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STUDIES ON THE PRODUCTION OF DIOSGENIN  
BY PLANT CELL CULTURES OF DIOSCOREA SPECIES

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1985



(ii)

DECLARATION

I HEREBY DECLARE THAT, EXCEPT WHERE OTHERWISE INDICATED, THIS THESIS IS THE RESULT OF MY OWN WORK.

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A C K N O W L E D G E M E N T S

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ABBREVIATIONS

g	gram(s) <u>or</u> unit of gravitational force
min	minute (s)
s	second (s)
hr	hour (s)
m	metre (s), milli ( $10^{-3}$ )
c	centi ( $10^{-2}$ )
$\mu$	micro ( $10^{-6}$ )
n	nano ( $10^{-9}$ )
M	molar
mol	mole(s)
l	litre (s)
$^{\circ}\text{C}$	degree(s) centigrade
E	Einstein
Ci	Curie (s)
cpm	counts per minute
dpm	disintegrations per minute
pH	negative log of the hydrogen ion concentration
rpm	revolutions per minute
psi	pounds per square inch
%	percentage
W/V	weight per volume (as percentage)
V/V	volume per volume (as percentage)
2,4-D	2,4-dichlorophenoxy acetic acid
TLC	thin layer chromatography
PTLC	preparative thin layer chromatography
GLC	gas liquid chromatography
BAT	blood agar test (medium)
MS/	

ABBREVIATIONS CONT

MS	Murashige and Skoog (medium)
PCV	packed cell volume
OD	optical density
sp. gr	specific gravity
ppm	parts per million
bp	boiling point
$\bar{X}$	mean
SE	standard error
Q	The studentised range
F	The variance ratio
SSR	shortest significant range

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ABSTRACT

The aim of this work was to investigate the growth of, and the diosgenin production by plant cell cultures of Dioscorea species.

Initially, attempts at raising plant cell cultures proved unsuccessful in three out of the four species of Dioscorea investigated. Eventually suspension cultures of Dioscorea composita were established using a Murashige and Skoog medium supplemented with  $1.4 \times 10^{-5} \text{ M}$  2,4-D.

The relationship between growth and diosgenin content of the cultures was investigated and evidence for an inverse relationship between growth and accumulation was obtained. A study of the depletion of the four main nutrients in the culture medium suggested that the growth of the cultures was limited by the concentration of inorganic phosphate. Sucrose, ammonium and nitrate were never limiting with respect to growth.

The above results suggested that the accumulation of diosgenin in suspension cultures of D. composita was regulated by the combined action of changes in the nutrient status of the cultures coupled to changes in growth rate. A hypothesis based on the competition between primary and secondary metabolism has been proposed to explain the mechanism of this regulation. Studies on stationary phase suspension cultures have also suggested that the level of diosgenin accumulation may be regulated by a feedback inhibition process.

A novel technique for the selection of cell lines yielding high levels of diosgenin was also developed. It was also shown that D. composita suspension cultures could be grown in a multi-litre fermentor (15 litres). While these techniques suggested it was possible to obtain high yielding strains of D. composita and to grow them on a large scale, it was concluded that the process would probably not be economically viable for the production of diosgenin.

Despite this, the above results show that the production of diosgenin in batch suspension cultures of D. composita would be a good model system to study the regulation of secondary metabolism.

## INTRODUCTION

Man has used compounds extracted from plants, as medicines, pigments, flavourings and perfumes for thousands of years (Mann, 1980). These compounds which include alkaloids, steroids, flavonoids and terpenoids still constitute a large and economically important section of the chemical industry (Staba, 1980). Although there has been a trend towards chemical synthesis for some of the simpler substances, plants are still an important source of many important products. These are usually either structurally complex such as the dimeric anti-tumour alkaloid, vinblastine or are very complicated mixtures such as onion or rose oil which depend not only on the individual components being present in the correct proportions to produce a commercially acceptable taste or smell but which often require the presence of trace components yet to be chemically identified. All these factors make the chemical synthesis of such compounds difficult and expensive. It is also advantageous that compounds from plant sources are not subject to the many stringent toxicological regulations which cover chemically synthesised compounds.

Many of the secondary plant products which are useful to man are extracted from plant material which is either grown in plantations or gathered from wild populations in the tropical and sub-tropical regions of the world. This has led to the cost and availability of these materials being vulnerable not only to variations in climate, crop yield and large scale infestations of insects and micro-organisms, but also to changes in /

in the political climate. Wild populations are also vulnerable to over exploitation. All of these factors have made the culture of plant cells an attractive proposition for the production of valuable secondary plant products. Such controlled culture processes can be continuous, are independent of climate and geographical location and can result in reliable yields of the product.

It is now almost twenty five years since Tulecke and Nickell, (1959), demonstrated that plant cells could be grown in submerged culture as suspensions. They also recognised that such cultures had great synthetic potential for the production of valuable natural products, (Nickell and Tulecke, 1960). In the intervening twenty five years however this potential has only been realised to a very limited extent.

In comparison with plant cell culture, the microbiological fermentation industry has over a similar period successfully harnessed the potential of micro-organisms to produce high yields of a wide range of valuable products. This success can be attributed to a combination of two factors, strain improvement and optimisation of growth and production conditions which have led to the discovery that, in general, regardless of the metabolite accumulated, eg antibiotic, alkaloid or microbial toxin, the relationship between growth and secondary metabolite production is an inverse one. The growth phase (trophophase) occurs first and after growth has ceased, usually through the depletion of a specific mineral nutrient in the culture medium, the production phase (idiophase) begins. Overall, although some attempts have been made to study the metabolic and genetic aspects of the regulation of microbial secondary/

secondary metabolism, the success of the industry has largely relied on an empirical approach (Aharonowitz, 1980).

#### ACCUMULATION OF SECONDARY METABOLITES BY PLANT CELL CULTURES

The approach generally adopted with plant cell cultures is a logical and empirical one. It consists of taking a plant known to accumulate a desired secondary product and placing it in culture, optimising the culture medium to encourage rapid growth in order to encourage accumulation of the products. However what happens in a great many cases is that the cultures fail to retain the synthetic abilities of the parent plant as expected. The cultures grow rapidly and accumulate large amounts of dry matter but very little if any of the desired compound. (Yeoman et al, 1982). From a literature search of the wide range of species tested for their ability to accumulate metabolites, characteristic of their parent, in culture, two general points emerge, they are:

- 1) Rapidly growing cultures accumulate cells and not secondary metabolites.
- 2) Certain cultures can accumulate secondary metabolites, usually in small amounts and occasionally in substantial amounts. Sometimes secondary products which accumulate are known precursors of the desired compound but are of no commercial value or structurally altered compounds which are not normally found in the intact plant.

On examination of this second group two common features emerge with respect to the type of cultures which accumulate secondary metabolites. The first is that slow growing mature cultures usually showing some degree of cellular differentiation tend to accumulate secondary products, the second point is that when 'undifferentiated' cultures accumulate secondary products they do so either when they have stopped growing (stationary phase) or when growth is slowing down (late linear phase).

#### RELATIONSHIP BETWEEN ACCUMULATION AND DIFFERENTIATION

The types of differentiation which have been correlated with the accumulation of secondary metabolites are varied and include organogenesis and embryogenesis. Hashimoto and Yamada, (1982), have shown that root differentiation in callus cultures of Hyoscyamus niger significantly promoted the synthesis of scopolamine. Similar effects on tropane alkaloid production have been reported in Atropa belladonna cultures by Thomas and Street (1970), in Datura innoxia by Hiraoda and Tabata, (1974), and in Scopolia parriflora by Tabata et al, (1972). Benjamin et al (1979), have reported that while callus cultures of Tylophora indica failed to produce substantial quantities of alkaloid, regenerated root and shoot cultures produced levels similar to the intact plant. The production of flavour compounds in onion cell cultures (Allium cepa) has been shown to be dependent on root and shoot differentiation (Turnbull et al, 1981; Freeman et al, 1974). Al-Abta et al (1979), have also shown that flavour compounds of celery cultures (Apium graveolens) cannot be detected in undifferentiated cultures but will accumulate in differentiated/

differentiated callus and differentiated suspension cultures when embryoids formed and the level of flavour compounds approaches that of the intact plant. Leaf organ cultures of Catharanthus roseus have been reported to accumulate a wide range of indole alkaloids including vindoline, (Krueger et al, 1982). This alkaloid has never been detected in 'undifferentiated' cultures of this species. Anderson et al (1982), have reported the accumulation of quinine and quinidine in leaf and root cultures of Cinchona ledgeriana while Heble and Staba, (1980), have reported diosgenin from shoot cultures of Dioscorea composita. There have also been two reports of digitoxin accumulation from leaf cultures of Digitalis purpurea (Hagimori et al, 1982) and Digitalis lanata (Liu and Staba, 1979). Komo et al (1982), have shown that the differentiation of shoots in cultures of Papaver somniferum is necessary before the spectrum of morphine alkaloids resembles that of the intact plant. In Citrus tissue cultures Brunet and Ibrahim, (1973), reported that light regimes which induce differentiation and lignification also stimulate flavinoid production. In contrast Bhatt et al (1983), found that dark grown differentiated cultures of Solanum nigrum produced significantly more solasidine than 'undifferentiated' callus.

The link between differentiation and accumulation has been further strengthened by a number of reports correlating the appearance of specialised cells, such as oil secretory glands with the production of specific secondary compounds.

Bricout et al (1978), has shown that colchicine can increase the level of essential oils in Mentha piperita cultures and that/

that the increase appears to be predominantly due to an increase in the number of secretory glands which store essential oil. Similar results linking the appearance of oil secretory glands and essential oil production have been reported for Ruta graveolens (Reinhard et al., 1968; Corduan and Reinhard, 1972) and for Pimpinella anisam and Ruta graveolens (Becker, 1970).

#### RELATIONSHIP BETWEEN GROWTH AND ACCUMULATION

One of the first examples of a plant cell culture producing substantial amounts of a secondary metabolite was the accumulation of diosgenin by callus and suspension cultures of Dioscorea deltoidea, (Kaul and Staba, 1968). Kaul et al (1969) then went on to show that the accumulation of diosgenin occurred in these cultures exclusively during the stationary phase. This inverse relationship between growth and diosgenin accumulation has been confirmed in cultures of D. deltoidea by a number of other workers, (Mehta and Staba, 1970; Tal and Goldberg, 1982; Taracanova et al, 1979). In cell cultures of Glycine max large changes have been reported in the activity of the enzymes of the phenyl-propanoid pathway prior to the accumulation of phenolics during stationary phase (Eahlbrock et al, 1971). Macheix et al (1981), have shown similar results in apple (Pyrus malus) suspension cultures derived from fruit parenchyma. The accumulation of phenolics during the stationary phase of growth in suspension cultures of Acer pseudoplatanus has been reported by Phillips and Henshaw, (1977) as has the accumulation of polyphenolics in cultures of )  
Paul's/

Paul's Scarlet Rose, (Davies, 1972a) and Camellia sinensis (Forrest, 1969). Jalal and Collin, (1979), have shown that phenolics in cultures of cocoa (Theobroma cacao), appeared during the stationary phase while Townsley, (1974), has shown that by roasting cultured tissue of Theobroma cacao, only stationary phase cells produced chocolate aromas indicative of cocoa secondary aromatic compounds. Mohammad and Collin, (1979), have reported that pigment formation in sugar beet callus (Beta vulgaris) also occurs late in the growth phase.

The relationship between growth and secondary metabolism has probably been studied more in alkaloid accumulating species than any other group. The accumulation of indole alkaloids has been shown to be inversely related to growth in two distinct cultured cell lines of Catharanthus roseus (Kurz et al, 1980; 1981). Roustan et al, (1982), has reported the accumulation of ajmalicine and serpentine to be a feature of the stationary phase of Catharanthus roseus cultures. Zenk et al (1977), in a study of serpentine accumulation in Catharanthus roseus have shown that while in flask cultures the accumulation begins during the linear phase of growth and reaches a maximum during the stationary phase, in fermenter culture all accumulation occurs during the stationary phase. In suspension cultures of Hyoscyamus muticus, Koul et al, (1983) have shown that tropane alkaloid accumulation is a feature of the stationary phase of growth. A similar result has been reported by Sharma and Khanna (1981), in cultures of Atropa belladonna. Both Khanna and Jain, (1972), and Radwan and Kokate, (1980), have reported that the accumulation of trigonelline is inversely proportional to growth in cultures of Trigonella foenum-graecum. Similarly Frishknecht and Bauman, (1980), have shown that the two purine alkaloids, caffeine and theobromine accumulated

maximally during stationary phase in cultures of Coffea arabica while Neumann and Muller, (1974), have reported similar results for alkaloid accumulation in cultures of Macleaya cordata. In an investigation of seven solanaceous species Lindsay and Yeoman (1983), obtained evidence for an inverse correlation between growth on the one hand and alkaloid accumulation and cell organisation on the other.

There is also evidence in the literature to suggest that accumulation of secondary metabolism can be induced if growth is artificially stopped by metabolic inhibitors. Mizukami et al, (1977), have shown that the addition of streptomycin sulphate can stimulate the accumulation of shikonin derivatives in cell cultures of Lithospermum erythrorhizon. Similarly Neumann and Muller, (1971), reported that D-threo-chloramphenicol while inhibiting protein synthesis and growth, increased the nicotine content of Nicotiana tabacum cultures. They also showed that Actinomycin.D can have a similar effect on growth and alkaloid accumulation in Macleaya cordata cultures. Aitchison, (1977, unpublished data) has shown that the addition of cyclohexamide to callus cultures of Capsicum frutescens at levels which inhibit protein synthesis, stimulated the incorporation of radioactively labelled phenylalanine and valine into capsaicin. Bricout et al (1978), has shown that the use of the cell division inhibitor colchicine can increase the levels of essential oils in callus cultures of Mentha piperita. As has been previously stated this has been correlated with the increase in the number of oil storage glands in the callus. This may suggest that colchicine acted to slow down or stop cell division which allowed more oil glands to form which in turn led to the increased accumulation/

accumulation of essential oils. Such a link between slow growth/differentiation and secondary metabolite accumulation has been proposed (Yeoman et al, 1982). It can be seen from the literature therefore that there is a general inverse relationship governing the accumulation of a wide range of secondary metabolites in plant cell culture. This relationship may be summarised as follows:

$$\text{Secondary metabolite accumulation and cell differentiation} \propto \frac{1}{\text{growth rate}}$$

If such a separation of growth and secondary metabolism is a feature of plant cells in general, then it also follows that there should be some temporal separation of primary metabolism from secondary metabolism. Such a relationship would require some regulatory mechanism to switch from one to another in order to ensure efficient use of common precursors and energy. It is also probable that such a mechanism would be one of the major factors which would determine the final yield of a secondary product in a plant cell culture.

It is one of the objectives of this project to study this relationship in diosgenin producing cultures of Dioscorea species as a part of an experimental programme which will include the development of strain selection techniques, and growth/accumulation studies in multi-litre fermentor vessels. The overall aim of the project being to investigate the feasibility of developing such cultures as a commercial source of diosgenin, which is used in the manufacture of steroid drugs, (Hardman, 1969).

Plant/

Plant cell cultures of Dioscorea were chosen as the experimental system for two reasons, firstly they can accumulate substantial amounts of diosgenin, ie 1 - 2% of culture dry weight (Kaul and Staba, 1968); (Kaul et al, 1969). Secondly it has been shown to be one of the few systems in which growth and accumulation of the secondary metabolite were completely separated. This should make the study of the relationship between primary and secondary metabolism easier than it would have been had the accumulation of diosgenin been growth linked.

The approach in this project may be briefly summarised as follows. Diosgenin producing species of Dioscorea were established in vitro and batch suspension cultures were used to determine:

- a) The relationship between culture growth and diosgenin accumulation.
- b) The effects on growth and diosgenin accumulation of the depletion of four major nutrients from the culture medium.
- c) The effects on growth and diosgenin accumulation of manipulating the culture medium of stationary phase cultures.

In parallel to these experiments a rapid method for screening cell lines was developed with the aim of detecting and isolating high yielding cell lines. The cultures were also grown/

grown in 15 litre fermentor vessels to study the problems of scaling the production of diosgenin by plant cell cultures from the laboratory to an industrial scale.

CHAPTER 2

MATERIALS AND METHODS

## CHAPTER 2 - SECTION 1

### PLANT CELL CULTURE METHODS

#### a) PLANT MATERIAL

The species of Dioscorea which were chosen as sources of explant material for the initiation of plant cell cultures are listed in Table 2.1.1. These were species known to accumulate large amounts (4 - 15% of tuber dry weight) of steroidal saponins containing diosgenin as the aglycone (Martin, 1969). The seeds were supplied by the USA Department of Agriculture, Federal Experimental Station, Managuez, Puerto Rico.

#### b) NUTRIENT MEDIA

The nutrient medium used in all the plant cell culture experiments was Murashige and Skoog medium (Murashige and Skoog, 1962). The powdered medium was obtained from Flow Laboratories, Irvine, Scotland. This was supplemented with sucrose and plant growth substances to give the complete medium. (The components and concentrations are listed in Table 2.1.2). This medium is hereafter referred to as MS medium.

The nutrient medium used in the growth of the bacterium Agrobacterium tumefaciens was yeast/mannitol medium. The components and concentrations of this medium are listed in Table 2.1.3.

The medium used in testing for microbial contamination were Nutrient Agar and Czapek Dox media. The components for these media are listed in Tables 2.1.4 and 2.1.5.

TABLE 2.1.1

SPECIES OF DIOSCOREA USED AS EXPLANT MATERIAL, THEIR GEOGRAPHICAL DISTRIBUTION AND DIOSCIN YIELD AS % TUBER DRY WEIGHT

<u>SPECIES</u>	<u>DISTRIBUTION</u>	<u>DIOSCIN YIELD</u> %
<u>DIOSCOREA COMPOSITA HEMSL</u>	CENTRAL AMERICA	13.0
<u>DIOSCOREA SPICULIFLORA HEMSL</u>	CENTRAL AMERICA	15.0
<u>DIOSCOREA FLORIBUNDA MART AND GAL</u>	CENTRAL AMERICA	10.0
<u>DIOSCOREA FRIEDRICHSTHALLI KNUTH</u>	CENTRAL AMERICA	4.0
<u>DIOSCOREA SYLVATICA ECKLON</u>	SOUTH AFRICA	6.0

TABLE 2.1.2THE COMPOSITION OF MURASHIGE AND SKOOG MEDIUMMINERAL SALTS

$\text{KNO}_3$	1900	mg/l
$\text{NH}_4\text{NO}_3$	1650	mg/l
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	mg/l
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	mg/l
$\text{KH}_2\text{PO}_4$	170	mg/l
FeNaE.D.T.A.	36.7	mg/l
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	mg/l
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	mg/l
$\text{H}_3\text{BO}_3$	6.2	mg/l
KI	0.83	mg/l
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	mg/l
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	mg/l
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	mg/l

ORGANICS

Sucrose	30000	mg/l
Glycine	2	mg/l
Inositol	100	mg/l
Thiamine HCL	0.1	mg/l
Pyridoxine HCL	0.5	mg/l
Nicotinic acid	0.5	mg/l

PLANT GROWTH SUBSTANCES

2,4-Dichlorophenoxy acetic acid

Kinetin

The amount of these substances varied according to the experiment. Concentrations are given in the relevant sections of the Results Chapter.

TABLE 2.1.3THE COMPOSITION OF YEAST MANNITOL MEDIUM

$K_2HPO_4$	0.5 g/l
$MgSO_4 \cdot 7H_2O$	0.2 g/l
NaCl	0.1 g/l
Mannitol	10.0 g/l
Yeast Extract	4.0 g/l
Calcium gluconate	0.5 g/l

TABLE 2.1.4THE COMPOSITION OF NUTRIENT AGAR MEDIUM

Beef Extract	3.0 g/l
Peptone	5.0 g/l
Agar	15.0 g/l

TABLE 2.1.5THE COMPOSITION OF CZAPEK DOX MEDIUM

$NaNO_3$	3.0 g/l
$K_2PO_4$	1.0 g/l
$MgSO_4 \cdot 7H_2O$	0.5 g/l
KCL	0.5 g/l
$FeSO_4 \cdot 7H_2O$	0.01 g/l
Sucrose	30.0 g/l
Agar	15.0 g/l

BOTH THIS MEDIUM AND NUTRIENT AGAR MEDIUM WERE SUPPLIED AS DRIED POWDERS FROM OXOID LTD

TABLE 2.1.6THE COMPOSITION OF PHOSPHATE BUFFERED SALINE

$\text{Na}_2\text{HPO}_4$	1.15 g/l
$\text{NaH}_2\text{PO}_4$	0.20 g/l
KCL	0.20 g/l
NaCl	8.00 g/l

THIS SOLUTION WAS ADJUSTED TO pH 7.0

All media were made up to the desired volume with glass distilled water and the pH was adjusted before autoclaving with 0.1M KOH. The MS medium was adjusted to pH 5.8 and the other media to pH 7.0. If solid medium was required then 10 g/l of agar (Oxoid No 3, Oxoid Ltd) was added prior to autoclaving. All media and instruments necessary for the initiation and maintenance of the cultures were sterilised by autoclaving at 120°C/15 psi for 15 min.

Prior to use all tissue culture glassware was soaked overnight in a 5% Solution (V/V) of Decon 90 detergent (Decon Laboratories Ltd). It was then rinsed 3 times with tap water, once with glass distilled water and dried in a hot air oven.

### c) CALLUS CULTURE

#### i) Description of explant material

The types of plant material used as explants to initiate callus cultures were as follows:

- a). seedlings (2-3 cm in length)
- b). Internodal and nodal stem segments
- c). Leaf discs, (from young leaves)
- d). Tuber discs

The above explants were prepared using all the species listed in Table 2.1.1 except Dioscorea spiculiflora.

The seeds of this species failed to germinate.

All explants except the seedlings, were prepared from 6 month old plants grown in Levingtons soilless compost ) (Fisons Ltd) in the departmental greenhouses. The seedlings were prepared from seed germinated on moist filter/

filter paper in pre-sterilised plastic Petri-dishes (9cm diameter, Sterilin Ltd).

ii) Sterilisation of explants

a) Seedlings

The seeds of the various Dioscorea species were firstly soaked in 90% ethanol (V/V with distilled water), containing a drop of detergent as a surfactant, for 1 min and then washed 3 times with distilled water. They were then sterilised in a 2% solution of sodium hypochlorite (W/V) (20% V/V commercial solution supplied by Mackay and Lynn Ltd), containing a few drops of detergent, for 20 min. Finally the seeds were washed 3 times with sterile distilled water.

b) Stem Segments

Stem segments 3-4 cm long containing internodal and nodal regions were cut from 3 month old plants. The cut ends were sealed with molten paraffin wax to prevent damage to internal tissue by the sterilisation procedure. This material was then sterilised in a similar manner to the seeds as described above.

c) Leaf Discs

Young leaves with intact petioles were cut from 3 month old plants. The petiole ends were then sealed with molten paraffin wax and the material was sterilised in a similar manner to the seeds as described above.

d) Tuber Discs

Tubers of all the species were scrubbed in a 1% (V/V) detergent solution, then soaked in 90% ethanol, containing a few drops of detergent for 5 mins. They were then washed 3 times with distilled water, and sterilised with a 5% sodium hypochlorite solution (W/V) containing a few drops of detergent. After 30 min they were washed 3 times with sterile distilled water.

All procedures which entailed sterilisation of explants, initiation of cultures, or maintenance of cultures were carried out in a purpose built 'Sterile Room' which was irradiated with ultraviolet light when not in use. It was also kept under positive air pressure which prevented dust and micro-organisms being carried in from adjoining rooms.

iii) Initiation of Callus Cultures

Sterile seeds were aseptically transferred to Petri-dishes which contained 3 layers of sterile moist filter paper (Whatman No 1). They were left to germinate until the seedlings were 2-3 cm long, then transferred to Petri-dishes containing callus initiation medium. The dishes were then sealed with parafilm (American Can Company).

The sterile stem segments were placed in a sterile Petri-dish and cut into discs 2-3 mm thick using a sterile scalpel. The discs were then transferred to Petri-dishes of callus initiation medium which were then sealed with Parafilm.

The sterile leaves were placed in a sterile Petri-dish and discs (1 cm diam). were cut out using a sterile cork borer. These were transferred to Petri-dishes containing callus initiation medium which were then sealed with Parafilm.

The tuber discs were prepared by cutting out cores of tissue from the sterile tubers using a sterile cork borer. The cores were then sliced into 23mm segments with a sterile scalpel and placed on callus initiation medium in Petri dishes. The dishes were then sealed with Parafilm.

All explants were kept in continuous light ( $200\mu\text{Em}^{-2}\text{S}^{-1}$ , from fluorescent tubes) at a temperature of  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

Any newly formed callus was isolated from the explant and placed on fresh culture medium in a Petri dish. Care was taken not to leave any new callus in contact with explants which showed signs of necrosis since the polyphenolic compounds produced by necrotic tissue can be inhibitory to growth of the callus.

iv) Propagation and maintenance of callus cultures

Once sufficient callus tissue was accumulated it was transferred to 100 ml of culture medium (solidified with agar) in wide necked 250 ml Erlenmeyer flasks capped by a double layer of aluminium foil.

Callus tissues were routinely sub-cultured every four weeks/

weeks to build up a stock of tissue for the initiation of suspension cultures and the long term maintenance of cell lines.

d) SUSPENSION CULTURES

i) Initiation of suspension cultures

Four week old callus cultures were divided into four and aseptically transferred to 50 ml of liquid culture medium in narrow necked 250 ml Erlenmeyer flasks capped by a double layer of aluminium foil. These flasks were then placed on an orbital shaking platform (orbital diameter 1.5 cm) and agitated at 100 rpm. After two weeks the initiated suspension cultures were sieved through a sterile stainless steel mesh (pore diameter 1mm) into a 100 ml measuring cylinder to remove large lumps of tissue. The fine tissue was allowed to settle in the measuring cylinder and the old medium was decanted off. The tissue was then transferred to 50 ml of fresh culture medium and returned to the shaking platform. The cultures were regularly sub-cultured every four weeks.

ii) Propagation of suspension cultures

Established suspension cultures were routinely sub-cultured by transferring 10 ml of four week old cultures to 50 ml of fresh culture medium using a sterile, 10 ml wide bore pipette. The flasks were then placed on the shaking platform as described above. All suspension and callus cultures were grown in a constant environment room, under constant light and temperature (see section 1c (iii)). Unless stated otherwise all experiments were carried out using/

using 60 ml suspension cultures in 250 ml Erlenmeyer flasks prepared as described above.

e) INDUCTION OF CROWN GALL TUMOURS

i) Growth of *Agrobacterium tumefaciens*

Cultures of *Agrobacterium tumefaciens* were maintained on yeast mannitol medium (Table 2.1.3) in the form of agar slopes (20 ml of medium in 50 ml culture tubes with screw caps). Stock cultures were stored at 4°C.

A bacterial culture suitable for the induction of crown gall tumours was prepared by aseptically transferring the bacteria from 2 agar slopes to 50 mls of liquid Yeast Mannitol medium in a 250 ml Erlenmeyer flask using a sterile loop. The culture was then agitated at 250 rpm, on an orbital shaking platform, (orbital dia, 1.5 cm) for 24 hr after which it was used in experiments to induce crown gall tumours. These cultures were used to infect intact plants "in-vivo" in the departmental greenhouse and tuber, leaf and stem tissue "in-vitro".

ii) Infection of intact plants

Intact plants were infected with *A. tumefaciens* using the method described by Butcher, (1977). In this, the internodal region of a stem, at least 5mm in diameter, was surface sterilised with 90% ethanol. A wound was then made in the stem tissue with a sterile syringe needle. The wound was then immediately covered with a moist cotton wool bandage to prevent the tissue drying out. After 48 hr the wound was uncovered and infected with a drop of a suspension culture of *A. tumefaciens* (grown/

(grown as described in part i above) using a syringe needle. The bandage was then replaced and kept on for a further 3 days. Plants which were successfully infected usually showed evidence of tumour formation after 14 days.

iii) Infection of tuber discs

Sterile tuber discs were prepared as described in Section 1 (iii) of this chapter. They were placed on MS medium (containing no growth substances) in Petri-dishes and left for 48 hours. After this period a drop of suspension culture of A. tumefaciens was placed onto the cut surface of the discs. They were then incubated under conditions as described in Section 1 (iii) of this chapter. The discs which were successfully infected usually showed evidence of tumour formation within 14 days.

iv) Infection of leaf discs

Sterile leaf discs were prepared as described in Section 1 (iii) of this chapter. They were placed on MS medium (containing no growth substances) in Petri-dishes and left for 48 hours. After this period each disc was dipped into a suspension culture of A. tumefaciens using sterile forceps and then replaced onto the MS culture medium. The discs were incubated as described above and those which were successfully infected usually showed evidence of tumour formation within 14 days.

v) Infection of stem discs

Sterile stem discs were prepared as described in Section 1 (iii) of this chapter. They were placed on MS medium (containing no growth substances) in Petri-dishes and left for 48 hours. After this period a drop of suspension culture/

culture of A. tumefaciens was placed on the cut surface of each stem disc. The discs were then incubated as described above and those which were successfully infected usually showed evidence of tumour formation after 14 days.

CHAPTER 2 - SECTION 2ANALYTICAL TECHNIQUESa) ANALYSIS OF CULTURE GROWTH

The techniques described below are those used to analyse the growth of suspension cultures used in the majority of experiments.

i) Cell viability

Cell viability was determined using the method of Widholm (1972). This test is based on the ability of living cells to remove the diacetate group from absorbed fluorescein diacetate leaving a molecule which fluoresces under ultraviolet irradiation.

A small sample of the culture (approximately 0.5 ml) was removed using a 10 ml wide bore pipette. Pieces of tissue were placed on a microscope slide and a drop of freshly prepared fluorescein diacetate solution was applied. A cover slip was placed on the tissue and gently squashed. The sample was then viewed using a Vickers 41 Photoplan fluorescence microscope to determine:

a) Total number of cells in field of view at 10 x magnification under white light illumination.

b) Total number of cells fluorescing in the same field of view at 10 x magnification under ultraviolet illumination. The average of six counts per sample was used to calculate the cell viability using the following formula:

Total/

$$\frac{\text{Total cells fluorescing}}{\text{Total cell number}} \times \frac{100}{1}$$

The fluorescein diacetate solution was prepared as follows:

A 0.5 m solution of fluorescein diacetate (Sigma. Chem Co Lt) was made up in acetone. This solution was added dropwise to distilled water until it turned faintly cloudy. This solution was prepared fresh for each batch of samples.

ii) Packed cell volume (PCV)

2 ml of the culture was removed using a 10 ml wide bore pipette and placed in a graduated centrifuge tube. The tube was then spun on a bench centrifuge (Gallenkamp Ltd) at 100 rpm for 2 min. Packed cell volume was then calculated by dividing the volume of the cell pellet after centrifugation by the volume of the sample before centrifugation and expressing the ratio as a percentage.

iii) Cell number

The tissue from the PCV determination was used to calculate cell number using a method adapted from that of Brown and Rickless, (1949).

The liquid was decanted from the centrifuge tube used in the PCV determination and replaced with 2 ml of 5% Chromic acid (W/V). The tube was then agitated to mix the cell pellet with the acid. The tissue was left to stand in the acid for 2 days at room temperature. The cells were then macerated by drawing them vigorously up and down a Pasteur/

Pasteur pipette. Having been suitably dispersed, small samples of the macerate were placed on the two counting grids of the haemocytometer (Modified Fuchs-Rosenthal) and the cell number was calculated under each.

The cell number of each macerate was determined six times using this procedure. The average figure was then used to calculate the cell number in the total sample by using the formula:

$$\frac{\text{Volume of macerate}}{\text{Volume under counting grid}} \times \frac{\text{Cell count}}{1}$$

This value was then calculated to give cell number per ml of culture.

iv) Fresh weight

After samples had been removed for all viability and cell number determinations, the remaining culture was filtered to separate the tissue from the culture medium. The filtration was carried out on a Buchner vacuum filter funnel using Whatman's No 53 filter paper. When the medium was required for further analysis it was stored frozen at  $-20^{\circ}\text{C}$ . The tissue was placed in preweighted foil caps and the fresh weight was determined on an Oertling balance.

v) Dry weight

Once the fresh weight had been determined the tissue was dried in an oven overnight at  $80^{\circ}\text{C}$ . It was then allowed to cool in a dessicator after which it was weighed to determine the dry weight. If this was not done immediately the sample was stored in the dessicator until the weighing was carried out.

b) ANALYSIS OF NUTRIENT DEPLETION

In order to correlate growth and diosgenin accumulation to utilisation of the major nutrients in the culture medium, the changes in the levels of four of these components were measured using various techniques. These nutrients were as follows:

i) Orthophosphate

This nutrient was assayed using the method of Allen, (1940).

Reagents:

60% Perchloric acid, (sp. gr 1.54), (BDH Ltd).

Ammonium Molybdate, (BDH Ltd), (8.3% W/V in distilled water).

Amidol reagent. (0.2 g, 2,4 - diaminophenol hydrochloride, (BDH Ltd), 4.0g Sodium metabisulphite, BDH LTD) made up in 20 ml of distilled water.

Method:

1 ml of each sample was mixed with 0.44 ml of 60% perchloric acid and left to stand for 2 mins. 4 ml of distilled water, 0.4 ml of amidol reagent and 0.2 ml of ammonium molybdate were added to the samples and mixed thoroughly. They were allowed to stand for 30 mins, then the absorbances of the samples were recorded at 620 nm on a Pye Unicam spectrophotometer. A separate calibration curve was used for each batch of samples analysed. They were prepared with  $\text{KH}_2\text{PO}_4$  (BDH Ltd) with a range of 0 - 150 mg orthophosphate/ml. An example of the curves is shown in Figure 2.2.1.

ii) Sucrose /

30.

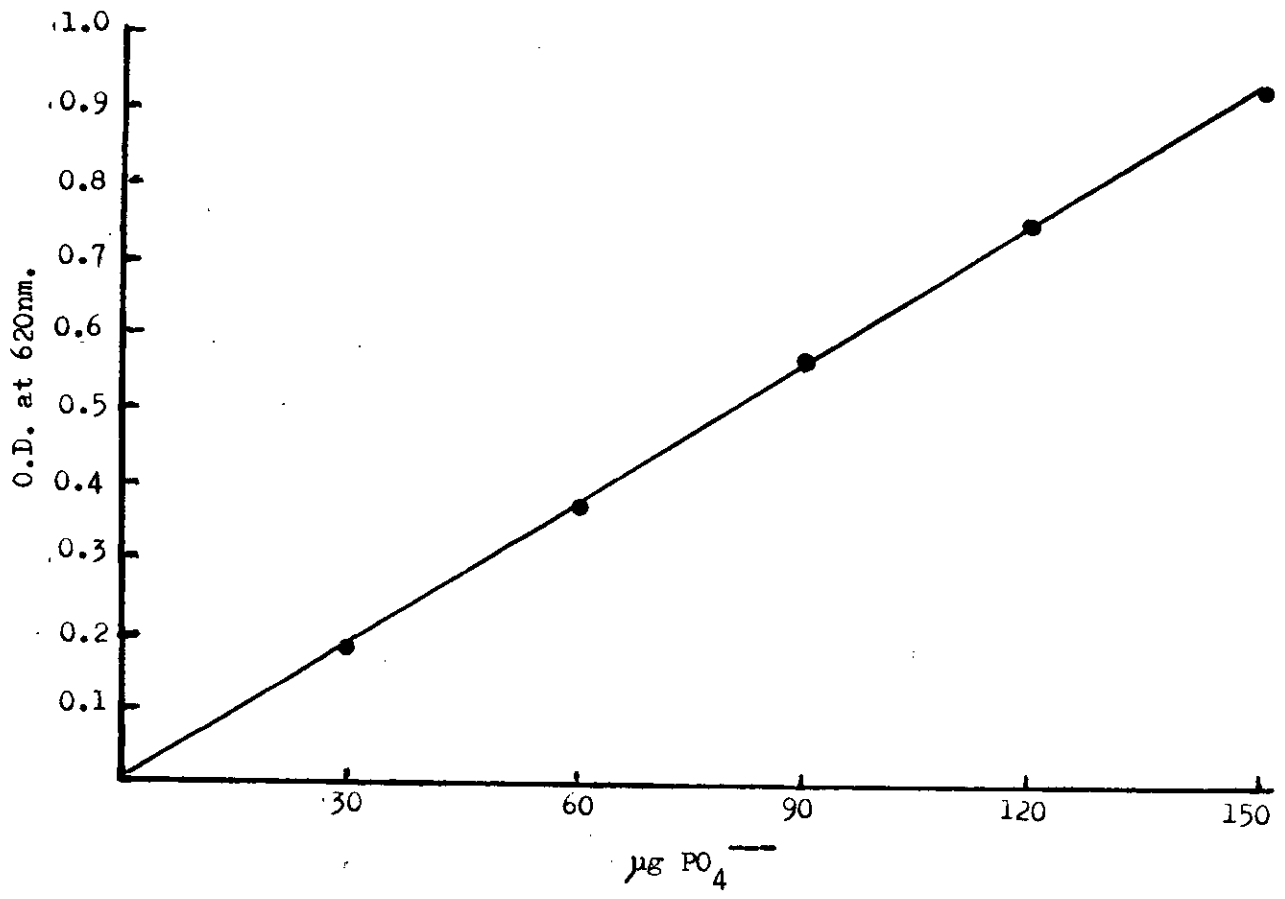


Figure 2.2.1. Orthophosphate Calibration Curve.

ii) Sucrose

This nutrient was assayed using a method based on that of Ashwell, (1957).

Reagents:

Anthrone reagent (0.2 g anthrone (Sigma Chem Co Ltd) was dissolved in 70% sulphuric acid).

Method:

5 ml of anthrone reagent was added to pre-chilled test tubes in an icebath. 1 ml of each suitably diluted sample was slowly layered on top of the anthrone reagent. When all the samples were prepared they were thoroughly mixed but kept in the icebath until they were all mixed. They were then placed in a boiling water bath for 10 min. After cooling the absorbances of the samples were recorded at 620 nm on a Pye Unicam Spectrophotometer. A separate calibration curve was used for each batch of samples analysed. These were prepared using Sucrose, (Analar grade, BDH Ltd), with a range of 0 - 100 mg sucrose/sample. An example of the curves is shown on Figure 2.2.2.

iii) Ammonium

This nutrient was assayed using a method based on that of Havilah et al, (1977).

Reagents:

Salicylate reagent. (34g Sodium salicylate) (BDH Ltd)  
0.24 g Sodium nitroprusside, (BDH Ltd), dissolved in )  
1 litre of distilled water).

Cyanurate/

32.

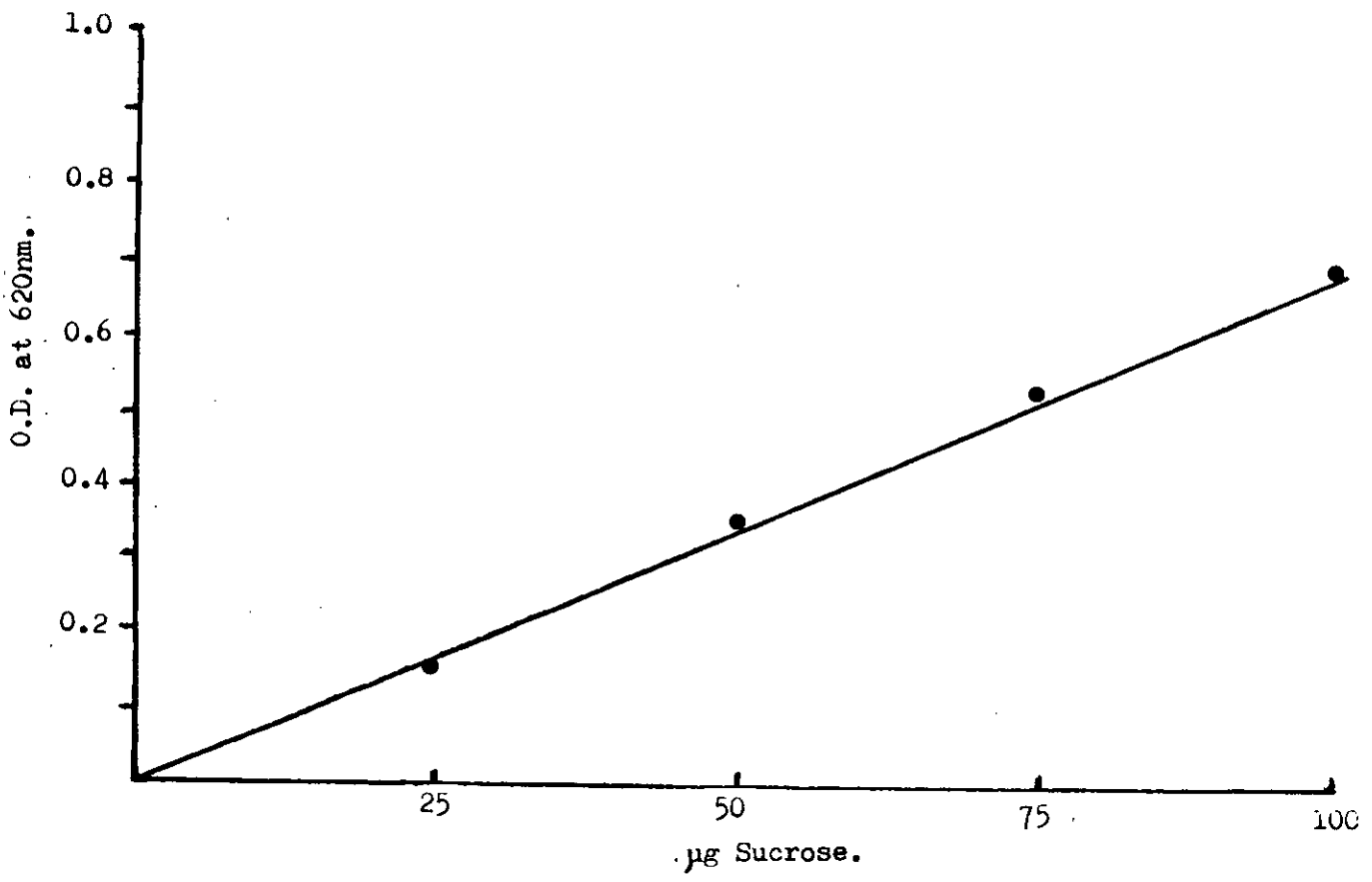


Figure 2.2.2. Sucrose Calibration Curve.

Cyanurate reagent (0.25 g Sodium dichloro-isocyanurate, (Sigma Chem Co Ltd), dissolved in 200 mls of distilled water. 15 ml of 10 M sodium hydroxide was then added and the solution made up to 1 litre with distilled water.

Method:

5 ml of salicylate and cyanurate reagents were added to 0.05 ml of sample and thoroughly mixed. They were then left to stand for 30 mins after which the absorbances of the samples were recorded at 660 nm on a Pye Unicam Spectrophotometer. A separate calibration curve was used for each batch of samples analysed. They were prepared using ammonium nitrate, (BDH Ltd) with a range of 0 - 10 mg of ammonium/50 ml. An example of the curves is shown on Figure 2.2.3.

iv) Nitrate

This nutrient was assayed using a method based on that of Wolley et al, (1960).

Reagents:

Acetic acid reagent. (20% acetic acid, V/V in distilled water, containing 0.2 ppm  $\text{Cu}^{++}$  as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , (BDH Ltd).

Powder mix:	100g	$\text{BaSO}_4$
	75 g	Citric acid
	12 g	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$
	4g	Sulphanilic acid
	2g	Powdered zinc
	2g	1-naphthylamine

All/

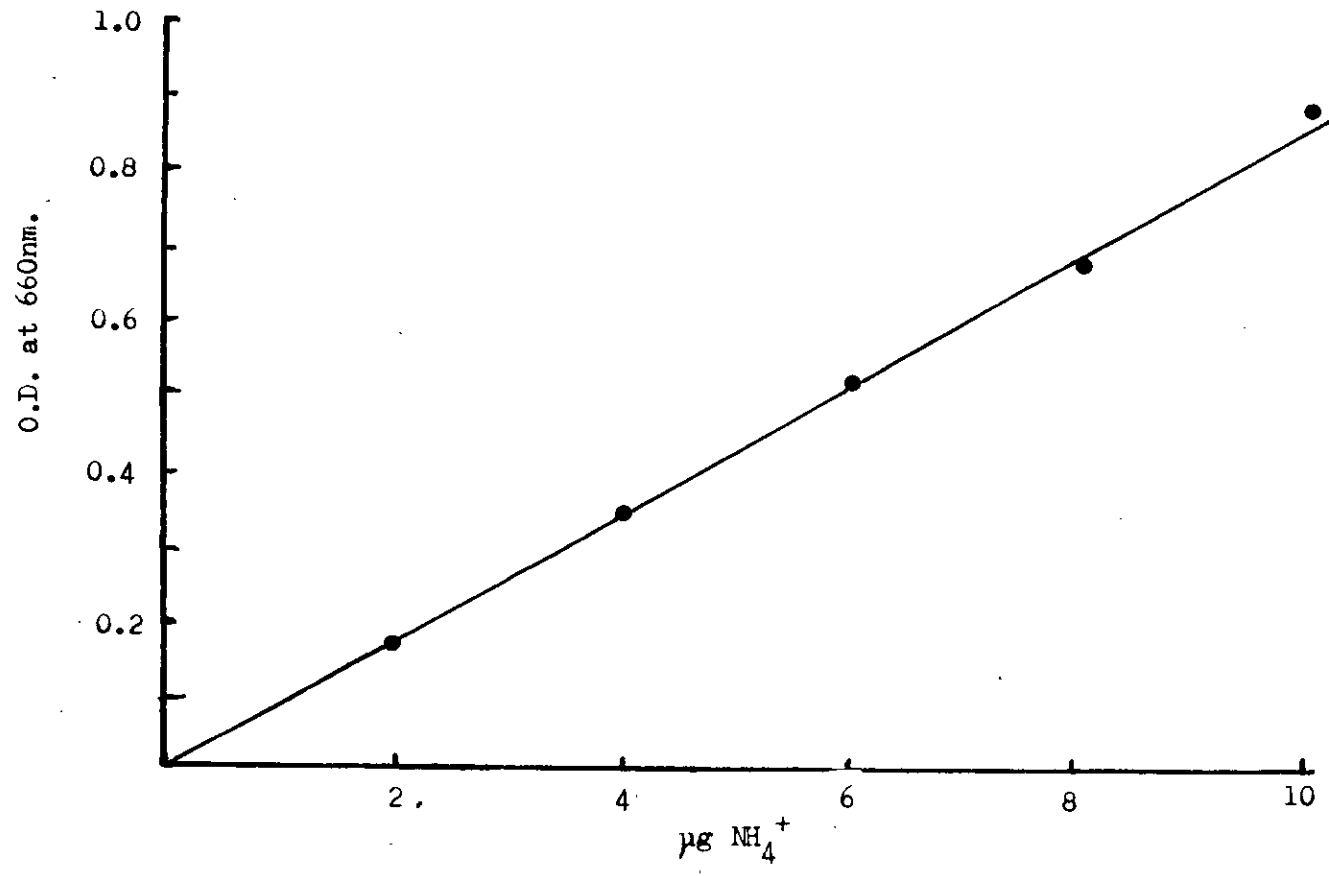


Figure 2.2.3. Ammonium Calibration Curve.

