferase.  
 2 = Nicotinic acid mononucleotide glycohydrolase.

## THE ACTIVITY OF QaPRT'ase IN PLANTS AND CULTURES OF *NICOTIANA*

The activity of the enzyme QaPRT'ase was determined in plants and in cultures of *Nicotiana*. Plants were divided into roots and shoots. The activity was determined in cultures of *N.tabacum* and *N.glauca* grown with and without illumination and in root cultures of *N.rustica*.

### The Activity Of QaPRT'ase In Plants Of *N.tabacum*

The activity of the enzyme QaPRT'ase was found to be greater in the roots than in the leaves of *N.tabacum*, which is consistent with previous reports (Mann & Byerrum 1974a, Saunders & Bush 1979, Wagner & Wagner 1984). The age of the plant material from which the enzyme was extracted was found to have a large effect on the activity of the enzyme. The specific activity of QaPRT'ase was much lower in roots of immature plants, twelve weeks after germination, than in plants just before flowering. Although the kinetics of alkaloid accumulation in the plant were not studied it has previously been found that nicotine synthesis is maximal in more mature plants (Dawson 1948), which would explain the increase in enzyme activity. However, the specific activity in the younger plants was lower than that reported in plants of the same age (Wagner & Wagner 1984) while the activity in the older plants was higher than that found in plants of *N.rustica* just before flowering (Mann & Byerrum 1974a). The activity of the enzyme can be determined either by measuring the amount of product formed, by spectroscopic or radio-chemical methods, or by measuring the amount of  $^{14}\text{C}$  released during the reaction when  $^{14}\text{C}$ -quinolinic acid is used as the substrate. The most sensitive method is to use  $^{14}\text{C}$ -quinolinic acid as the substrate and to determine the activity incorporated into the product. This technique in conjunction with a spectroscopic method was used in this study and in the work conducted by Wagner and Wagner (1984). However, in *N.rustica* plants (Mann & Byerrum 1974a) the activity of the enzyme was determined by measuring the evolution of  $^{14}\text{C}$  which is less sensitive. This fact in conjunction with the use of a different species and a different extraction technique may account for the difference in activity between mature plants of *N.tabacum* in the present study and mature plants of *N.rustica*. However, the only variation of note between

this work and that of Wagner and Wagner (1984) was the cultivar used. The decreased activity found in this study was therefore not due to a difference in technique and may be attributed to a biochemical difference between the tissues examined.

The protein extracted from the roots and leaves in the previous study (Wagner & Wagner 1984) was over two-fold greater than that extracted from similar tissue in this study. Therefore it is possible that in the current study incomplete extraction of enzyme activity was achieved. There is also the possibility that differences were due to the cultivar and the conditions under which the plants were grown. The biggest difference observed between the results presented here and those previously reported (Wagner & Wagner 1984) was in the kinetics of the enzyme reaction. The enzyme reaction was found to be linear for 5min and to have reached completion by 10min while the reaction has previously been reported to be linear for up to 60min and was only reaching completion after an assay period of 3hours (Wagner & Wagner 1984). When the reaction was allowed to proceed for 90min the amount of NaMN formed was found to decrease thus the product of the enzymatic reaction was being metabolised. For the synthesis of NAD, NaMN is converted to nicotinic acid adenine dinucleotide (NaAD) a reaction that requires ATP. In the assay system employed no ATP was present since endogenous ATP would have been removed at the gel filtration stage of the extraction and ATP was not added to the assay buffer since it inhibits the activity of QaPRT'ase (Hadwinger *et al* 1963). Therefore NaMN formed in the reaction could not be converted into NaAD. However, an enzyme that converts NaMN directly into nicotinic acid has been reported in tobacco (Wagner *et al* 1986). This enzyme NaMN glycohydrolase requires the presence of water and splits NaMN into nicotinic acid and ribose 5-phosphate. A higher activity of NaMN glycohydrolase in the present study would account for the differences between the kinetics of QaPRT'ase in the two investigations. A higher activity of NaMN glycohydrolase may occur due to slight differences in the pH or temperature of the assay system or because the enzyme is susceptible to degradation by proteases which were inhibited by the addition of PMSF to buffers in this study but were not used by Wagner and Wagner (1984). There is also the possibility that complete extraction of NaMN glycohydrolase was achieved but that only partial extraction of QaPRT'ase was achieved. The kinetics of the QaPRT'ase catalyzed reaction were not studied in any of the other tissues used thus it is not

possible to determine whether the kinetics determined in extracts from these plants are due to the cultivar used or are attributable to the extraction and assay procedures employed. This area requires detailed analysis since it has great implications in the synthesis of the alkaloids.

Despite the discrepancies in the kinetics of the reaction it was found that, as has previously been reported, the activity of the enzyme QaPRT'ase was higher in the roots than in the leaves. Also of interest was the increase in the activity of the enzyme in the roots of mature plants. The kinetics of alkaloid accumulation in plants needs to be followed in conjunction with studies on plant age and activity of QaPRT'ase to determine if there is a correlation between these two parameters. Since QaPRT'ase activity was lower in the aerial parts of the plant the enzyme activity was determined in cultures grown in the light or in the dark to determine whether light may influence its activity. The results of this study are now discussed.

#### **The Activity Of QaPRT'ase In Suspension Cultures Of *N.tabacum* And Callus Cultures Of *N.glauca* Grown In Either The Light Or In The Dark.**

Suspension cultures of *N.tabacum* grown either heterotrophically, photoheterotrophically or mixotrophically were lyophilized prior to extraction as described by Wagner & Wagner (1984). No activity was detectable in the extracts from any of these cultures, even though the heterotrophic culture-line synthesised alkaloids. The extracts themselves were black in colour which suggests that phenolic compounds, such as chlorogenic acid, were present in large quantities and may therefore have caused the loss of any enzyme activity that was present. This problem has been pointed out when using enzyme extracts from tobacco leaves where the addition of leaf extract to an extract from roots inhibited the activity of QaPRT'ase (Mann & Byerrum 1974a). In future work with suspension cultures, compounds that bind phenolic compounds or that inhibit polyphenol oxidases should be added to the extraction buffer in an attempt to overcome this problem.

Callus cultures of *N.glauca* were extracted without being lyophilized. The extract from cultures grown in the dark had a specific QaPRT'ase activity

that was similar to the specific activity of the enzyme in mature plants of *N.tabacum*, but there was a large amount of variation in the activity of the extract between replicates. Due to the difference in species a direct comparison is not an accurate reflection on the situation in the plant and that in culture of *N.glauca*. The activity of the enzyme in cultures grown on the same medium in the light was reduced by 96% when compared with the activity of cultures grown in the dark. The specific activity of the enzyme in callus cultures of *N.tabacum*, that are under optimal alkaloid producing conditions, has been reported to be approximately 30% of that found in the roots of immature plants (Wagner *et al* 1986a). The activity of QaPRT'ase in callus cultures of *N.tabacum* was found to increase by up to ten-fold on transfer of callus to medium promoting alkaloid synthesis and then to decline as the culture cycle progressed. Since the activity of the enzyme was only measured at one time, during the stationary phase, in cultures of *N.glauca* it is not possible to determine whether QaPRT'ase activity correlated with nicotine synthesis.

Although further studies of QaPRT'ase activity are required, particularly with respect to the kinetics of the reaction and to the activity of the enzyme during a culture cycle, the results show that cultures of *N.glauca* grown in the dark have a much higher specific activity of the enzyme than do cultures grown in the light.

#### The Alkaloid Content And The QaPRT'ase Activity Of Callus Cultures Of *N.glauca* Grown In The light Or The Dark.

There was a 96% reduction in the specific activity of QaPRT'ase when callus cultures of *N.glauca* were grow in the light. The alkaloid content of these cultures grown in the light was 78% less than that of similar cultures grown in the dark. This suggests that the activity of QaPRT'ase is a factor that is involved in determining the alkaloid content of cultures of *N.glauca* and that the activity of the enzyme itself is influenced by the light conditions under which the cultures are grown. The question therefore arises as to why should light regulate this enzyme and how such regulation is achieved?