All the powder mix components were supplied by BDH Ltd. The powder mix must be left for 15 days before use.

Method:

9 ml of acetic acid reagent was added to 1 ml of suitably diluted sample. A constant amount of powder mix (0.8g) was added to each, shaken for 15 s, left to stand for 3 min and shaken again for 15s. After standing for 3 more min the tubes were centrifuged at 1000g for 3 min. The absorbances of the clear supernatants were then recorded at 520 nm on a Pye Unicam Spectrophotometer. A separate calibration curve was used for each batch of samples analysed. They were prepared using Potassium nitrate (BDH Ltd) with a range of 0 - 100 mg nitrate/sample. An example of the curves is shown on Figure 2.2.4.

c) EXTRACTION OF DIOSGENIN FROM TISSUE AND CULTURE MEDIUM

Diosgenin is present in plant tissue as a number of glycosides the most common being dioscin (Figure 2.2.5.) The extraction procedure used therefore was a two step one, firstly hydrolysis to remove the sugar moieties followed by extraction using an organic solvent to remove the free diosgenin. The method is based on that of Morris et al, (1958), which was designed for the routine assay of the diosgenin content of Dioscorea tubers.

i) Hydrolysis

Dried tissue was refluxed in 1M hydrochloric acid for 4 hrs (100 ml of acid for every 0.5 g of dry tissue). The mixture was then cooled down, filtered through Whatman's No 53 filter paper in a Buchner funnel and the hydrolysed tissue was washed 3 times with 100 ml of distilled water.

The/

36.

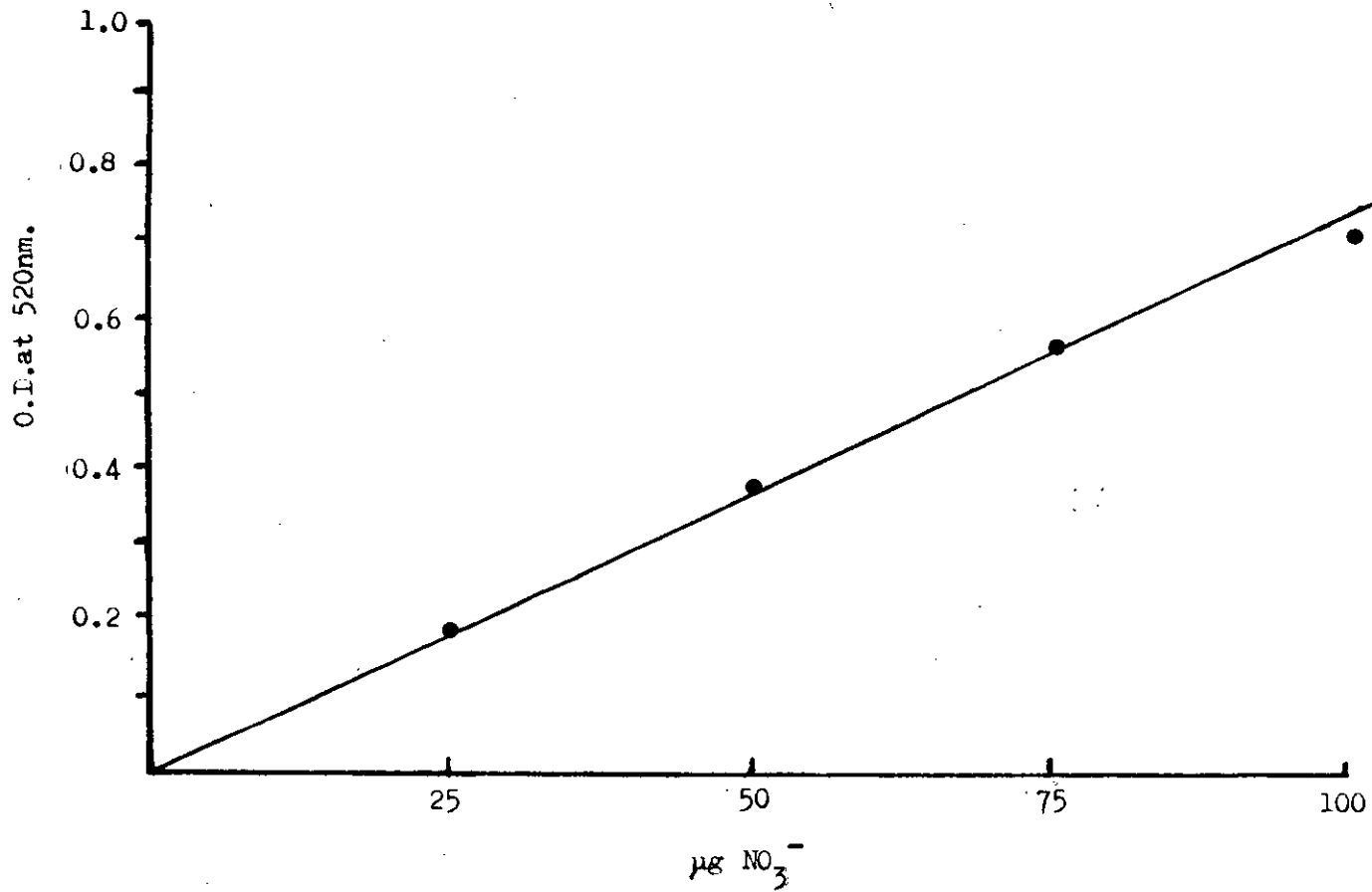


Figure 2.2.4. Nitrate Calibration Curve.

The tissue was finally dried overnight in an oven at 80°C.

ii) Extraction

Following hydrolysis, the tissue was then extracted with Chloroform (80 ml/sample) for 8 hrs in a Soxhlet extraction apparatus. The chloroform extracts were evaporated to dryness in-vacuo at 40°C. The residues were redissolved in 0.5-1.0 ml of chloroform. These solutions were then used for qualitative and quantitative analysis using various chromatographic techniques.

iii) Culture medium

When culture medium was analysed for steroid content, it was carried out on batches of 100 ml. The solutions were acidified (pH 1.0) with 5M hydrochloric acid and refluxed for 2 hrs. They were cooled and extracted twice with 200 ml of petroleum ether (bp 60° - 80°C). The solvent-extracts were combined and evaporated to dryness in-vacuo at 40°C. The residues were redissolved in 0.5 - 1.0 ml of chloroform for subsequent analysis.

iv) Preparation of 25R-spirostan - 3,5-diene

The acidcatalysed hydrolysis of saponins in Dioscorea tissue described in Section 2c of this chapter and in Figure 2. 2.5. as well as breaking the bond between diosgenin and the sugar molecules is also reported to cause to a limited extent the dehydration of the free diosgenin. This small side reaction produces the artificial steroid 25R-spirostan-3,5-diene, Figure 2.2.5. (Morris, 1958, Rothrock et al, 1957). This compound was/

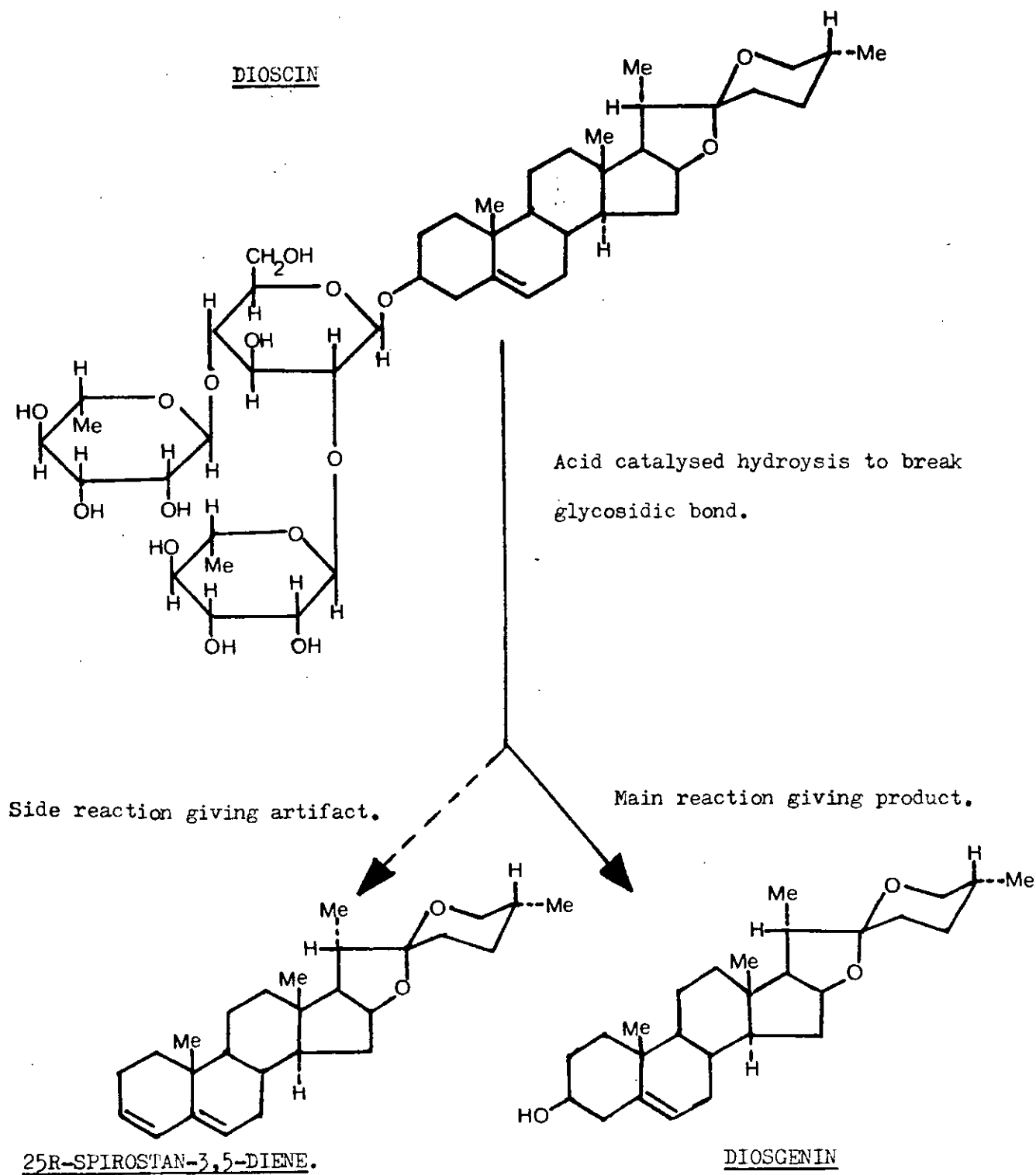


Figure 2.2.5. The acid catalysed hydrolysis of dioscin to produce diosgenin and the dehydration side reaction which produces the artifact 25R-Spirostan-3,5-diene.

was not available commercially therefore it was necessary to prepare a sample for use as a TLC Standard. Normally the molarity of the acid used in the hydrolysis is 1 to 2 molar which maximises this reaction while minimising the dehydration reaction, (Morris, 1958) however, to prepare a sample of 25R-spirostan-3,5-diene, 6M hydrochloric acid was used.

Firstly, the commercial grade diosgenin (Sigma Chem Co Ltd) was purified to remove an impurity. This impurity gave an identical colour reaction to diosgenin with both anisaldehyde and antimony trichloride reagents and ran on TLC with a lower R<sub>f</sub> than diosgenin. A dihydroxy sapogenin with these characteristics, called pennogenin has been reported as an impurity in commercial grade diosgenin (Sofowora et al, 1974 Marquardt, 1978).

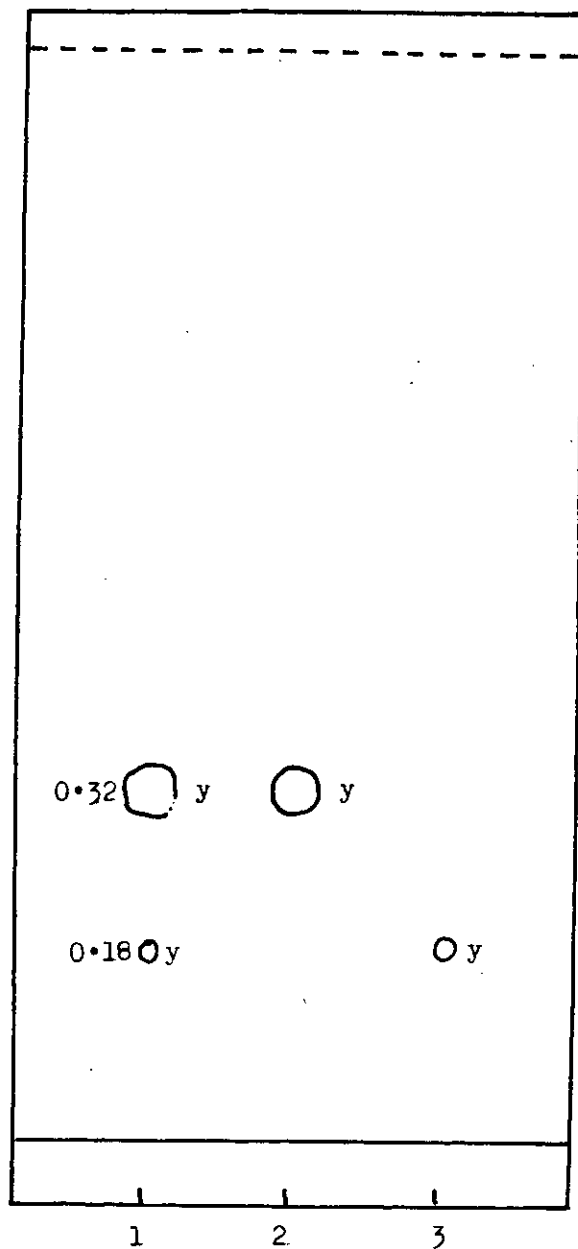
This impurity was removed from 100 mg sample of diosgenin by preparative TLC (PTLC). The diosgenin (in chloroform solution) was streaked along the origin of 4 silica gel TLC plates (0.5 mm thickness, 20 cm x 20 cm). The plates were developed in benzene: ethyl acetate (3:1). Standards of the commercial diosgenin were applied on either side of the main sample and after development they were sprayed with anisaldehyde reagent to visualise the various components on the chromatogram. The zones corresponding to the compounds were scraped off the TLC plates and they were then eluted from the silica with chloroform. The chloroform solutions were reduced in-vacuo to 2 ml and transferred to vials.

Figure/

Figure 2.2.6 shows a diagram of a TLC of the isolated compounds and the original mixture which were run on a 0.2mm analytical silica gel TLC plate developed in benzene: ethyl acetate (3:1) and sprayed with anisaldehyde reagent. It can be seen that the PTLC removed the impurity from the commercial grade diosgenin, 95 mg of which was recovered. The R<sub>f</sub> values for each compound were 0.32 for diosgenin and 0.18 for the impurity.

The 25R-spirostan-3,5-diene was then prepared by refluxing 80 mg of the purified diosgenin in 6M HCL for 8 hrs. The solution was then cooled, neutralised with 10M ammonia solution and extracted 3 times with chloroform. The chloroform extract was dried with anhydrous sodium sulphate and evaporated to dryness in-vacuo. The extract was then redissolved in 1 ml of chloroform and the product was separated from the starting material by PTLC. The extract was loaded onto 3 silica gel TLC plates (0.5 mm thickness, 20 cm x 20 cm) and developed in benzene: ethyl acetate (3:1). The compounds were detected by spraying a 5mm band down one side of the plate with anisaldehyde reagent and heating. Two yellow bands were visible, one with a high R<sub>f</sub> value and one with a lower R<sub>f</sub> value. These bands were scraped off and the compounds eluted from the silica gel with chloroform. The chloroform was reduced in-vacuo to 2 ml and transferred to vials.

Figure 2.2.7 describes a thin layer chromatogram of the isolated compounds from the above reaction. They were run on a 0.2 mm analytical silical gel TLC plate, developed in benzene: ethyl acetate (3:1), and sprayed with Anisaldehyde/



1. Crude diosgenin.

Spot colour

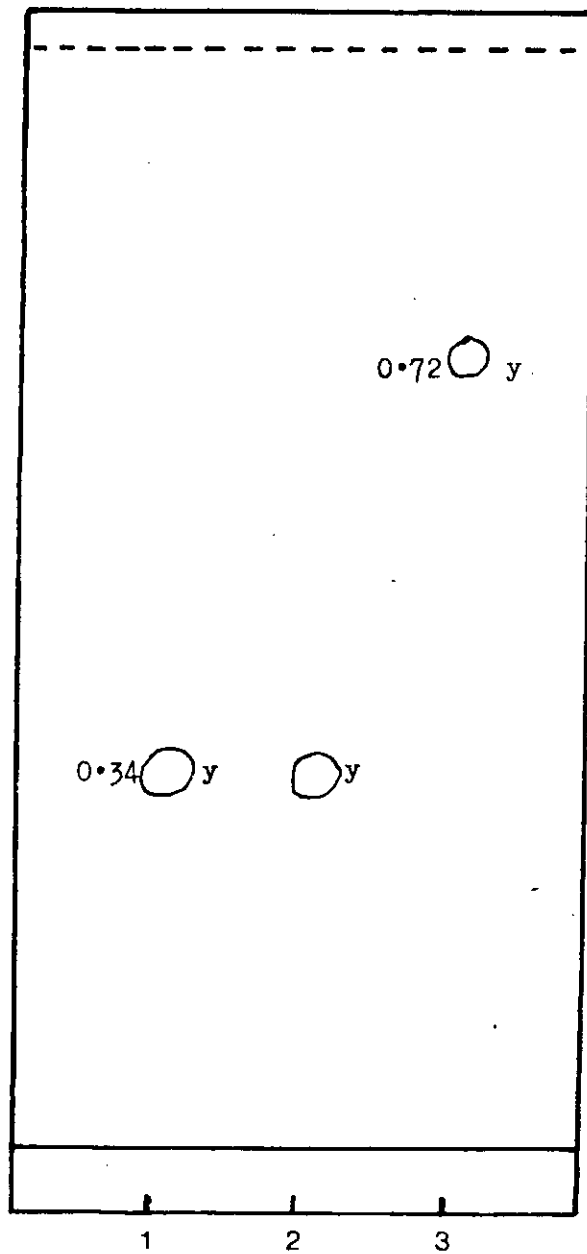
2. Diosgenin (after P.T.L.C.)

y=yellow

3. Impurity (after P.T.L.C.)

v=violet

Figure 2.2.6. T.L.C. of diosgenin purification procedure sprayed with anisaldehyde reagent.



1. Diosgenin standard.
2. Recovered diosgenin from reaction.
3. 25R-spirostan-3,5-diene from reaction.

Spot colour

y=yellow

v=violet

Figure 2.2.7. T.L.C. of products from the acid catalysed dehydration of diosgenin.

Anisaldehyde reagent. It can be seen that the PTLC separated the 25R-spirostan-3,5-diene (Rf 0.72) from the unreacted diosgenin (Rf 0.34). The reaction gave 33 mg of the diene and 47 mg of unreacted diosgenin. This represented a yield of 41% for the diene.

d) REAGENTS FOR THE DETECTION OF STEROIDS

Two reagent sprays were used for the detection of steroids on thin layer chromatography plates.

i) Anisaldehyde Reagent

This reagent was prepared by mixing 0.5 ml of anisaldehyde (BDH Ltd) in 50 ml of glacial acetic acid, then adding 1 ml of concentrated sulphuric acid. The solution was thoroughly mixed and stored in a glass reagent bottle. The reagent was applied to TLC plates using an aerosol driven spray unit (Shandon Chromatography Ltd). The plates were then heated in an oven for 5 min at 90°C. Diosgenin gave a yellow spot with this reagent while sterols gave dark violet spots.

ii) Antimony trichloride reagent

This reagent was prepared by dissolving antimony trichloride (BDH Ltd) in chloroform until a saturated solution was formed. The solution was stored in the dark in a well stoppered brown glass bottle. For use the reagent was applied to TLC plates from an all-glass spray unit (Gallenkamp Ltd) driven by air from a hand pump. The plates were heated in an oven at 100°C for 10 min. Diosgenin gave a red spot while sterols gave a violet colour.

e) QUANTITATIVE ANALYSIS OF PLANT AND CULTURE EXTRACTS

The individual steroid components of the various extracts were separated and identified using thin layer chromatographic techniques. Two types of TLC plate were used: 'homemade' silica gel plates prepared in the laboratory and commercially prepared silica gel plates.

i) 'Homemade' silica gel TLC plates

These plates were used for routine analysis especially the initial investigations of extracts where Rf values were not critical. They were prepared as follows:

Glass plates (20 cm x 20 cm) were firstly cleaned with acetone and placed on a TLC plate spreader (Shandon Unoplan). 30 g of silica gel 60 (E Merck, Darmstadt) was placed in a Buchner Flask and mixed with 70 ml of distilled water. The mixture was then thoroughly degassed and poured into the applicator which had been pre-set with a feeler gauge to 0.25 mm or 0.5 mm depending on the type of plate required. The applicator was then drawn across the glass plate on the spreader. After coating, the plates were left to dry horizontally, before use the plates were activated by heating in an oven at 100°C for at least 6 hr.

ii) Commercial silica gel TLC plates

Since the 'homemade' plates were sometimes not always uniform resulting in inconsistent Rf values, commercially prepared plates were used for critical qualitative and all quantitative analysis. The plates which were used were 20 cm x 20 cm plastic TLC plates coated with 0.2/

0.2 mm silica gel 60 (E Merck, Darmstadt). The nature of the plates allowed them to be cut in sections easily if only a small number of samples were being run. The translucent nature of the plastic backing also allowed them to be used on a gel scanner for quantitative work.

iii) Method

1 - 10 ml of chloroform extracts were applied to the TLC plates. The plates were developed in one of two solvent systems, either that described by Bennett et al, (1963) composed of benzene: ethyl acetate (3:1 V/V) or that described by Bennett and Heftmann (1962), composed of chloroform: methanol: water (97:2:0.2, V/V). Chromatography tanks were left for 30 min after addition of solvent to allow the tank to saturate with solvent vapour. Fresh solvent was used at the beginning of each day's analyses. Steroids were detected by using the reagent sprays described in section (2).

f) QUALITATIVE ANALYSIS OF PLANT AND CULTURE EXTRACTS

Gas liquid chromatography was chosen initially as the method for quantifying the steroid content of the various extracts. Although the method was developed and tested with some of the early extracts, for various reasons it was replaced by the use of densitometric thin layer chromatography. The details of both techniques are given below.

i) Gas Liquid chromatography (GLC)

The chromatograph used for this work was a Pye Series 104 Gas Chromatograph. A 1.5 m x 4mm glass column was used packed with diatomite CQ (Phase separations Ltd) as/

as the support phase and 3% OV-17 (Phase Separations Ltd) as the liquid phase. The carrier gas was nitrogen and the separated components were detected using a flame ionisation detector. Samples were injected as chloroform solutions using a microlitre glass syringe (Hamilton).

ii) Densitometric thin layer chromatography

The equipment used for this technique was a Helena Laboratories Gel Scanner/Densitometer working in the transmission mode. Samples were analysed as follows:

Fourteen samples plus three standard diosgenin samples were run on a 20 cm x 20 cm commercial TLC plate which had been developed in benzene:ethyl acetate (3:1). The steroids were visualised with antimony trichloride reagent. The plate was then placed on the densitometer and scanned past the fixed light source from origin to solvent front. The translucent nature of the TLC plates used allowed the beam to pass through the spots being analysed, through the plate, to the detector. The diosgenin content of the unknowns was calculated by comparing their absorbances with a calibration curve of absorbances prepared from the standard diosgenin samples ran on each TLC plate. Each unknown was run twice so the figure used to calculate the diosgenin content was an average of two absorbance figures. A separate standard curve was prepared for each TLC plate run.

Further details of the development of both the above quantitative analysis techniques is given in the following results chapter.

g) IDENTIFICATION OF MICROBIAL CONTAMINATION OF PLANT CELL CULTURES

Microbial contamination of plant cell cultures is usually fairly obvious in callus cultures where the causative organism can be seen growing in colonies on the surface of the agar. It is more difficult in the case of suspension cultures, however, where the contaminating organism is dispersed by agitation throughout the liquid medium which is sometimes naturally turbid.

In order to make a positive identification of suspected microbial contamination, two methods were adopted. The first involved staining a sample of culture medium using Gram's Staining method which will stain bacteria and differentiate between Gram positive and Gram negative forms. This procedure will also stain yeast and other fungi.

The second procedure involved plating out samples of the suspected culture medium onto Petri-dishes containing microbiological culture media to attempt to induce the formation of colonies of any contaminating organisms. Two media were used, these were, Nutrient Agar medium which is a general microbiological medium which will support the growth of bacteria and fungi and Czapek Dox medium which is a general medium for the cultivation of fungi.

i) Gram's Staining Method

The sample to be tested was smeared onto a microscope slide which was then passed a few times through a Bunsen burner flame to fix the sample to the slide.

The slide was then flooded with methyl violet and left for 5 min. The stain was then washed off with iodine solution which was left to act for 2 min. The iodine was drained from the slide and the sample was decolourised with acetone for not more than 5s. The slide was then, immediately washed with water and counterstained with carbol fuchsin for 30 s. The slide was then washed with water, blotted and left to dry in the air. Gram positive organisms appear blue under the microscope while Gram negative organisms are pink.

ii) Plating of contaminated samples on microbiological culture media

1 ml of the suspected medium was transferred with a sterile pipette to Petri-dishes of nutrient Agar and Czapek Dox medium. These dishes were then incubated under the same conditions as the plant cell cultures for 1 week. If the medium is contaminated, then colonies of the organism(s) responsible should appear within that time.

Gram's staining method can then be employed if further identification of the organism is necessary.

The components of Nutrient Agar and Czapek Dox medium are described in section 1b of this chapter.

h) PREPARATION OF BLOOD/AGAR TEST MEDIUM FOR THE DETECTION OF SAPONINS

The blood agar medium used for the haemolytic test for saponins was a mixture of MS medium and defibrinated horse blood solidified with 1% agar (W/V). The MS medium was as described in/

in Section 1b of this chapter except for the addition of mannitol at a concentration of 4%. The horse blood was supplied sterile by Gibco-Biocult Ltd., Paisley.

To prepare the test medium the MS medium, mannitol and agar were mixed and sterilised by autoclaving. It was then allowed to cool to just above its setting temperature and then mixed with the blood at the desired ratio. The blood/agar medium was then poured into Petri-dishes (9cm dia) on its own to set or it was used to form a layer on top of an already set agar medium containing standard MS medium. Whichever of the above forms were used the total volume of medium in the Petri-dishes was 30ml.

i) QUALITATIVE AND QUANTITATIVE TECHNIQUES FOR THE DETERMINATION OF RADIOLABELLED STEROIDS

i) Autoradiography

Autoradiography was carried out using Cronex 4 X-ray film (Du Pont UK Ltd). The TLC plates were exposed to the film for 3 weeks. The film was developed in Polycon variable contrast developer (May and Baker Ltd) for 4 mins, stopped in a bath of 1% (V/V distilled water) acetic acid for 30 sec, rinsed in distilled water for 30 sec and fixed in Perfix high speed fixer (May and Baker Ltd) for 15 min.

ii) Scintillation Counting

The amount of radioactivity in each spot detected by autoradiography was quantified as described in Section 3 of the results chapter using a Beckman LS 7000 Scintillation counter. The scintillation fluid was Ready-Solv-NA which is a pre-mixed cocktail based on trimethylbenzene/

trimethylbenzene and supplied by Beckman Ltd.

Each sample was counted for 10 min. and the results expressed as cpm (No 1). Each sample was then recounted after the addition of 10 ml (1560 dpm) of toluene internal standard (Amersham International) and the results expressed as cpm (No 2). The background count was then subtracted from these figures which were used in the following equation to calculate the counting efficiency.

$$\text{Counting efficiency} = \frac{\text{cpm (No 2)} - \text{cpm (No 1)}}{\text{dpm internal standard added}} \times \frac{100}{1}$$

The counting efficiency was then used to correct CPM (No 1) to give the actual amount of radioactivity in each sample expressed as disintegrations per minute (dpm). This was then calculated and expressed as dpm per gram dry weight.

#### j) STATISTICAL ANALYSES

Standard errors were calculated and student t-test were performed according to Parker (1973). In the cases where data was in the form of percentages, the values were subjected to arcsin transformation, and statistical manipulations were carried out on the transformed values, which were ultimately converted back to percentages. This was done because percentage values do not conform to the normal distribution which means that statistical methods which assume that sample data are normally distributed about a mean cannot be applied to their analysis. The transformations carried out in this thesis used the tables found in Fisher and Yates (1963).

In the experiments which consisted of a random block containing several different treatments, the statistical significance of any differences between treatment means was tested by analysis of variance. This consisted of a preliminary analysis to calculate the variance ratio  $F$  which if equal to or greater than the tabulated value for  $P = 0.05$  revealed that there are significant differences between treatment means. The second part of the analysis consisted of testing the significance of individual differences between treatment means. This was done by a multiple range test using shortest significant ranges (SSR). Both the analysis of variance described above and the calculation of correlation coefficients used in the discussion were carried out as described by Parker (1973).



CHAPTER 3

EXPERIMENTAL RESULTS

The results chapter falls into three main parts. The first part, which contains sections one and two, describes two vital tasks which laid the groundwork for the majority of the experimental programme. The first of these was the initiation of friable proliferating callus cultures which were required for the establishment of suspension cultures. The second task was the development of quantitative and qualitative assay techniques which could isolate, detect and calculate the levels of diosgenin produced by the Dioscorea plant cell cultures.

The experiments in section three make up the second main part of the results chapter. Using the techniques developed in the previous two sections, the relationship between culture growth and diosgenin accumulation was investigated. The aim of this was to achieve a better understanding of the relationship between primary and secondary metabolic processes which in turn might lead to ways of increasing the yield of diosgenin in plant cell cultures.

The last part of the results chapter, which consists of sections four and five, contains experiments in which techniques were developed which are vital to the commercial exploitation of any secondary metabolic process in the plant cell culture. The first of these was the development of a rapid screening technique for the detection of diosgenin which could be used to select high yielding cell lines capable of producing commercially viable yields of diosgenin. The second set of experiments contained in section five investigated/

investigated the growth of the Dioscorea plant cell cultures., and the accumulation of diosgenin in multi-litre fermentors. Such systems provide some measure of the ease by which a plant cell culture system could be transferred from small scale laboratory experiments (such as those described in section 3) to commercial processes ranging from hundreds to thousands of litres in volume.

R E S U L T S    S E C T I O N    1

THE INITIATION AND ESTABLISHMENT OF PLANT  
CELL CULTURES FROM VARIOUS DIOSCOREA SPECIES

The aim of the work described in this section was the initiation of friable, proliferating callus cultures of various Dioscorea species. The establishment of these cultures was vital since they were required for the initiation of suspension cultures on which the majority of the experimental programme was to be based. With this in mind and taking into account the well documented problems of obtaining 'undifferentiated' callus from monocotyledonous plants it was decided to adopt three strategies for the initiation of callus.

The initial approach which is described in part 1 of this section was to initiate callus from seedlings of the Dioscorea species listed in Section 1a of Chapter 2. The seeds used for this purpose were also used to raise mature plants in the departmental greenhouse. Once such material was available it was used for the second approach which was the initiation of callus from leaf, stem and tuber tissue. This work is described in Part 2 of this Section. The aim of the final approach is described in Part 3 of this Section and was to establish crown gall cultures of the various Dioscorea species since this type of culture is well known to be friable and a good source of tissue for the initiation of suspension cultures.

SECTION 1 - PART 1

THE INITIATION AND ESTABLISHMENT OF PLANT  
CELL CULTURES FROM SEEDLINGS OF DIOSCOREA  
SPECIES

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Ten seeds each of D. composita, D. floribunda, D. friedrichsthalli and D. sylvatica were sterilised, germinated and placed on the initiation medium as described in Section 1c of Chapter 2. The culture medium used for the initiation of callus was Murashige and Skoog medium (MS Medium) as described in Section 1b of Chapter 2. The plant growth substance used was 2,4-dichlorophenoxy acetic acid (2,4-D) at  $1.4 \times 10^{-5}$  M. No cytokinin was used as previous work had shown (Kaul et al 1968, Mehta 1970) that this was not necessary for the initiation of callus cultures from Dioscorea species. The individual procedures for each species are described below.

i) THE INITIATION OF CALLUS FROM D.COMPOSITA SEEDLINGS

After 4 weeks on the initiation medium the hypocotyl region and the roots of the seedlings had begun to swell. The swollen hypocotyl region began to break open after 8 weeks and signs of friable callus appeared. No further change was noticed in the swollen roots. All the seedlings were then transferred to fresh MS initiation medium and grown for another 8 weeks. At the end of this period a small amount of callus had grown very slowly and there was some signs of root regeneration from within the band of callus around the hypocotyl region of the seedlings. It was at this point that it was decided to separate the part of the seedling which had callused from the remainder since some of the latter tissue seemed to be turning necrotic and the phenolic substances produced were probably exerting an inhibitory effect on the growth of callus. This operation was carried out with a sterile scalpel and the callused hypocotyl/

hypocotyl segments were then transferred to fresh MS medium. At the end of another 8 week culture period it was apparent that the callus had again grown very slowly and there was more root regeneration than observed previously. It was obvious that callus initiation was not proceeding according to the literature since Mehta and Staba (1970) obtained proliferating 'undifferentiated' callus from seedlings within 3-4 weeks. The only difference observed between the procedure used here and that of Mehta and Staba was the use of the standard Murashige and Skoog (1962) medium as described in Section 1b of Chapter 2 with  $1.4 \times 10^{-5}$  M 2,4-D. Mehta and Staba used the same 2,4-D concentration in a modified MS medium designated revised tobacco medium (RT medium). This medium is described by Kaul and Staba (1968) and the constituents are listed in table 3.1.1

To compare the two media half the callus/root material was transferred to MS medium and half to RT medium and grown for a further period of 8 weeks. The treatments were examined at the end of this period and both had produced small amounts of slow growing callus with extensive root regeneration. This suggested that no advantage would be gained by using the more complex RT medium and MS medium was used in all further experiments.

The two main problems still unresolved were the regeneration of roots and the slow growth of the callus. In an attempt to solve the latter problem the regenerating callus was transferred to MS initiation medium containing 5% (V/V) coconut milk which is recognised as aiding the initiation and rapid proliferation of callus (Yeoman and Macleod, 1973).

The/

TABLE 3.1.1

THE COMPONENTS OF REVISED TOBACCO MEDIUM  
(KAUL AND STABA, 1968)

---

$\text{KNO}_3$	1,900	mg/l
$\text{NH}_4\text{NO}_3$	1,650	mg/l
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	mg/l
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	mg/l
$\text{KH}_2\text{PO}_4$	170	mg/l
Fe E.D.T.A.	37.6	mg/l
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	mg/l
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	mg/l
$\text{H}_3\text{BO}_3$	6.2	mg/l
KI	0.83	mg/l
$\text{Na}_2\text{MnO}_4 \cdot 2\text{H}_2\text{O}$	0.25	mg/l
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	mg/l
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	mg/l
Sucrose	30	g/l
Myo-inositol	5	g/l
Nicotinamide	2	mg/l
Thiamine HCL	1	mg/l
Pyridoxine $\text{PO}_4$	1	mg/l
Ca-Pantothenate	1	mg/l
Choline Chloride	1	mg/l
Biotin	1	mg/l
Riboflavin	0.5	mg/l
Folic acid	0.5	mg/l
Cyanocobalamin	0.0015	mg/l
2,4-dichlorophenoxy acetic	3	mg/l

The tissue was cultured on this medium for 8 weeks. When compared with tissue which had been cultured on standard MS initiation medium it was clear that the amount of callus produced was small on both media and root regeneration was more extensive in the presence of coconut milk, accordingly the use of coconut milk was discontinued.

The next experiment was designed to eliminate the regeneration of roots since it was thought that if this problem was solved first then subsequent attempts to increase the growth of the callus would stand a better chance of success. Pieces of regenerating callus were placed into 50 ml of liquid MS initiation medium and agitated on a rotary shaker at 100 rpm. It was hoped that in these 'suspended' cultures the absorption of 2,4-D would be greater leading to enhanced callus formation. The experiment was run for 4 weeks after which it became apparent that the effect of suspending the tissue in the initiation medium had the opposite effect to the desired one. Callus formation almost completely disappeared and roots were proliferating at such a rate that a solid mat formed after 4 weeks which became immobile within the flasks. These masses of roots could be regularly sub-cultured every 4 weeks. This was an interesting phenomenon, but did not assist in the production of callus, therefore, attention returned to the manipulation of cultures on a solid medium.

By the time work on the 'suspended' cultures had been completed, enough of the slow growing regenerating callus had been accumulated so that a range of culture conditions could be tested. The experiment consisted of growing the regenerating callus/

callus on MS medium containing 18 different combinations of the plant growth substances, 2,4-D and kinetin. The combinations and concentrations of the plant growth substances used are set out in Table 3.1.2. The experiment was run for 8 weeks after which the effects of the various treatments were compared. On examination, 2 major effects were apparent. Firstly if the cultures were compared in the treatments where the concentration of 2,4-D was increasing, ie across the columns in table 3.1.2, it could be seen that the first two concentrations,  $1.4$  and  $1.8 \times 10^{-5}$  M had little effect while  $2.3 \times 10^{-5}$  M promoted callus formation but still extensive root formation. Secondly in the treatments where kinetin was increasing ie down the columns in table 3.1.2 there were no differences between the effects of 2,4-D alone or with increasing concentrations of kinetin. These results suggest that callus formation was promoted at concentrations of 2,4-D of  $2.3 \times 10^{-5}$  M and that kinetin at concentrations between  $1 \times 10^{-7}$  M and  $1 \times 10^{-5}$  M did not modify this effect.

The effect of 2,4-D concentration on callus formation was further investigated by transferring regenerating callus to MS medium containing a variety of concentrations of 2,4-D above and below  $2.5 \times 10^{-5}$  M. The range of concentrations used is set out in Table 3.1.3, as before the cultures were grown for 8 weeks after which the effects of the various treatments were compared. The first two treatments  $1.4$  and  $1.8 \times 10^{-5}$  M as in previous experiments, produced little callus formation and extensive root growth, while the third treatment, at  $2.3 \times 10^{-5}$  M produced, as before increased callus/

TABLE 3.1.2

CONCENTRATIONS OF 2,4-D AND KINETIN USED TO INITIATE CALLUS FROM DIOSCOREA SEEDLINGS

KINETIN	2,4 - D		
	$1.4 \times 10^{-5} M$	$1.3 \times 10^{-5} M$	$2.3 \times 10^{-5} M$
0	1	2	3
$1 \times 10^{-7} M$	4	5	6
$5 \times 10^{-7} M$	7	8	9
$1 \times 10^{-6} M$	10	11	12
$5 \times 10^{-6} M$	13	14	15
$1 \times 10^{-5} M$	16	17	18

THE NUMBERS IN THE TABLE REFER TO TREATMENT NUMBER

TABLE 3.1.3

THE CONCENTRATIONS OF 2,4-D USED TO INITIATE CALLUS FROM DIOSCOREA SEEDLINGS

TREATMENT	2,4-D CONCENTRATION
1	$1.4 \times 10^{-5} M$ (3)
2	$1.8 \times 10^{-5} M$ (4)
3	$2.3 \times 10^{-5} M$ (5)
4	$2.7 \times 10^{-5} M$ (6)
5	$3.6 \times 10^{-5} M$ (8)
6	$4.5 \times 10^{-5} M$ (10)

THE FIGURES IN BRACKETS ARE THE CONCENTRATIONS IN PARTS PER MILLION

callus formation but similar levels of root growth. The fourth treatment ( $2.7 \times 10^{-5} \text{M}$ ) however, produced a few relatively large areas of callus which were free of any root regeneration. The last 2 concentrations at 3.6 and  $4.5 \times 10^{-5} \text{M}$  both produced very little growth and caused necrosis of the tissue. These results show that the optimum concentration of 2,4-D for the production of 'undifferentiated' callus from D. composita seedlings was  $2.7 \times 10^{-5} \text{M}$ . Higher concentrations were toxic while lower concentrations failed to prevent substantial root regeneration.

Each of the six separate areas of callus free root regeneration was subcultured onto fresh MS medium containing  $2.7 \times 10^{-5} \text{M}$  2,4-D and grown for 4 weeks. This produced enough tissue in the Petri-dish cultures to enable the transferring of tissue to medium in conical flasks to bulk up the callus. Before this was done, however, a small experiment was carried out to determine whether the 2,4-D concentration could be reduced to the level reported in the literature for the maintenance of established D. composita callus cultures, ie  $1.4 \times 10^{-5} \text{M}$ . In this experiment pieces of callus were transferred to Petri-dishes containing 1.0, 1.4 and  $1.8 \times 10^{-5} \text{M}$  2,4-D and cultured for 4 weeks. In this experiment it was found that the level of 2,4-D could be reduced to  $1.4 \times 10^{-5} \text{M}$  without regeneration.

This concentration of 2,4-D was then adopted for the growth of all callus and suspension cultures of D. composita.

The callus cultures of D. composita produced were then transferred to 100 ml of MS medium in 250 ml Erlenmeyer flasks and  
a/

a stock was built up as described in Section 1 (c) of Chapter 2. This stock was used to initiate suspension cultures of D. composita, as described in Section 1(d) of Chapter 2 and these cultures were used in the majority of the experiments described in this thesis.

ii) THE INITIATION OF CALLUS FROM D. FLORIBUNDA SEEDLINGS

The initial response of the D. floribunda seedlings to the sequence of treatments for the initiation of callus was identical to that of D. composita up until the sub-culture to liquid MS medium, except D. floribunda produced less callus and more roots.

The pieces of D. floribunda regenerating callus responded differently than D. composita to 'suspended' culture. Instead of producing a tangled mat of roots they responded by forming compact pellets of callus (approximately 5mm in dia) covered with small roots. The remainder of the sequence of treatments failed to alter the morphology of the tissue which showed that the culture conditions which eventually produced friable proliferating callus from D. composita seedlings was not optimal for D. floribunda. No further attempt was made to initiate callus from this tissue.

iii) THE INITIATION OF CALLUS FROM D. SYLVATICA SEEDLINGS

The seedlings of D. sylvatica were subcultured through the same sequences of treatments as previously described for D. composita and D. floribunda. Initially the seedlings produced small amounts of callus from swollen hypocotyls in the same way as the other 2 species except there were no signs of any regeneration. The tissue remained free of regeneration but/

but grew very slowly through all the treatments in the initiation sequence until the experiment with varying concentrations of 2,4-D and kinetin. It was found that the highest concentration of 2,4-D ( $2.3 \times 10^{-5}M$ ) did cause a small increase in the growth of the callus but it also altered the appearance of the callus which went from being friable to growing as compact spherical tissue masses. Kinetin did not modify the effect of 2,4-D in any way. Continuous subculturing of this slow growing callus at 2.3 and  $2.7 \times 10^{-5}M$  did not succeed in reverting this compact growth habit to a friable one or in speeding up its growth.

Despite the fact that the above initiation procedure succeeded in producing 'undifferentiated' callus of D. sylvatica and no regeneration problems were encountered, the callus produced was too slow growing to be used experimentally. No further experiments were carried out on this tissue.

iv) THE INITIATION OF CALLUS FROM D. friedrichsthalli SEEDLINGS

The seedlings of D. friedrichsthalli responded to the sequence of callus initiation treatments in a similar fashion to D. floribunda seedlings except they grew more slowly. At the end of the sequence the tissue consisted of small compact pellets of callus covered with roots and like D. floribunda no further attempts were made to establish callus from this tissue.

Although the initiation of callus from seedlings of D. composita, as described above was eventually successful no callus had been produced by the time mature plants of the/

the various Dioscorea species were ready in the departmental greenhouse. It was therefore thought wise to attempt to initiate callus from as many alternate types of explant as possible. As a result of this, attempts were made to raise callus from various organs of mature Dioscorea plants. These procedures are described in the following part of this Section.

SECTION 1 - PART 2

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THE INITIATION OF PLANT CELL CULTURES  
FROM MATURE TISSUE OF VARIOUS  
DIOSCOREA SPECIES

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The plants of D. composita, D. floribunda, D. sylvatica, and D. friedrichsthalli which were used in the following experiments were grown from seed in the departmental greenhouse and were approximately 6 months old. They were used to prepare sterile leaf discs, tuber discs, nodal and internodal stem discs for explant material as described in Section 1c of Chapter 2.

The initiation experiments consisted of culturing the explants on MS medium containing varying concentrations of 2,4-D and kinetin and 2,4-D and zeatin. The 2,4-D/kinetin treatments are numbered 1 to 24 and are shown in Table 3.1.4. The 2,4-D/zeatin treatments are displayed in a similar manner in Table 3.1.5. The experiments were carried out in Petri-dishes (9 cm diam) with 5 explants to a dish and 2 dishes per treatment.

i) CALLUS INITIATION FROM LEAF DISCS

The leaf discs of the 5 species were plated out onto Petri-dishes containing the various combinations of plant growth substances listed in Table 3.1.4 and 3.1.5. and cultured for 4 weeks. At the end of this time there was no sign of any callus formation in any of the treatments, all the discs however remained green. They were then transferred to fresh medium and cultured for another 4 weeks at the end of which there was still no sign of any callus formation and some of the discs, especially those on treatments with high 2,4-D concentrations, were beginning to turn brown at the edges. After another transfer and four weeks of culture the leaf discs were re-examined but no callus had formed and the majority of the tissue was beginning to turn necrotic.

Both/

TABLE 3.1.4

2,4-D AND KINETIN CONCENTRATIONS USED TO INITIATE CALLUS FROM MATURE TISSUE OF DIOSCOREA PLANTS

	2,4-D			
KINETIN	$1.4 \times 10^{-5} \text{M}$	$1.8 \times 10^{-5} \text{M}$	$2.3 \times 10^{-5} \text{M}$	$2.6 \times 10^{-5} \text{M}$
0	1	2	3	4
$1 \times 10^{-7} \text{M}$	5	6	7	8
$5 \times 10^{-7} \text{M}$	9	10	11	12
$1 \times 10^{-6} \text{M}$	13	14	15	16
$5 \times 10^{-6} \text{M}$	17	18	19	20
$1 \times 10^{-5} \text{M}$	21	22	23	24

TABLE 3.1.5

2,4-D AND ZEATIN CONCENTRATIONS USED TO INITIATE CALLUS FROM MATURE TISSUE OF DIOSCOREA PLANTS

	2,4-D			
KINETIN	$1.4 \times 10^{-5} \text{M}$	$1.8 \times 10^{-5} \text{M}$	$2.3 \times 10^{-5} \text{M}$	$2.6 \times 10^{-5} \text{M}$
0	1	2	3	4
$1 \times 10^{-7} \text{M}$	5	6	7	8
$5 \times 10^{-7} \text{M}$	9	10	11	12
$1 \times 10^{-6} \text{M}$	13	14	15	16
$5 \times 10^{-6} \text{M}$	17	18	19	20
$1 \times 10^{-5} \text{M}$	21	22	23	24

Both 2,4-D/kinetin and 2,4-D/Zeatin experiments were terminated at this stage with no callus having been initiated from leaf discs of any of the Dioscorea species

ii) CALLUS INITIATION FROM NODAL STEM DISCS

The nodal stem discs were plated out as described above and cultured for 4 weeks when the explants were examined, no callus formation could be detected in any of the plant growth substance regimes. All the nodal stem discs however remained green so they were subcultured onto fresh MSmedium containing the appropriate combinations of plant growth substance and cultured for 4 weeks.

When the explants were examined it could be seen that there were some signs of root formation. This regeneration was seen in explants of all 4 species which were growing on treatments containing the highest concentrations of 2,4-D ie  $2.6 \times 10^{-5}$  M. No difference was noticed in the amount or state of the regeneration with any concentration of kinetin or zeatin. The remaining treatments had no effect on the explants at all, although they remained green. All explants including those which showed signs of regeneration were transferred to fresh MS medium and cultured for another 4 weeks. The explants which had shown signs of root regeneration did not develop any further and began to show signs of browning as did all the other explants.

In an attempt to prevent the root tissue from dying it was carefully removed from the explant using a sterile scalpel and transferred to fresh MS medium but after a further 2 weeks the tissue had died. None of the other explants produced/

produced callus so experiments with the nodal stem discs were stopped.

### iii) CALLUS INITIATION FROM INTERNODAL STEM DISCS

The internodal stem discs were plated out onto Petri-dishes containing the various plant growth substance treatments. After four weeks of culture no callus could be seen in either the 2,4-D/kinetin or the 2,4-D/zeatin experiments but as with the nodal stem discs all the internodal tissue was still green. The discs were transferred to fresh MS medium and cultured for another 4 weeks at the end of which there was still no callus formation in any of the treatments. It was noticeable however that in contrast to the nodal discs, the internodal tissue showed no root formation in response to the highest concentrations of 2,4-D ( $2.6 \times 10^{-5} M$ ). Another difference which was apparent between the two types of stem tissue was that the internodal discs grew brown and necrotic faster than the nodal tissue. The nodal tissue was cultured for 3 months before it died with the internodal discs becoming badly necrotic after 2 months and were dead around 1 week into their third subculture. Experiments were therefore stopped on internodal stem discs as well as nodal stem discs without callus being successfully produced.

### iv) CALLUS INITIATION FROM TUBER DISCS

The tuber discs of each species were plated onto medium in Petri-dishes containing the various treatments and were cultured for 4 weeks after which it could be seen that as in the other experiments no callus had formed during this period on any of the treatments. All explants however were still viable with no signs of browning.

They were then transferred to fresh MS medium and cultured for a further 4 weeks. When the tuber discs were examined it could be seen that certain treatments had produced small amounts of callus in all four species. Treatments 3,7,11, 15,19 and 23 in both the 2,4-D/kinetin and 2,4-D/zeatin experiments had produced a very small amount of callus material around the edge of the explants. Treatments 4,8, 12,16,20 and 24 in both the 2,4-D/kinetin and 2,4-D/zeatin experiments had produced slightly more callus material which covered the surface of the explants. When these responses were examined within a group of treatments where the 2,4-D concentration remained the same and the kinetin or zeatin concentration varied, ie 3,7,11,15,19 and 23 then it could be seen that the cytokinins had no effect on the callus formation. This suggests that only the 2 concentrations of 2,4-D,  $2.3 \times 10^{-5}M$  and  $2.6 \times 10^{-5}M$ , were capable of inducing callus formation in the tuber discs. All the remaining treatments in the 2,4-D/kinetin and 2,4-D/zeatin experiments had no effect on the tuber discs.

None of the discs including the callusing ones were showing any signs of browning so they were all transferred to fresh MS medium and cultured for another 4 weeks.

When the tuber discs were examined at the end of this culture period, it was apparent that the callus which had been produced during the previous culture period had not grown any further and that the explants themselves had started to turn necrotic. The explants which had not produced callus during/

during the previous culture period had turned brown and looked dead.

The callus material produced in the previous culture period was then carefully removed from the browning explants and placed on fresh MS medium containing 2,4-D at the concentration at which it had been produced. None of the callus however which was subcultured grew in the subsequent 4 weeks and it eventually became necrotic and died. None of the other explants which were subcultured at the same time as the callus survived therefore all attempts to produce callus from tuber discs of Dioscorea species were unsuccessful. No attempt could be made to repeat these experiments which led to some callus formation due to lack of tuber material.

The results presented in the first two parts of this section seem to confirm the difficulty in obtaining friable, proliferating callus from monocotyledonous species such as Dioscorea. Only one out of four species of Dioscorea seedlings yielded any callus and even then it was necessary to use twice the reported concentration of 2,4-D (Mehta, 1970) in the initiation medium. All attempts to raise callus from mature explants including tuber tissue were unsuccessful despite the reported initiation of callus from tubers of Dioscorea deltoidea (Chaturvedi, 1976).

The tissues used as explants in callus initiation are usually either seedlings or pieces of mature plant such as leaf or stem tissue. In both cases these tissues are characterised/

characterised by a particular cellular organisation which is governed by two major regulatory controls. The first is the overall chemical environment of the tissue, ie gradients that have been set up by long distance transport of substances from other parts of the plant. The second is the local chemical exchange that exists between adjacent cells. The breakdown of these two levels of control by exogenous plant growth substances alone or in combination with wound reactions, leading to a breakdown in cellular organisation, is the key factor in the initiation of callus. (Yeoman and Forche, 1980).

The above results suggest that it is perhaps the difficulty in overcoming such regulatory constraints in monocotyledons such as Dioscorea that is the cause of the problems in the initiation of callus.

The fact that callus was eventually initiated from seedlings but not from any mature tissue explant suggests that although the level of regulatory constraint on cellular organisation may have been on average greater than that which is found in most dicotyledons, it was less in the seedlings of D. composita than it was in any of the mature explants. This supports the view that seedlings which have a high growth potential, are a more dependable source of callus in some cases, than mature explants whose growth response can vary widely depending on the physiological status of the plant before excision (Yeoman and Forche, 1980).

It could also be seen that in the case where callus was successfully/

successfully initiated that the level of 2,4-D necessary for subsequent maintenance of the callus could be lowered below that which was necessary for the actual initiation process, without the original level of cellular organisation being re-established. This suggests that the processes involved in the breakdown of the cellular organisation of the explant can be thought of as overcoming an energy barrier, past which the level of disorganisation represented by the callus can be maintained at a more lower energy level. At the same time as the above experiments began on the initiation of callus from mature tissue explants, the same material was used in an attempt to raise crown gall cultures of the Dioscorea species. This was carried out to provide an alternative source of friable callus should the orthodox methods described in parts 1 and 2 of this Section prove unsuccessful. The cultures were to be initiated from crown gall tumours raised on mature plants and isolated parts of mature plants which had been infected with Agrobacterium tumefaciens. These procedures are described in the following part of this section.

S E C T I O N   1   -   P A R T   3

THE INFECTION OF PLANTS AND ISOLATED TISSUE  
OF VARIOUS DIOSCOREA SPECIES WITH THE CROWN  
GALL BACTERIUM AGROBACTERIUM TUMEFACIENS

The results in this part of Section 1 describe attempts to raise crown gall tumours by infecting various Dioscorea species with the bacterium Agrobacterium tumefaciens, The aim of these experiments being to use the tumours to initiate crown gall cultures which in turn could be used as a source of friable callus of the Dioscorea species. The results firstly describe attempts to infect whole plants of various Dioscorea species and secondly attempts to infect isolated tissues from Dioscorea plants 'in-vitro'.

i) The STRAINS OF A. TUMEFACIENS

Three strains of A. tumefaciens were used in the following experiments, 2 tumorigenic strains and 1 non-tumorigenic strain.

- a) A. tumefaciens C 58 is a tumorigenic nopaline strain which contains a single  $120 \times 10^6$  dalton plasmid.
- b) A. tumefaciens B 6 is a tumorigenic octopine strain which contains 2 large plasmids of similar size, only one of which is a  $T_1$  plasmid.
- c) A. tumefaciens II BNV6 is a non-tumorigenic strain which contains a plasmid of about  $25 \times 10^6$  daltons.

ii) THE INFECTION OF MATURE DIOSCOREA PLANTS WITH A. TUMEFACIENS

Six month old plants of D. composita, D. floribunda, D. sylvatica and D. friedrichsthalli which had been grown from seed in the departmental greenhouse were used in this experiment. One plant of each species was infected with each strain of A. tumefaciens using the technique of Butcher (1977) as described in section 1g of Chapter 2. One tobacco plant/

plant (*Nicotiana tabacum* var. 'White Burley') and one tomato plant (*Lycopersicum esculentum* var. 'Ailsa Craig') were also infected with each strain of bacterium as controls. The infected plants were all examined weekly for 2 months for any signs of crown gall tumour production.

It was found that both tumourigenic strains were unsuccessful in inducing crown gall tumours on any of the four Dioscorea species. This was not due to any lack of virulence since both strains produced tumours on the control tobacco and tomato plants within 3 weeks. It was also found that strain IIBNV6 did not, as expected, induce tumours on any of the four Dioscorea species or the two species employed as controls. Tumour production in the control species would have implicated the presence of a tumourigenic organism other than A.tumefaciens, such as wound tumour virus.

These results suggest that the strains of A.tumefaciens used in this experiment were incapable of producing crown gall tumours in the Dioscorea species which were tested.

Having failed to induce crown gall tumours in whole plants of Dioscorea species, a similar protocol was used in an attempt to induce tumours by infecting tissues of mature plants 'in-vitro'.

iii) THE 'IN-VITRO' INFECTION OF ISOLATED TISSUES OF DIOSCOREA SPECIES WITH A.TUMEFACIENS

Six month old plants of D.composita, D.floribunda, D.sylvatica and D.friedrichsthalli were used to prepare the tissue for these

experiments. Tuber discs, leaf discs and nodal stem discs were inoculated with each strain of A.tumefaciens as described in Section 1g of Chapter 2. Potato tuber discs (Solanum tuberosum var 'Desiree') and tobacco (Nicotiana tabacum var 'White Burley') were used as controls. Tuber, leaf and nodal stem discs of all species were also prepared and plated out uninoculated, as a second set of controls. The infected tissues were all examined weekly for 2 months and any signs of crown gall tumour production were recorded.

It was found that, as with whole plants, both tumourigenic strains were unsuccessful in inducing tumours on tuber discs in any of the four species of Dioscorea over the two months of the experiment. The strains did however induce tumorous tissue on the potato disc controls which supports the view that they were virulent. Some proliferating tissue was also seen earlier in both infected and uninfected controls. The tissue in the uninfected controls did however turn necrotic and die while that in the infected controls continued to grow until the end of the experiment. This suggests that the initial proliferation in the uninfected controls was a temporary wound response.

Similar results to those described above were found following attempts to induce tumours on leaf discs and nodal stem sections of the four species of Dioscorea.

The foregoing results suggest that the two virulent strains of A.tumefaciens used were incapable of successfully inducing crown gall tumours on any of the 4 species of Dioscorea tests. This occurred regardless of whether the infection was carried out on

whole plants or isolated tissue in-vitro.

These results confirm the present view that, despite the report of crown gall tumours on a species of Dioscorea (Stehle, 1954), the host range of A.tumefaciens does not extend to the induction of tumours in monocotyledons (De Cleene, 1976; Butcher 1977).

Despite the problems encountered in establishing friable callus, once it was produced, good suspension cultures of D.composita were quickly initiated and established. This operation successfully concluded the first part of the preliminary work necessary before the main experimental programme could begin. The second part consisted of the development of qualitative and quantitative analytical techniques necessary to identify and quantify the levels of diosgenin produced in the suspension cultures of D.composita. The next Section of this Chapter describes these procedures.

S E C T I O N    2

THE DEVELOPMENT OF THE QUALITATIVE  
AND QUANTITATIVE TECHNIQUES FOR THE  
IDENTIFICATION AND ESTIMATION OF  
DIOSGENIN

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In the previous section the development of the plant cell culture systems which were required for the majority of the experimental programme were described. The majority of the experimental programme however was also dependent on the ability to identify and calculate the levels of diosgenin produced in the cell cultures. The development of such qualitative and quantitative analytical techniques are described in the following section.

The section consists of two main parts, the first contains the development of various thin layer chromatography systems which were used to separate and detect diosgenin and related compounds from various Dioscorea plant extracts.

The second part of this section describes the development of quantitative assay techniques for diosgenin. Two methods are developed, one using gas liquid chromatography and a second involving a densitometric thin layer chromatographic technique.

S E C T I O N   2   -   P A R T   1

THE DEVELOPMENT OF THE QUALITATIVE TECHNIQUES  
FOR THE SEPARATION AND DETECTION OF DIOSGENIN  
IN PLANT CELL CULTURES OF DIOSCOREA SPECIES

1 a) QUALITATIVE ANALYSIS OF PLANT EXTRACTS FOR  
DIOSGENIN AND RELATED STEROIDS

Thin layer chromatography was chosen as the method for analysing the various plant extracts for diosgenin and related steroids because it allowed the rapid handling of large numbers of individual samples. The colour reactions of a large number of steroids to common TLC spray reagents are also well documented, as are the solvent systems necessary for their resolution (Bennett, 1962, Heftmann, 1975).

i) The behaviour of a number of steroid standards on TLC developed using 3 different solvent systems

Before any plant extracts were analysed, the behaviour of a number of steroid standards were investigated on TLC using 3 different solvent systems. These were:

- 1 Benzene:ethyl acetate, 3:1, as described by Bennett et al, (1962).
- 2 Chloroform: toluene, 9:1, as described by Bennett et al, (1962).
- 3 n-hexane:acetone, 8:1, as described by Brain et al, (1968).

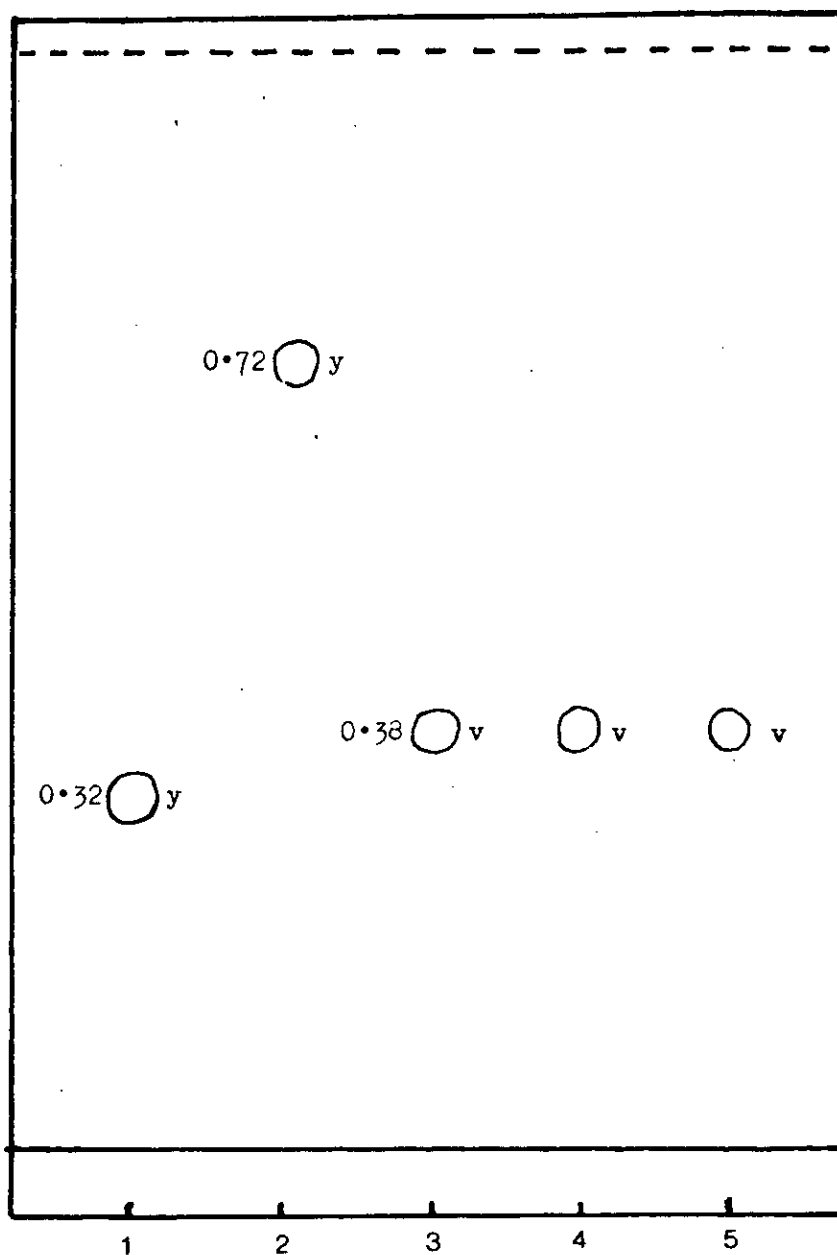
The steroid standards used in this experiment were diosgenin, 25 $\alpha$ -spirostan-3,5-diene, cholesterol, sitosterol and stigmasterol. 5 mg samples of each were run on 0.2mm analytical silica gel TLC plates using the solvent systems described above. The plates/

plates were sprayed with anisaldehyde reagent after development to visualise the spots.

Fig 3.2.1 shows a diagram of the TLC of the standard samples developed in benzene:ethyl acetate, (3:1) from which it can be seen that the diosgenin moved with a Rf of 0.32 in this solvent system while the 25R-spirostan-3,5-diene moved with a Rf of 0.72. The three sterols, cholesterol, sitosterol and stigmasterol separated from the other 2 compounds but did not separate from each other and all had Rf values of 0.38.

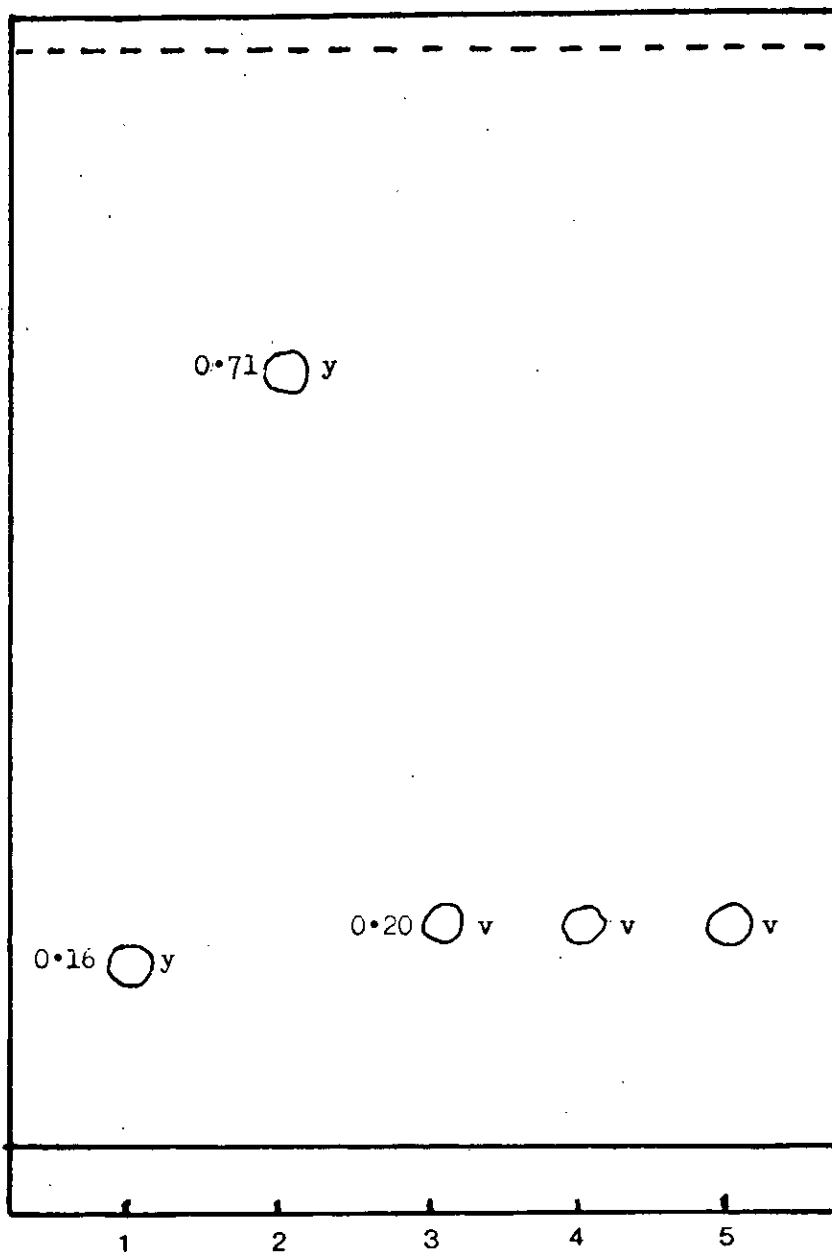
Figure 3.2.2 shows a diagram of the TLC of the standard samples developed in chloroform:toluene (9:1), from which it can be seen that the diosgenin moved with a Rf of 0.16 in this solvent system while the 25R-spirostan-3,5-diene moved with a Rf of 0.71. Once again the 3 sterols did not separate from each other but with a Rf of 0.20 were separated from the other two compounds.

Figure 3.2.3, shows a diagram of the TLC of the standard samples developed in n-hexane:acetone (8:1) from which it can be seen that the diosgenin moved with a Rf of 0.28 while the 25R-spirostan-3,5-diene moved with a Rf of 0.72. The three sterols as in the previous 2 solvents systems did not separate from each other but did separate from the other 2 compounds. All 3 sterols had a Rf value of 0.33.



1.Diosgenin.	<u>Spot colour</u>
2.25R-Spirostan-3,5-diene.	y=yellow
3.Cholesterol.	v=violet
4.Sitosterol.	
5.Stigmasterol.	

Figure 3.2.1. T.L.C. of various steroid standards developed in a benzene:ethyl acetate(3:1) solvent system.



1. Diosgenin.	<u>Spot colour</u>
2. 25R-Spirostan-3,5-diene.	y=yellow
3. Cholesterol.	v=violet
4. Sitosterol.	
5. Stigmasterol.	

Figure 3.2.2. T.L.C. of various steroid standards developed using a chloroform:toluene(9:1) solvent system.

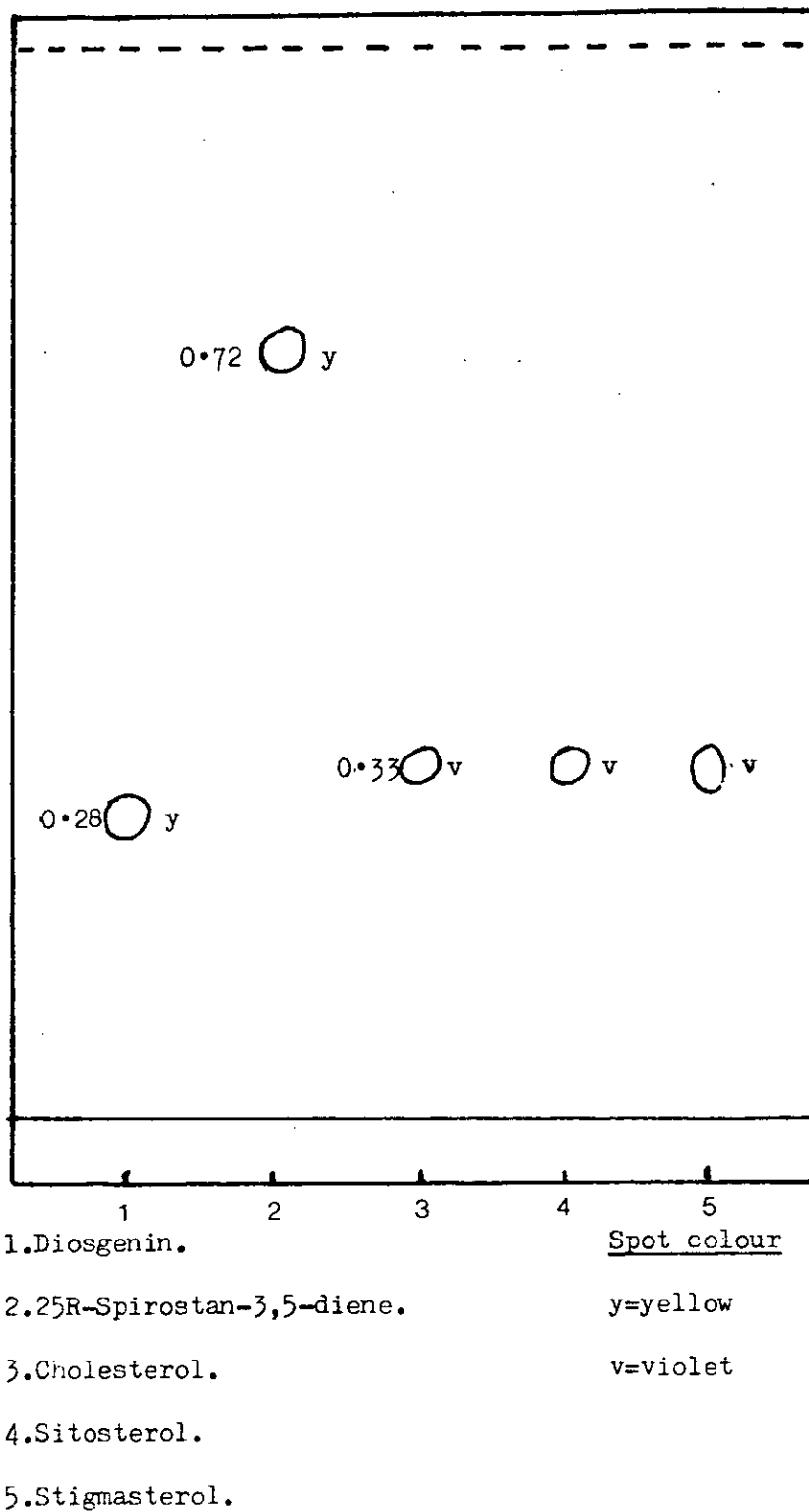


Figure 3.2.3. T.L.C. of various steroid standards developed using a n-hexane:acetone(8:1) solvent system.

ii) Qualitative investigation of the steroid content of various Dioscorea and Trigonella whole plants and plant cell cultures

These experiments were designed to test the ability of the TLC technique and the various solvent systems to separate and detect diosgenin in whole plant samples and plant cell cultures of various species. The plants and cultures used in these experiments and the dry weight of each tissue was as follows:

TISSUE	DRY WEIGHT (g)
1 <u>Dioscorea composita</u> tuber	5.48
2 <u>Dioscorea composita</u> differentiated callus	0.90
3 <u>Dioscorea floribunda</u> differentiated callus	0.33
4 <u>Dioscorea sylvatica</u> differentiated callus	0.52
5 <u>Trigonella foenum-graecum</u> seedlings*	1.18
6 <u>Trigonella foenum-graecum</u> suspension culture	0.62
7 <u>Trigonella foenum-graecum</u> callus	1.73

The plant material was grown in the departmental greenhouse. The Dioscorea composita tuber was 6 months old and the Trigonella foenum-graecum seedlings were 1 week old. The seedlings were germinated in trays of Levington's compost. The 3 differentiated Dioscorea callus cultures were grown as described in section 1 of this chapter. The Trigonella foenum-graecum callus and suspension cultures were initiated and established as described in Section 1 Chapter 2.

The Trigonella foenum-graecum plants and plant cell cultures were chosen as extra test tissues to compare with Dioscorea !

\* Trigonella foenum-graecum seeds were supplied by Thompson and Morgan (Ipswich) Ltd.

as the presence of diosgenin had been reported in both (Fazli 1968, Khanna 1973, Brain 1976).

All tissue was harvested and the diosgenin extracted as described in Sections 2a and 2c of Chapter 2. The resulting chloroform extracts were chromatographed on 0.2mm analytical T.L.C. plates which were developed in the 3 solvent systems described in Part 1(2) of this Section and sprayed with anisaldehyde reagent. The results were as follows.

1) The Benzene:ethyl acetate solvent system

a) T.L.C. of Dioscorea and Trigonella plant and plant cell culture extracts

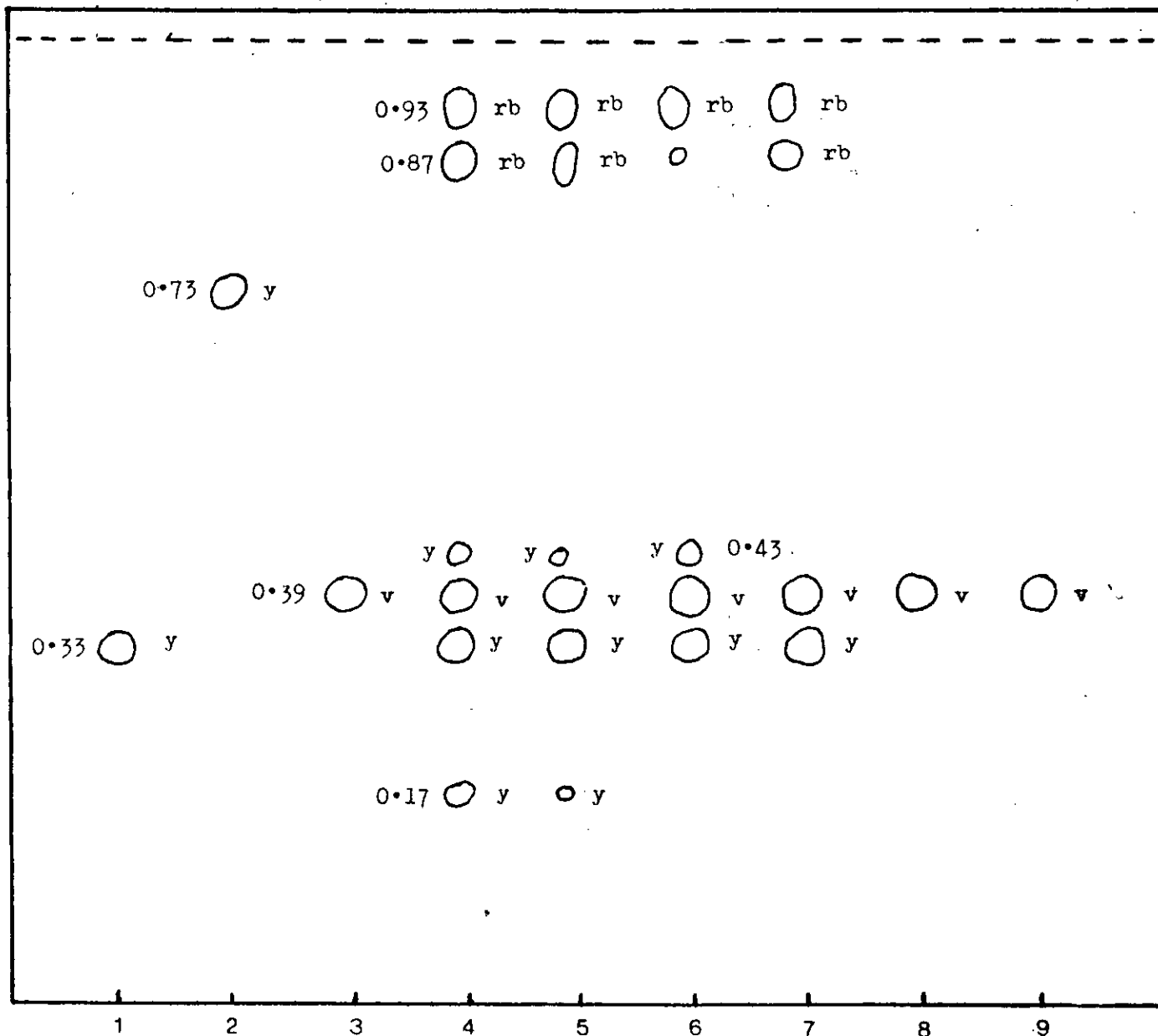
Diagrams of the T.L.C.'s of the Dioscorea and Trigonella extracts run in this solvent system are shown in Figure 3.2.4 and Figure 3.2.5 respectively. It can be seen from the results presented in these figures that 2 compounds were detected in all extracts. One compound stained yellow with anisaldehyde reagent and 'co-run' with diosgenin ( $R_f$  0,33), while the other compound stained violet and 'co-ran' with all 3 sterols ( $R_f$  0.39). The two exceptions to this were the Trigonella suspension culture and callus culture extracts which did not contain the compound which 'co-ran' with diosgenin. The extracts also contained two compounds which stained reddish brown with  $R_f$  values of 0.93 and 0.87. These compounds however, did not 'co-run' with any of the standards and therefore could not be identified. Two other yellow spots were present in the T.L.C. of the Dioscorea extracts. One compound with an  $R_f$  value of 0.43 was present in the D.composita tuber extract, the D.composita and D.floribunda differentiated callus extracts but absent from the

Figure 3.2.4. T.L.C. of Dioscorea and plant cell culture extracts developed in benzene:ethyl acetate(3:1)

1. Diosgenin standard.
2. 25D-spirostan-3,5-diene st.
3. Cholesterol standard.
4. D. composita tuber extract.
5. D. composita differentiated callus extract.
6. D. floribunda differentiated callus extract.
7. D. sylvatica differentiated callus extract.
8. Sitosterol standard.
9. Stigmasterol standard.

Spot Colour

y=yellow, v=violet, rb=reddish brown.



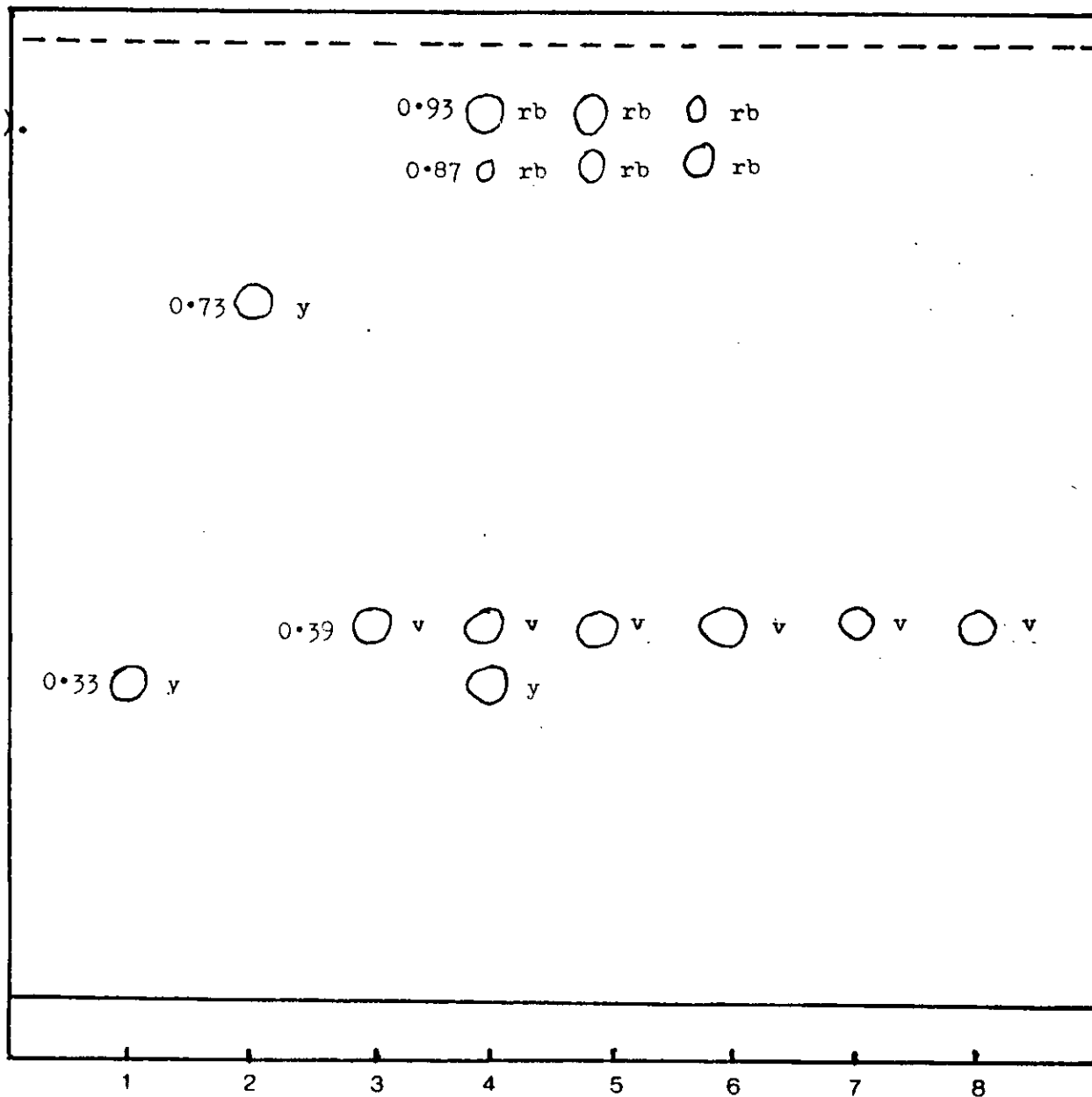
92.

Figure 3.2.5.

T.L.C. of Trigonella foenum-graecum plant and plant cell culture extracts developed in benzene:ethyl acetate(3:1).

1. Diosgenin standard.
2. 25R-spirostan-3,5-diene standard.
3. Cholesterol standard.
4. T. foenum-graecum seedling extract.
5. T. foenum-graecum suspension culture extract.
6. T. foenum-graecum callus culture extract.
7. Sitosterol standard.
8. Stigmasterol standard.

Spot Colour y=yellow, v=violet,  
rb=reddish brown.



D.sylvatica extract. The other compound ( $R_f$  0.17) was only present in the D.composita extracts. Neither of these compounds 'co-ran' with any of the standards although the compound with the  $R_f$  value of 0.17 did 'co-run' in the benzene:ethyl acetate solvent system with the impurity in the commercial diosgenin described in Figure 2.1.2. This was tentatively described as pennogenin by comparison with published data.

## 2) The Chloroform:toluene Solvent System

### a) T.L.C. of Dioscorea and Trigonella plant and plant cell culture extracts

Diagrams of the T.L.C.'s of the Dioscorea and Trigonella extracts run in this solvent system are shown in Figures 3.2.6 and 3.2.7 respectively. It can be seen that the chromatograms are in essence identical with those shown in Figures 3.2.4 and 3.2.5 in that the same number of compounds were detected in the same order although the  $R_f$  values were different.

## 3) The n-hexane:acetone solvent system

### a) T.L.C. of Dioscorea and Trigonella plant and plant cell culture extracts

Diagrams of the T.L.C.'s of the Dioscorea and Trigonella extracts run in this solvent system are shown in Figures 3.2.8 and 3.2.9 respectively. It can be seen that the same number of compounds has again chromatographed in the same order as that shown in Figures 3.2.4 and 3.2.5 except that as before the  $R_f$  values were different.

Figure 3.2.6.

T.L.C. of Dioscorea plant and plant cell culture extracts developed in chloroform:toluene (9:1).

1. Diosgenin standard.
2. 25R-spirostan-3,5-diene st.
3. Cholesterol standard.
4. D. composita tuber extract.
5. D. composita differentiated callus extract.
6. D. floribunda differentiated callus extract.
7. D. sylvatica differentiated callus extract.
8. Sitosterol standard.
9. Stigmasterol standard.

Spot Colour y=yellow, v=violet, rb=reddish brown.

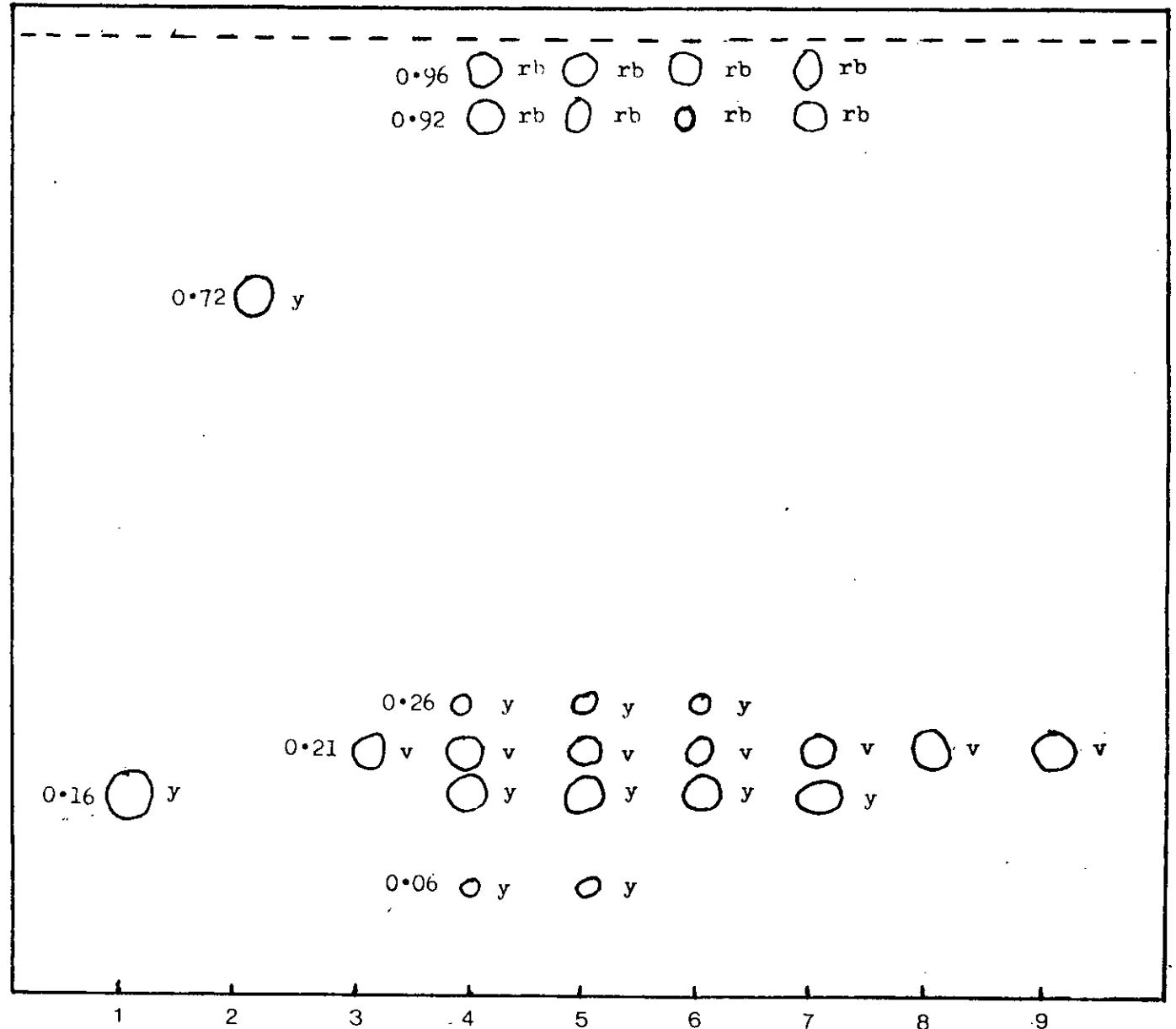


Figure 3.2.7.