## **REGULATION OF QaPRT'ase BY LIGHT: AN INDIRECT LINK BETWEEN PHOTOSYNTHESIS AND THE ACTIVITY OF THE ENZYME?**

From the results of the present investigation and those of previous studies (Mann & Byerrum 1974a, Saunders & Bush 1979, Wagner & Wagner 1984, Wagner *et al* 1986a,b) it appears that the enzyme QaPRT'ase is a possible determinant of alkaloid synthesis. The activity of QaPRT'ase, and consequently alkaloid synthesis, are both suppressed in chlorophyllous tissues. A possible reason for such inhibition and a process through which enzyme activity can be controlled are now discussed.

### **A Possible Reason For The Inhibition Of QaPRT'ase In Chlorophyllous Tissues.**

Photosynthesis in green plants is defined as "the synthesis of carbohydrates from CO<sub>2</sub>, as a source of carbon, and water, as a hydrogen donor, using sunlight energy trapped by chlorophyll, catalyzed by various enzymes in the chloroplasts" (Henderson's Dictionary of Biological Terms 1979). The carbon fixed through photosynthesis, in chlorophyllous tissues, is then used to support the metabolic activities of the plant. For the export of carbohydrates from the chloroplast production must exceed the chloroplasts' own demand for carbohydrates. This can only be achieved by returning newly synthesised intermediates to the Calvin cycle so as to regenerate ribulose bis phosphate for further CO<sub>2</sub> fixation. Therefore a delicate balance must be struck between recycling, export and internal storage of carbohydrate in the chloroplast (Walker 1976). In non-chlorophyllous tissues the metabolism of carbohydrates does not have the same effect on the carbon fixing potential of the plant. Hence in chlorophyllous tissues processes that have common substrates with photosynthesis should be under strict metabolic control so as to allow photosynthesis to proceed at optimal levels.

Alkaloid synthesis in tobacco is dependent on the availability of nicotinic acid, which is a common precursor to all of the alkaloids. Nicotinic acid is a constituent of the pyridine nucleotide cycle (PNC) (Ryrie & Scott 1969), which is responsible for the synthesis and salvage of NAD (Gholson 1966). The

constituents of the PNC are unable to cross the inner chloroplast membrane (Heber 1974) therefore it is necessary for the PNC to be present in the chloroplasts and the cytosol, since both have a requirement for pyridine cofactors. The synthesis of pyridine nucleotides is dependent on the conversion of quinolinic acid into NaMN, the first compound in the PNC on the synthetic pathway to NAD (Waller & Dermer 1981). Quinolinic acid is synthesised from glyceraldehyde-3-phosphate (G-3-P) and aspartate (Fleeker & Byerrum 1967). G-3-P is a constituent of the Calvin cycle (the carbon fixing cycle of photosynthesis) and an intermediate in glycolysis (the sequence of reactions through which carbohydrates are converted into pyruvate with the concomitant production of ATP) (Stryer 1981). Therefore for the synthesis of quinolinic acid fixed carbon must be transported out of the chloroplast, in the form of triose phosphates (Sharkey *et al* 1986), and as a result the amount of G-3-P available in the chloroplast for recycling, via the Calvin cycle, to ribulose bis phosphate will be reduced. Hence the operation of the PNC in photosynthetic tissues at a level that was capable of sustaining pyridine cofactors at a metabolically active concentration would allow photosynthetic carbon fixation to occur to a greater degree than if there was a high level of NAD synthesis. However, in the non-photosynthetic tissues NAD synthesis could proceed at a higher rate without affecting the photosynthetic carbon fixing potential of the plant or tissue. In this case quinolinic acid is synthesised using G-3-P that results from the metabolism of sucrose, that has been transported from the leaves or in the case of cultured cells that is supplied by the medium.

Through such differences in the degree of NAD synthesis in photosynthetic and non-photosynthetic tissues the amount of nicotinic acid available for alkaloid synthesis would also be different. In non-photosynthetic tissues with an increased level of NAD synthesis nicotinic acid could be removed from the PNC without a deleterious effect on primary metabolism. The activity of the PNC could be regulated by the enzyme QaPRT'ase, that converts quinolinic acid into NaMN. If this enzyme had a low activity in photosynthetic tissue quinolinic acid would not be metabolised as rapidly and therefore the amount of G-3-P required for its synthesis would be reduced. In non-photosynthetic tissues a higher activity of QaPRT'ase would allow the synthesis of more nicotinic acid and enhance the potential for alkaloid synthesis without deleterious effects on other metabolic processes.

The activity of QaPRT'ase has been found to be greater in roots of species that produce pyridine alkaloids (Mann & Byerrum 1974a, Saunders & Bush 1979, Wagner & Wagner 1985). Within these plants the activity is greater within the roots, while in the leaves the activity is similar to the activity in the roots of non-alkaloid producing species (Wagner *et al* 1986). Therefore it is possible that the activity of QaPRT'ase is regulated in such a way as to allow photosynthesis to proceed, in chlorophyllous tissues, at an optimal rate. The question that then needs to be considered is how can such regulation be achieved.

### **A Possible Mechanism For The Regulation Of QaPRT'ase.**

It has been found that QaPRT'ase activity is low in leaves and high in roots of plants that synthesise pyridine alkaloids (Mann & Byerrum 1974a, Wagner & Wagner 1985, Wagner *et al* 1986). In callus cultures of *N.glauca* QaPRT'ase activity was undetectable in cultures grown in the light while cultures maintained in the dark had an enzyme activity similar to that in the roots of plants *N.tabacum*. On moving cultures from the dark into the light alkaloid accumulation decreased but not to the same extent as seen in cultures continuously grown in the light. Cultures moved from the light into the dark began to accumulate alkaloids but not to the same degree as cultures continuously grown in the dark. In suspension cultures it was found that light inhibited alkaloid accumulation and that this inhibition was not directly associated with photosynthesis. In differentiated cultures light had a less pronounced effect on alkaloid synthesis than it did in disorganized callus and suspension cultures.

These results suggest that light is in some way having a direct effect on the activity of the enzyme, rather than causing substrate limitation through competition between a photosynthetic requirement for G-3-P and the synthesis of quinolinic acid which also requires G-3-P. On transfer of cultures into the light chloroplast biogenesis occurs within a few days (Cannon *et al* 1985) thus creating a potential for photosynthesis. In cultured cells this potential is not realized, unless the correct cultural conditions are provided, whereas in the plant photoautotrophy is rapidly attained. For photoautotrophic



growth photosynthesis must be functioning at as high a level as possible. Therefore if processes that compete for compounds involved in photosynthetic CO<sub>2</sub> fixation are suppressed at the time of chloroplast biogenesis the full photosynthetic potential can be realized more rapidly. One such competing process could be the PNC and therefore the operation of this cycle at a minimal level could be advantageous to further carbon fixation. The activity of the PNC is determined to a great extent by the synthesis of NaMN from quinolinic acid thus a low activity of QaPRT'ase will lead to reduced synthesis of the pyridine nucleotides. The regulation of QaPRT'ase could be achieved in one of two ways: direct regulation of enzyme activity by light through a phytochrome mediated process, or through the regulation of the expression of the gene encoding the enzyme, at one of several levels. Direct regulation of the enzyme activity through phytochrome is unlikely since the inhibition of alkaloid synthesis in cultured roots grown in the light did not correspond to the inhibition seen in other cultures. The determination of enzyme activity can be achieved by regulation of gene expression through: posttranslational, translational, transcription or genomic factors (Becker 1986). Any one of these controlling factors could bring about changes in the concentration of the enzyme of interest.