T.L.C. of Trigonella foenum-graecum

plant and plant cell culture extracts  
developed in chloroform:toluene(9:1)

1. Diosgenin standard.
2. 25R-sp irostan-3,5-diene standard.
3. Cholesterol standard.
4. T. foenum-graecum seedling  
extract.
5. T. foenum-graecum suspension  
culture extract.
6. T. foenum-graecum callus culture  
extract.
7. Sitosterol standard.
8. Stigmasterol standard.

Spot Colour y=yellow, v=violet,  
rb=reddish brown.

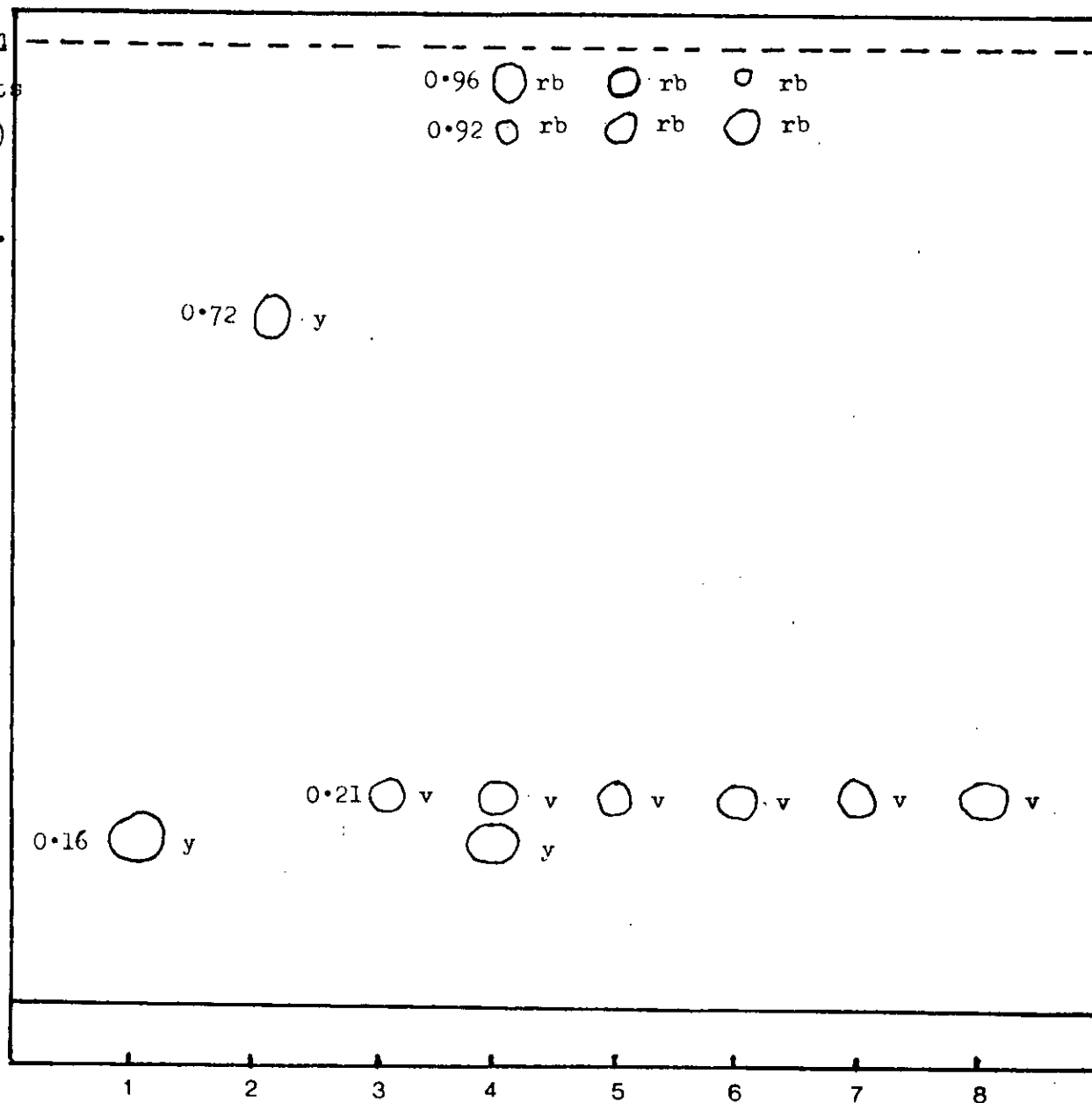


Figure 3.2.8.

T.L.C. of Dioscorea plant and plant cell culture extracts developed in n-hexane:acetone (8:1)

1. Diosgenin standard.
2. 25R-spirostan-3,5-diene standard.
3. Cholesterol standard.
4. D. composita tuber extract.
5. D. composita differentiated callus extract.
6. D. floribunda differentiated callus extract.
7. D. sylvatica differentiated callus extract.

8. Sitosterol standard.

9. Stigmasterol standard.

Spot Colour y=yellow, v=violet, rb=reddish brown.

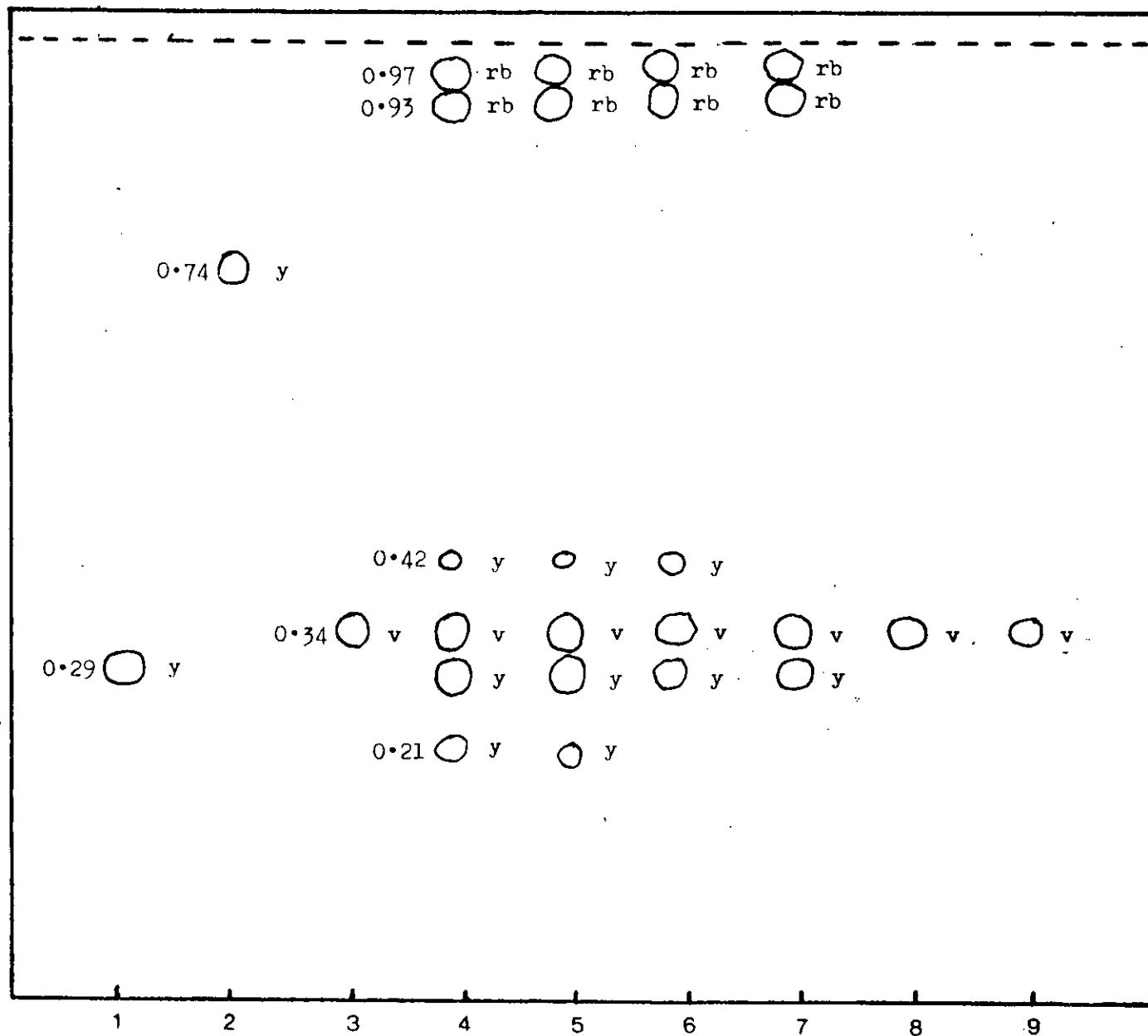


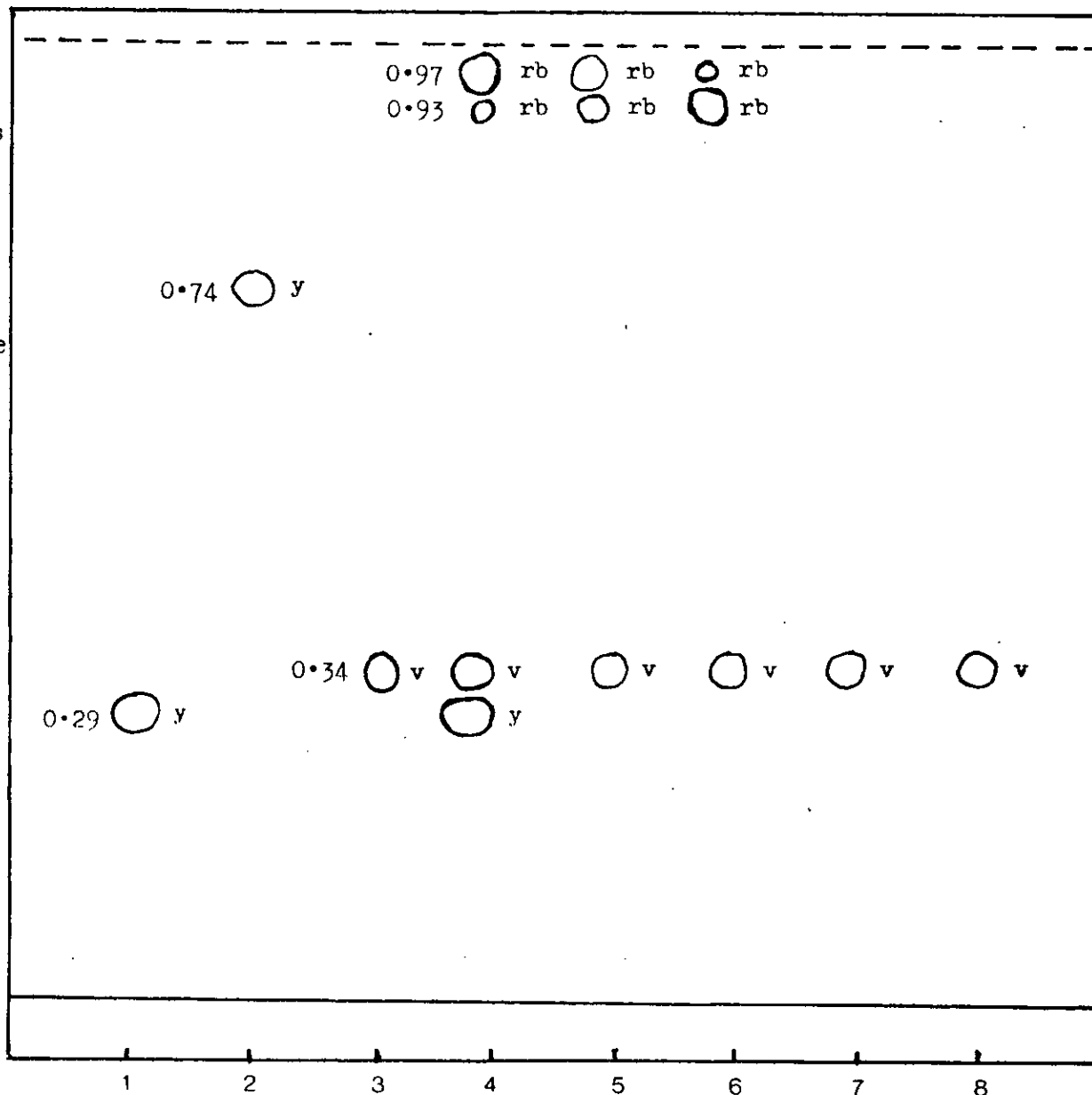
Figure 3.2.9.

T.L.C. of Trigonella foenum-graecum

plant and plant cell culture extracts developed in n-hexane:acetone (8:1)

1. Diosgenin standard.
2. 25R-spirostan-3,5-diene standard.
3. Cholesterol standard.
4. T. foenum-graecum seedling extract.
5. T. foenum-graecum suspension culture extract.
6. T. foenum-graecum callus culture extract.
7. Sitosterol standard.
8. Stigmasterol standard.

Spot Colour y=yellow, v=violet, rb=reddish brown.



The following conclusions may be drawn from the above experiments.

1) Thin layer chromatography of the various steroid standards showed that all the solvent systems used were capable of separating diosgenin from plant sterols such as sitosterol and stigmasterol. Such a separation was important since these two compounds are likely to be the other main steroids, apart from diosgenin, encountered in Dioscorea plant cell cultures (Kaul 1969, Stohs 1975).

Of the 3 systems examined which separated diosgenin from the sterols, the benzene:ethyl acetate system showed the best separation from the baseline while maintaining a good separation from 25R-spirostan-3,5-diene. For this reason the benzene:ethyl acetate solvent system was chosen for all subsequent thin layer chromatography, for both qualitative and quantitative analysis.

2) The results confirm that diosgenin may be extracted from a variety of plant tissues, separated from other steroidal constituents and successfully identified.

3) The results also show that no 25R-spirostan-3,5-diene was detected in any of the extracts. This suggests that little or no diosgenin had been lost during the acid catalysed hydrolysis reaction.

One question which arose from the above experiment was that although the results suggest that the hydrolysis of the saponin to yield diosgenin occurred without significant loss from dehydration to 25R-spirostan-3,5-diene, it did not show that

the hydrolysis reaction was complete and that no saponin remained in the tissue.

To investigate the question the tissues from the previous experiment were re-hydrolysed and re-extracted as described in Sections 2a and 2c of Chapter 2. The resulting chloroform extracts were run on 0.2mm analytical silica gel T.L.C. plates developed in benzene:ethyl acetate (3:1) and sprayed with anisaldehyde reagent. A 10, 25, 50ml sample of each extract was run as described above and no diosgenin or sterols could be detected in any of the extracts.

This suggests that the hydrolysis and extraction procedures described in Section 2 of Chapter 2 successfully removed all the diosgenin and sterols from the tissues employed.

Once the techniques of qualitative analysis had been developed it was vital to be able to quantify the amount of diosgenin extracted from the plant cell cultures. The next part of this Section describes the development of a quantitative analytical technique for the measurement of diosgenin in plant cell extracts.

S E C T I O N   2   -   P A R T   2

THE DEVELOPMENT OF THE TECHNIQUES FOR THE  
QUANTIFICATION OF THE LEVELS OF DIOSGENIN  
IN PLANT CELL CULTURES OF DIOSCOREA SPECIES

1a) GAS LIQUID CHROMATOGRAPHY OF DIOSGENIN AND RELATED STEROIDS

The first technique chosen to develop a quantitative method for the assay of diosgenin in cell cultures of Dioscorea species was gas liquid chromatography. This technique had been used successfully in a number of laboratories (Kaul 1968, 1969; Mehta 1970; Stohs 1975).

Despite the fact that G.L.C. was shown to be useful as a quantitative technique, the time of analysis per sample was too long to deal with large numbers of samples. In addition the persistent failure of the instrument prevented the completion of the analytical programme and therefore the method was abandoned.

The next part of this Section describes the development of a thin layer densitometer technique which eventually became the regular method for determining the level of diosgenin in the cell cultures.

1 b) DENSITOMETRIC THIN LAYER CHROMATOGRAPHY OF DIOSGENIN

Densitometry was chosen as the replacement quantitative technique for GLC for a number of reasons. Firstly the technique allows a large number of samples to be run per TLC plate and secondly, because it is a TLC method it combines qualitative and quantitative analysis on the same plate. The technique has also been applied successfully to estimate the levels of diosgenin in plant tissue on a number of occasions (Blunden, 1967, Brain, 1968, Lockwood, 1974).

i) Characterisation of the diosgenin standard curve for densitometry

Since the solvent system for the separation of diosgenin on thin layer chromatography had already been optimised (Part 1 of this Section), the only procedure which remained to be carried out before the densitometric method was used was the preparation of a diosgenin standard curve. To prepare a standard curve, a range of diosgenin concentrations were spotted onto a 0.2mm analytical silica gel TLC plate (Translucent plastic backed). The range of values were 0.15 mg in 2.5 mg increments, with each value repeated 3 times. The plate was developed in benzene:ethyl acetate (3:1) and sprayed with antimony trichloride reagent. The plate was then scanned on the densitometer in the transmission mode as described in section 2fii of Chapter 2.

The results of this experiment are shown in Figure 3.2.15 from which it can be seen that the relationship between the integrator reading and diosgenin concentration seemed to be linear over the range 0 - 2.5 mg. The linearity then/

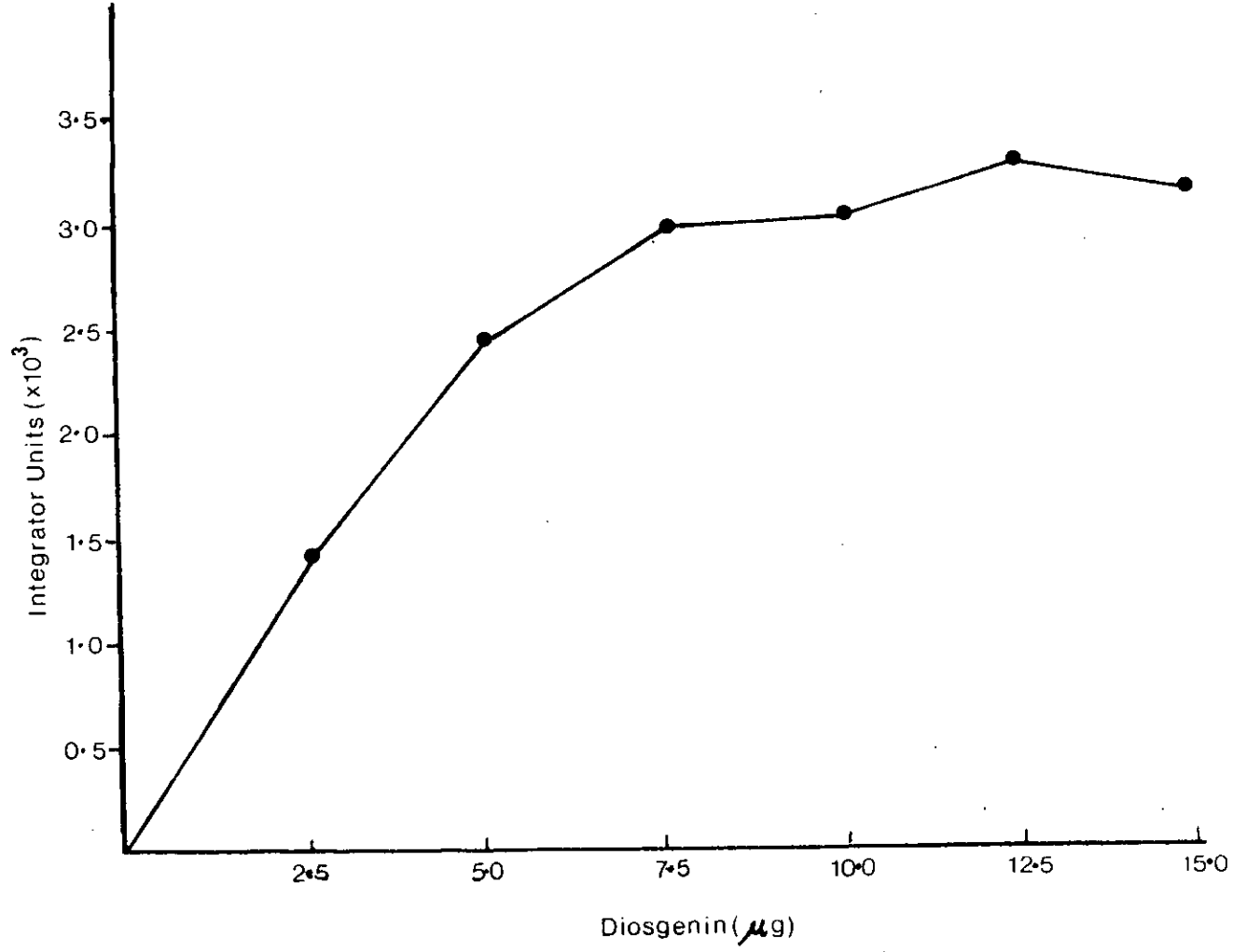


Figure 3.2.15. The relationship between integrator reading and diosgenin concentration over the range 0-15 $\mu\text{g}$ . Each point is the mean of 3 values.

then started to tail off between 2.5 and 7.5 mg until between 7.5 mg and 15 mg there was practically no difference between integrator values. The above results suggest that the only useful part of the standard curve described in figure 3.2.15 was between 0 and 2.5 mg.

To further investigate the possibility of using the relationship between integrator reading and diosgenin concentration over this range the above experiment was repeated. This time a range of diosgenin concentrations were spotted onto TLC plates in the range 0.- 3 mg with 0.5 mg increments and each value was repeated 9 times. The plates was developed in benzene:ethyl acetate (3:1) and sprayed with antimony trichloride reagent. The plates were then scanned as described above and the results were expressed as a mean of the 9 replicates with standard error at 95% confidence limits.

These results are shown in Figure 3.2.16 where it can be seen that the relationship between integrater reading and diosgenin concentration was linear over the range investigated. A similar relationship was found over such a range by Brain (1968). It can be seen from Figure 3.2.16 that the error at each value over 9 replicates was small. In an attempt to keep errors to a minimum a separate standard curve was calculated for every TLC plate used.

Once both the plant cell culture system and the analytical techniques had been developed, they were then applied to carry out the main experimental programme which is described in the remaining three sections of this Chapter.

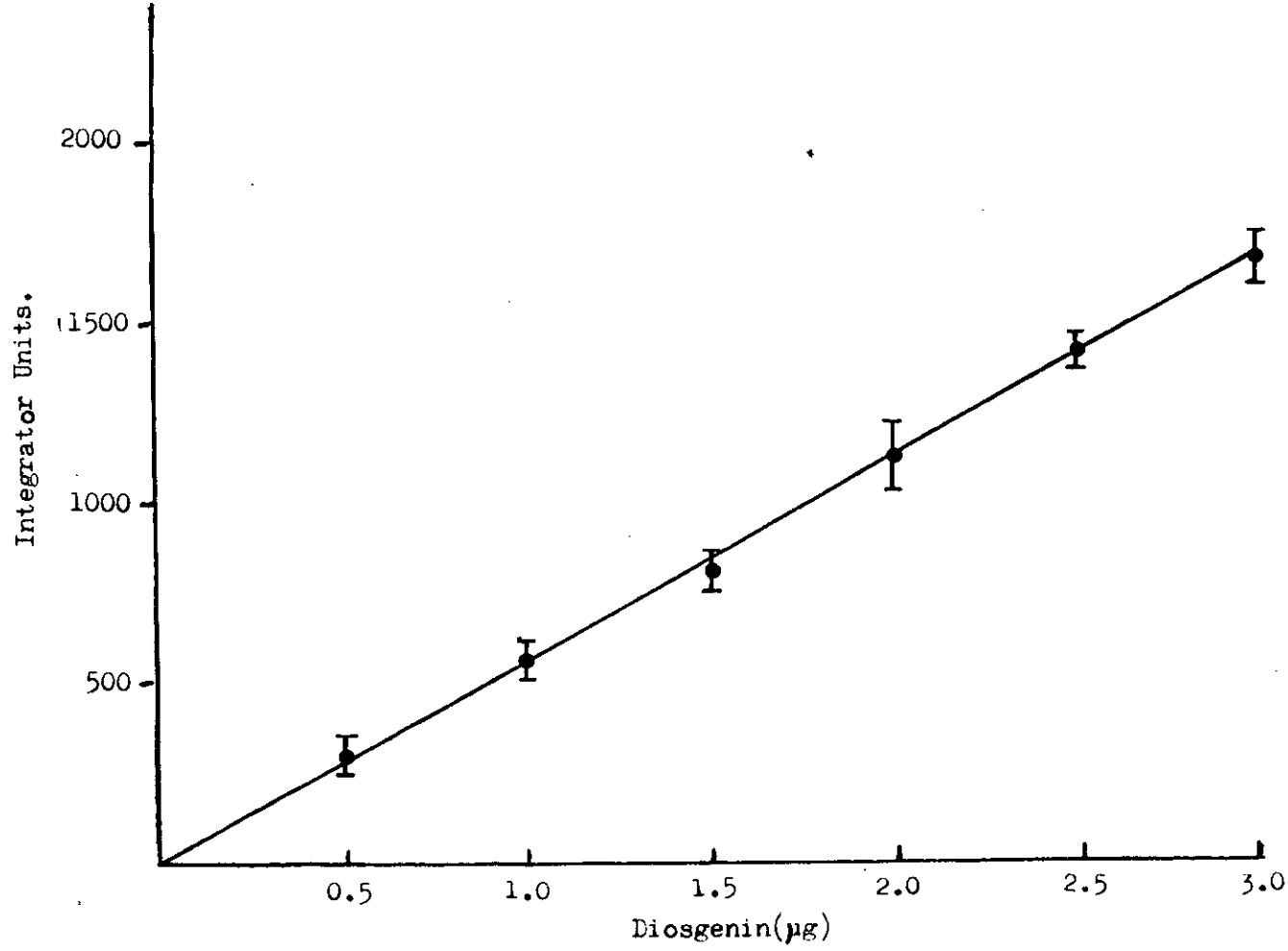


Figure 3.2.16. The relationship between integrator reading and diosgenin concentration over the range 0-3 $\mu\text{g}$ . Each point is a mean of 9 separate values. Vertical bars represent standard error (95% confidence limits).

RESULTS SECTION 3

GROWTH AND DIOSGENIN ACCUMULATION IN  
D. COMPOSITA BATCH SUSPENSION CULTURES

In the following six parts of Section 3 experiments are described in which the relationship between growth and diosgenin accumulation in suspension cultures of D. composita is examined. In this study the factors which are thought to regulate secondary metabolism were investigated and consideration is given to how these may be manipulated to produce high yields of diosgenin.

The experiment in Part 1 of Section 3 was designed to investigate the relationship between growth and diosgenin accumulation in batch cultures over a 10 week period. 5 parameters were employed, cell number, packed cell volume, dry weight, fresh weight and cell viability.

Part 2 of Section 3 contains a study in which the effects of nutrient depletion on culture growth and diosgenin accumulation were examined. The effects of depletion of orthophosphate, ammonium, nitrate and sucrose in the culture medium on growth and diosgenin accumulation were followed over a 10 week period.

In parts 3 - 5 experiments are described in which attempts were made to increase the yield of diosgenin by feeding stationary phase batch suspension cultures with various combinations of sucrose, 2,4-D and kinetin or sucrose and abscisic acid or sucrose and rhamnose.

Finally in part 6 of this Section, experiments are described in which a study was made of the competition between primary and secondary metabolism for common precursors using two <sup>14</sup>C radiolabelled precursors of diosgenin, mevalonic acid and cholesterol.

S E C T I O N    3    -    P A R T    1

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THE RELATIONSHIP BETWEEN GROWTH AND  
DIOSGENIN ACCUMULATION IN D. COMPOSITA  
BATCH SUSPENSION CULTURES

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An essential prerequisite to any study in which plant cell cultures are manipulated for optimum yield of a secondary metabolite is to establish the relationship between growth and accumulation.

The results of this part of Section 3 examine the characteristics of growth and diosgenin accumulation in batch suspension cultures of D. composita and how they relate to each other.

The experiment was carried out using 55, 60 ml suspension cultures prepared as described in Section 1 (d) of Chapter 2. These freshly inoculated cultures contained approximately 20,000 cells/ml with a packed cell volume of 11%. Growth and diosgenin accumulation were measured every 7 days over a 10 week period and 5 replicates were harvested at each time point.

The growth of the cultures was measured with respect to changes in cell number, packed cell volume, dry weight, fresh weight and cell viability. Diosgenin accumulation was measured as described in Sections 2 c - f of Chapter 2.

#### i) CHANGES IN CELL NUMBER

The changes in cell number over the 10 week culture period are shown in Figure 3.3.1 (a) from which it can be seen that the total cell number increased from an average initial inoculum value of about 20,000 cells/ml to an average of about 78,000 cells/ml in the first week. This represents an approximately 4-fold increase, with a cell doubling time of 3.6 days. Cell number increased at/

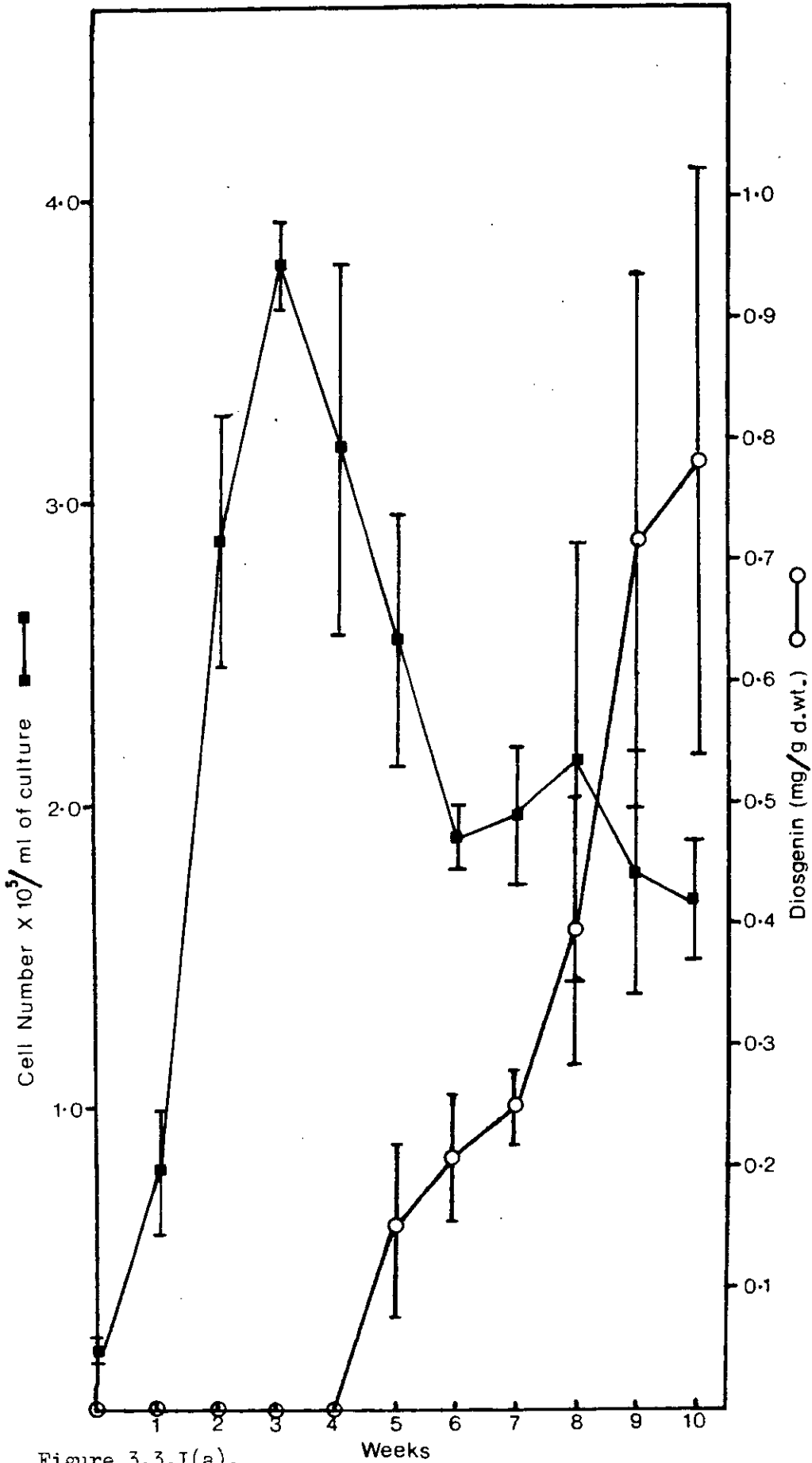


Figure 3.3.1(a).

Cell number and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

at more or less the same rate until the end of the second week (a 3.7 fold increase and cell doubling time of 3.8 days). This resulted in a cell count of around 288,000 cells/ml of culture.

The maximum value for cell number was reached at the end of the third week with an average of about 280,000 cells/ml of culture. This represents a 1.3-fold increase and a doubling time of 10.8 days which suggests a slowing down of cell division between weeks 2 and 3.

From the maximum cell number at week 3 there was a significant decrease over the next 3 weeks in which the cell number was halved to 190,000 cells/ml at week 6. From the sixth week to the end of the experiment there was no further significant decline in cell number which remained at about half of the maximum value attained at week 3.

In Figure 3.3.2 (a) the cell number has been calculated as the logarithm to the base 10 and plotted against time to give a semi-log plot from which it can be seen there is a straight line relationship between  $\log_{10}$  cell number and time over the first 2 weeks of culture. This suggests that the increase in cell number was exponential during the early stages of culture growth and that no measurable lag phase could be detected. However, care must be taken with such an interpretation, since it is based on only 3 points which is the absolute minimum necessary/

necessary for such a calculation. Ideally many more points would be required to make firm statements about the rates of cell division.

ii) CHANGES IN PACKED CELL VOLUME (PCV)

The changes in packed cell volume over the 10 week culture period are shown in Figure 3.3.1 (b) from which it can be seen that packed cell volume increased to just over 13% of the total culture volume from an initial figure of 11% during the first week. In the following week PCV increased by 1.6-fold to over 21% and by the end of week 3 had reached just over 31%, a 1.5-fold increase. PCV reached a maximum value at the end of week 4, with a figure of 34%, and remained more or less constant at this value until the end of the experiment.

If PCV is represented on a semi-log plot (Figure 3.3.2(b)) in a similar way to cell number, it can be seen that the increase in packed cell volume over the early stages of culture growth show a different pattern to that of cell number. The results suggest a 2-phase pattern with a slower increase over the first week followed by a faster increase over the next two weeks with the straight line relationship that it is exponential. Again caution must be used in such an interpretation as it is only based on a few points.

iii) CHANGES IN DRY WEIGHT

The changes in dry weight over the 10 week culture period are shown in Figure 3.3.1 (c) from which it can be seen that from an initial average figure of 57 mg/culture, dry weight increased 2.5-fold to 147 mg in the first week./

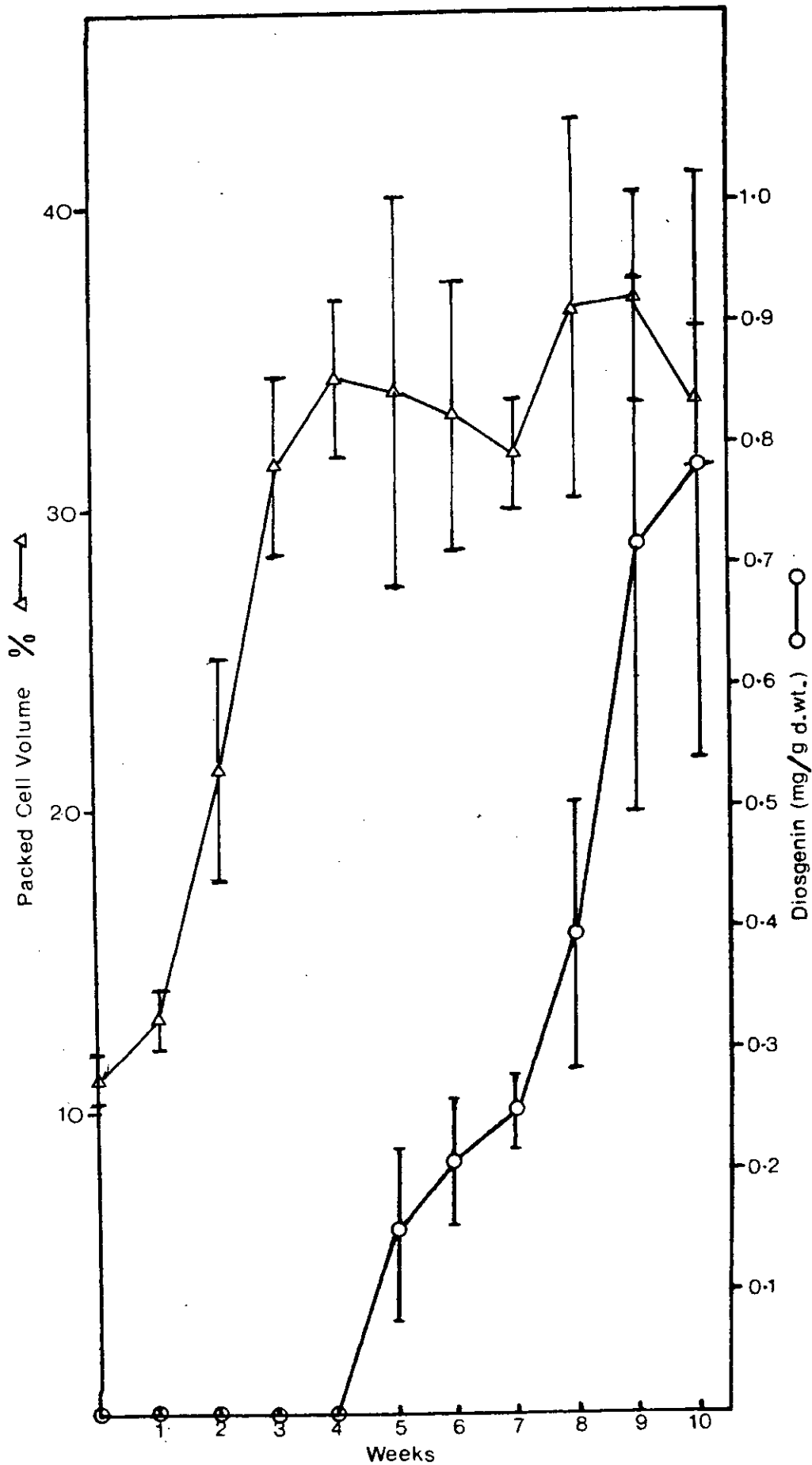


Figure 3.3.I(b). Packed cell volume and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

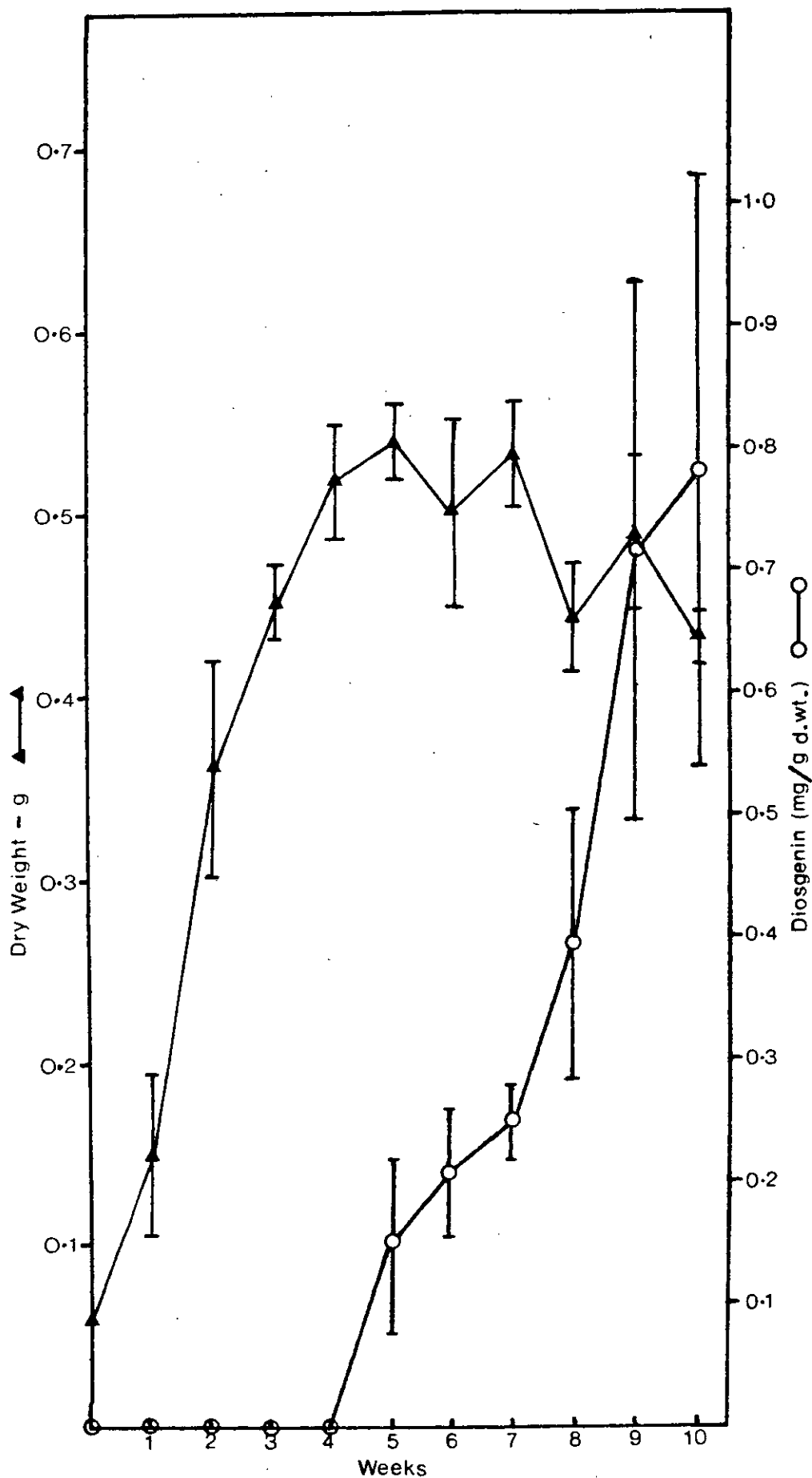


Figure 3.3.1(c).

Dry weight and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

week. By the end of the second week, dry weight had increased to 361 mg/culture, a comparable gain of just under 2.5-fold. At the end of week 3, the average dry weight had risen to 450 mg which at a 1.2-fold increase is much less of an increase than the previous 2 weeks. This pattern continued into week 4 where dry weight increased again to reach its maximum figure of 515 mg. This increase at 1.1-fold is comparable to that of the previous week.

The average dry weight/culture by the end of week 5 was 536 mg, however, this was not significantly different from the value for week 4. The dry weight/culture levelled off between weeks 5 and 7, remaining around 530 mg. There was however a significant drop in dry weight at week 8 but it levelled off once more at around 450 mg until the end of the experiment.

Figure 3.3.2 (c) illustrates the semi-log plot of dry weight against time. It can be seen from this that, like cell number, there is a straight line relationship between  $\log_{10}$  dry weight and time over the first two weeks of culture growth. This suggests that the initial increase in dry weight was exponential with no measurable lag phase, though caution must again be applied to this interpretation since it is based on only a few points.

#### iv) CHANGES IN FRESH WEIGHT

The changes in fresh weight over the 10 week culture period are shown in Figure 3.3. 1 (d). It can be seen from/

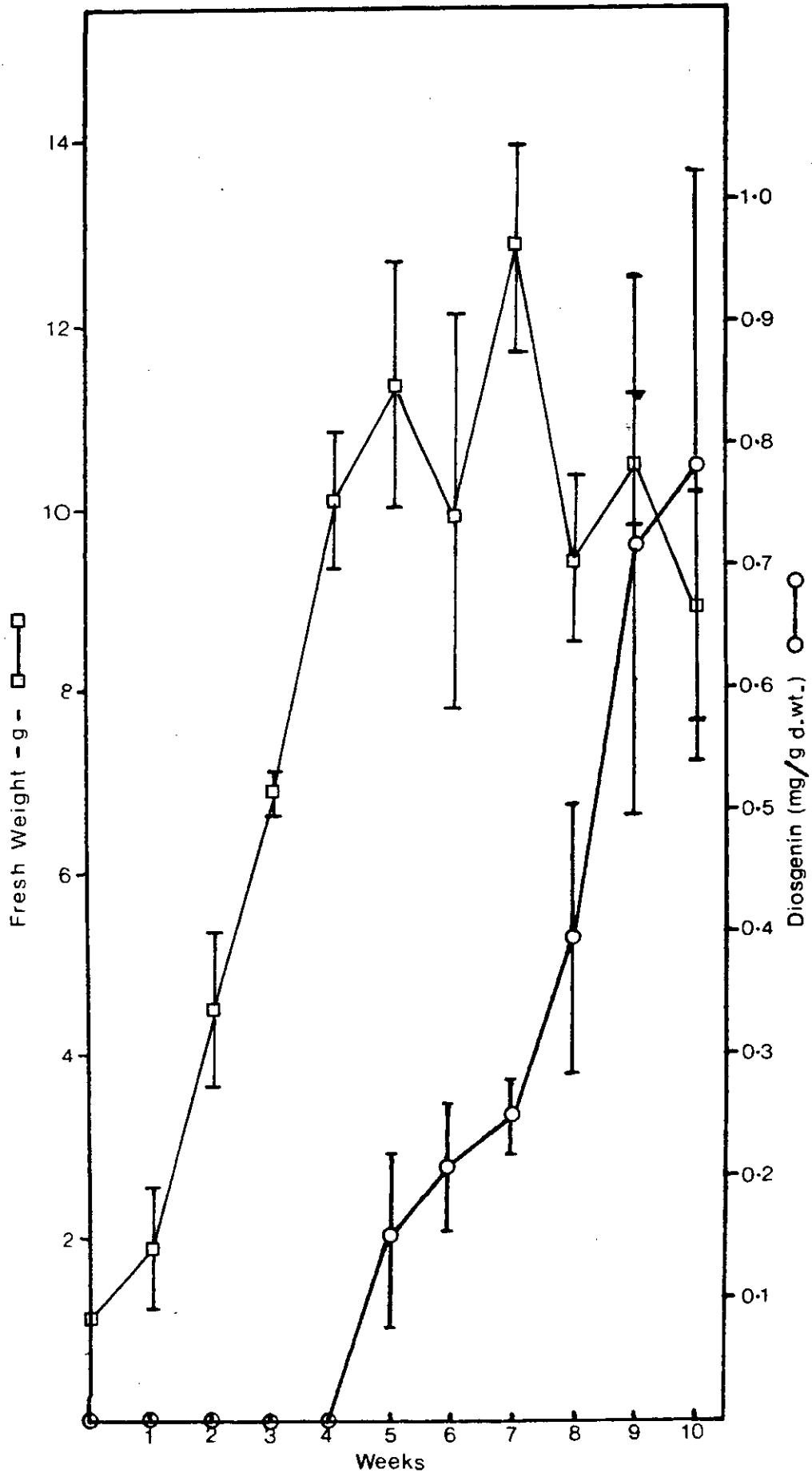


Figure 3.3.1(d). Fresh weight and diosgenin production in suspension / cultures of D. composita. Bars represent standard error (95%) confidence limits.

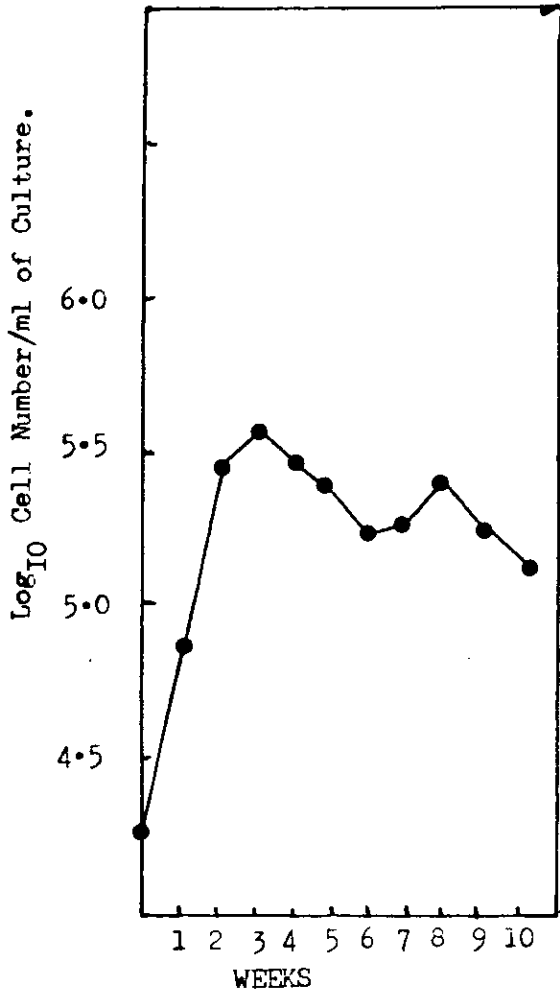


Figure 3.3.2(a). Semi-log plot of  $\log_{10}$  cell number against time in suspension cultures of D. composita.

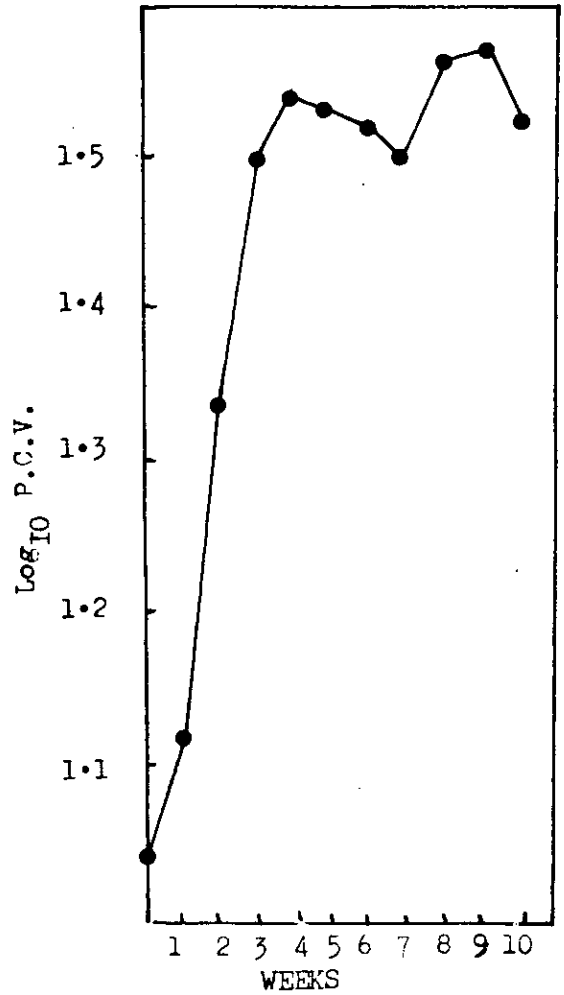


Figure 3.3.2(b). Semi-log plot of  $\log_{10}$  P.C.V. against time in suspension cultures of D. composita.

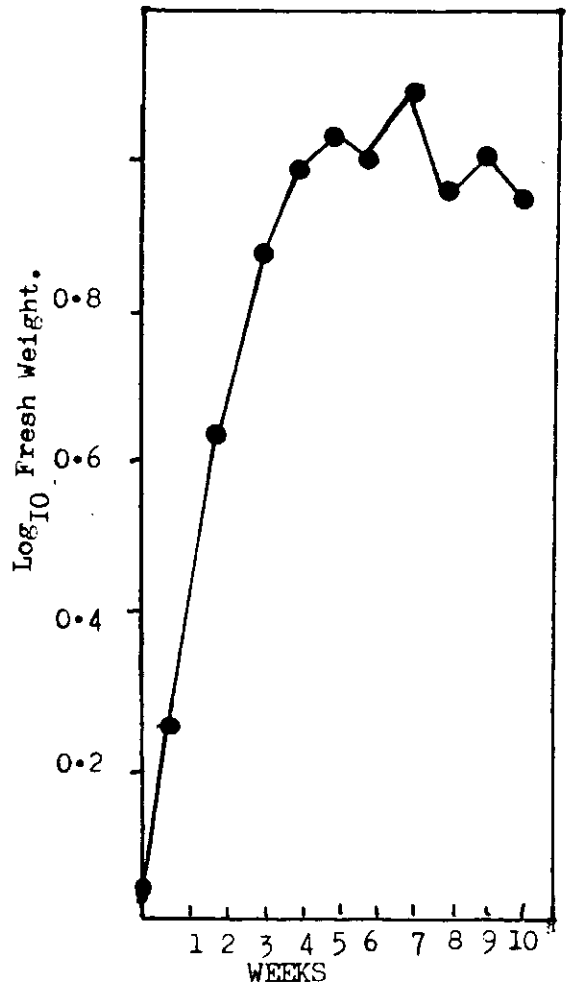
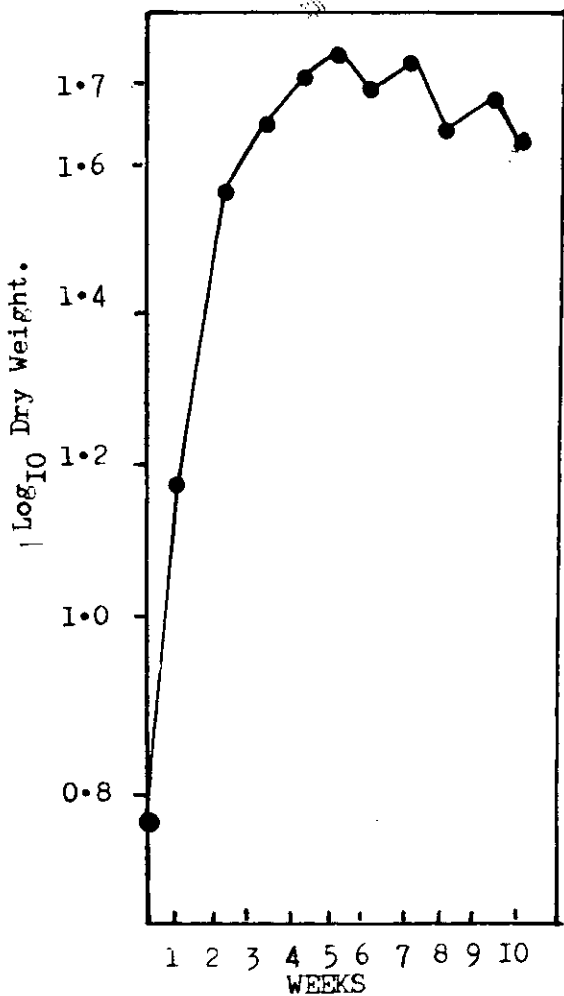


Figure 3.3.2(c). Semi-log plot of  $\log_{10}$  dry weight against time in suspension cultures of *D. composita*.

Figure 3.3.2(d). Semi-log plot of  $\log_{10}$  fresh weight against time in suspension cultures of *D. composita*.

from this, that from an initial value of 1.1g, fresh weight increased by 1.7-fold in the first week to 1.9g. By the end of the second week, it had increased to 4.5g a 2.5-fold increase and by the end of week 3 to 7.6g, a 1.6-fold increase. The decrease in the size of the fresh weight gain between weeks 2 and 3 continued to the end of week 4 where a 1.3-fold increase resulted in a value of 10.1 g.

Fresh weight reached 11.3 g by the end of week 5 with the level of increase down again to 1.1-fold. There was no significant difference between the average fresh weight values for weeks 4 and 5 which suggests the maximum value was reached between those 2 times.

Fresh weight then levelled off at 12g with no significant changes until week 8 when there was a decrease to approximately 9g. The average figure then remained more or less the same until the end of the experiment.

Figure 3.3.2 (d) illustrates the semi-log plot of  $\log_{10}$  fresh weight against time. It can be seen from this that the pattern is most similar to that of the semi-log plot of packed cell volume (Figure 3.3.2 (b)) where the results suggest a lag phase before any increase begins. In the case of fresh weight however, one cannot detect any period of exponential increase after the lag phase since any straight line can only be drawn between 2 points. Of all the parameters however, the results suggest that fresh weight increased more gradually than any of the others.

v) CHANGES IN CELL VIABILITY

The changes in cell viability over the 10 week culture period are shown in Figure 3.3.3. It can be seen from this that the cell viability remained on average between 84 and 91% over the first 4 weeks of culture. The average cell viability then gradually declined to reach a figure of 75% at the end of the experiment. The error margins in the data however suggest that there was little significant change in the cell viability of the cultures during the entire experiment.

vi) DIOSGENIN ACCUMULATION

The yield of diosgenin/culture is shown in Figure 3.3.4 and as can be seen, it did not accumulate during the first 4 weeks of culture. Accumulation began during the fifth week and by the end of that week had reached an average yield of  $80\mu\text{g/culture}$ . By the sixth week the yield had increased 1.4-fold to  $111\mu\text{g/culture}$ . The average value by the end of week 7 was  $149\mu\text{g/culture}$ , a 1.3-fold increase. The increase over the next 7 days was 1.1-fold giving a value at the end of week 8 of  $169\mu\text{g/culture}$ . After a period of four weeks of quite similar increases, diosgenin accumulation increased 2.0-fold over week 9, resulting in a yield of  $340\mu\text{g/culture}$ . Once this maximum value had been reached the average yield fell slightly during the last week to give a final yield of diosgenin of  $324\mu\text{g/culture}$ .

The yield of diosgenin/g dry weight of culture is shown in Figures 3.3.1 (a), 3.3.1 (b), 3.3.1 (c) and 3.3.1 (d), where it is compared to changes in cell number, PCV, dry weight/

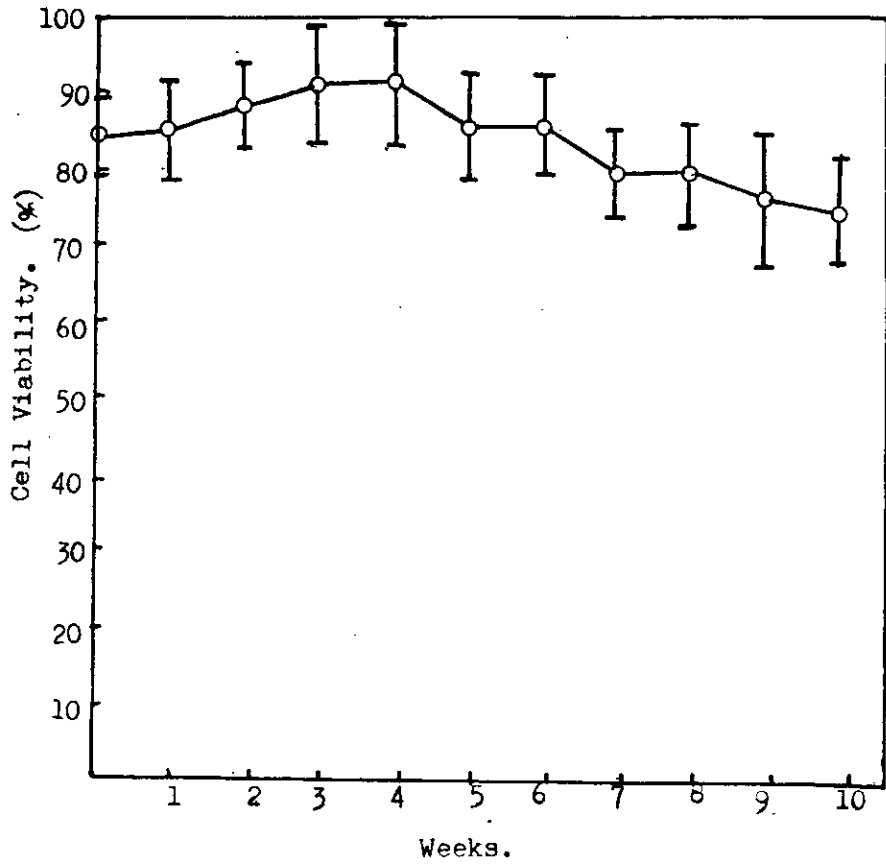


Figure 3.3.3. Cell viability in suspension cultures of D. composita over a ten week period.

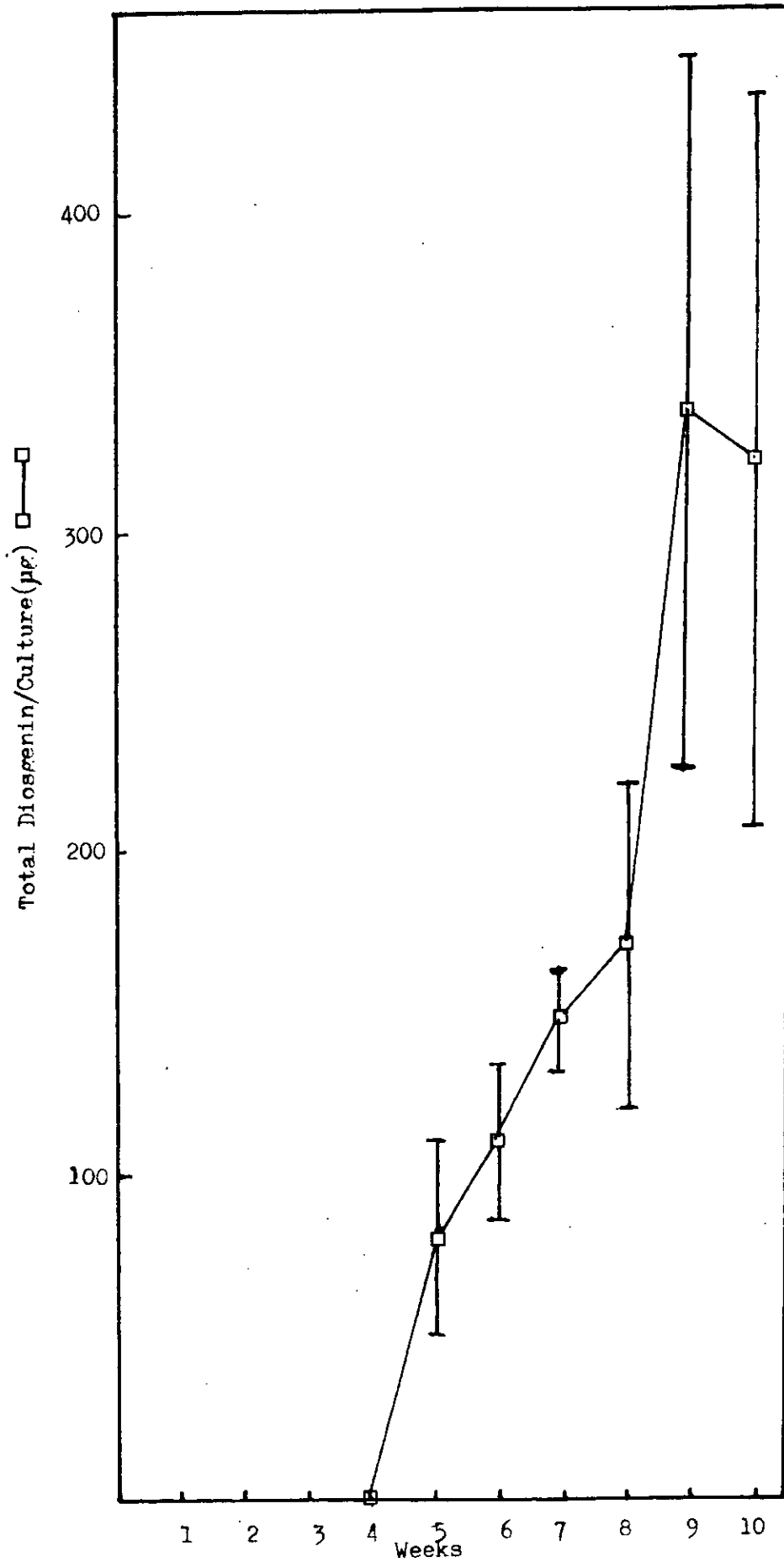


Figure 3.3.4. The yield of diosgenin( $\mu\text{g}/\text{flask}$ ) in suspension cultures of *D. composita*.

weight and fresh weight respectively. The overall pattern of diosgenin accumulation expressed in this way is similar to that in Figure 3.3.4.

It can be seen from Figure 3.3.1 (a) that diosgenin did not start to accumulate until week 5 which is after cell number had reached its maximum value. Cell number was in fact decreasing during the first 2 weeks of diosgenin accumulation. The rest of the accumulation occurred when the cell number was relatively stable.

In Figure 3.3.1 (b) it can be seen that diosgenin did not start to accumulate until after packed cell volume had reached its maximum value. From that point onwards packed cell volume stayed more or less constant during the entire period of diosgenin accumulation.

In Figure 3.3.1 (c) it can be seen that diosgenin did not start to accumulate until after dry weight had reached its maximum value.

The first 3 weeks of diosgenin accumulation occurred with dry weight staying more or less constant. Dry weight then fell slightly over the eighth week and then levelled off over the next while the yield of diosgenin was increasing over both those weeks. Both dry weight and diosgenin yield remained constant over the last week of the experiment.

In Figure 3.3.1 (d) it can be seen that diosgenin did not/

not start to accumulate until after fresh weight had reached its maximum value. The majority of the diosgenin accumulation occurred with constant fresh weight except during week 8 when there was a small decrease which then levelled off until the end of the experiment.

The yield of diosgenin/cell is shown in Figure 3.3.5 (a) from which it can be seen that from a zero value at week 4, the yield of diosgenin/cell increased to 5.2pg/cell by week 5. By week 6 this had increased by 1.8-fold to 9.6 pg/cell. By comparison cell number during these 2 weeks had fallen by 1.7 fold.

The yield of diosgenin/cell increased by 1.3-fold to 12.6 pg/cell by the end of week 7 and by 1.1-fold to 13.4 pg/cell by week 8. This apparent levelling off of diosgenin accumulation occurred when cell number had also levelled off. Cell number then remained fairly constant till week 10, however, diosgenin yield/cell increased 2.4 fold to 31.5pg/cell during week 9 then levelled off at that value until the end of the experiment at week 10.

A diagram of a thin layer chromatogram of the extracts from the above experiment is shown in Figure 3.3.5 (b) from which it can be seen that the data presented confirms the pattern of diosgenin accumulation described above in the quantitative results. It also shows that unlike diosgenin, the plant sterols were present in the suspension cultures at all times during the culture period.

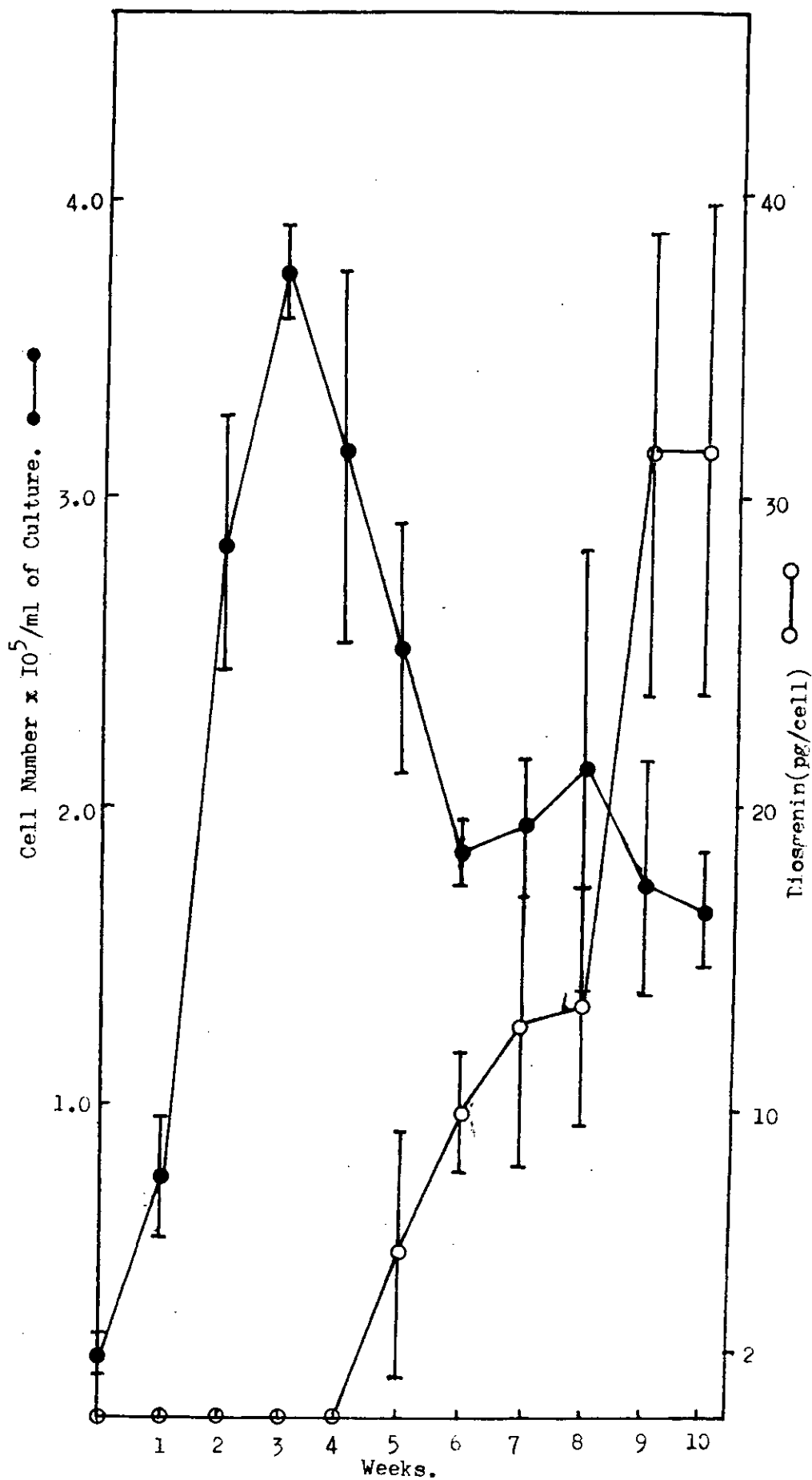
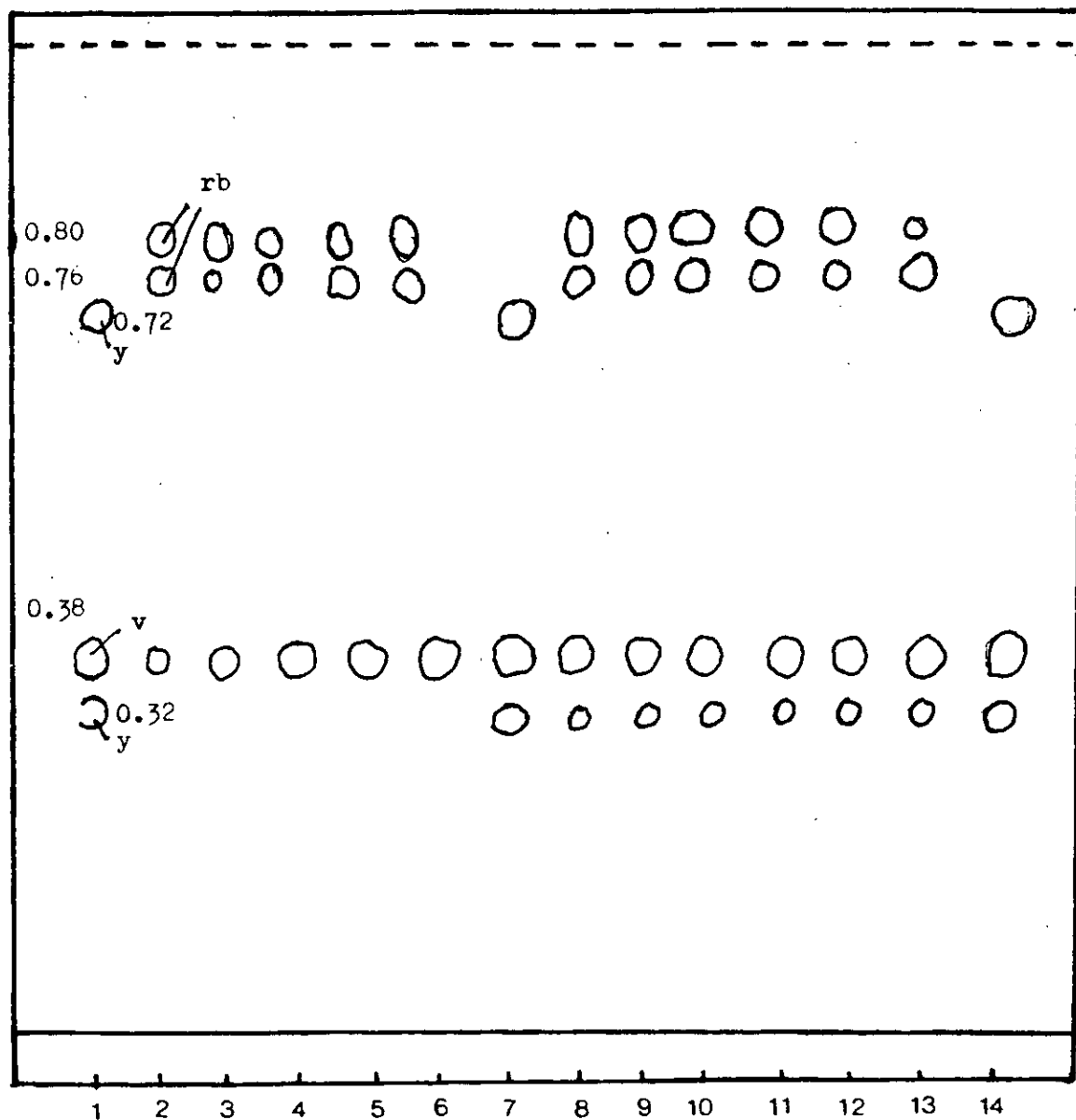


Figure 3.3.5(a). Diosgenin accumulation (pg/cell) and cell number in suspension cultures of *D. composita*.

Sample

1. Standard mixture

2. Day 0 extract.

3. Week 1 extract.

4. Week 2 extract.

5. Week 3 extract.

6. Week 4 extract.

7. Standard mixture.

Sample

8. Week 5 extract.

9. Week 6 extract.

10. Week 7 extract.

11. Week 8 extract.

12. Week 9 extract.

13. Week 10 extract.

14. Standard mixture.

v=violet, y=yellow, rb=reddish brown.

standard mixture=diosgenin, cholesterol and 25R spirostan-3,5-diene.

Figure 3.3.5.(b) Diagram of the T.L.C. of chloroform extracts of

D. composita suspension culture grown over 10 weeks,  
sprayed with anisaldehyde reagent.

No diosgenin was detected in the culture medium at any time during the experiment.

vii) DIOSGENIN ACCUMULATION IN THE PATTERN OF CULTURE GROWTH

It can be seen from the results presented in this section that diosgenin does not begin to accumulate in the suspension cultures until all aspects of cell growth have ceased.

The accumulation of the secondary product, in this case, diosgenin, occurs exclusively in the stationary phase which suggests that growth and metabolite production are separated in time.

One question which arises directly from this experiment is that if diosgenin accumulation is a characteristic of comparatively old stationary phase cultures, how is it affected by the depletion of essential nutrients in the culture medium ?

SECTION 3 - PART 2

THE EFFECTS OF NUTRIENT DEPLETION ON THE  
GROWTH AND DIOSGENIN ACCUMULATION IN  
D. COMPOSITA BATCH SUSPENSION CULTURES

In the previous part of this Section, the relationship between growth and diosgenin accumulation was investigated in batch suspension cultures of D.composita over a ten week period. It was found that growth and accumulation were completely separated in time. Culture growth had ceased with respect to the parameters studied by the fourth week of culture, whereas diosgenin accumulation did not reach its maximum value until the ninth week of culture.

The aim of the experiment described in this part of Section 3 was to study the depletion of four major nutrients supplied from the culture medium over a similar culture period to the previous experiment and assess how this affected growth and diosgenin accumulation.

The nutrients studied were phosphate, ammonium, nitrate and sucrose. The experiments was carried out using 33, 60ml-suspension cultures, prepared as previously described. 'Growth', diosgenin accumulation and nutrient depletion were measured every 7 days over a 10-week period. Three replicates were harvested at each point. The depletion of the nutrients from the culture medium was measured by use of the techniques described in Section 2(b) of Chapter 2.

#### i) CHANGES IN CULTURE GROWTH

The changes in the growth of the cultures over the 10-week period with respect to cell number, packed cell volume, dry weight and fresh weight are shown in Figures 3.3.6a, 3.3.6b, 3.3.7a and 3.3.7b, respectively. It can be seen from Figure!

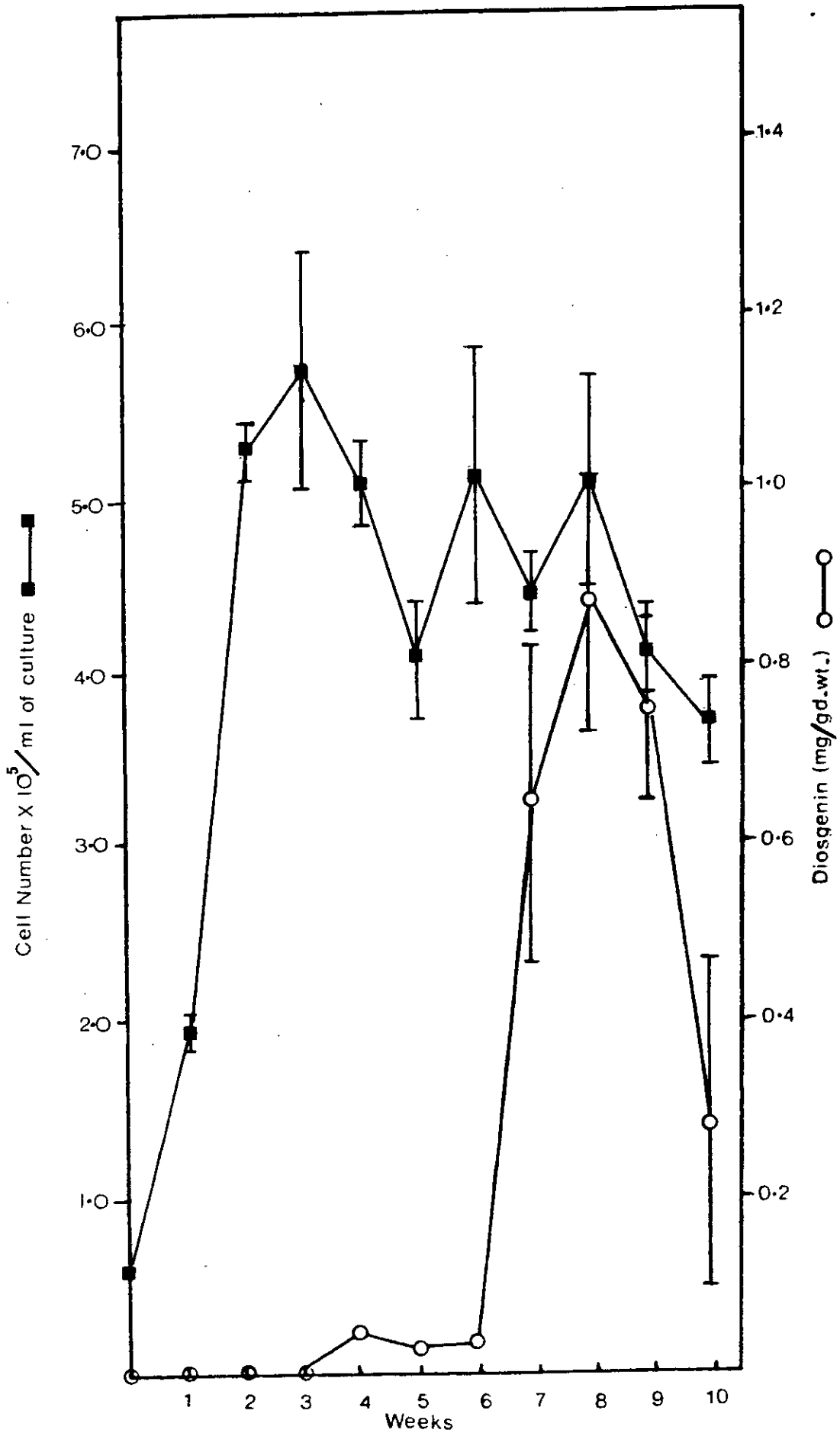


Figure 3.3.6a. Cell number and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

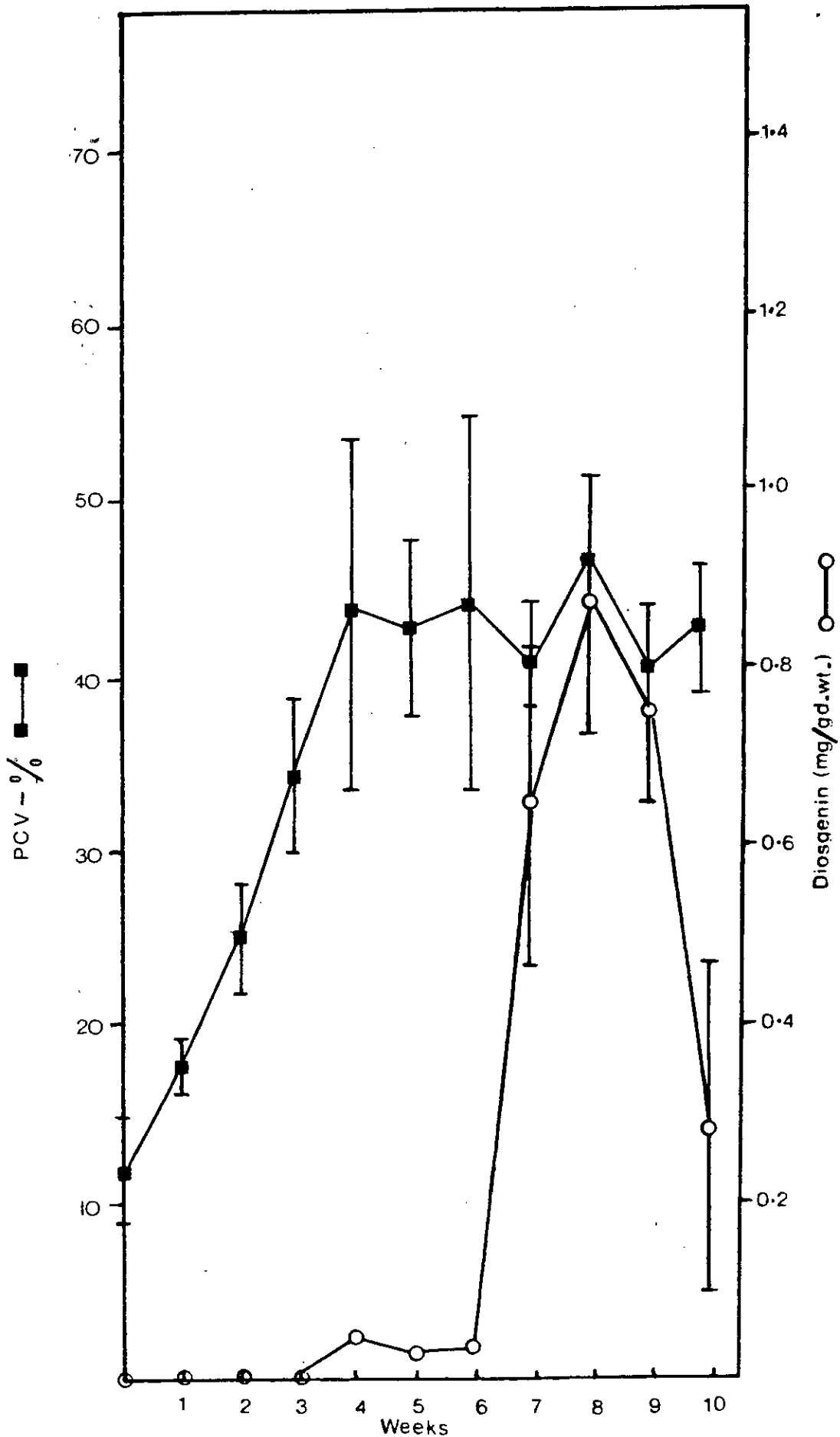


Figure 3.3.6b. Packed cell volume and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

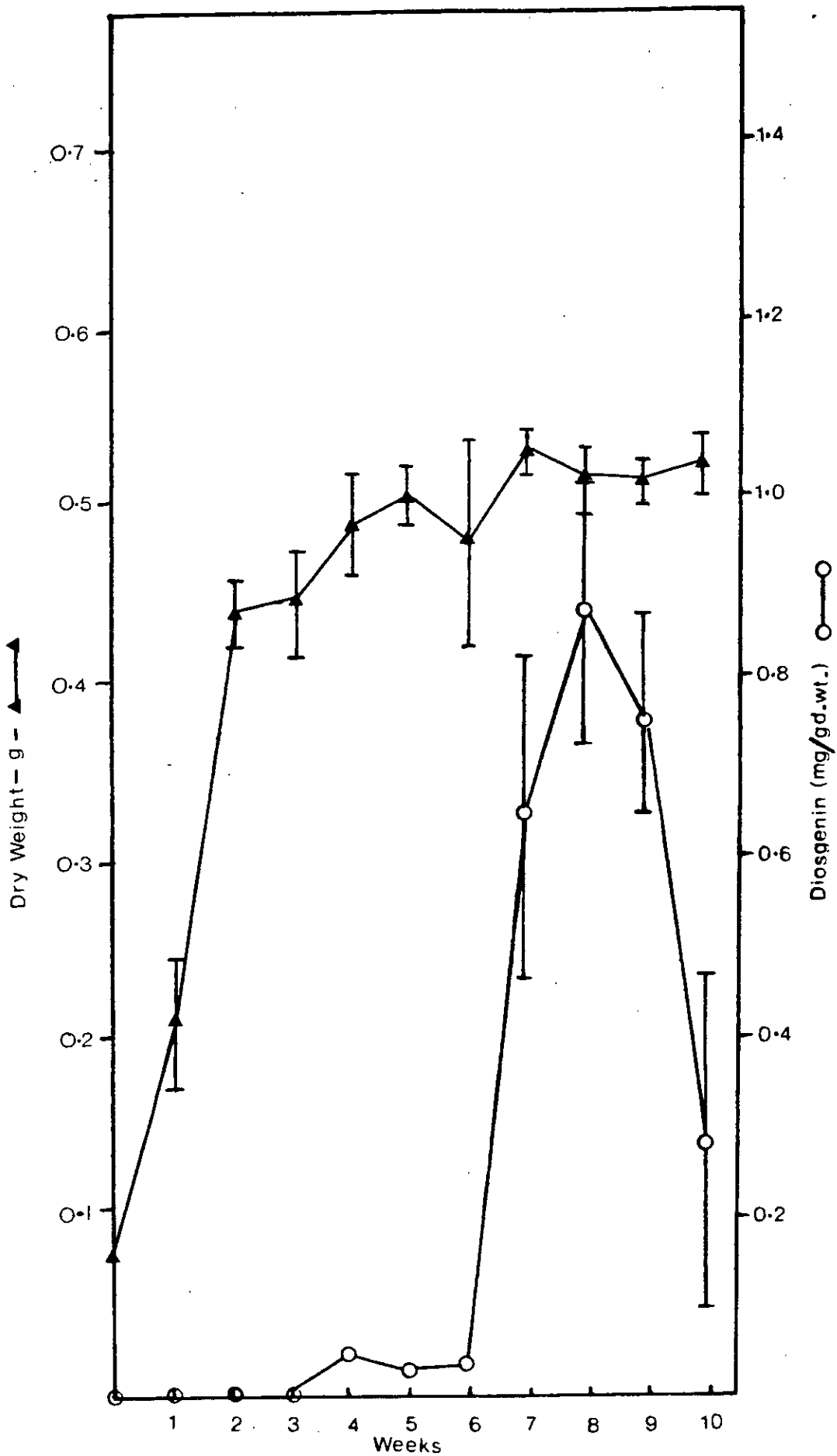


Figure 3.3.7a. Dry weight and diosgenin production in suspension cultures of *E. composita*. Bars represent standard error (95%) confidence limits.

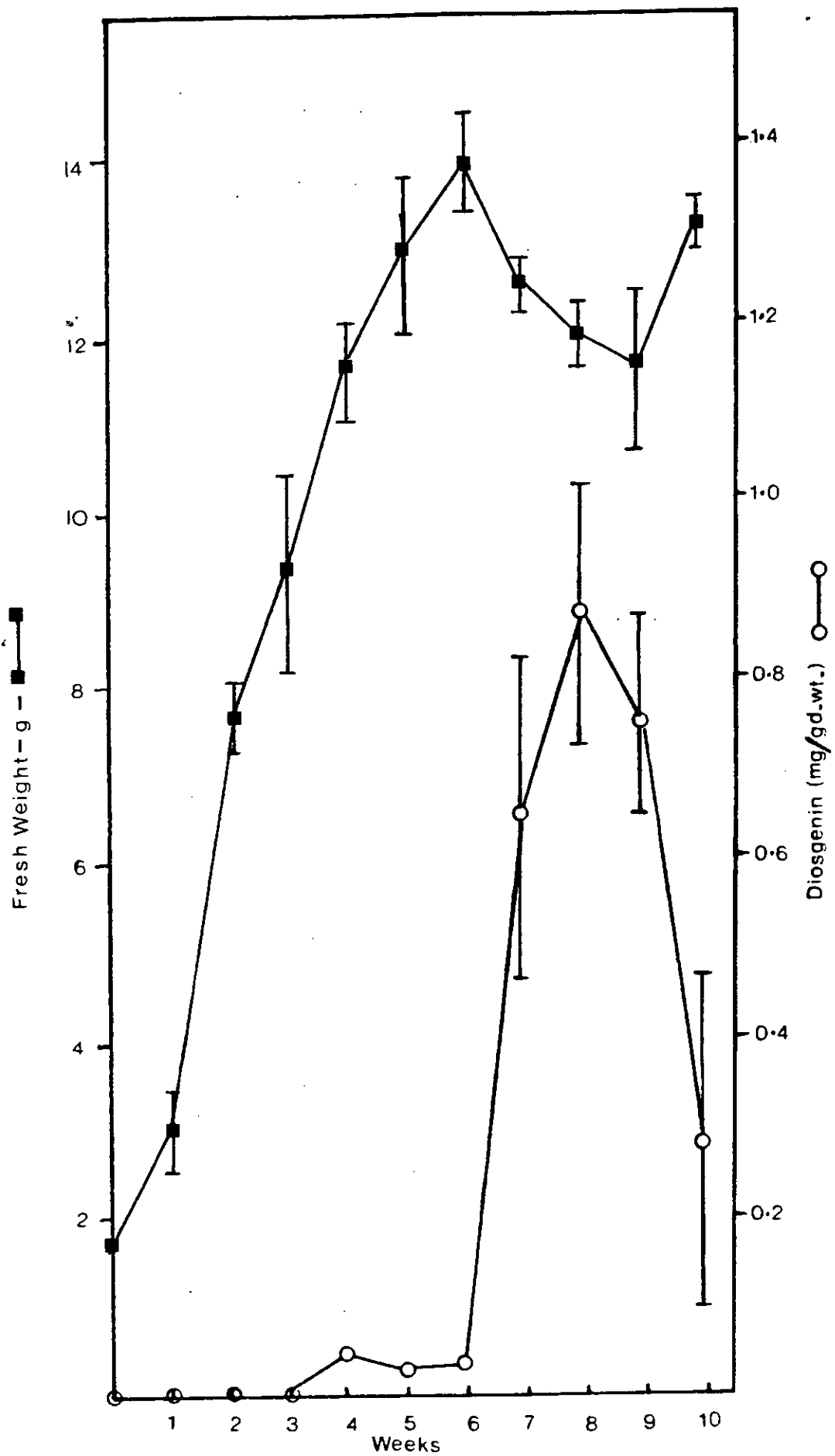


Figure 3.3.7b. Fresh weight and diosgenin production in suspension cultures of *I. composita*. Bars represent standard error (95%) confidence limits.