All cultures, except root cultures, grown in the light were chlorophyllous and therefore contained chloroplasts. Cultures that were transferred from the light to the dark retained some chlorophyll during the five week experiment, while cultures transferred into the light became chlorophyllous. Root cultures grown in the light contained small amounts of chlorophyll while shoot cultures had high chlorophyll contents regardless of illumination. Chloroplast biogenesis occurs within three days of illumination of tissues (Cannon *et al* 1985). Chloroplast biogenesis and thereafter the presence of chloroplasts may induce changes in the cell that cause a decrease in the concentration of the enzyme QaPRT'ase thus reducing the synthesis of PNC constituents. This being the case the removal of G-3-P from the Calvin cycle and the glycolytic pathway will be reduced therefore enhancing the cells capacity for photosynthesis and growth. In non-chlorophyllous tissues there would not be this decrease in enzyme concentration and therefore the availability of nicotinic acid for alkaloid synthesis would not be restricted.

In summary it is possible that the metabolic differences between chlorophyllous and non-chlorophyllous tissues cause a change in the concentration of the enzyme QaPRT'ase through control of gene expression. The reduced activity of the enzyme in chlorophyllous tissues allows optimal operation of primary metabolism, photosynthesis and other growth associated processes, since G-3-P is not being removed from the Calvin cycle and/or the glycolytic pathway. In non-chlorophyllous tissues there is no photosynthetic activity and the metabolism of sucrose produces G-3-P which may be used to form quinolinic acid which is fed into the PNC thus enhancing the potential for alkaloid synthesis.

## CONCLUSIONS.

1. Light inhibits the synthesis of alkaloids in suspension cultures of *N.tabacum* and callus cultures of *N.glauca*. The inhibition of alkaloid synthesis was less pronounced in root cultures than in unorganized cultures. Alkaloid synthesis in shoot cultures is low regardless of illumination.
2. The inhibition of alkaloid synthesis in suspension cultures of *N.tabacum* is not accounted for by photosynthetic activity.
3. The lack of accumulation of alkaloids in illuminated callus and suspension cultures is not attributable to photochemical degradation of the alkaloids.
4. Suspension cultures of *N.tabacum* have the ability to transform exogenous nicotine into nornicotine, the degree of nicotine transformation apparently being dependent on either the chlorophyll content or the photosynthetic activity of the cells.
5. In callus cultures of *N.glauca* the activity of the enzyme QaPRT'ase decreases in the light, while in the dark the activity is comparable to the roots of *N.tabacum* plants.

## FUTURE WORK.

The investigations undertaken in this thesis, and the results obtained, open up many avenues for future research into the regulation of alkaloid synthesis in tobacco. The major areas requiring further detailed investigation and the objectives of such investigations are considered.

1. Determination of the activity of QaPRT'ase and the alkaloid content in the range of cell cultures employed along with the kinetics of such changes after transfer from conditions promoting alkaloid synthesis to conditions suppressing alkaloid synthesis and *vice versa*. Through this study the presence, or absence, of a correlation between enzyme activity and alkaloid synthesis could be established.
2. Characterization of the enzyme NaMN glycohydrolase in roots and in cultured material of *Nicotiana*. The presence and activity of this enzyme has great implications for the possible genetic manipulation of cells and plants with respect to changing alkaloid synthesis, since this enzyme bypasses the pyridine cofactors going directly to nicotinic acid.
3. Determination of the absolute amount of QaPRT'ase present in tissues capable of alkaloid synthesis and in tissues in which synthesis does not occur. If a decrease in enzyme concentration is found in tissues in which synthesis does not occur an investigation into the level of gene regulation of the enzyme should be undertaken.
4. An investigation in the enzymology of the transformation of nicotine to nornicotine. This transformation is controlled by a single dominant gene which has a high frequency of mutation. Identification of the gene may allow genetic manipulation to introduce a more stable gene into plants for commercial use.

The investigations described above will lead to a greater understanding of the regulation of alkaloid biosynthesis in tobacco and the regulation of primary and secondary metabolism in general. At present this field

offers a great potential for research, since at present "the factors that control the onset of secondary metabolism are generally not well understood" (Haslam 1986).

## APPENDIX 1


The chlorophyll content and fluorescence profiles of two cultures grown on MSS medium supplemented with 1.8% fructose. The culture from which fluorescence profile (a) was obtained had a chlorophyll content of  $11.98\mu\text{g g}^{-1}$  fr wt, while the culture from which profile (b) was obtained had a chlorophyll content of  $18.46\mu\text{g g}^{-1}$  fr wt. The fluorescence profile obtained with the culture with the lower chlorophyll content showed that that culture was more photosynthetically competent than the culture with the higher chlorophyll content. Therefore it can be concluded that chlorophyll content does not necessarily equate with the photosynthetic capacity of a culture.