3.3.6a that cell number increased from an initial average of 62,000 cells/ml of culture to a maximum average value of 577,000 cells/ml of culture by the end of week 3. There was, however, no significant difference between this value and that of week 2. Maximum cell number was therefore reached sometime during the third week from inoculation.

Although there was some variation in the average cell number after the end of week 3 it can be seen that the overall trend was downwards. The average cell number at the end of the experiment was 367,000 cells/ml of culture.

The changes in packed cell volume over the 10-week culture are shown in Figure 3.3.6b. From this it can be seen that P.C.V. increased steadily from an average initial figure of 12% of the total culture volume to a maximum average figure of 44% by the end of week 4. This value was, however, not significantly different from the previous value. This suggests that the cultures therefore reached their maximum value with respect to P.C.V. during the fourth week from inoculation and did not change significantly during the remainder of the culture period.

It can be seen from Figure 3.3.7a that dry weight increased rapidly from an initial average value of 82mg/culture to an average value of 442mg/culture by the end of the second week. This five-fold increase was followed by less dramatic increases resulting in the maximum growth with respect to dry weight being reached at approximately the end of the fourth week. Dry weight stayed relatively constant during the remaining six weeks of the experiment.

The changes in fresh weight are shown in Figure 3.3.7b.

It can be seen from this that fresh weight steadily increased from an average initial value of 1.66g/culture to a maximum value of approximately 13g/culture around the end of the fifth week. The trend for fresh weight over the latter part of the experiment was downwards apart from a small increase during the last week.

#### ii) DEPLETION OF NUTRIENTS

The depletion of phosphate, sucrose, ammonium and nitrate from the nutrient medium over the ten week period is shown in Figures 3.3.8a, 3.3.8b, 3.3.9a and 3.3.9b, respectively.

It can be seen from Figure 3.3.8a that 90% of the phosphate in the nutrient medium was absorbed by the cells in the first 7 days. By the end of the second week, 98.5% of the phosphate had been absorbed. The amount of phosphate stayed at this low level for the remainder of the experiment.

The depletion of sucrose from the culture medium was very similar to that of phosphate. It can be seen from Figure 3.3.8b that 86% of the sucrose present at inoculation had been absorbed by the cells during the first two weeks. It took another three weeks however for the remaining sucrose to reach 1-2% which was approximately the value it stayed at until the end of the experiment.

The depletion of ammonium from the nutrient medium is shown in Figure 3.3.9a. It can be seen from this that approximately 60% of the ammonium had been absorbed by the end of the first week. The amount absorbed increased to an average of 89% by

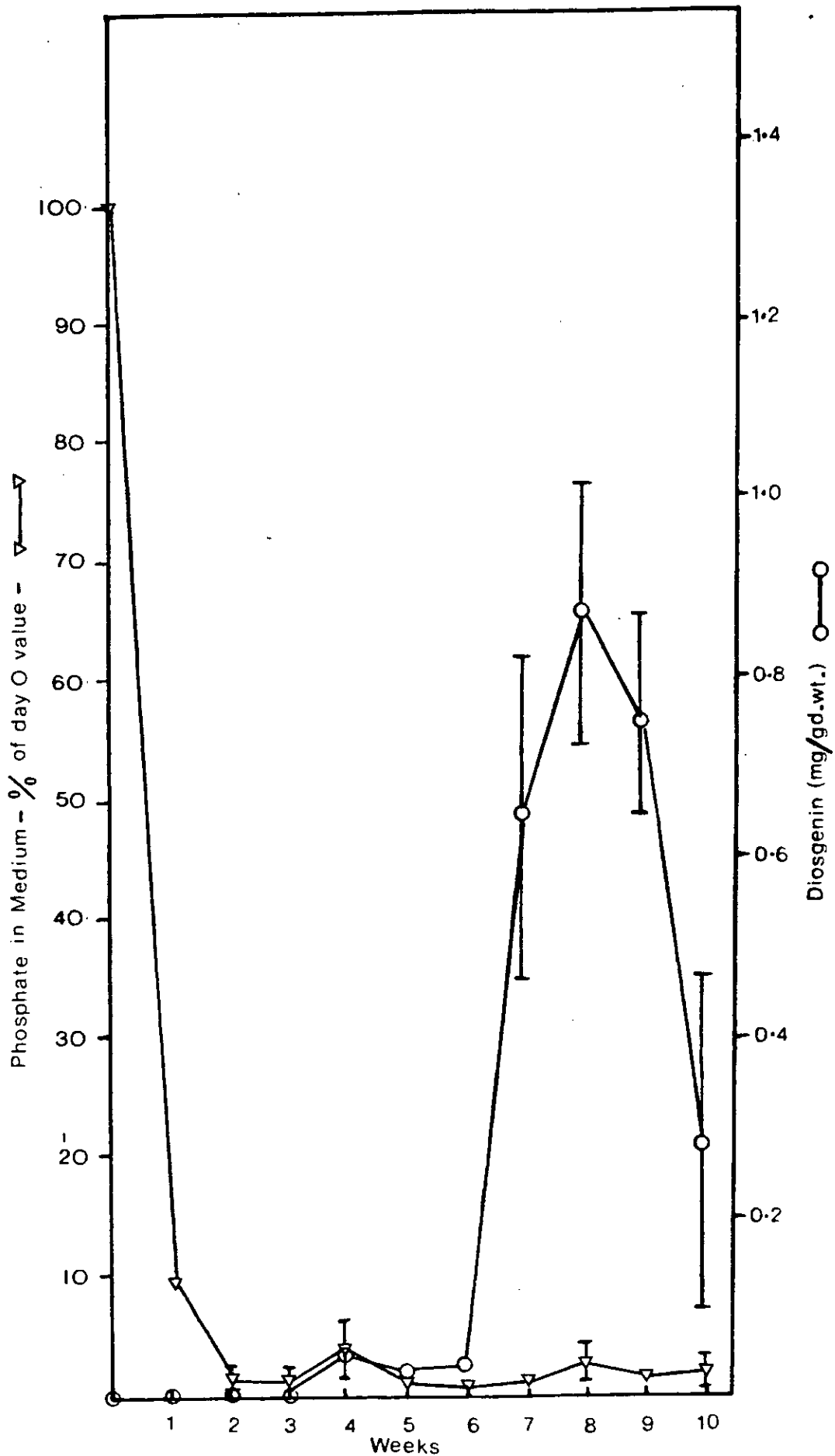


Figure 3.3.8a. Phosphate depletion and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

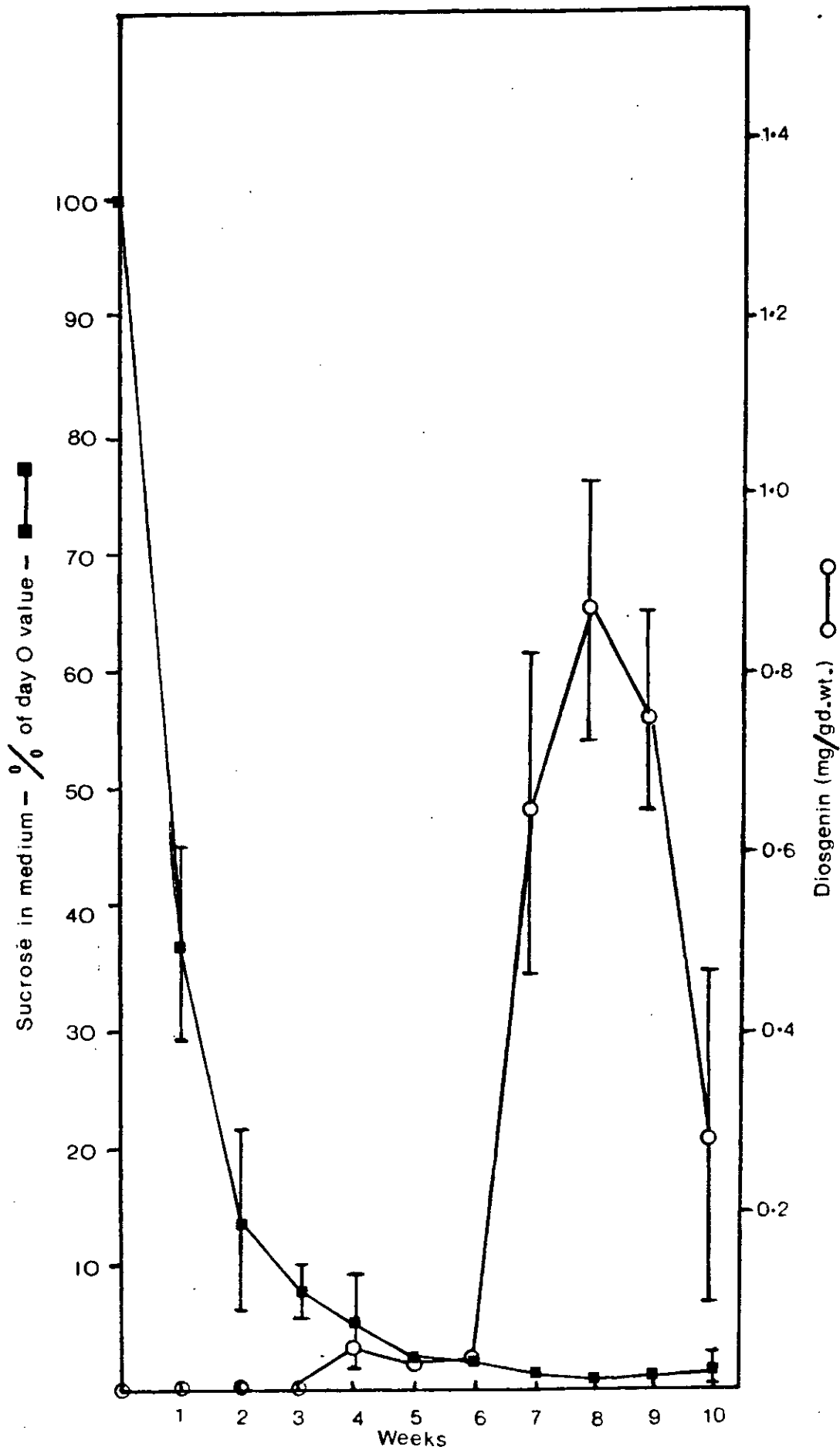


Figure 3.3.8b. Sucrose depletion and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

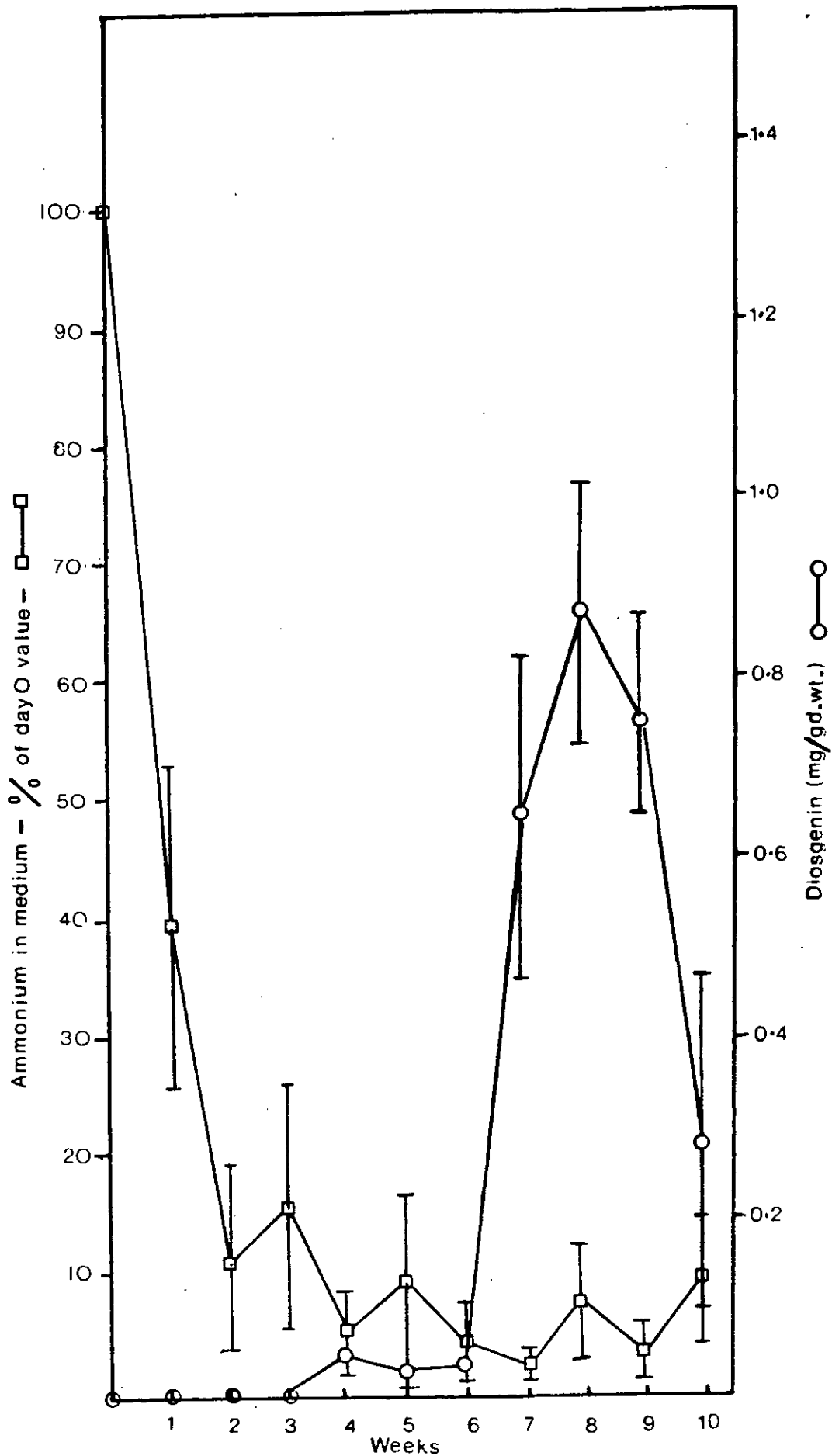


Figure 3.3.9a. Ammonium depletion and diosgenin production in suspension cultures of *D. composita*. Bars represent standard error (95%) confidence limits.

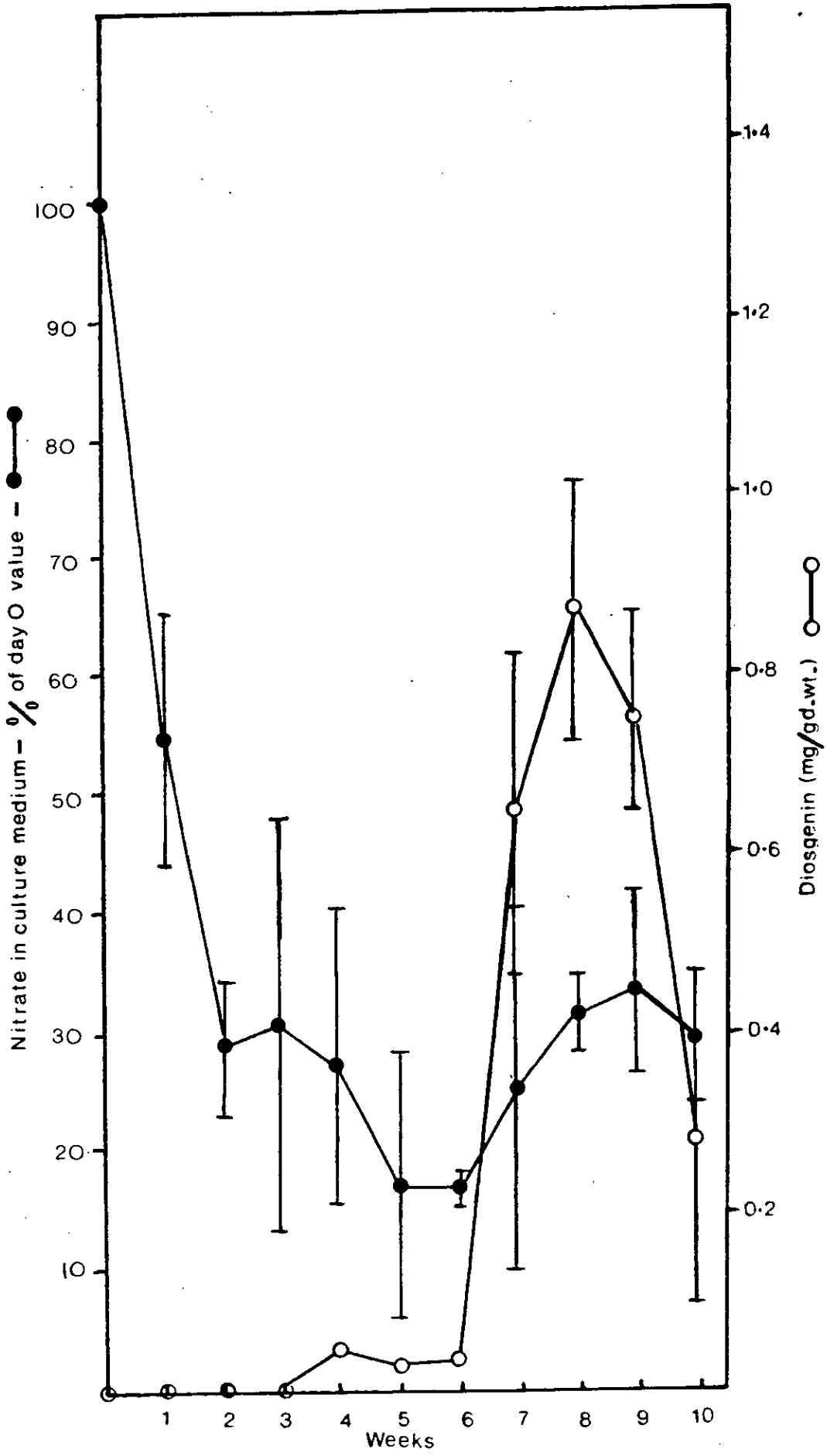


Figure 3.3.9b. Nitrate depletion and diosgenin production in suspension cultures of L.composita. Bars represent standard error (95%) confidence limits.

the end of the second week. After which although the average values fluctuate, there was no further significant decrease in the levels of ammonium. The important feature of this was that between the second and the tenth week of culture there was always a significant level of ammonium left in the culture medium, i.e. between 5 and 10%.

It can be seen from Figure 3.3.9b that the depletion of nitrate from the nutrient medium was similar to that of ammonium. Considerable amounts of nitrate were absorbed over the first two weeks, in this case 71%. Significant levels (20-30%), however, remained in the nutrient medium until the end of the experiment.

### iii) DIOSGENIN ACCUMULATION

The yield of diosgenin/culture is shown in Figure 3.3.10 from which it can be seen that diosgenin did not accumulate during the first three weeks of culture. Accumulation began during the fourth week and remained at between 11 to 21 $\mu$ g/culture for three weeks. There was then a substantial increase in the yield of diosgenin during the seventh week resulting in an average yield of 342 $\mu$ g/culture. The yield remained at this level until the end of the ninth week, after which it fell substantially reaching an average of 142 $\mu$ g/culture by the end of the tenth week.

The yield of diosgenin/gram dry weight of culture is shown in Figures 3.3.6a, 3.3.6b, 3.3.7a and 3.3.7b where it is compared with changes in cell number, P.C.V., dry weight and fresh weight

respectively. It can be seen from these figures that in every case the bulk of the diosgenin accumulated after each of the growth parameters had reached its maximum value.

The yield of diosgenin/cell is described in Figure 3.3.11 from which it can be seen that it is similar in pattern to the yield of diosgenin expressed per culture and per gram dry weight of culture. The initial yield of diosgenin was approximately 0.5pg/cell for the first three weeks of accumulation. This relatively low yield coincided with a period of decline in cell number. The yield however increased substantially to an average of 13pg/cell by the end of the seventh week of culture growth. The yield remained at this level for another two weeks. This period of relatively high diosgenin accumulation occurred during a period when cell number was relatively constant. The fall in yield/cell during the last week of the experiment coincided with a small decline in cell number. No diosgenin was detected in the culture medium at any point in the experiment.

iv) THE RELATIONSHIP BETWEEN NUTRIENT DEPLETION, GROWTH AND DIOSGENIN ACCUMULATION

It can be seen by comparing Figure 3.3.8a with Figures 3.3.6 (a-b) and 3.3.7(a-b) that the nutrient medium was almost devoid of phosphate by the time cell number or any of the other growth parameters studied had reached their maximum values.

The depletion of sucrose in the culture medium is shown in Figure 3.3.8b. When this is compared to Figure 3.3.6a it can be seen that 10-15% of the sucrose remained in the culture medium

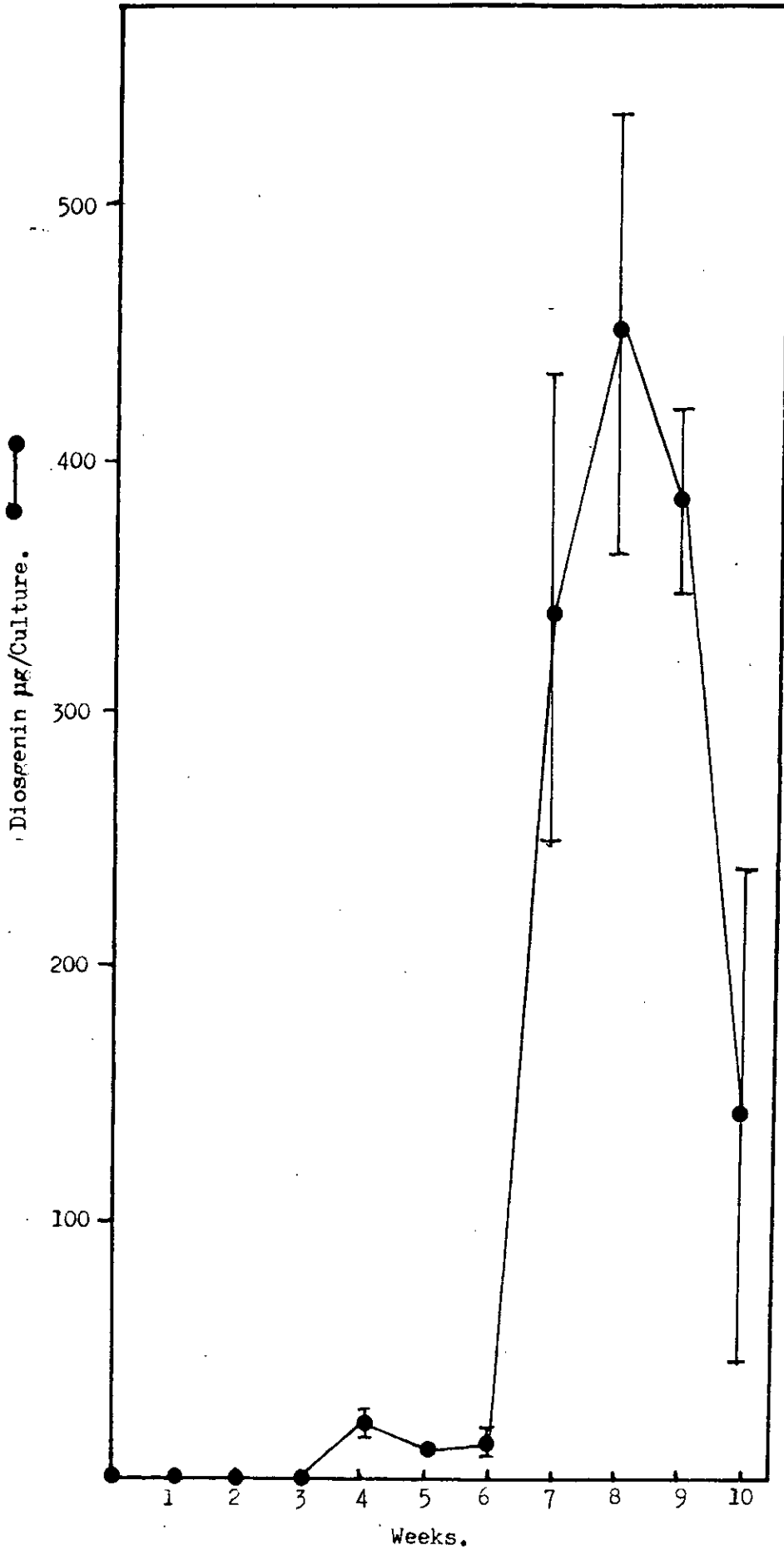


Figure 3.3.10. The yield of diosgenin ( $\mu\text{g}/\text{flask}$ ) in suspension cultures of D. composita.

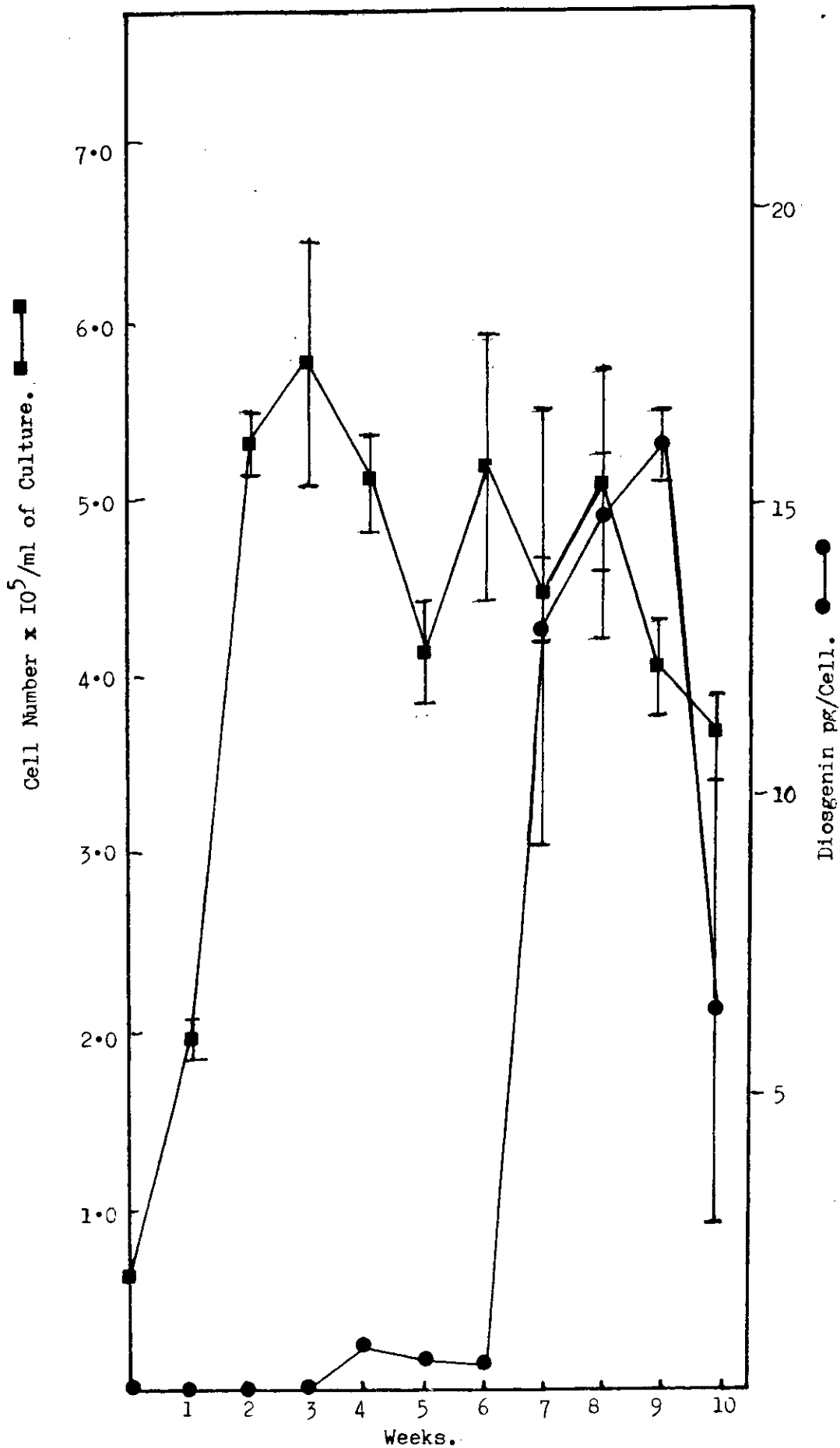


Figure 3.3.II. Cell number and the yield of diosgenin/cell in suspension cultures of *D. composita*.

by the time cell number reached its maximum value. If Figure 3.3.8b is compared to Figures 3.3.6b and 3.3.7(a-b) it can be seen that the increases in packed cell volume, dry weight and fresh weight, respectively, closely followed the decrease in sucrose in the medium and by the time stationary phase had been reached the medium was practically devoid of sucrose.

It can be seen by comparing Figure 3.3.9a with Figures 3.3.6(a-b) and 3.3.7(a-b) that ammonium was never limiting with respect to cell number, packed cell volume, dry weight or fresh weight. Comparison of Figure 3.3.9b with Figures 3.3.6(a-b) and 3.3.7(a-b) reveals that nitrate was also never limiting with respect to any of the growth parameters measured.

It can be seen from Figures 3.3.8(a-b) that the nutrient medium was practically devoid of both phosphate and sucrose before diosgenin began to accumulate. By comparison it can be seen from Figures 3.3.9(a-b) that significant amounts of ammonium and nitrate remained in the nutrient medium during the accumulation of diosgenin.

In summary, it can be seen from the results presented above that as in the previous experiment diosgenin accumulation does not begin until all aspects of cell growth have ceased, i.e. the accumulation occurs during the stationary phase.

It can also be seen that in terms of the major nutrients in the culture medium, nitrogen measured as nitrate and ammonium, never became limiting at any time during the experiment. The results suggest, however, that phosphate and carbon, as sucrose, did become limiting, phosphate by the end of the second week<sup>1</sup>

and sucrose by the end of the fifth.

One question which arises from the results of this experiment is whether the eventual yield of diosgenin is limited by the low levels of sucrose in the nutrient medium during stationary phase, since ultimately this is the source of carbon for steroid biosynthesis.

SECTION 3 - PART 3

THE EFFECT OF SUCROSE AND PLANT GROWTH  
SUBSTANCES ON GROWTH AND DIOSGENIN  
ACCUMULATION OF STATIONARY PHASE  
D. COMPOSITA BATCH SUSPENSION CULTURES

In the previous two parts of this Section, it has been shown that growth and diosgenin accumulation in batch suspension cultures of D. composita are separated, in time, in such a way that diosgenin does not begin accumulating until stationary phase. It has also been shown that by this time the level of sucrose remaining in the culture medium is extremely low.

The aim of the experiment described in this part of Section 3 was to investigate if the low level of sucrose in the culture medium during stationary phase was affecting the accumulation of diosgenin. Such a situation was a possibility since the carbon skeleton of diosgenin is derived from sucrose via the isoprenoid pathway.

The results in this part of Section 3 describe the effects on culture growth and diosgenin accumulation of adding sucrose to stationary phase batch suspension cultures of D. composita and how the effects were modified by plant growth substances. The plant growth substances used were 2,4-D and kinetin, which along with sucrose were added singly or in various combinations as set out in Table 3.3.1. The final concentrations of these compounds are set out in Table 3.3.2.

The experiment was carried out using 40, 60 ml suspension cultures prepared as described in Section 1 (d) of Chapter 2. The cultures were allowed to grow for 5 weeks, after which a sterile 5ml solution of each of the treatments listed in Table 3.3.1 was added to each of 5 replicates. The control (treatment 1) consisted of distilled water. The cultures were then grown for another 4 weeks. At the end of the

TABLE 3.3.1

THE COMBINATIONS OF SUCROSE AND PLANT GROWTH SUBSTANCES  
USED IN THE STATIONARY PHASE ADDITION EXPERIMENTS

TREATMENT	ADDITIONS		
	SUCROSE	2,4-D	KINETIN
1	-	-	-
2	-	-	+
3	-	+	-
4	-	+	+
5	+	+	+
6	+	+	-
7	+	-	+
8	+	-	-

TABLE 3.3.2

THE FINAL CONCENTRATIONS OF SUCROSE AND PLANT GROWTH  
SUBSTANCES USED IN THE STATIONARY PHASE ADDITION EXPERIMENTS

ADDITIONS	FINAL CONCENTRATIONS (MOLARITY)
SUCROSE	$7.3 \times 10^{-2} \text{M}$
2,4-D	$1.0 \times 10^{-6} \text{M}$
KINETIN	$1.0 \times 10^{-5} \text{M}$

experiment, cell number, packed cell volume, dry weight, fresh weight and the amount of diosgenin accumulated were measured as described in Sections 2(a), 2(d) and 2(e) of Chapter 2.

The experiment was designed as a randomised block experiment with the statistical significance of any differences between treatment means being tested by analysis of variance (incorporating a multiple range test). These operations were carried out as described by Parker (1973).

i) THE EFFECTS ON CULTURE GROWTH

The effects of the various treatments on cell number and packed cell volume are shown in Figure 3.3.12(a) and Figure 3.3.12(b). It can be seen that the various treatments described in Tables 3.3.1 and 3.3.2 produced values which on average differed from those of the control. The analysis of variance data, however, presented in Table 3.3.11 and 3.3.12 of the Appendix show that no treatments actually differed significantly from the control value.

The effects of the various treatments on dry weight are shown in Figure 3.3.13(a). It can be seen that treatments 5, 6, 7 and 8 produced a marked increase in dry weight compared to the average control value of 524mg/culture. Sucrose alone (treatment 8) produced a dry weight of 1010mg/culture while sucrose and kinetin (treatment 7) produced a value of 1020mg/culture. Sucrose and 2,4-D (treatment 6) produced a value of 960mg/culture while sucrose and both plant growth substances together (treatment

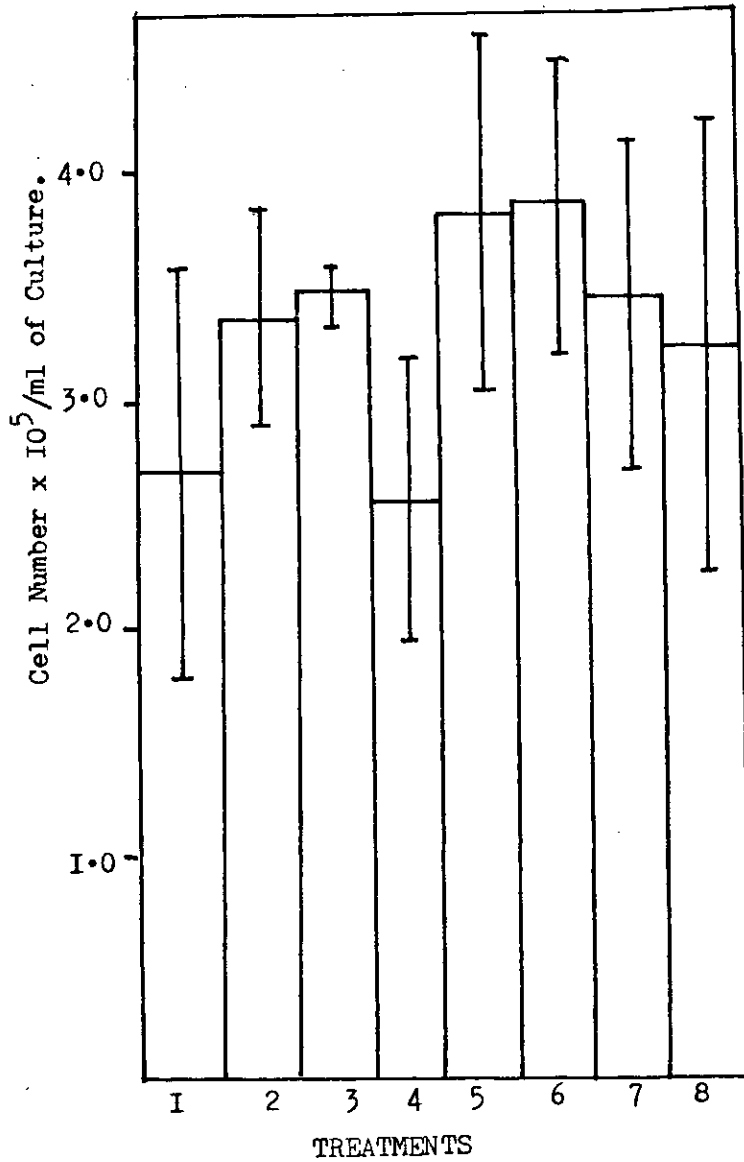


Figure 3.3.12(a). The effect of sucrose and plant growth substances on cell number of stationary phase suspension cultures of D. composita. The bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

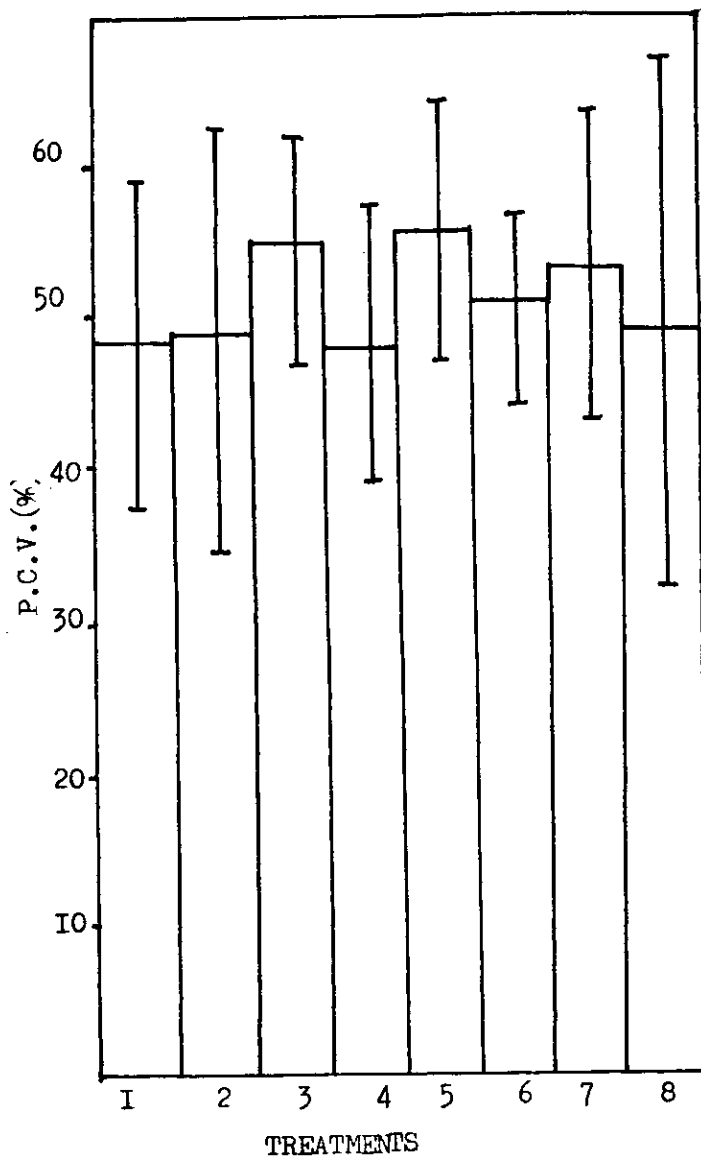


Figure 3.3.I2(b). The effect of sucrose and plant growth substances on P.C.V. of stationary phase suspension cultures of stationary phase suspension cultures of *D. composita*. The bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

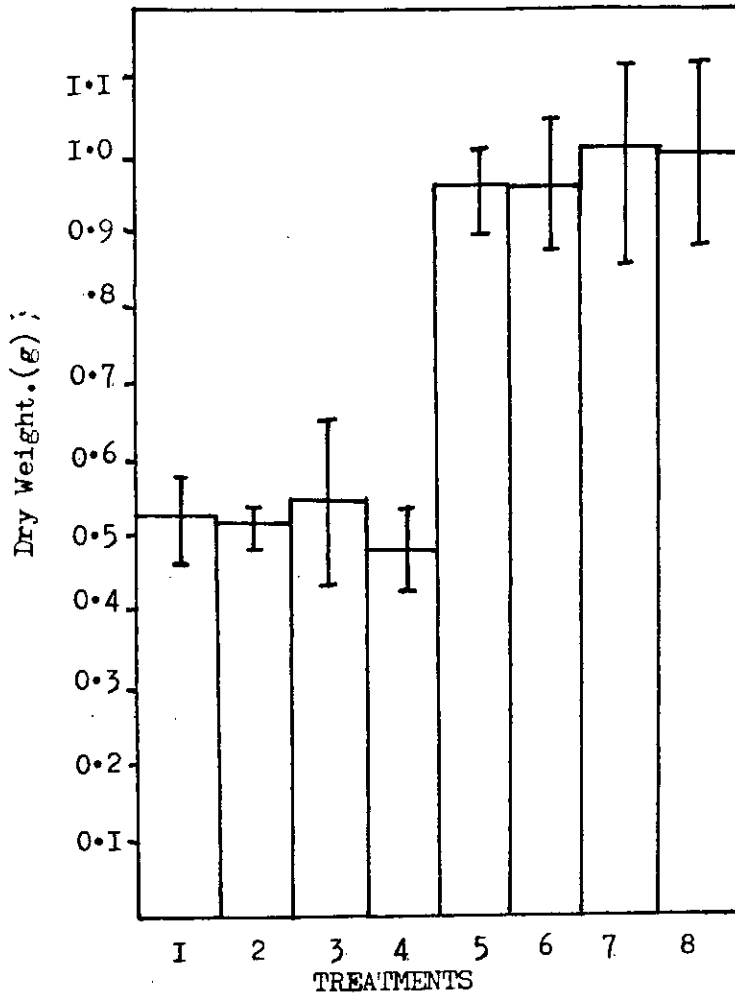


Figure 3.3.13(a). The effect of sucrose and plant growth substances on dry weight of stationary phase suspension cultures of *D. composita*. The bars represent standard error (95% confidence limits).

The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

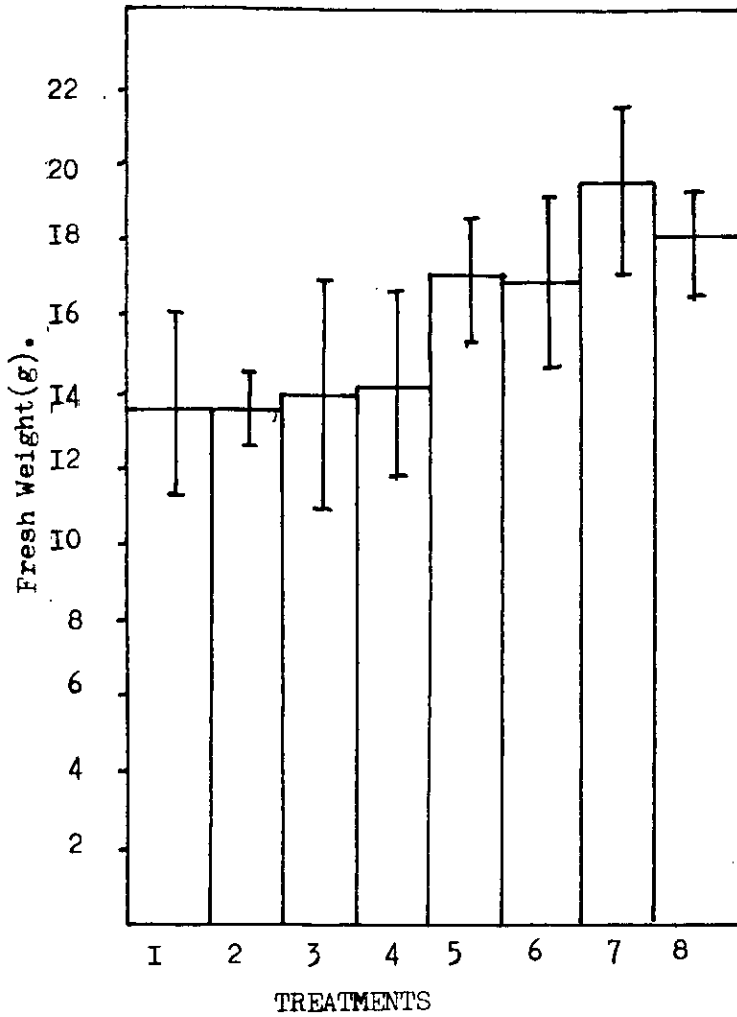


Figure 3.3.13(b). The effect of sucrose and plant growth on fresh weight of stationary phase suspension cultures of *D. composita*. The bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

5) produced an average dry weight of 958mg/culture. The three other treatments (2,3 and 4) varied little from the control value. The analysis of variance data presented in Table 3.3.13 of the Appendix confirms that treatments 5-8 differed significantly from the control and treatments 2-4.