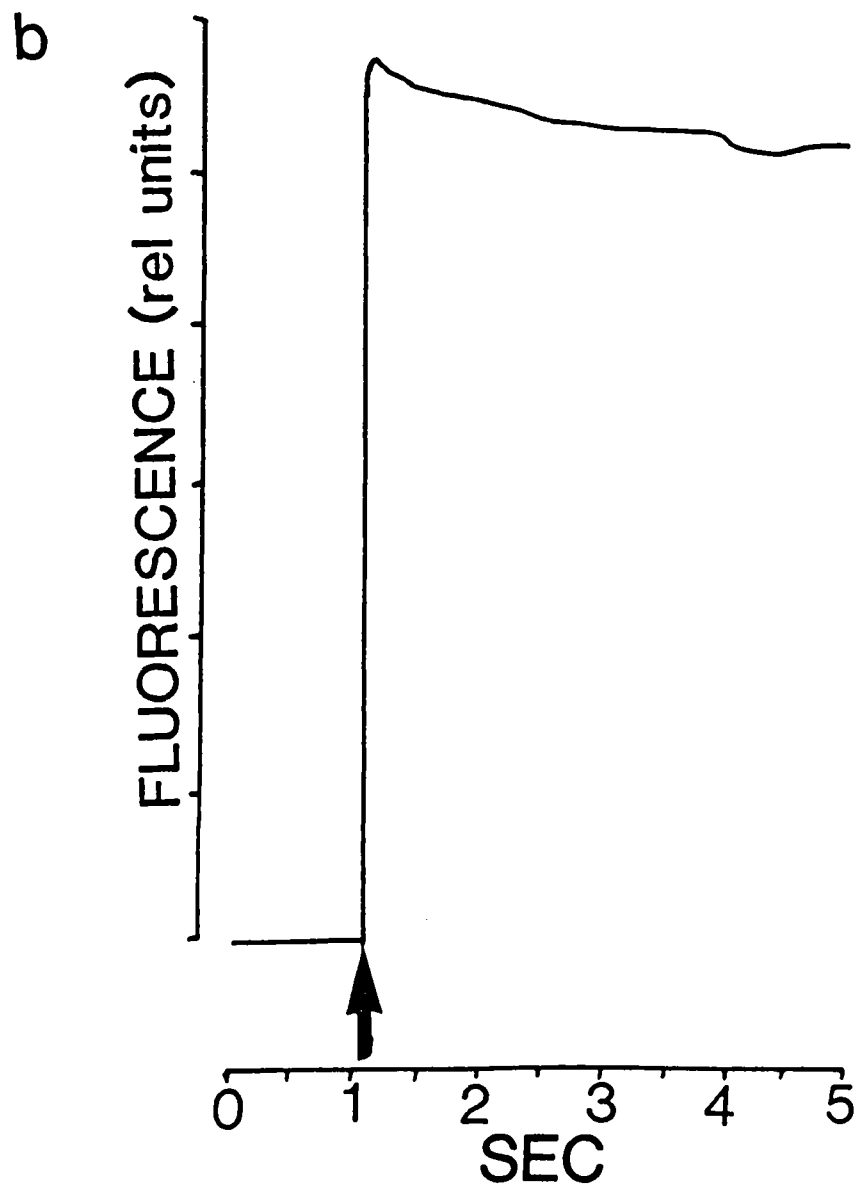
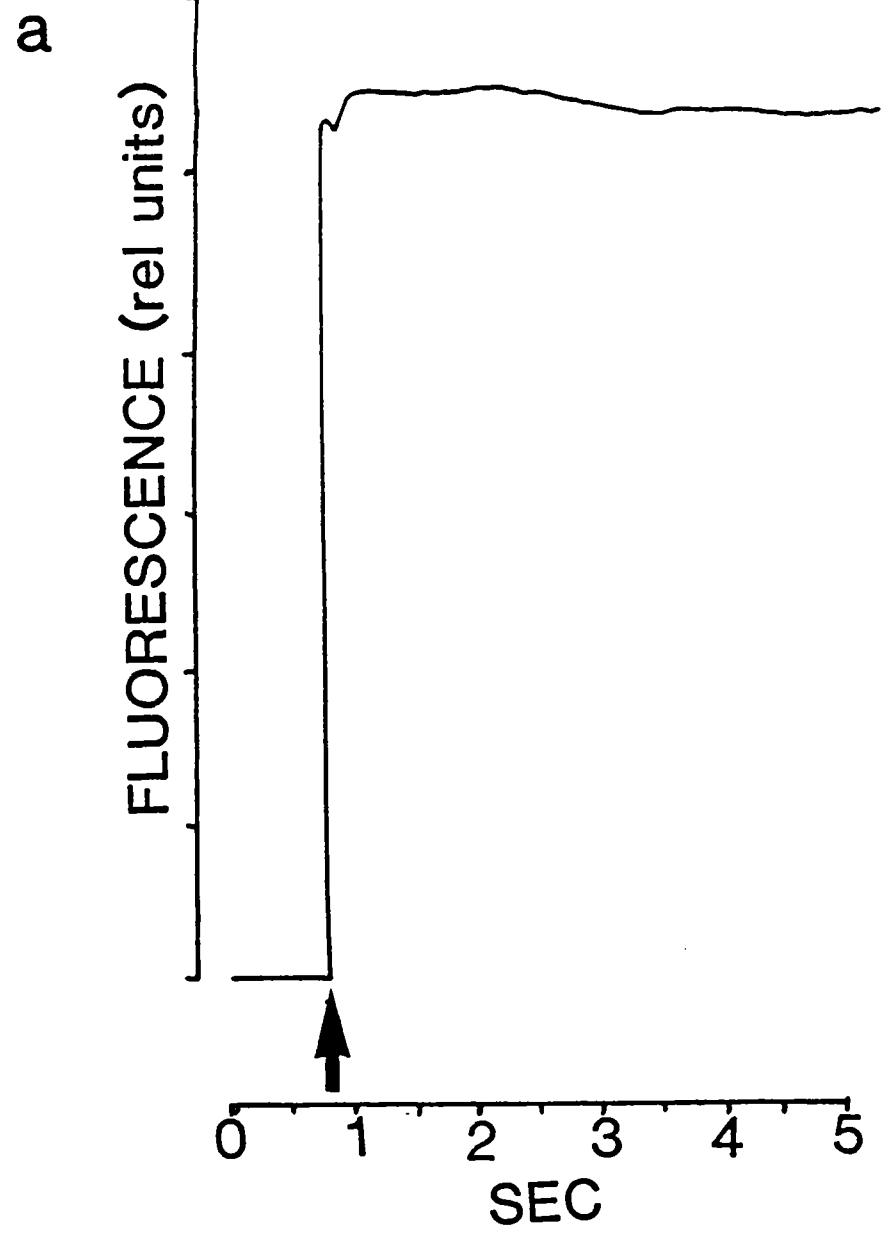
**The Chlorophyll Fluorescence Profiles Of Cultures Grown In The Same Media But That Have Different Chlorophyll Contents.**

Cells were separated from their medium and blotted dry. Approximately 2g was then incubated in the dark for 5min, before being given a 2.5sec flash of light followed by continuous illumination at  $300\mu\text{E m}^{-1} \text{sec}^{-1}$ . The fluorescence emission was recorded at a time-base of  $0.5\text{sec cm}^{-1}$  and a sensitivity of  $0.1\text{V cm}^{-1}$  during the period of constant illumination.

a. The fluorescence profile of a culture with a chlorophyll content of  $12 \mu\text{g g}^{-1}$  fr wt.

b. The fluorescence profile of a culture with a chlorophyll content of  $18 \mu\text{g g}^{-1}$  fr wt.

 = Activation of the LED light source.





## APPENDIX 2

During the process of selecting for photosynthetic cultures the chlorophyll fluorescence properties of the cells of several culture-lines were recorded at regular intervals. Cultures with fluorescence profiles that indicated increasing photosynthetic activity were transferred to media with a decreased percentage of fructose. The process was then repeated until cells capable of growth in carbohydrate free medium were established. The fluorescence profiles from the most photosynthetically capable culture-line at five such screening are shown.

### The Chlorophyll Fluorescence Profiles Of The Most Photosynthetically Competent Culture-line At Five Screening Times.

Cells were separated from their medium and blotted dry. Approximately 2g of cells were then incubated in the dark before being given a 2.5sec flash of light followed by constant illumination at  $300\mu\text{E m}^{-2} \text{sec}^{-1}$ . During the period of constant illumination the fluorescence emissions were recorded at a time base of  $0.5\text{sec cm}^{-1}$  and an appropriate sensitivity.


a. = Cells grown in MS medium containing 3% sucrose with a chlorophyll content of  $9\mu\text{g g}^{-1}$ . The fluorescence signal was detected at a sensitivity of  $1\text{mV cm}^{-1}$ .

b. = Cells grown in MS medium containing 2% sucrose, with a chlorophyll content of  $13\mu\text{g g}^{-1}$ . The fluorescence profile was detected at a sensitivity of  $50\text{mV cm}^{-1}$ .

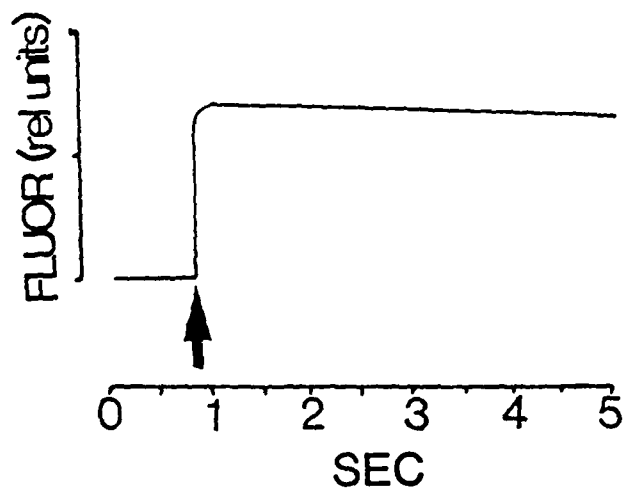
c = Cells grown in MSS medium containing 1.4% fructose with a chlorophyll content of  $20\mu\text{g g}^{-1}$  fr wt. The fluorescence emissions were detected at a sensitivity of  $0.1\text{V cm}^{-1}$ .

d = Cells grown in MSS medium containing 1% fructose, with a chlorophyll content of  $28\mu\text{g g}^{-1}$  fr wt. The fluorescence profile was detected at a sensitivity of  $0.2\text{V cm}^{-1}$ .

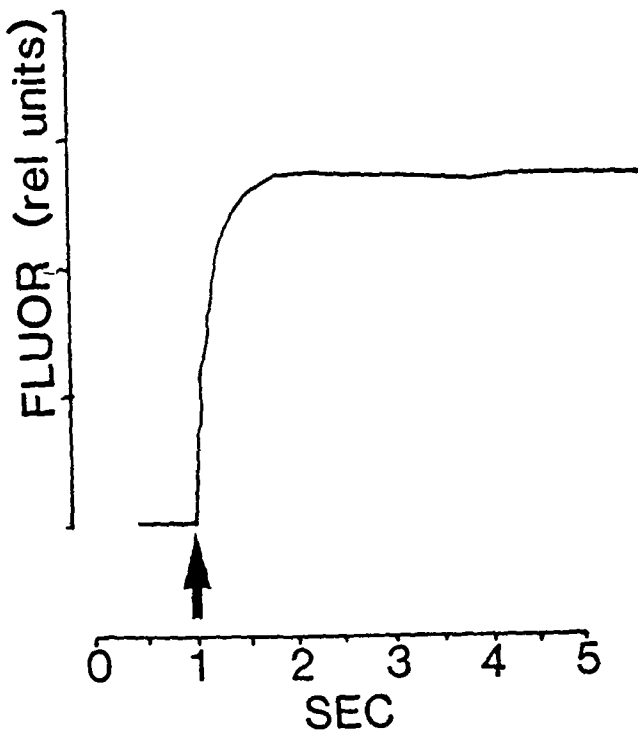
e. = Cells grown in the "flat-bed" system with MSS medium containing 0.2% fructose. The culture had a chlorophyll content of  $39\mu\text{g g}^{-1}$  fr wt, and the fluorescence signal was captured at a sensitivity of  $0.2\text{V cm}^{-1}$ .

 = Activation of the LED light source.