The effects of the various treatments on fresh weight are shown in Figure 3.3.13(b). It can be seen that, as with dry weight, treatments 2-4 varied little in terms of fresh weight from the control value of 13.7g/culture while treatments 5-8 all showed an increase in average fresh weight/culture. Sucrose (treatment 8) produced a fresh weight of 17.9g/culture while sucrose and kinetin (treatment 7) produced 19.4g/culture. Sucrose and 2,4-D (treatment 6) produced 16.8g/culture while sucrose and both plant-growth substances together (treatment 5) resulted in a fresh weight of 17.0g/culture. Again the analysis of variance data presented in Table 3.3.14 of the Appendix confirms that the differences between treatments 5-8 and the control and treatments 2-4 were statistically significant.

Overall it can be seen that the addition of sucrose and plant growth substances to stationary phase suspension cultures of D.composita did cause some changes in the growth of the cultures. It did not however, affect all of the growth parameters studied.

Sucrose, 2,4-D and kinetin added singly or together in the combinations and concentrations shown in Tables 3.3.1 and 3.3.2 caused no detectable changes in the cell number or packed cell volume of the cultures when compared to the control. A significant effect was detected however in dry and fresh weight where

the addition of sucrose resulted in an almost doubling of dry weight and a significant increase in fresh weight. In neither case was the increase modified in any way by the addition of 2,4-D or kinetin singly or together along with sucrose. This suggests that the effect in both cases was entirely due to sucrose. The two plant growth substances also caused no detectable changes in dry weight or fresh weight when added singly or together in the absence of sucrose.

ii) THE EFFECTS ON DIOSGENIN ACCUMULATION

The effects of the various treatments on diosgenin accumulation are shown in Figures 3.3.14(a), 3.3.14(b) and 3.3.14(c), where the yield is expressed as  $\mu\text{g/g}$  culture dry weight,  $\mu\text{g/culture}$  and  $\text{pg/cell}$  respectively. It can be seen from these that there are differences in the average diosgenin yield of the control and the various treatments described in Tables 3.3.1 and 3.3.2. The analysis of variance data, however, presented in Tables 3.3.15, 3.3.16 and 3.3.17 of the Appendix shows that there were no statistically significant differences in the yield of diosgenin between the control (treatment 1) and the other treatments regardless of whether the yield is expressed as  $\mu\text{g/g}$  culture dry weight,  $\mu\text{g/culture}$  or  $\text{pg/cell}$ .

Overall it can be seen that although sucrose alone caused increases in dry and fresh weight when added to stationary phase suspension cultures of D.composita, the results suggest that neither this or any other treatment involving 2,4-D or kinetin, produces any significant effect on the yield of

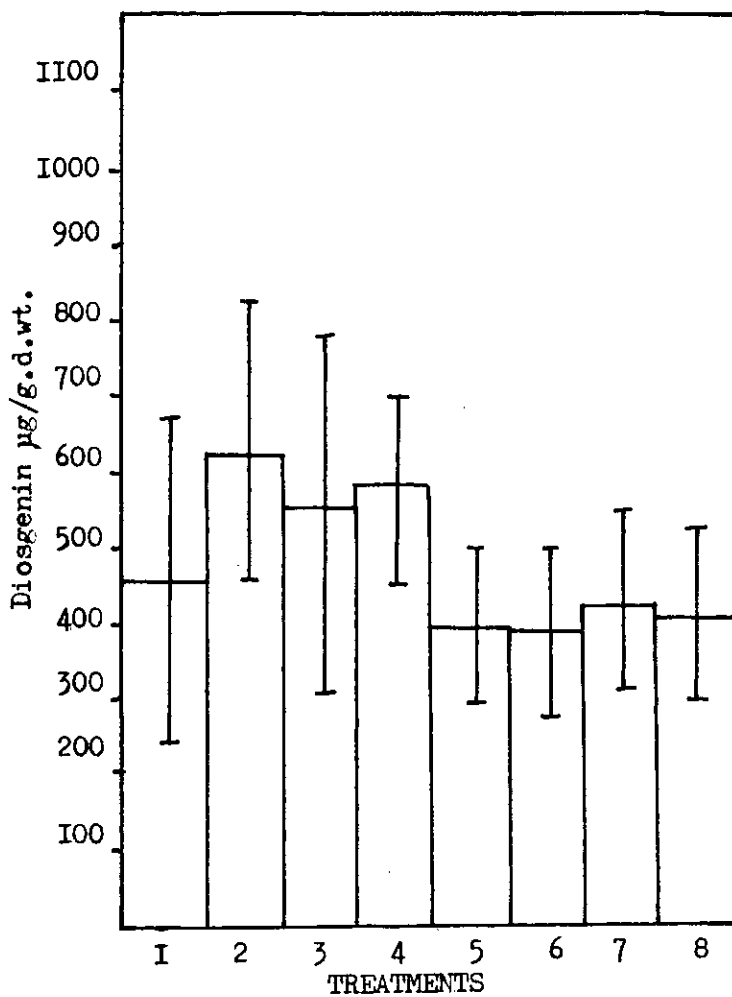


Figure 3.3.14(a). The effect of sucrose and plant substances on diosgenin accumulation ( $\mu\text{g/g}$  dry weight) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

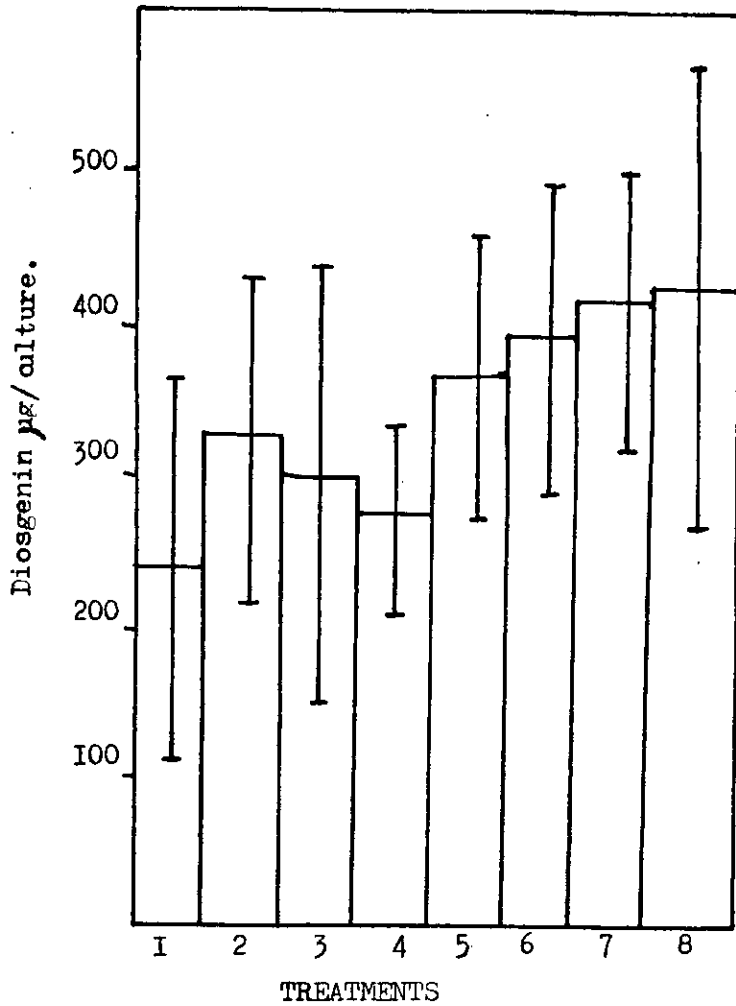


Figure 3.3.14(b). The effect of sucrose and plant growth substances on diosgenin accumulation ( $\mu\text{g/g}$  culture) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

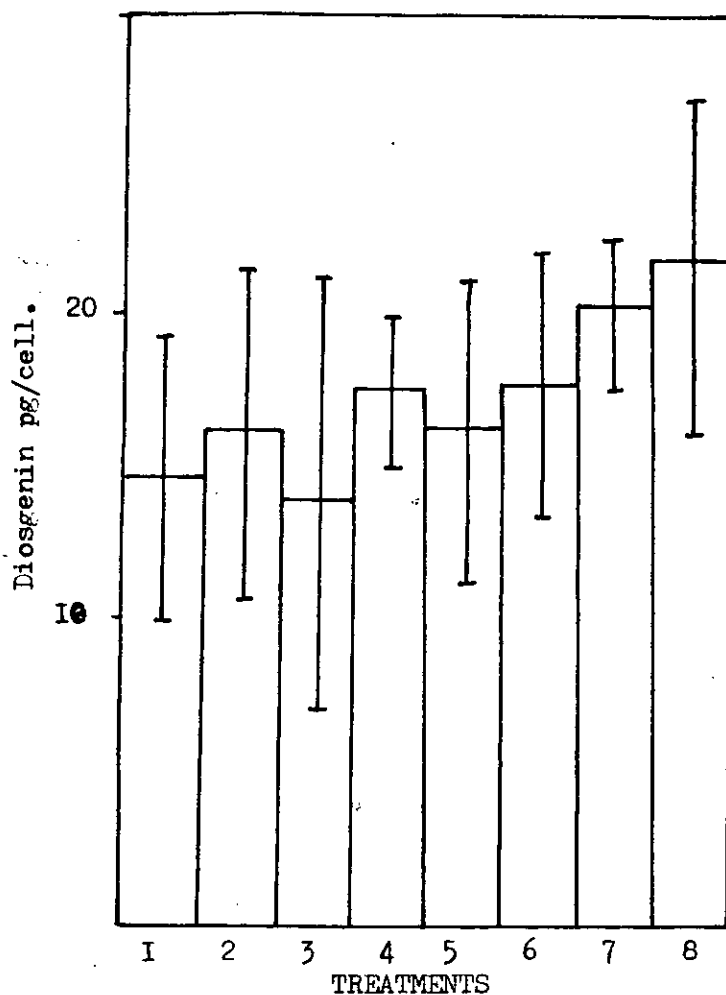


Figure 3.3.I4(c) The effect of sucrose and plant growth substances on diosgenin accumulation (pg/cell) of stationary phase suspension cultures of *D. composita*. The bars represent standard error (95% confidence limits.) The treatment numbers refer to those described in Tables 3.3.1. and 3.3.2.

diosgenin regardless of whether it was on a per gram dry weight, per culture or per cell basis.

If, as the above results suggest, that the main effect of adding sucrose to the stationary phase suspension cultures was to increase dry weight then the two most probable explanations would either be that sucrose was absorbed from the culture medium and stored or it was utilised for some aspect of cell wall synthesis. Although both alternatives would mean that the added sucrose was not utilised by secondary metabolism only the latter would involve its active utilisation in a primary metabolic pathway. If the added sucrose was metabolised for cell wall synthesis, then the only way such a treatment would be effective in raising the yield of diosgenin would be by preventing it being used by primary metabolism and diverting it to secondary metabolism. The following part of this section describes the use of the plant growth regulator, abscisic acid in an attempt to inhibit the primary metabolism of stationary phase suspension cultures of D.composita and thus make added sucrose available to secondary metabolic processes such as diosgenin synthesis.

S E C T I O N    3    -    P A R T    4

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THE EFFECT OF ABSCISIC ACID AND SUCROSE  
ON GROWTH AND DIOSGENIN ACCUMULATION OF  
STATIONARY PHASE D. COMPOSITA BATCH  
SUSPENSION CULTURES

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In the previous part of this Section, it was shown that the addition of sucrose to stationary phase suspension cultures of D. composita resulted in a doubling of culture dry weight and an increase in fresh weight. It had no stimulatory effect on the yield of diosgenin despite being added at a time when previous experiments had shown diosgenin started to accumulate. If the accumulation of dry matter, as suggested, was due to utilisation of the sucrose for cell wall synthesis, then a successful enhancement of the yield of diosgenin would only be possible if the sucrose was diverted away from primary metabolic processes such as cell wall synthesis to secondary processes such as diosgenin synthesis.

The plant growth regulator abscisic acid has been implicated in the induction of dormancy in higher plants which physiologically represents an inhibition or slowing down of primary metabolic processes. The aim of the experiment discussed in this part of Section 3 was to use abscisic acid to inhibit the primary metabolism of stationary phase suspension cultures of D. composita and thus allow added sucrose to be utilised for diosgenin synthesis.

The effect of abscisic acid was studied at two concentrations in the culture medium, these were  $1 \times 10^{-5}$  M and  $1 \times 10^{-7}$  M. It was added singly and along with sucrose (final concentration, 1.5g/culture) in the combinations shown in table 3.3. 3. The experiment was carried out using 30, 60ml suspension cultures prepared as described in Section 1 d of Chapter 2. The cultures were allowed to grow for five weeks, after which  
a/

TABLE 3.3.3

THE COMBINATIONS OF SUCROSE AND ABSCISIC ACID  
USED IN THE STATIONARY PHASE ADDITION EXPERIMENT

TREATMENT	ADDITIONS		
	SUCROSE	ABSCISIC ACID ( $10^{-5}$ M)	ABSCISIC ACID ( $10^{-7}$ M)
1	-	-	-
2	+	-	-
3	-	+	-
4	-	-	+
5	+	+	-
6	+	-	+

TABLE 3.3.4

THE COMBINATIONS OF SUCROSE AND RHAMNOSE USED  
IN THE STATIONARY PHASE ADDITION EXPERIMENT

TREATMENT	ADDITIONS	
	SUCROSE	RHAMNOSE
1	-	-
2	+	-
3	-	+
4	+	+

a sterile 5ml solution of each of the treatments listed in Table 3.3.3 was added to each of 5 replicate cultures. The control (treatment 1) consisted of distilled water. The cultures were then grown for another four weeks. At the end of the experiment, cell number, packed cell volume, dry weight, fresh weight and the amount of diosgenin accumulated were measured as described in Sections 2a, 2c, 2d and 2e of Chapter 2.

This experiment and the following one described in Part 5 of Section 3 were carried out together in the same randomised block experiment using the same distilled water and sucrose controls. The statistical significance of any differences between treatment means was tested using analysis of variance (incorporating a multiple range test) as described by Parker (1973).

i) THE EFFECTS ON CULTURE GROWTH

The effects of the various treatments described in Table 3.3.3 on cell number and packed cell volume are shown in Figure 3.3.15 (a) and Figure 3.3.15(b). As in the previous experiment, the analysis of variance data presented in Tables 3.3.18 and 3.3.19 of the Appendix shows that there were no statistically significant differences between any of the treatments and the controls with respect to the above growth parameters.

The effects of the various treatments on dry weight are shown in Figure 3.3.16(a). It can be seen that treatments 2, 5 and 6 produced substantial increases in the average dry weight

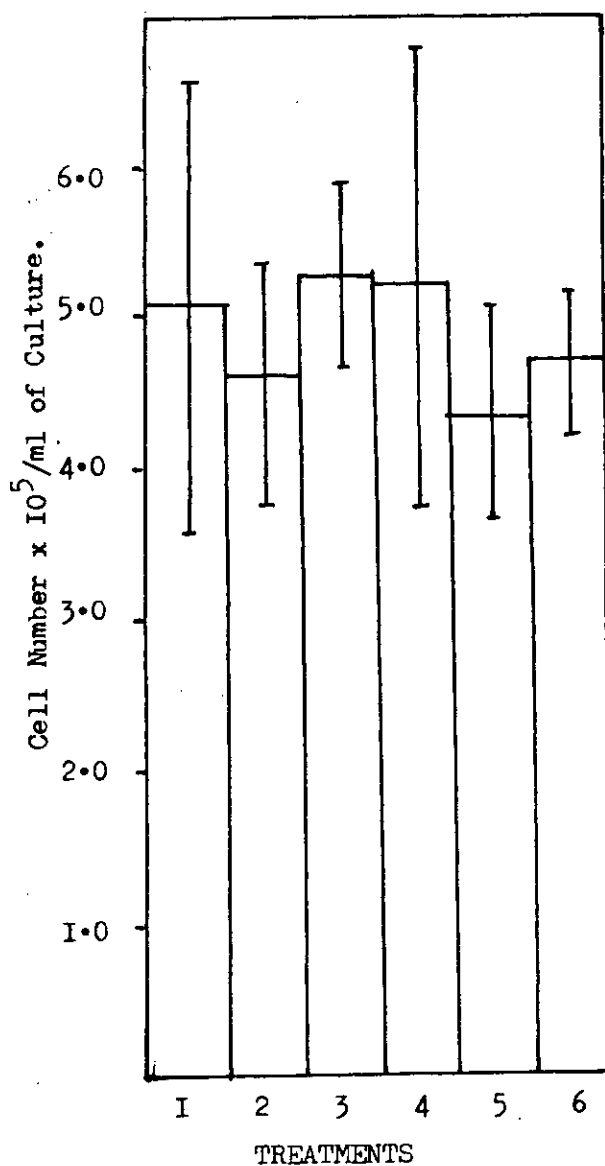


Figure 3.3.15(a). The effect of abscisic acid and sucrose on cell number of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

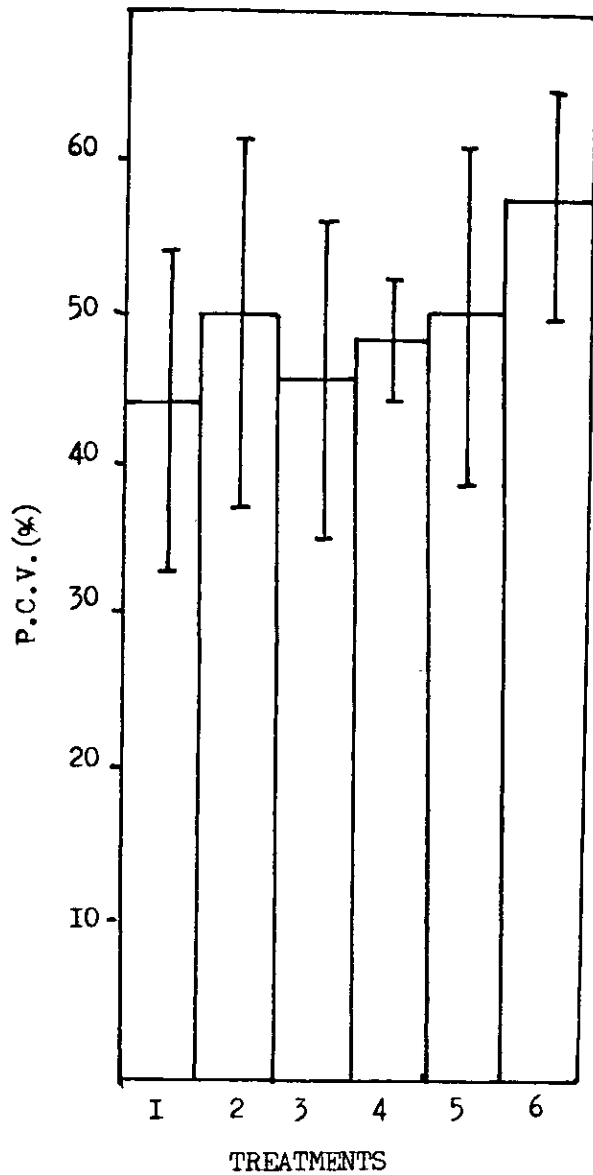


Figure 3.3.15(b). The effect of abscisic acid and sucrose on P.C.V. of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

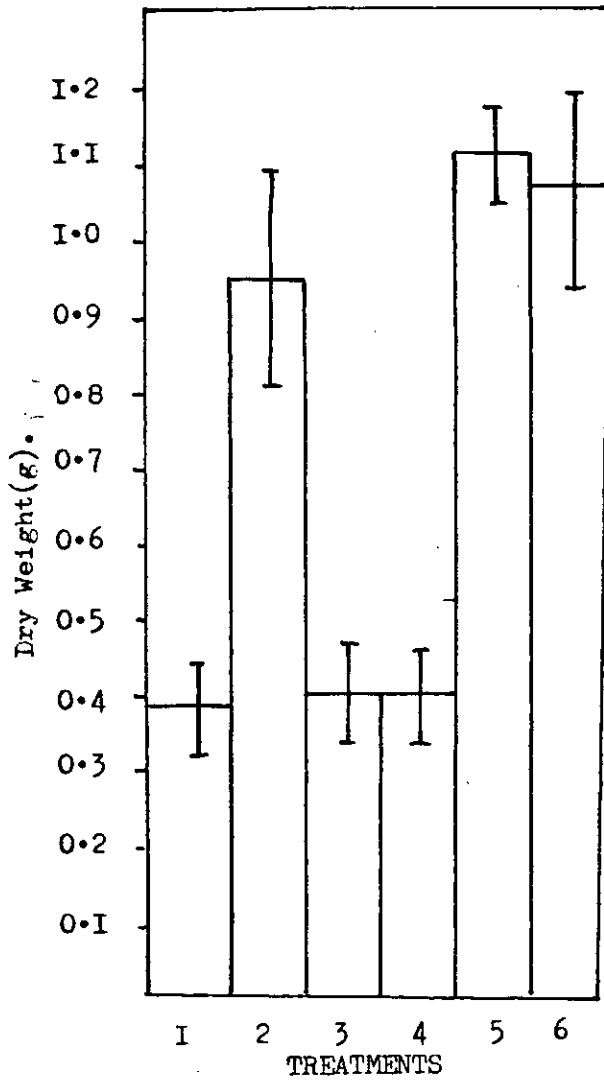


Figure 3.3.16(a) The effect of abscisic acid and sucrose on dry weight of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

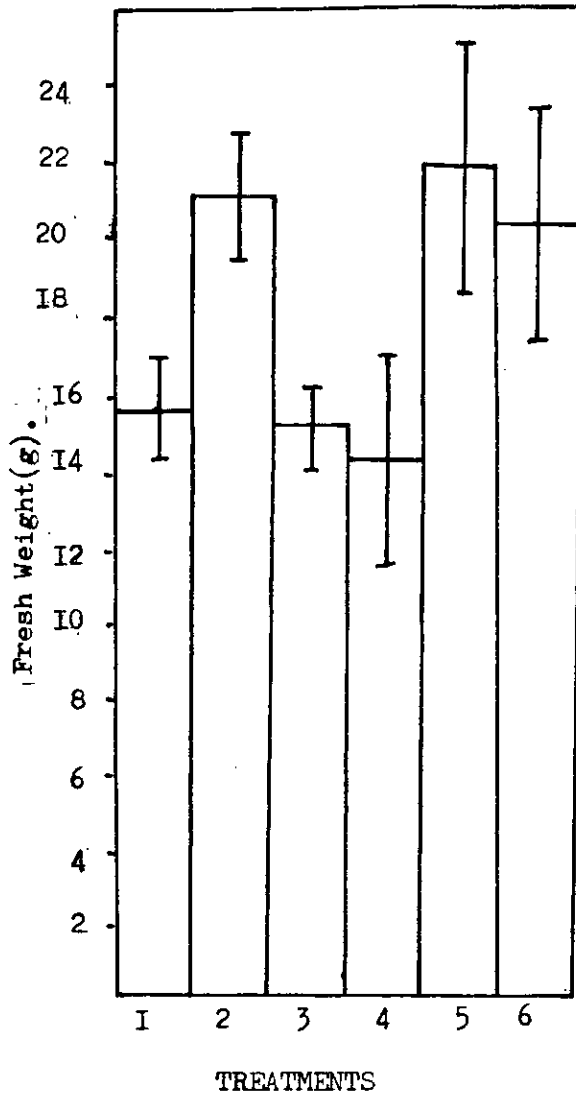


Figure 3.3.16(b). The effect of abscisic acid and sucrose on fresh weight of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

compared with the distilled water control value of 0.394g/culture. The sucrose control (treatment 2) produced a dry weight of 0.958g. The sucrose/abscisic acid at  $1 \times 10^{-5} \text{M}$  (treatment 5) raised the average dry weight to 1.106g/culture while the sucrose/abscisic acid at  $1 \times 10^{-7} \text{M}$  gave a value of 1.084g/culture. The two abscisic acid treatments (3,4) varied little from the distilled water control value (No.1). The analysis of variance data presented in Table 3.3.20 of the Appendix shows that the differences between treatments 2, 5 and 6 and the distilled water control were statistically significant. It also shows that the values for treatments 5 and 6 were significantly larger than the sucrose control (treatment 2).

The effects of the various treatments on fresh weight are shown in Figure 3.3.16(b). It can be seen that treatments 2, 5 and 6 once again resulted in substantial increases in fresh weight when compared to the distilled water control of 15.7g. The sucrose control (treatment 2) gave an average fresh weight of 21.2g. The sucrose/abscisic acid at  $1 \times 10^{-5} \text{M}$  (treatment 5) resulted in a fresh weight of 22.9g while the sucrose/abscisic acid at  $1 \times 10^{-7} \text{M}$  gave 20.6g. Treatments 3 and 4 varied little from the distilled water control. The statistical significance of the differences between treatments 2, 5 and 6 and the distilled water control are presented in the analysis of variance data in Table 3.3.21 of the Appendix.

Overall the above results confirm the previous findings that the addition of sucrose to stationary phase suspension cultures of D.composita results in a doubling of dry weight, an increase

in fresh weight and has no effect on cell number or packed cell volume. The results also show that abscisic acid, at the two concentrations studied, had no effect at all on the growth of the cultures.

However, when abscisic acid was added with sucrose it resulted in an even greater increase in dry weight than with sucrose alone. This effect was not found in the fresh weight values. This suggests that abscisic acid, at both concentrations studied, can interact with sucrose to produce larger increases in dry weight than would be found with sucrose alone.

#### ii) THE EFFECTS ON DIOSGENIN ACCUMULATION

The effects of the various treatments described in Table 3.3.3 on diosgenin accumulation are shown in Figures 3.3.17(a), 3.3.17(b) and 3.3.17(c). Where the yield is expressed at  $\mu\text{g/g}$  culture dry weight,  $\mu\text{g/culture}$  and  $\text{pg/cell}$  respectively.

It can be seen from Figure 3.3.17(a) that all the treatments (2-6) resulted in yields of diosgenin which were lower than the distilled water control value of  $876\mu\text{g/g}$  dry weight. The analysis of variance data presented in Table 3.3.22 of the Appendix however shows that only treatments 2,5 and 6 were significantly different from the distilled water control (treatment 1). The sucrose control (treatment 2) gave a yield of  $490\mu\text{g/g}$  dry weight. The sucrose/abscisic acid at  $1 \times 10^{-5}\text{M}$  (treatment 5) gave a yield of  $547\mu\text{g/g}$  dry weight while the sucrose/abscisic acid at  $1 \times 10^{-7}\text{M}$  (treatment 6) gave a yield of  $464\mu\text{g/g}$  dry weight.

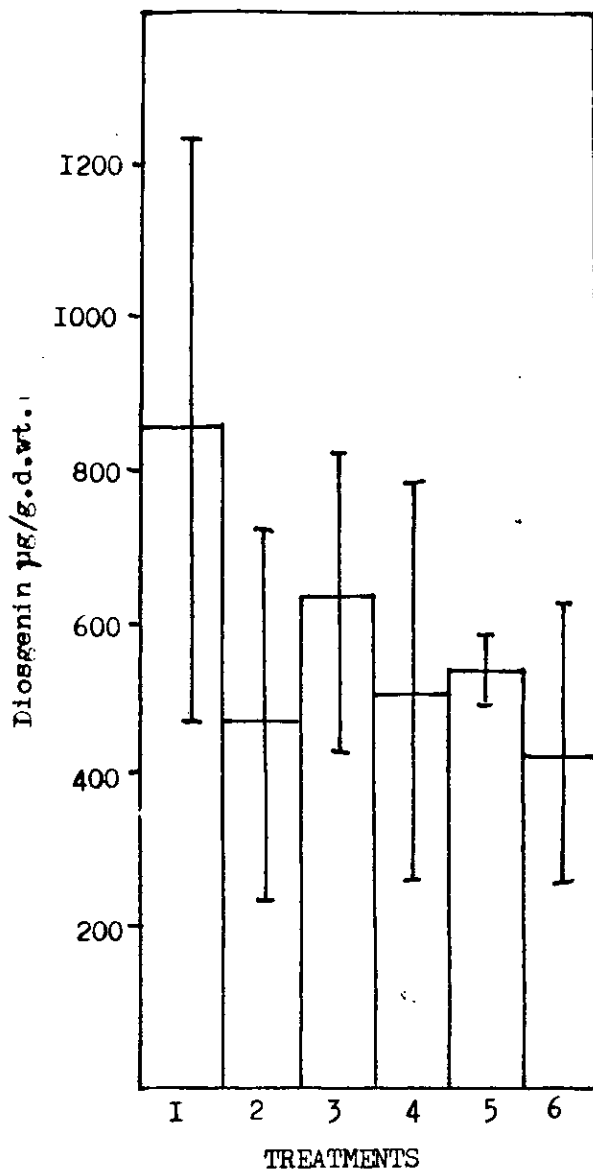


Figure 3.3.17(a). The effect of abscisic acid and sucrose on diosgenin accumulation ( $\mu\text{g/g d.wt.}$ ) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

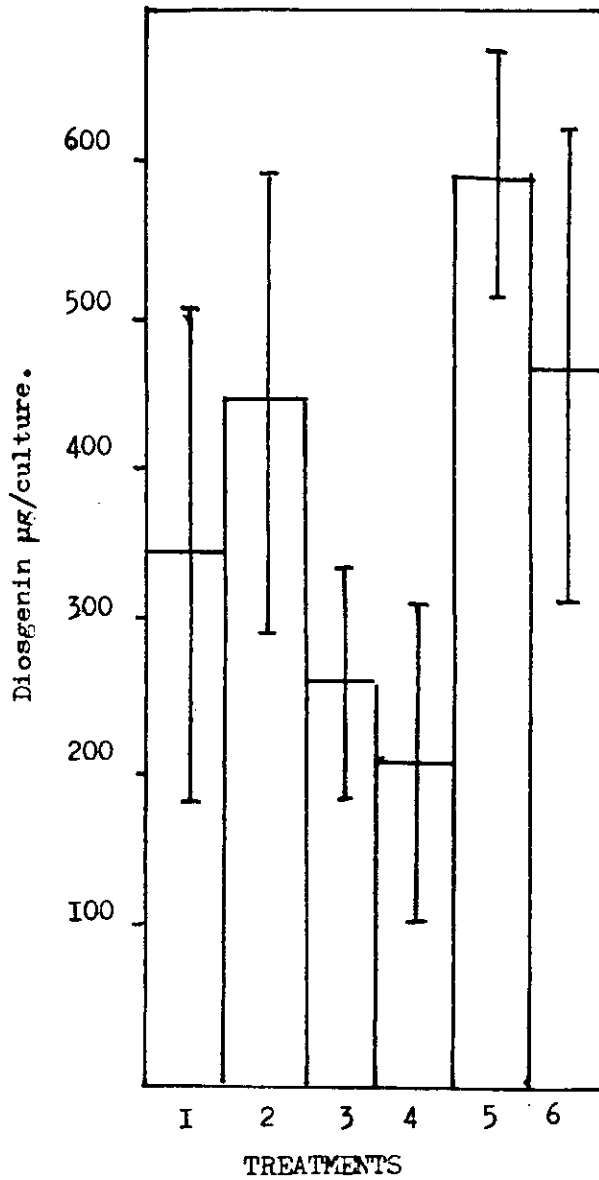


Figure 3.3.I7(b). The effect of abscisic acid and sucrose on diosgenin accumulation ( $\mu\text{g}/\text{culture}$ ) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

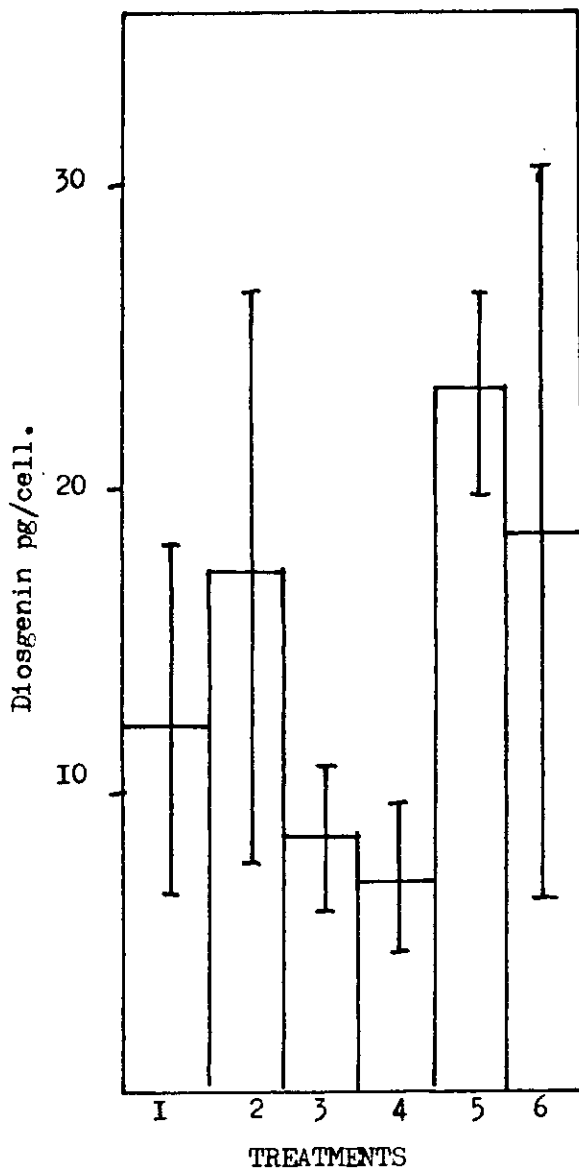


Figure 3.3.17(c). The effect of abscisic acid and sucrose on diosgenin accumulation (pg/cell) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.3.

The effects of the various treatments on diosgenin yield/culture are presented in Figure 3.3.17(b). It can be seen from this that treatments 2, 5 and 6 resulted in a substantial increase in the average yield of diosgenin/culture compared with the distilled water control value (treatment 1) of 347 $\mu$ g/culture. Treatments 3 and 4 produced average yields below that of the distilled water control. The analysis of variance data presented in Table 3.3.23 of the Appendix, however, shows that only treatment 5 was significantly different from the distilled water control. This treatment which consisted of sucrose/abscisic acid at  $1 \times 10^{-5}$  M produced a yield of 601 $\mu$ g/culture.

The effects of the various treatments on diosgenin yield/cell are presented in Figure 3.3.17(c). It can be seen from this that again treatments 2, 5 and 6 have resulted in increases in the yield of diosgenin when compared to the distilled water control value of 12pg/cell. Once again however the analysis of variance data presented in Table 3.3.24 of the Appendix shows that only the value for treatment 5 was significantly different from the distilled water control. This treatment resulted in a yield of 23pg/cell.

It can be seen from the above results that sucrose and abscisic acid at  $1 \times 10^{-5}$  M almost doubled the yield of diosgenin/cell when added to stationary phase suspension cultures of D.composita. Neither sucrose nor abscisic acid (at  $1 \times 10^{-5}$  M or  $1 \times 10^{-7}$  M) produced such an enhancement of yield when added singly. Sucrose and abscisic acid at  $1 \times 10^{-7}$  M added together also failed to increase the yield of diosgenin/cell. A similar

pattern was found when the yield diosgenin/culture was calculated. This would be expected, however, since cell number/culture did not change during the experiment. When the relative yield of diosgenin/g culture dry weight is considered it can be seen that the yields for the sucrose control and both sucrose/abscisic acid treatments were significantly lower than the distilled water control. This is consistent, however, with the large increases in dry weight recorded for these treatments. The decrease is in fact less than would be expected due to the almost doubling of the diosgenin yield on a per cell basis.

Overall, therefore, it can be seen that not only can abscisic acid interact with sucrose to bring about changes in dry and fresh weight when added to stationary phase suspension cultures of D.composita, they can also interact to increase the absolute yield of diosgenin. The results in fact suggest that abscisic acid interacted with sucrose in such a way that both primary and secondary metabolism were stimulated. The vast majority of available carbon however was still utilised by primary metabolic processes.

Both this experiment and the previous one were both designed to enhance diosgenin accumulation by channelling precursor into secondary metabolism. The accumulation of most secondary metabolites is the result of a balance between synthesis and degradation. The accumulation of diosgenin depends on storage as a glycoside. It is therefore possible, if any of the material for the glycosylation reactions was limiting, then any diosgenin synthesised and not immediately converted to its glycoside could be degraded.

The following part of this Section describes an experiment which investigates the effect on diosgenin accumulation of adding one of the sugars involved in the glycosylation of diosgenin, rhamnose, to stationary phase suspension cultures of D.composita.

S E C T I O N    3    -    P A R T    5

THE EFFECT OF RHAMNOSE AND SUCROSE ON  
GROWTH AND DIOSGENIN ACCUMULATION OF  
STATIONARY PHASE D. COMPOSITA BATCH  
SUSPENSION CULTURES

It was suggested at the end of the previous part of this Section that if the availability of substances involved in the glycosylation of diosgenin were limiting, then any diosgenin not immediately converted to glycosides such as dioscin, may be liable to degradation which in turn would reduce accumulation.

The sugars involved in the glycosylation reactions which produce dioscin are glucose and rhamnose, one glucose and two rhamnose molecules for every diosgenin molecule. It is very unlikely that the level of such an ubiquitous metabolite such as glucose would ever be limited enough to affect the glycosylation of diosgenin. Rhamnose on the other hand is a relatively uncommon sugar in the plant cell and is only found in small quantities in pectin and hemicellulose.

The results presented in this part of Section 3 describe the effects on culture growth and diosgenin accumulation of adding rhamnose and sucrose to stationary phase batch suspension cultures of D. composita.

The rhamnose and sucrose were added singly and together at levels which produced final concentrations of 300 mg/culture and 1.5g/culture respectively. The various combinations are described in Table 3.3.4 . The experiment was carried out using ten 60 ml suspension cultures prepared as described in section 1d of Chapter 2. Only ten flasks were required, since, as previously described, this experiment was carried out as a part of the same randomised block experiment which contained the abscisic acid experiment described in part 4 of this section, therefore it shared the same distilled water and sucrose/

sucrose controls.

The cultures were grown for five weeks, after which a sterile 5ml solution of each treatment listed in Table 3.3.4 was added to each of 5 replicate cultures. The cultures were then grown on for 4 weeks. At the end of the experiment, cell number, packed cell volume, dry weight, fresh weight and the amount of diosgenin accumulated were measured as described in Sections 2a, 2c, 2d and 2e of Chapter 2. The statistical significance of any differences between treatment means was as for the previous experiment, tested using analysis of variance (incorporating a multiple range test) as described by Parker, (1973).

i) THE EFFECTS ON CULTURE GROWTH

The effects of the various treatments described in Table 3.3.4 on cell number are shown in Figure 3.3.18(a). It can be seen from this that both the addition of rhamnose (treatment 3) and sucrose/rhamnose (treatment 4) resulted in a substantial decrease in the average cell number when compared to both the distilled water control ( $5.11 \times 10^5$  cells/ml of culture) and the sucrose control ( $4.62 \times 10^5$  cells/ml of culture). The analysis of variance data presented in Table 3.3.18 of the Appendix confirmed that the value for the rhamnose treatment (No.3) of  $0.25 \times 10^5$  cells/ml of culture and the sucrose/rhamnose treatment (No.4) of  $2.1 \times 10^5$  cells/ml of culture were both significantly different from the control values. The difference in cell number between treatments 3 and 4 were also statistically significant.

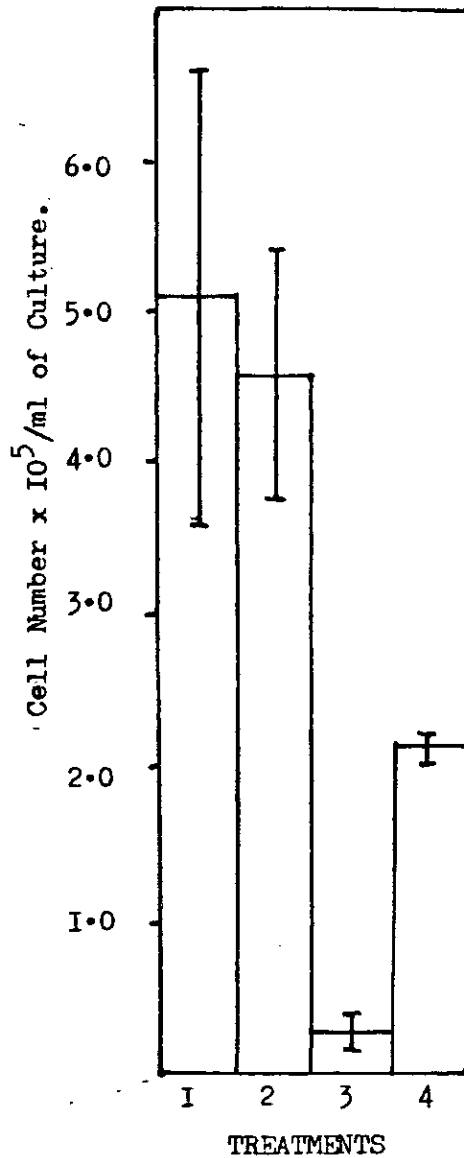


Figure 3.3.18(a). The effect of rhamnose and sucrose on cell number of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95%-confidence limits). The treatment numbers refer to those described in Table 3.3.4.

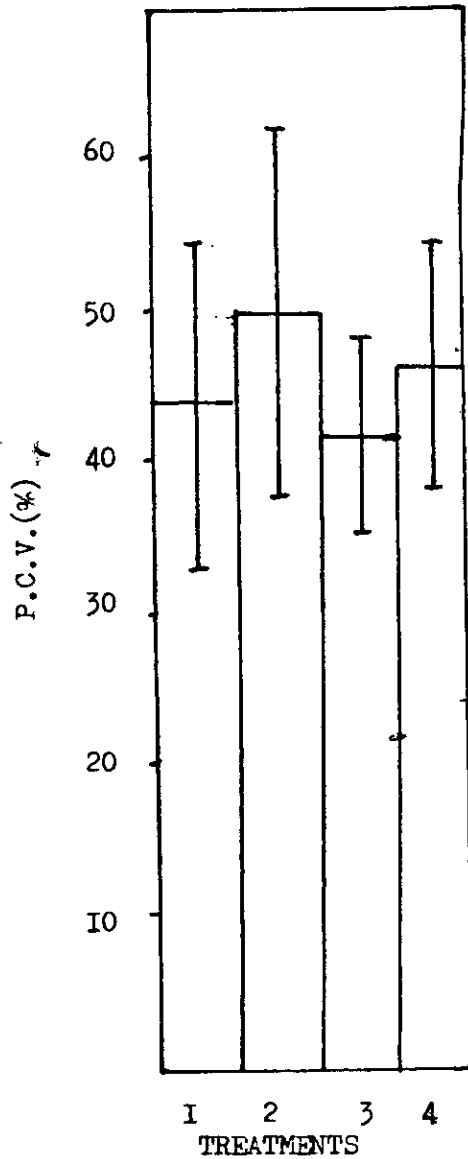


Figure 3.3.18(b). The effect of rhamnose and sucrose on the P.C.V. of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.4.

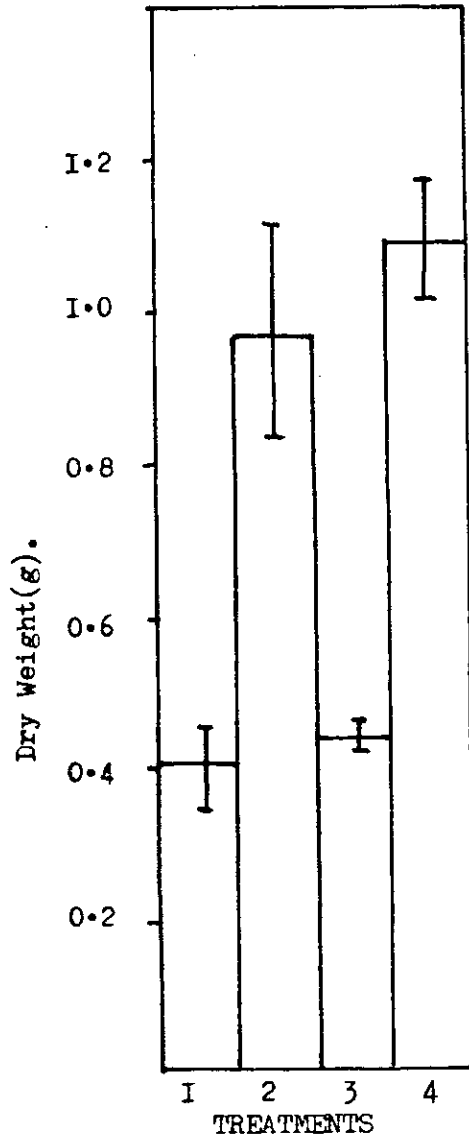


Figure 3.3.19(a). The effect of rhamnose and sucrose on dry weight of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% - confidence limits). The treatment numbers refer to those described in Table 3.3.4.