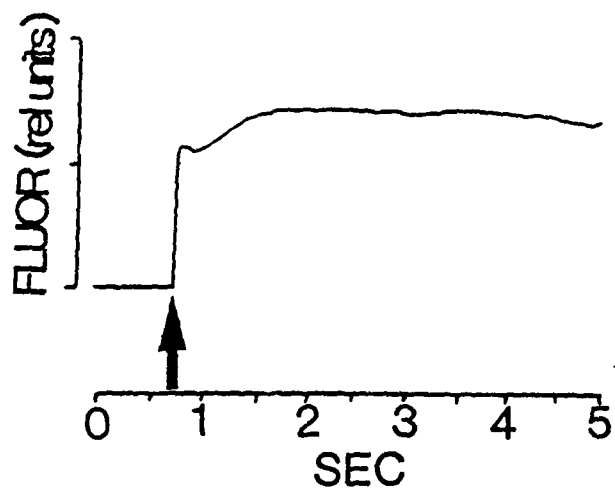
a



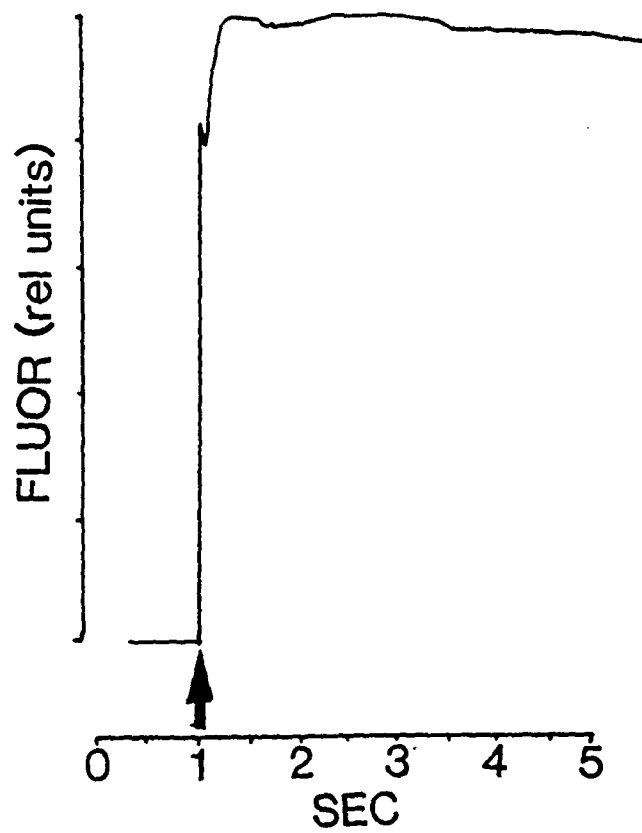
b



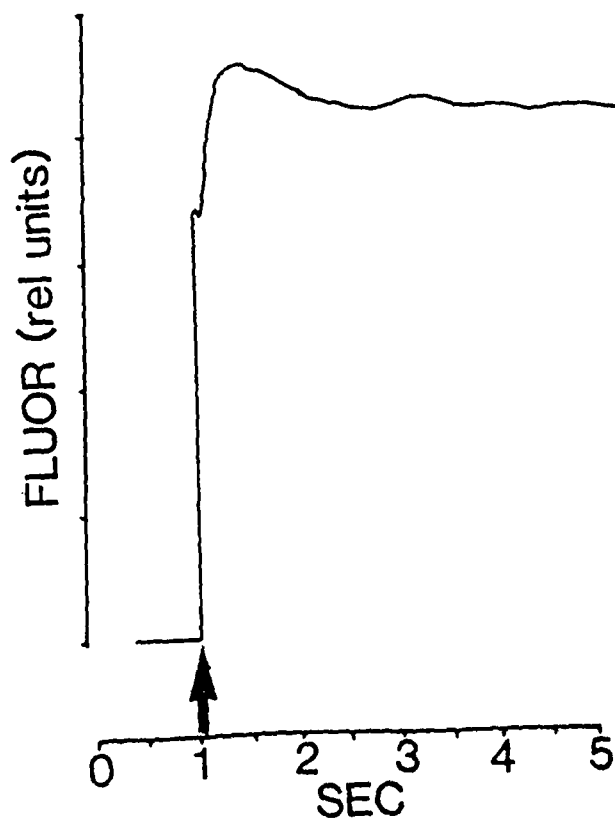
c



d



e



### APPENDIX 3

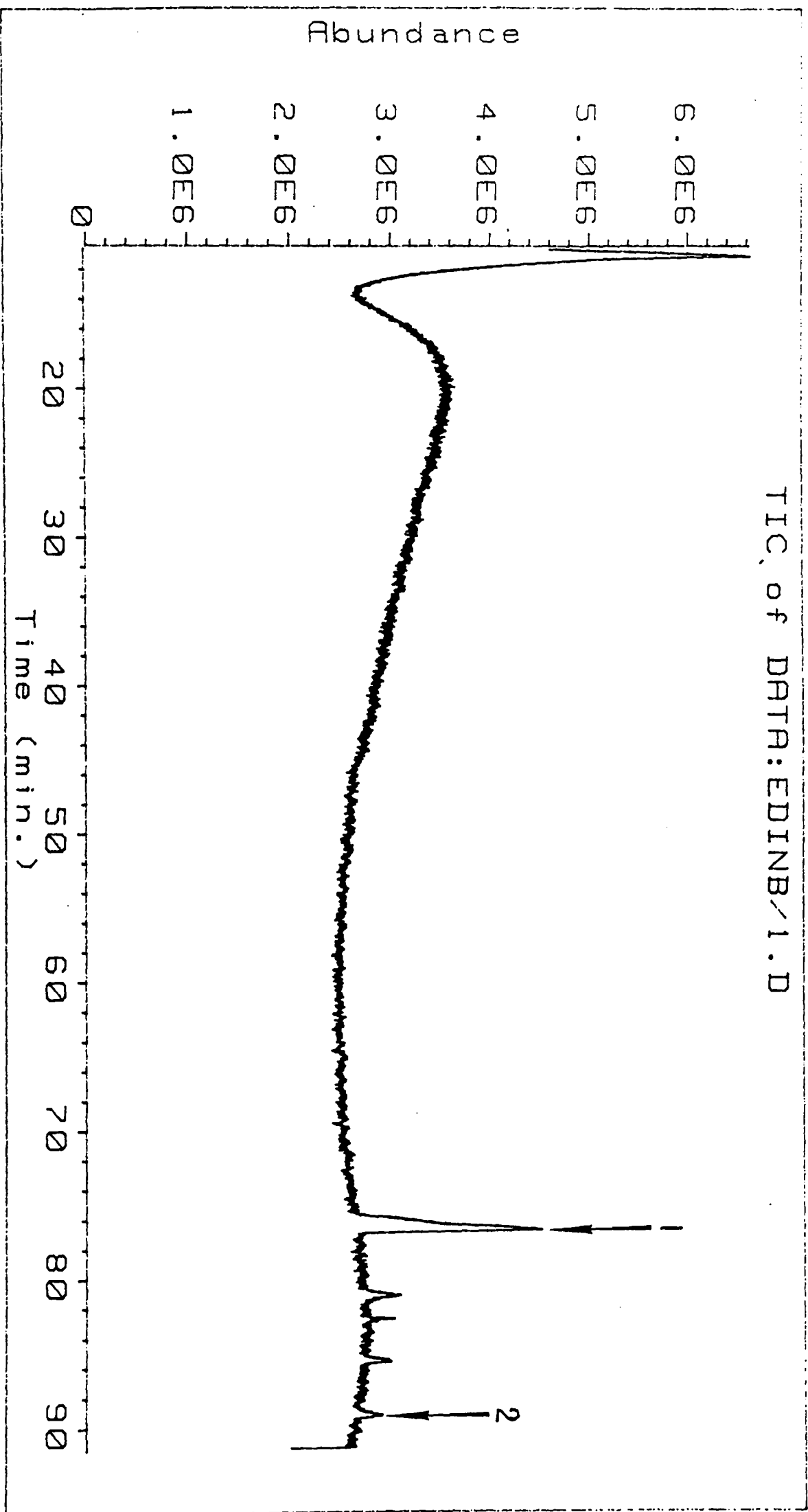
The identification of the compound that was extracted with the tobacco alkaloids and detected by HPLC analysis. The HPLC fraction containing the unknown compound was collected and sent for GC-MS analysis at Rothmans International Services, Basildon. When analysed by GC-MS four peaks were found, two of which were identified. The main peak was identified as di-(2-ethyl-hexyl)-phthalate while the smaller peak was identified as an amide.

GC-MS Chromatogram From Which The Unknown Compound That Was Extracted With The Alkaloids Was Identified.

The GC-MS chromatogram obtained with an injection of the HPLC fraction collected at the retention time of the unknown alkaloid that was extracted with the tobacco alkaloids.

1 = di-(2-aethyl-hexyl)-phthalate.  
2= amide.

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## APPENDIX 4

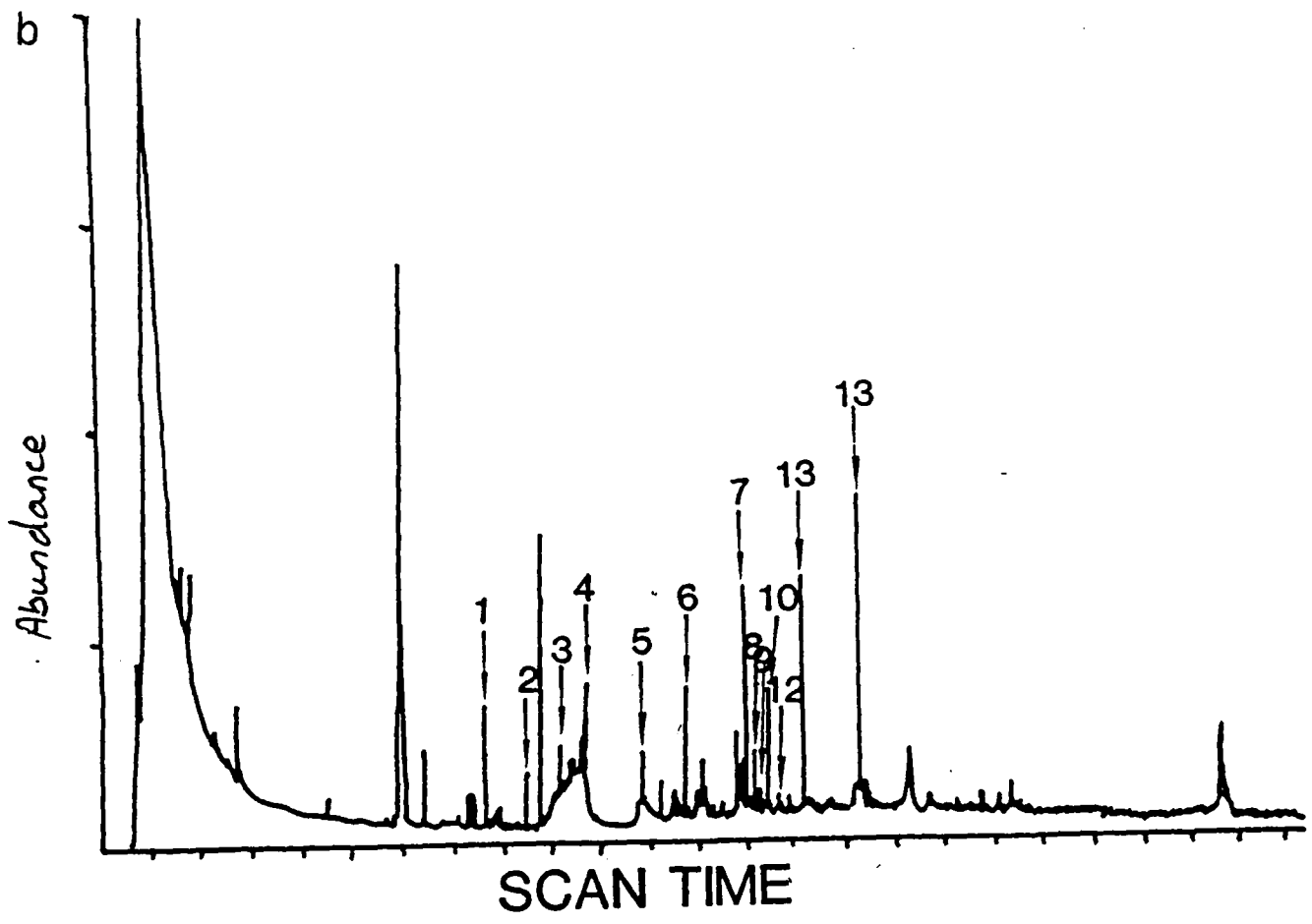
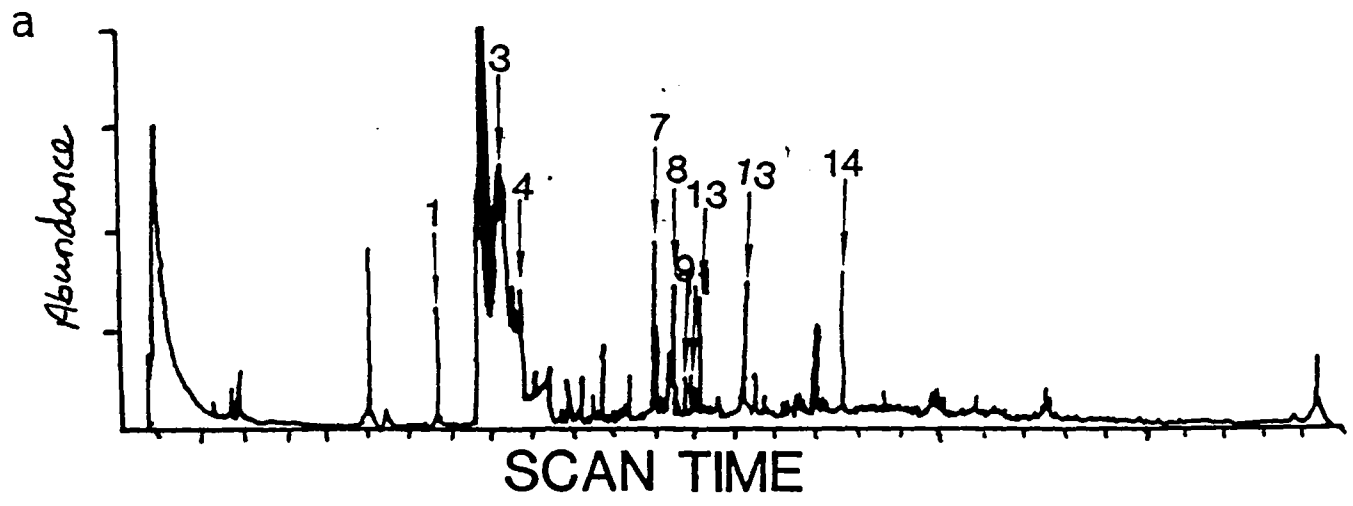
Analysis of extracts from the cells (a) and the medium (b) of cultures grown in B<sub>5</sub> medium supplemented with 3% sucrose and 2.5mM nicotine. Analysis by GC-MS was carried out by Rothmans International Services: Chemical Research, Bremen.

The chromatograms obtained after GC-MS analysis of B<sub>5</sub> cultures grown with 2.5mM nicotine in the medium.

a = Cellular extract.  
b = Medium extract.

1 = Nicotine.  
2 = Myosmine.  
3 = Nornicotine.  
4, 5, 6, & 7 = Phthalate.  
8 = N-Formylnornicotine.  
9 = Unknown alkaloid.  
10 = N-Nitrosonornicotine.  
11 = N-Acetylnornicotine.  
12 = Unknown alkaloid.  
13 = Phthalate.  
14 = Epoxy-cembatrien-ol.





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## PUBLICATION

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CHANGES IN GROWTH AND NUTRITIONAL REQUIREMENT OF CULTURED  
NICOTIANA TABACUM CV. WISCONSIN-38 CELLS WITH INCREASING  
DEPENDENCY UPON PHOTOSYNTHESIS FOR THE PROVISION OF ORGANIC  
CONSTITUENTS.

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ABSTRACT

Suspension cultures of Nicotiana tabacum with differing degrees of dependency upon photosynthesis for the provision of organic constituents were established. At one extreme cells were grown in the dark, with 3% fructose (w/v) in the medium, while at the other extreme cells were grown in the light with 1% fructose supplementation. Growth rates and nutritional requirements of the two culture lines were determined. Cells grown in the dark were totally dependent upon an exogenous carbon source (other than CO<sub>2</sub>), since they had no photosynthetic capacity, for the provision of organic constituents and were regarded as heterotrophic. Cultures grown in the light showed increased photosynthetic activity as the culture period progressed reaching a maximum slightly above the compensation point. Initially these cells grew by utilizing carbon in the medium and as this became depleted (almost fully by day 15) sustained themselves through photosynthetic carbon fixation. This culture line was regarded as mixotrophic.

INTRODUCTION

In Nicotiana tabacum nicotine is synthesised chiefly in the roots in vivo, and in tobacco cell cultures it has been reported that there is a reversible suppression of nicotine synthesis upon transfer from dark to light which increases as the intensity of illumination increases (Ohta & Yatazawa 1978). It is thus feasible to suggest that different metabolic states exist according to the amount of illumination. Although differences in the activities of several enzymes have been reported between roots and shoots (Feth et al 1986; Wagner et al 1986) there is very little information regarding their regulation. In order to determine whether or not light (either directly or via photosynthesis) has a regulatory effect upon one of these enzymes, quinolinic acid phosphoribosyl transferase, cultures have been established with differing degrees of dependency upon light (photosynthesis) for the provision of organic constituents for growth.

Cultures with a total dependence upon nutrients in the medium for the provision of organic constituents (heterotrophic) and cultures dependent upon both nutrients in the medium and photosynthetic carbon

fixation (mixotrophic) have been established. In this presentation changes in growth and nutritional requirements between heterotrophic and mixotrophic cultures are reported.

#### MATERIALS AND METHODS

##### Suspension cultures of *Nicotiana tabacum* Cv.

Wisconsin-38 were initiated from callus and grown in Murashige & Skoog basic salts medium (Murashige & Skoog 1962) supplemented with: biotin ( $0.05 \text{ mg l}^{-1}$ ); glutamine ( $200 \text{ mg l}^{-1}$ ); glycine ( $0.2 \text{ mg l}^{-1}$ ); inositol ( $100 \text{ mg l}^{-1}$ ); nicotinic acid ( $0.05 \text{ mg l}^{-1}$ ); pyroxidine-HCl ( $0.05 \text{ mg l}^{-1}$ ); thiamine ( $0.1 \text{ mg l}^{-1}$ );  $10^{-5} \text{ M}$  NAA and  $10^{-6} \text{ M}$  kinetin. Before transfer each cell line was screened for chlorophyll content (Harborne 1976). Lines with least chlorophyll were supplemented with 3% fructose and placed in the dark while those with higher chlorophyll contents were placed in the light with 2% fructose in the medium. All cultures were shaken at 90rpm on a rotary shaker and the temperature maintained at  $25^{\circ}\text{C}$ . The former cell lines were maintained at 3% fructose supplementation. The latter cell lines were periodically screened for  $\text{O}_2$  uptake (respiration) and evolution (photosynthesis) using oxygen electrode and further photosynthetic characterization was achieved using chlorophyll fluorescence. The most photosynthetically efficient cells at each screening were moved to medium supplemented with 0.2% less fructose and the process repeated until 1% fructose supplementation was attained.

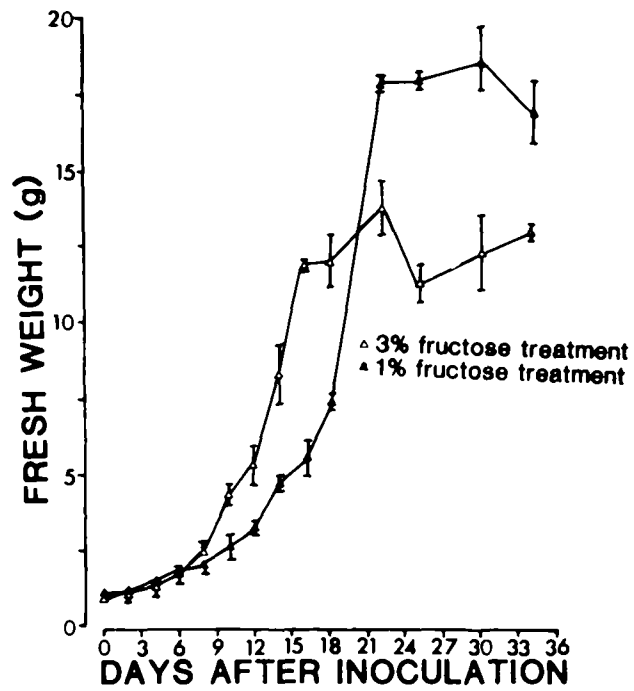
So as to determine the difference between cultures supplemented with 3% and those with 1% fructose, 48 flasks per treatment were inoculated with 1.5-2g wet weight of cells, and grown as above. Three flasks per treatment were harvested at regular intervals. Fresh weight (Hall & Yeoman 1986); chlorophyll content; respiration and photosynthetic rates were determined. The medium was retained and the fructose and phosphate concentration determined according to Asnwell (1957) and Allen (1940) respectively.

#### RESULTS AND DISCUSSION

It can be seen from the data presented in Fig. 1 that fresh weight increased gradually until day 6 in both treatments, then there was a rapid increase until day 16 in cultures supplemented with 1% fructose. In the other treatment the growth rate was maximal between days 18 and 22 and the final biomass yield was greater. In both cases respiration (Fig. 2) increased after inoculation, to a maximum and then showed a gradual overall decline. The initial increase may be a response to a combination of: wounding, dilution and increased metabolic activity prior to cell growth. The latter assumption is supported by the fact that respiration rates peaked prior to the increase in fresh weight in both treatments. Both chlorophyll and photosynthetic activity were undetectable in cells grown in the dark. In cells grown in the light net photosynthesis was substantially below the compensation point until day 8 (Fig. 3) when it increased to and remained near to the compensation point. In these cultures there

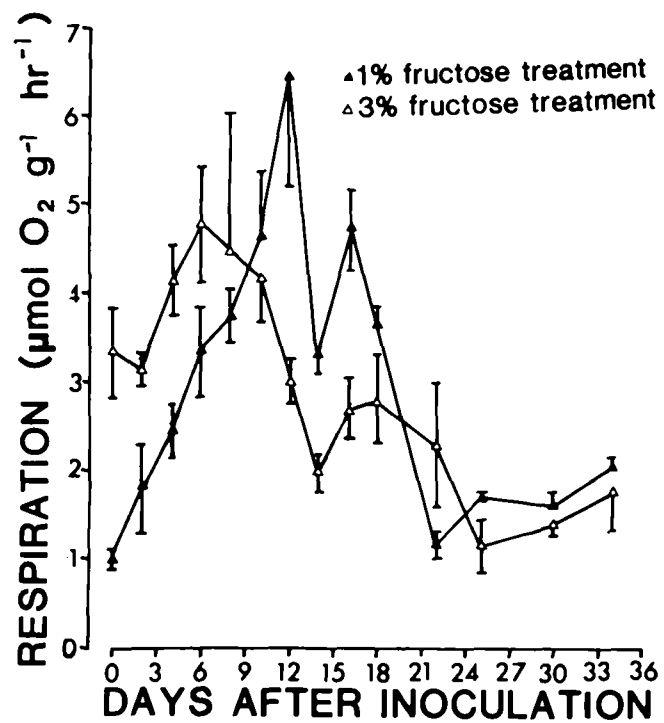


Fig. 1.  
Changes in fresh weight of *N. tabacum* cell culture supplemented with 1% or 3% fructose over 36 days.



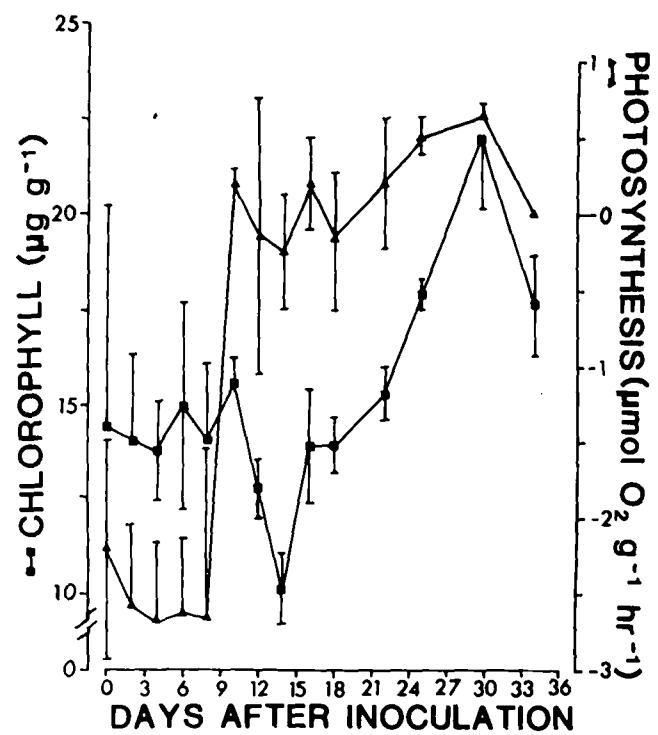
Each point is the mean of 3 replicates

Fig. 2.  
Respiration rates of cells cultured with 1% or 3% fructose over a 36 day period.



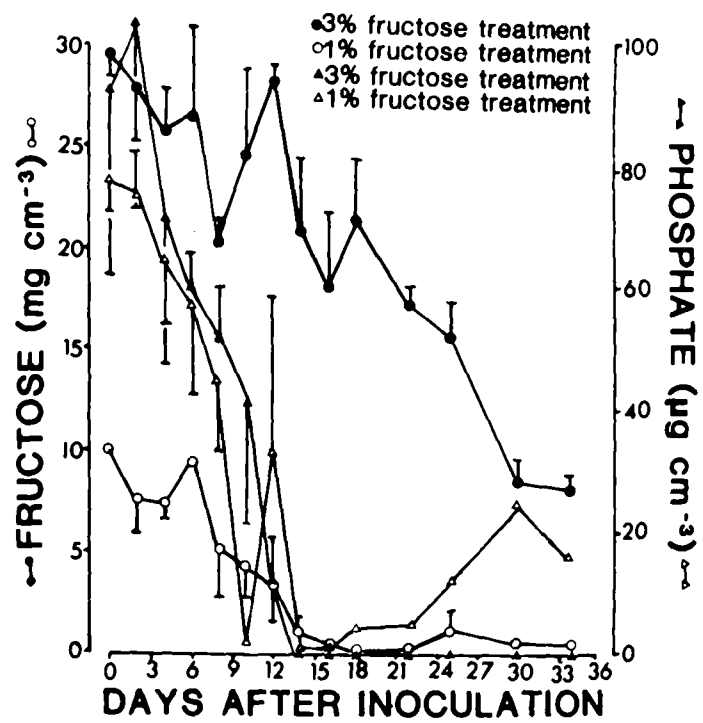
Each point is the mean of 3 replicates

Fig. 3.  
Photosynthetic characteristics of cells cultured with 1% fructose for 36 days.



Each point is the mean of 3 replicates

Fig. 4.  
Nutrient depletion of the media by cultured cells during a culture cycle.



Each point is the mean of 3 replicates

was a slight decrease in chlorophyll content until day 16 (Fig. 3) which may have been due to a dilution effect caused by unbalanced growth (La Rosa *et al* 1984). After day 16 there was an increase in chlorophyll content until day 30, which may be associated with increased cellular organisation during the stationary phase and/or a release from suppression of chlorophyll synthesis by fructose in the medium (Edelman & Hanson 1971). As can be seen from Fig. 4 the fructose concentration in the media in the 1% treatment was negligible by day 18 and was thus probably limiting growth. In cultures with 3% fructose the carbon source did not become limiting. In both treatments phosphate was rapidly depleted from the medium (Fig. 4). In the 1% fructose treatment the phosphate concentration in the medium increased towards the end of the time course, as the cells began to senesce, indicating that it had not become limiting. However, in cultures supplemented with 3% fructose phosphate was undetectable after day 14 and may thus have become limiting later in the culture cycle.

From this work it can be concluded that cells grown in medium supplemented with 3% fructose exhibit heterotrophic characteristics and those with 1% fructose exhibit mixotrophic characteristics. As cells were moved from the former state to the latter so the limiting nutrient appears to shift from phosphate to fructose.

The mixotrophic cells obtained can be moved towards a total dependence upon photosynthesis for the provision of organic constituents (photoautotrophy) by employing a flat bed system (Lindsey & Yeoman 1983) which ensures that nutrient fluctuations are minimized. Using cultures with differing degrees of dependence upon photosynthesis (light) for growth the effect of illumination is being investigated upon the activity of quinolinic acid phosphoribosyl transferase, and subsequently nicotine synthesis.

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