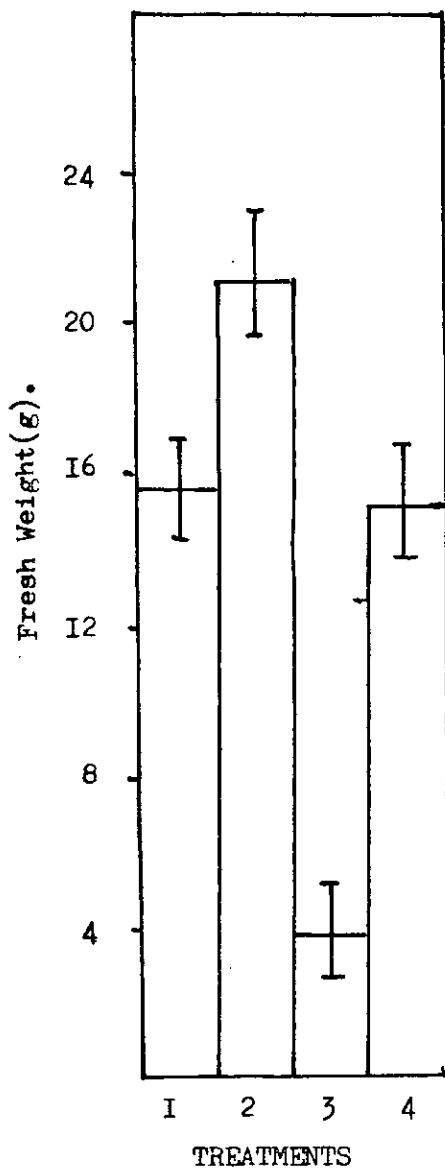


Figure 3.3.19(b). The effect of rhamnose and sucrose on fresh weight of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% - confidence limits). The treatment numbers refer to those described in Table 3.3.4.

It can be seen from Figure 3.3.18(b) that the various treatments had little effect on packed cell volume. This was confirmed by the analysis of variance data presented in Table 3.3.19 of the Appendix.

The effects of the various treatments on dry weight are shown in Figure 3.3.19(a). It can be seen from this that the addition of sucrose (treatment 2) and rhamnose/sucrose resulted in a substantial increase in dry weight when compared to the distilled water control value of 0.349g/culture (treatment 1). The addition of rhamnose had little effect. The analysis of variance results presented in Table 3.3.20 of the Appendix shows that the sucrose control value (treatment 2) of 0.958g/culture and the sucrose/rhamnose treatment (No.4) value of 1.076g/culture were both significantly different from the distilled water control. It also showed that the value for treatment 4 was significantly larger than that for treatment 2.

It can be seen from Figure 3.3.19(b) that the addition of sucrose resulted in an increase in fresh weight while rhamnose/sucrose had little effect. Rhamnose added singly reduced fresh weight substantially compared to the distilled water control value of 15.7g. The analysis of variance data presented in Table 3.3.21 of the Appendix confirmed that the above differences were statistically significant.

In summary, the addition of rhamnose to stationary phase cultures of D.composita seems to have toxic effects on certain aspects of growth. It caused a 20-fold decrease in cell number. The addition of sucrose alongside rhamnose while

reducing this effect still resulted in a halving of cell number. Rhamnose also caused a large decrease in fresh weight which was totally reversed if sucrose was added with rhamnose. Other aspects of growth however do not seem to be adversely affected i.e. packed cell volume and dry weight. In fact rhamnose seems to interact with sucrose to increase dry weight to a greater extent than would be found with sucrose alone.

ii) THE EFFECTS ON DIOSGENIN ACCUMULATION

The effects of the various treatments described in Table 3.3.4 on diosgenin accumulation are described in Figures 3.3.20(a), 3.3.20(b) and 3.3.20(c), where the yield is expressed as  $\mu\text{g/g}$  culture dry weight,  $\mu\text{g/culture}$  and  $\text{pg/cell}$  respectively.

It can be seen from Figure 3.3.20(a) that the average yield of diosgenin/g dry weight dropped in all the treatments when compared to the average value of the distilled water control (treatment 1) which was  $876\mu\text{g}$ . The sucrose control (treatment 2) fell to  $490\mu\text{g}$ , the rhamnose treatment (No.3) to  $59\mu\text{g}$  and the sucrose/rhamnose treatment (No.4) to  $165\mu\text{g}$ . The analysis of variance data presented in Table 3.3.22 of the Appendix, however, shows that only treatments 3 and 4 were significantly different from the distilled water control. The difference between treatments 3 and 4 was also statistically significant.

It can be seen from Figure 3.3.20(b) that the addition of rhamnose (treatment 3) and sucrose/rhamnose (treatment 4) both caused a decrease in the average yield of diosgenin/culture.

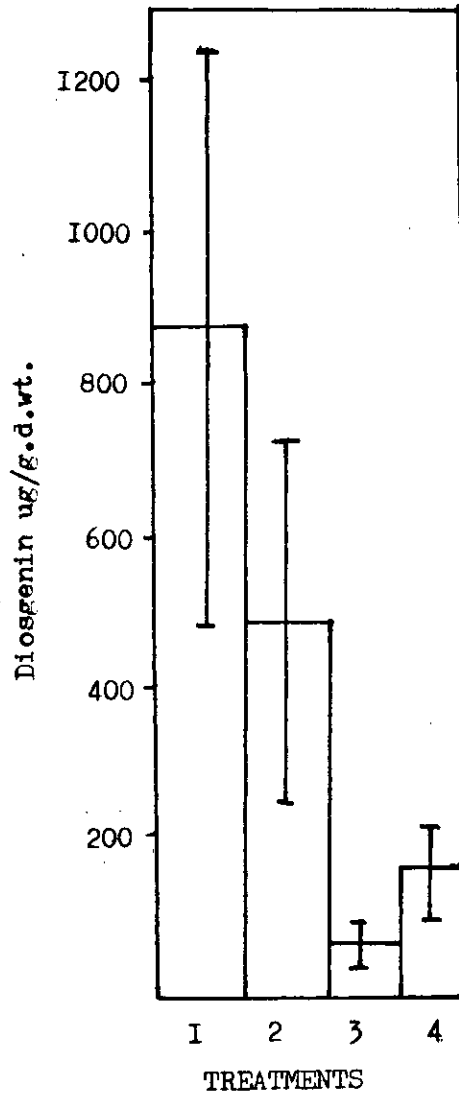


Figure 3.3.20.(a). The effect of rhamnose and sucrose on diosgenin accumulation (mg/g d.wt.) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.4.

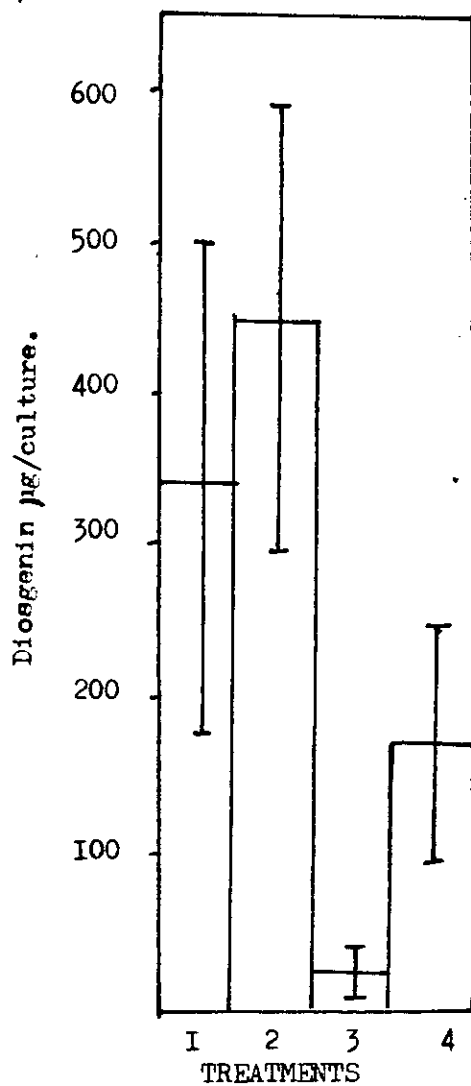


Figure 3.3.20(b). The effect of rhamnose and sucrose on diosgenin accumulation ( $\mu\text{g}/\text{culture}$ ) of stationary phase suspension cultures of D. composita. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.4.

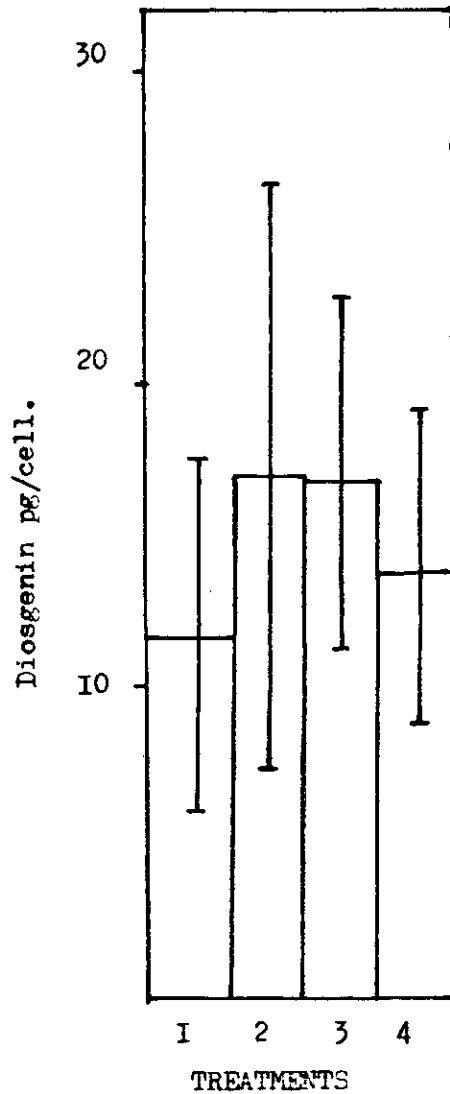


Figure 3.3.20(c). The effect of rhamnose and sucrose on diosgenin accumulation (pg/cell) of stationary phase suspension cultures of *D. composita*. The vertical bars represent standard error (95% confidence limits). The treatment numbers refer to those described in Table 3.3.4.

The former resulted in a yield of 25 $\mu$ g/culture and the latter 175 $\mu$ g/culture compared with the distilled water control value of 347 $\mu$ g/culture and the sucrose control value of 450 $\mu$ g/culture. The analysis of variance data presented in Table 3.3.23 of the Appendix shows that only the rhamnose treatment resulted in a yield which was significantly different from the distilled water control.

The effects of the various treatments on the yield of diosgenin/cell are shown in Figure 3.3.20(c). From this and the analysis of variance data presented in Table 3.3.24 of the Appendix it can be seen that none of the treatments produced any changes in the yield of diosgenin/cell which were statistically significant.

It can be seen that the above results suggest that rhamnose and sucrose administered singly or in combination, at the concentrations shown, had no effect on the yield of diosgenin/cell in stationary phase suspension cultures of D.composita. The decrease in diosgenin yield/culture and yield/g dry weight in both rhamnose and sucrose/rhamnose treatments was consistent with the drop in cell number caused by these two treatments. This suggests that the decrease in diosgenin accumulation was not caused directly by rhamnose but indirectly through its adverse effects on growth.

The experiments previously described in this section have shown that diosgenin does not begin to accumulate until stationary phase. One would then expect the demand for precursors by primary metabolic processes to decline and their availability

to secondary metabolism to increase. The experiments with stationary phase cultures however have shown that despite the onset of secondary metabolic processes during stationary phase, primary metabolism is still active and will utilise the majority of any sucrose added at this time in the culture cycle.

These experiments have highlighted the fact that although primary and secondary processes are often regarded as completely separate phenomena, they are in fact closely inter-related by virtue of sharing common precursors. This relationship inevitably leads to a competition for common precursors and results in primary metabolism being one of the major factors which regulates the accumulation of secondary metabolites.

The work presented in the following part of this Section describes attempts to measure the competition for common precursors between primary and secondary metabolism in suspension cultures of D.composita. This involved following the fate of radioactively labelled precursors of diosgenin which had been added to suspension cultures over a period which included the onset of diosgenin accumulation.

SECTION 3 - PART 6

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THE INCORPORATION OF RADIOACTIVELY  
LABELLED MEVALONIC ACID AND CHOLESTEROL  
INTO BATCH SUSPENSION CULTURES OF  
D. COMPOSITA

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All secondary metabolic processes share common precursors with pathways in primary metabolism, (Mannitto, 1981) eg amino acids in alkaloid and protein synthesis and also phenylpropanoid and protein synthesis. Diosgenin is biogenetically a terpenoid, therefore its synthesis is an example of where competition is possible between terpenoid and fatty acid metabolism for acetate. The situation is however more complicated than that for not only does diosgenin synthesis compete for acetate with fatty acid synthesis, which is a relatively distant metabolic pathway, it also competes with a pathway which is not only much closer in metabolic terms but constitutes a part of its own biosynthetic pathway. This situation arises because diosgenin's carbon skeleton is derived wholly from plant sterols, probably cholesterol (Schulte, 1975) Mannitto, 1981) which, although they look like secondary metabolites, they have more in common with primary metabolism. They are recognised as having a key role to play in maintaining the structural integrity of plant cell membranes (Grunwald, 1975). This satisfies what is generally accepted as the definition of primary metabolism, ie carrying out a function vital to an organism's survival (Luckner, 1972).

The experiment described in this part of section 3 was designed to study the competition for common precursors between sterol synthesis (primary metabolism) and diosgenin synthesis (secondary metabolism.) The period of the culture growth cycle was chosen such that the experiment would start when growth was slowing down but diosgenin accumulation had not begun and continue through the period of diosgenin accumulation/

accumulation. In this way it was hoped to detect any change in demand for the common precursors between the two types of metabolism by detecting changes in the incorporation pattern of  $^{14}\text{C}$ -labelled mevalonic acid and cholesterol into plant sterols and diosgenin.

The experiment was carried out using 24, 60 ml suspension cultures prepared as described in section 1d of chapter 2. After 3 weeks growth the radioactive precursors were administered to six flasks. Three flasks were given  $1\mu\text{Ci}$  each of DL- $[2 - ^{14}\text{C}]$  - mevalonic acid as its DBED salt\* while the other three flasks were given  $1\mu\text{Ci}$  each of  $[4 - ^{14}\text{C}]$  - cholesterol. This procedure was repeated with another six flasks at the end of the fourth, fifth and sixth weeks of the culture period. Each group of six flasks was left for 7 days after which the cultures were harvested and the dry weight of the tissue determined as described in Section 2a of Chapter 2. The dried tissue was then extracted as described in Section 2c of Chapter 2 with one modification. This consisted of a chloroform extraction step prior to acid hydrolysis, as well as the normal one after. The first extraction removed free sterols which was especially useful in the cholesterol experiment as this removed unincorporated radiolabelled material from the tissue. The second extraction removed diosgenin and sterols which were present in the tissue as glycosides before acid hydrolysis.

Samples of the two resulting chloroform solutions from both/

\* DBED = NN-Dibenzylethylenediamine. Both  $^{14}\text{C}$ -labelled compounds were supplied by the Radio Chemical Centre, Amersham.

both experiments were run on 0.25mm analytical silica gel TLC plates (Merck, 20cm x 20 cm, glass backed). They were developed in benzene:ethyl acetate (3:1). Two sets of plates were run for all the samples in the experiment. One set was sprayed with anisaldehyde reagent to visualise diosgenin and sterols, the other set was used to analyse the incorporation of the  $^{14}\text{C}$ -labelled compounds into the respective steroids, qualitatively and quantitatively.

A qualitative picture of the incorporations was obtained by preparing autoradiographs of the unsprayed set of TLC plates. Once the autoradiography was complete, the two areas where mixtures of steroid standards had been run were visualised with anisaldehyde reagent and compared to both the autoradiographs and the other set of TLC plates which had been completely sprayed with anisaldehyde reagent. Once all the qualitative information had been gained, quantitative information was obtained by using the autoradiographs to detect and scrape off the zones on the TLC plates, corresponding to diosgenin and sterols, into vials of scintillation fluid (15ml). In this way the amount of radioactivity incorporated into each group of steroids was determined by scintillation counting. The autoradiography and scintillation counting were carried out as described in Sections 2 (i) of Chapter 2.

1) THE INCORPORATION OF  $^{14}\text{C}$  MEVALONIC ACID INTO DIOSGENIN  
AND RELATED STEROIDS

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1 a) TLC OF THE STEROIDS VISUALISED BY  
ANISALDEHYDE

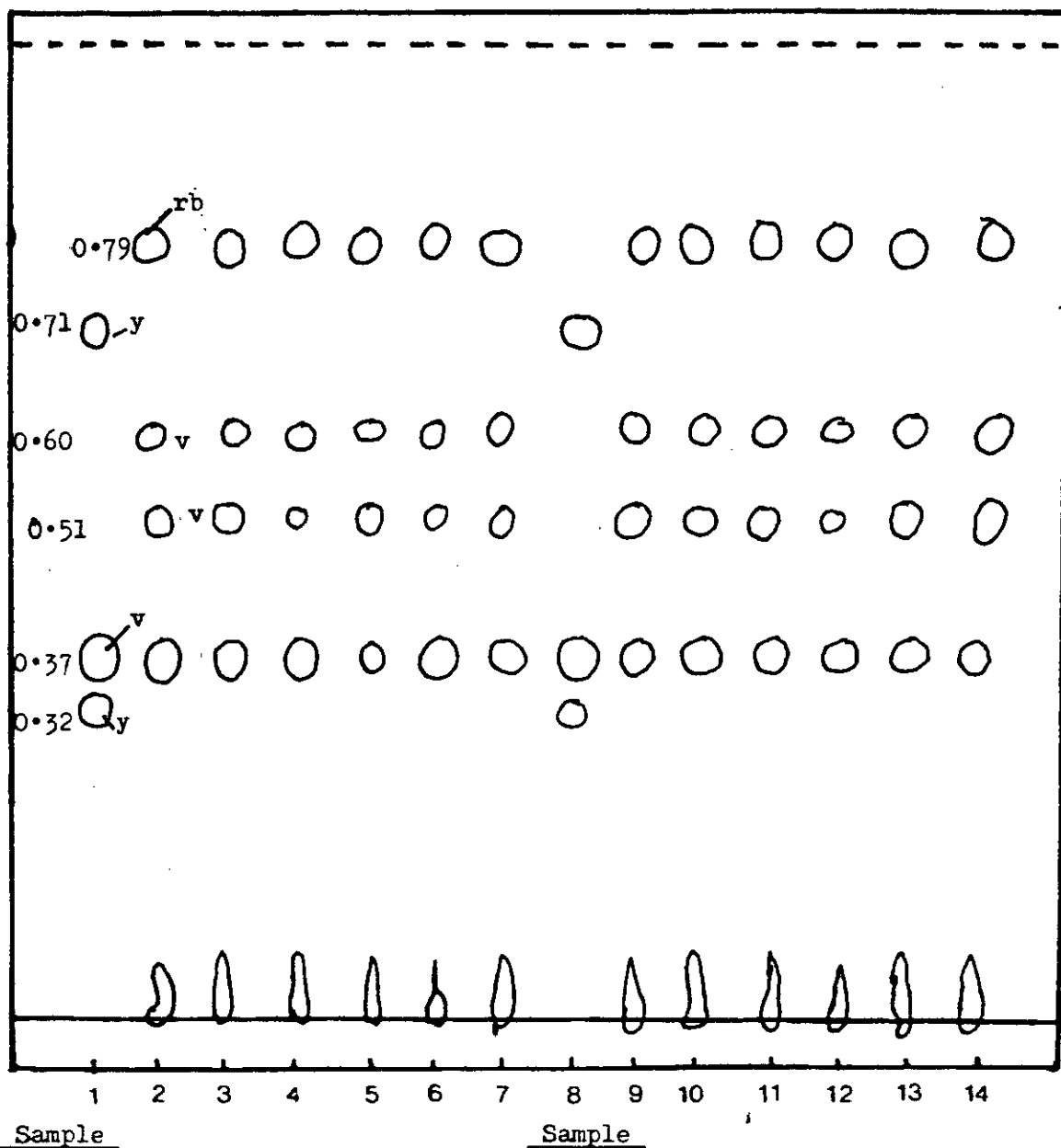
i) The first chloroform extraction

A diagram of the TLC of the samples from the first chloroform extraction of suspension cultures of D. composita which had been administered  $^{14}\text{C}$ -mevalonic acid is shown in figure 3.3.21(a).

It can be seen from this that the mixture of steroid standards containing diosgenin, cholesterol, stigmasterol, sitosterol and 25R-spirostan-3,5-diene were all visualised by anisaldehyde reagent. Diosgenin and 25R-spirostan-3,5-diene both gave yellow colours with Rf values of 0.32 and 0.71 respectively while the 3 sterols ran as one violet spot with an Rf value of 0.37.

Four spots were detected in all the samples from the first chloroform extraction. 3 gave violet colours with anisaldehyde and 1 gave a reddish brown colour. The violet spot had Rf values of 0.37, 0.51 and 0.60 while the reddish brown spot was 0.79. The violet spot at 0.37 co-ran and had an identical colour to the 3 sterol standards which ran together.

These results suggest that the samples from the first chloroform extraction of the cultures harvested at weeks 4,5,6 and 7 contained plant sterols, but not diosgenin or 25R-spirostan-3,5 diene. The other 3 compounds detected with Rf values of 0.51, 0.60 and 0.79 did not co-run with any of the available standards therefore they could not be identified.  
The/



1. Standard mixture.

2. Week 4, replicate 1.

3. Week 4, replicate 2.

4. Week 4, replicate 3.

5. Week 5, replicate 1.

6. Week 5, replicate 2.

7. Week 5, replicate 3.

8. Standard mixture.

9. Week 6, replicate 1.

10. Week 6, replicate 2.

11. Week 6, replicate 3.

12. Week 7, replicate 1.

13. Week 7, replicate 2.

14. Week 7, replicate 3.

○=strong spot, ○=faint spot, v=violet spot, y=yellow spot,

rb=reddish brown spot.

Figure 3.3.2I(a) T.L.C. of the first chloroform extraction of suspension cultures of *D. composita* after the incorporation of  $^{14}\text{C}$  mevalonic acid, sprayed with anisaldehyde reagent.

The 2 compounds with Rf values of 0.51 and 0.60 did however show an identical colour reaction, on spraying with anisaldehyde reagent, to that shown by cholesterol, sitosterol and stigmasterol which suggests they may be sterols.

ii) The second chloroform extraction

A diagram of the TLC of the samples from the second chloroform extraction of suspension cultures of D. composita which had been administered  $^{14}\text{C}$  mevalonic acid is shown in Figure 3.5.21(b).

It can be seen from this that the mixture of steroid standards containing diosgenin, cholesterol, sitosterol, stigmasterol and  $25\text{R}$ -spirostan-3,5-diene behaved identically, in terms of Rf values and colour reaction to anisaldehyde reagent, to that described in the previous figure.

Four spots were detected in all the samples from the second chloroform extraction, while 1 faint spot was detected in all the samples except numbers 2,4 and 6. Of the 4 spots detected in all the samples, the yellow spot with an Rf value of 0.32 and the violet spot with an Rf value of 0.37 co-ran with and had identical anisaldehyde colour reactions to that of diosgenin and sterol standards respectively. The faint yellow spot with an Rf value of 0.71 which was detected in all but 3 samples co-ran with and had an identical anisaldehyde colour reaction to that of the  $25\text{R}$ -spirostan-3,5-diene standard.

These results suggest that the samples from the second chloroform extraction of cultures harvested at 4,5,6 and 7 weeks/

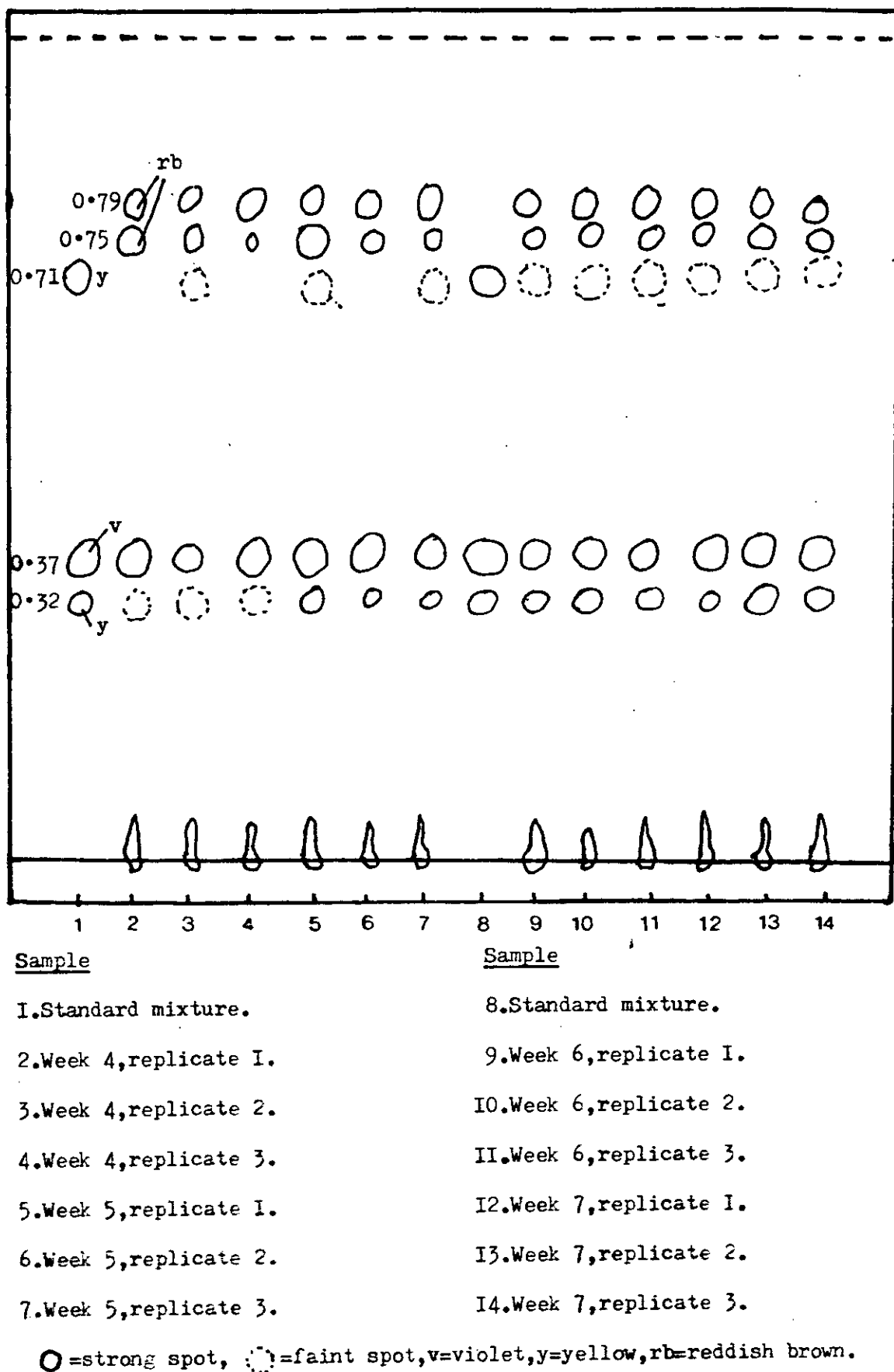


Figure 3.3.21(b). T.L.C. of the second chloroform extraction of suspension cultures of *D. composita* after the incorporation of  $^{14}\text{C}$ -mevalonic acid, sprayed with anisaldehyde reagent.

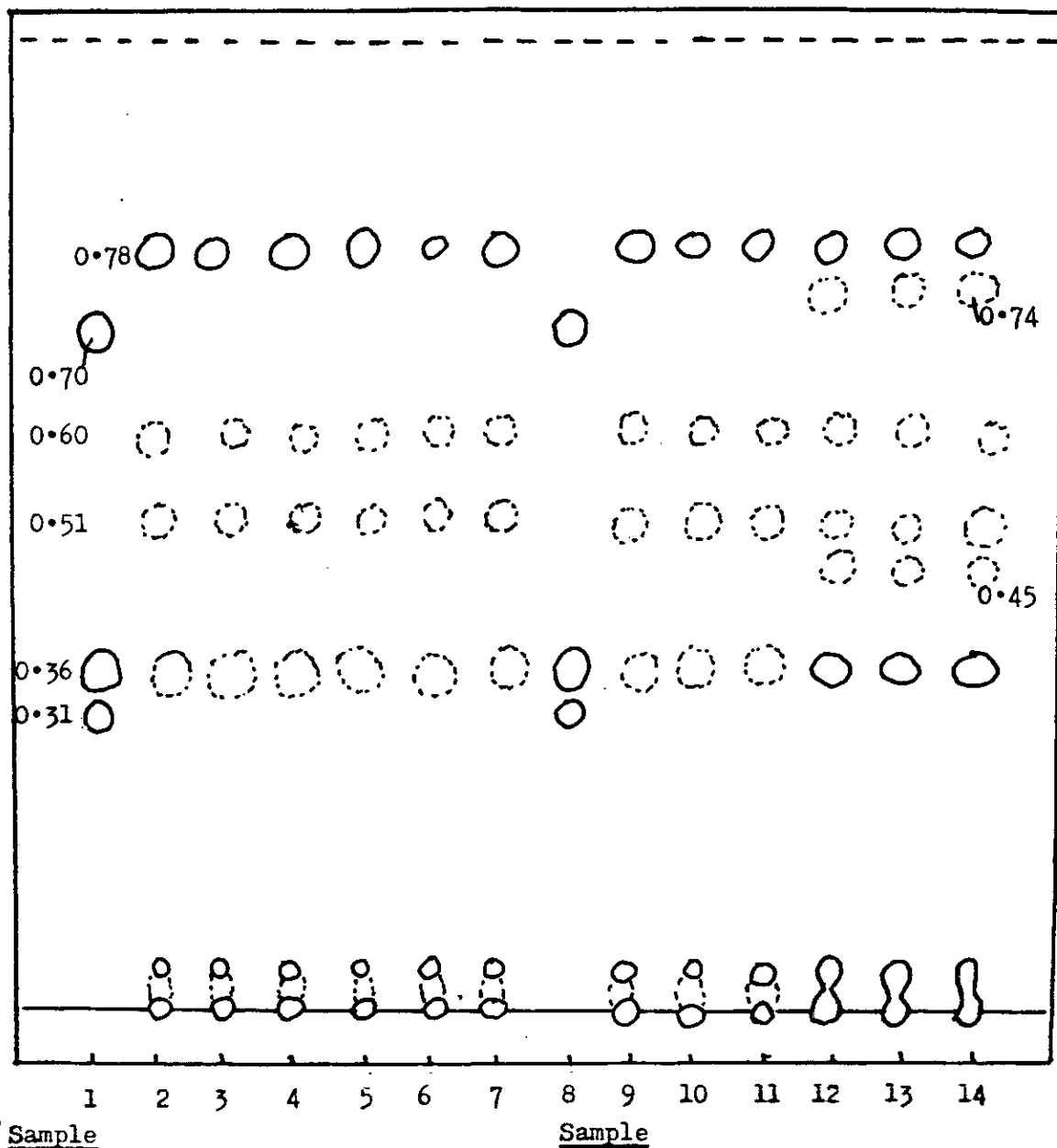
weeks contained diosgenin and plant sterols with the amount of diosgenin at week 4 comparatively lower than that at weeks 5,6 and 7. The presence of plant sterols in this extraction and the first chloroform extraction suggest that they were present in the Dioscorea composita culture tissue as steryl glycosides and free sterols. The detection of trace amounts of 25R-spirostan-3,5-diene for the first time in some of the extracts suggests that some dehydration of diosgenin had occurred during the acid catalysed hydrolysis. The 2 compounds detected with Rf values of 0.75 and 0.79 which had given reddish brown anisaldehyde colour reactions could not be identified as they did not co-run with any of the available standards. The compound with the Rf value of 0.78 could however have been identical to the compound detected in the first chloroform extract which gave the same anisaldehyde colour reaction and a comparable Rf value of 0.79. If they were the same compound, then as with the sterols, its detection in both extractions suggests that it was present in the culture tissue as a glycoside and a free compound.

1 b) AUTORADIOGRAPHY OF THE THIN LAYER CHROMATOGRAMS OF THE EXTRACTS OF THE PLANT CELL CULTURES WHICH HAD BEEN ADMINISTERED  $^{14}\text{C}$ -MEVALONIC ACID

i) The first chloroform extraction

The autoradiograph of the first chloroform extraction of the suspension cultures of D. composita after being given  $^{14}\text{C}$ -mevalonic acid is presented in figure 3.3.22 (a).

It can be seen from this that five radioactive spots were detected in the samples from the cultures extracted at weeks/



1. Standard mixture.

2. Week 4, replicate 1.

3. Week 4, replicate 2.

4. Week 4, replicate 3.

5. Week 5, replicate 1.

6. Week 5, replicate 2.

7. Week 5, replicate 3.

8. Standard mixture.

9. Week 6, replicate 1.

10. Week 6, replicate 2.

11. Week 6, replicate 3.

12. Week 7, replicate 1.

13. Week 7, replicate 2.

14. Week 7, replicate 3.

○ = strong spot, ◌ = faint spot.

Figure 3.3.22(a). Autoradiograph of the first chloroform extraction of suspension cultures of *D. composita* after the incorporation of  $^{14}\text{C}$ -mevalonic acid.

weeks 4,5 and 6 while 7 radioactive spots were detected in the cultures extracted at week 7. Of the 5 spots detected in the samples from weeks 4,5 and 6, only the spot with the Rf value of 0.36 coincided with any of the known standards, in this case, the 3 sterols which ran as one spot. The Rf values of 3 of the 4 other spots were comparable with unknowns detected on TLC with anisaldehyde reagent (figure 3.3.21a while the other 2 radioactive spots coincided with the 2 violet spots with Rf values of 0.60 and 0.51. The last radioactive spot at 0.04 could not be matched with any TLC spot in figure 3.3.21a but this may have been due to the thick brown streaks masking spots near the origin of the TLC plate. In the extract from cultures harvested at week 7, 5 of the radioactive spots were identical to those discussed for weeks 4, 5, and 6 with the exception that the radioactive spot corresponding to the sterol standard was much stronger in week 7 than in the other samples. The two radioactive spots which were only detected in week 7 samples had Rf values of 0.74 and 0.45, and did not correspond to any spot detected on TLC plates with anisaldehyde reagent.

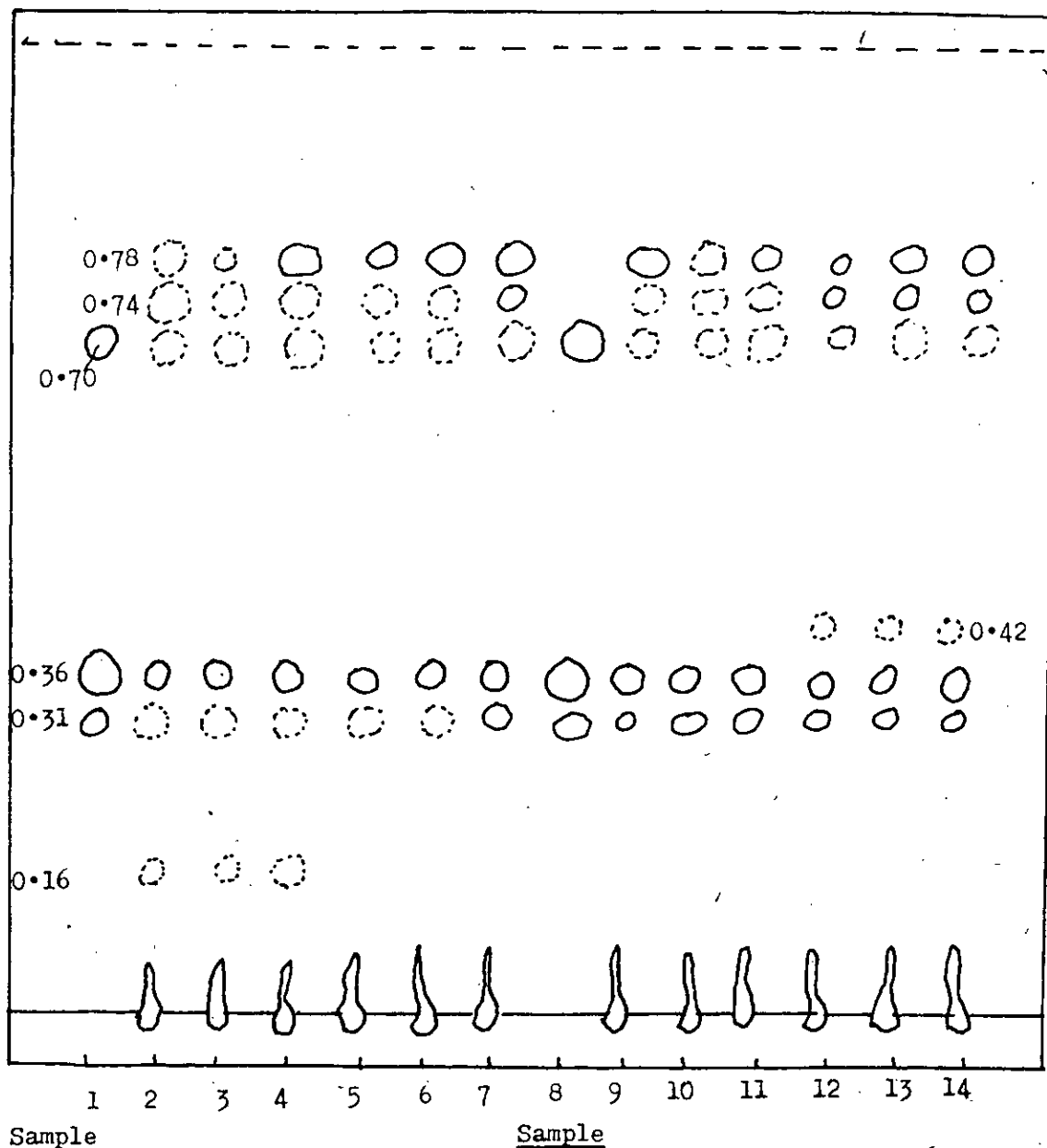
These results suggest that the D. composita suspension cultures were synthesising free sterols during weeks 4,5,6 and 7 of their culture period and that the maximum accumulation over this period was during week 7. Whether this was due simply to more free sterols being synthesised or to a change in the balance between synthesis and degradation could not be ascertained in this experiment. The overall pattern of incorporation of  $^{14}\text{C}$  mevalonic acid remained the same over weeks 4,5 and 6 but changed during week 7, the precise nature of this change in metabolism remained unclear as none of the other radioactive spots could be identified.

ii) The second chloroform extraction

The autoradiograph of the second chloroform extraction of the suspension cultures of D. composita after being administered  $^{14}\text{C}$  mevalonic acid is shown in Figure 3.3.22 (b).

It can be seen from this that 5 radioactive spots were detected in all the samples from the cultures extracted at weeks 4,5,6 and 7 and while one additional spot was detected in both week 4 and week 7, samples, they had differing Rf values. Of the 5 spots detected in all the samples, the spots with Rf values of 0.31, 0.36 and 0.70 co-ran with the diosgenin, plant sterols and  $25\text{R}$ -spirostan-3,5-diene standards respectively. The Rf values of the other 2 spots were comparable with unknowns detected on TLC with anisaldehyde reagent (Figure 3.3.21b) The radioactive spots at 0.78 and 0.74 were both similar to the reddish brown spots at 0.79 and 0.75 in Figure 3.3.21.b. Neither the radioactive spot with the Rf values of 0.16 in the samples from week 4 nor the spot with the Rf value of 0.42 in the samples from week 7 could be matched with any of the standards or any unknown spot detected on TLC with anisaldehyde reagent.

These results suggest that the D. composita suspension cultures were synthesising steryl glycosides and diosgenin over the 4 week period of the experiment and while there seems to be little difference in the intensity of the sterol spots, those corresponding to diosgenin seem to show some increase from the beginning of the experiment to the end. This is supported by the TLC data in figure 3.3.21(b). The detection of the radioactive spot corresponding to  $25\text{R}$ -spirostan-3,5-diene also supports data from Figure 3.3.21b. that suggests that some dehydration of diosgenin had taken place/



1. Standard mixture.

2. Week 4, replicate 1.

3. Week 4, replicate 2.

4. Week 4, replicate 3.

5. Week 5, replicate 1.

6. Week 5, replicate 2.

7. Week 5, replicate 3.

8. Standard mixture.

9. Week 6, replicate 1.

10. Week 6, replicate 2.

11. Week 6, replicate 3.

12. Week 7, replicate 1.

13. Week 7, replicate 2.

14. Week 7, replicate 3.

○=strong spot, ⊙=faint spot.

Figure 3.3.22(b). Autoradiograph of the second chloroform extraction of suspension cultures of *D. composita* after the incorporation of  $^{14}\text{C}$ -mevalonic acid and T.L.C.

place during the acid catalysed hydrolysis.

Overall the pattern of incorporation remained more or less the same except for the two spots in week 4 and 7 which could not be identified.

2) THE INCORPORATION OF  $^{14}\text{C}$  CHOLESTEROL INTO  
DIOSGENIN AND RELATED STEROIDS

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2 a) TLC OF THE STEROIDS VISUALISED BY  
ANISALDEHYDE REAGENT

i) The first chloroform extraction

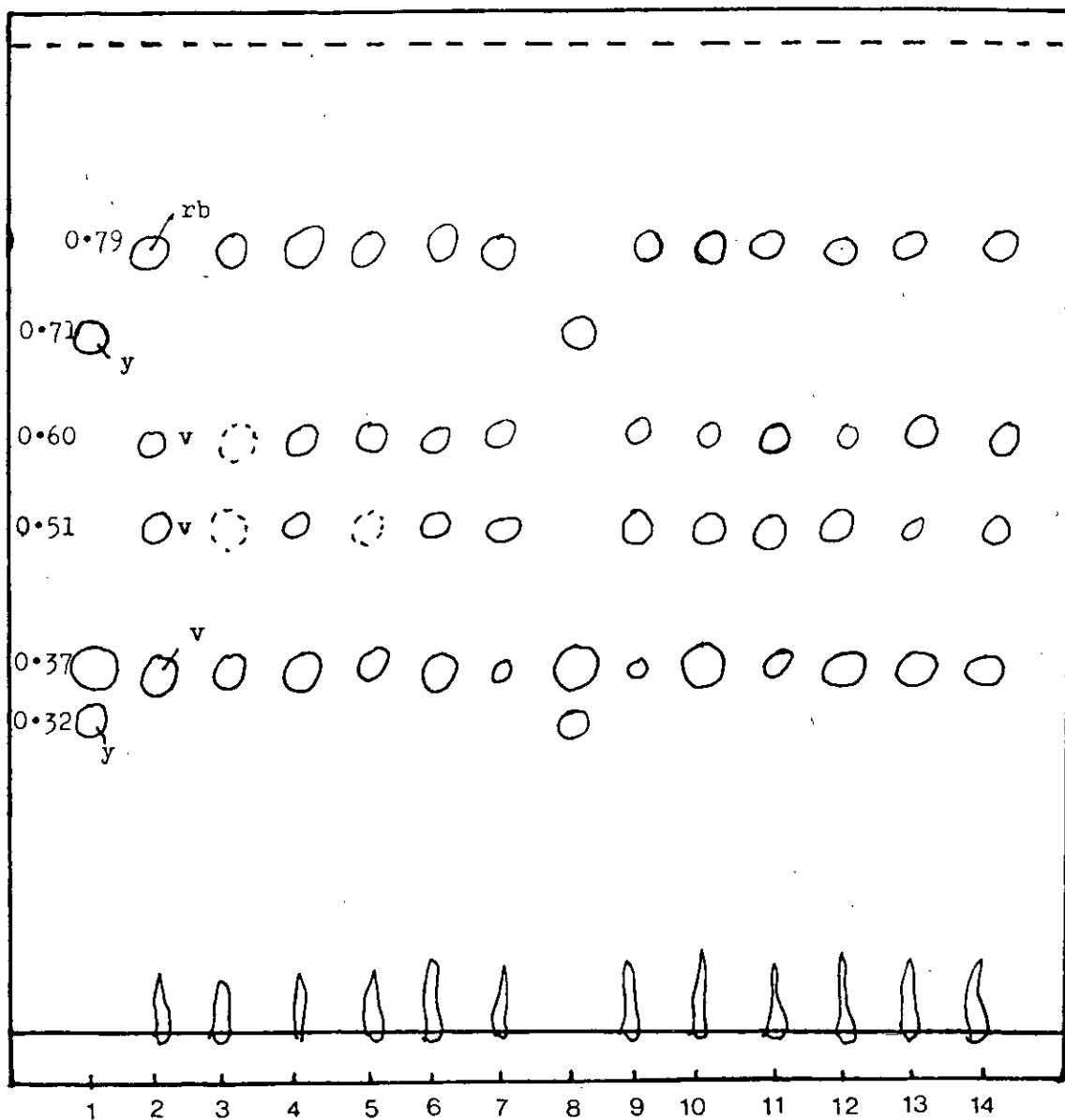
A diagram of the TLC of the samples from the first chloroform extraction of suspension cultures of D. composita after being administered  $^{14}\text{C}$  cholesterol is shown in Figure 3.3.23(a).

It can be seen from this that the mixture of steroid standards showed similar Rf values to those obtained in Figures 3.3.21(a) and 3.3.21(b).

Four spots were detected in all the samples from the second chloroform extraction, 3 gave violet colours with anisaldehyde and had Rf values of 0.37, 0.51, and 0.60 while 1 spot was reddish-brown with an Rf value of 0.79. The violet spot at 0.37 co-ran with the sterol standard and was the only spot of the 4 which did co-run with any of the standards.

These results suggest that the first chloroform extraction of the cultures harvested at 4,5,6 and 7 weeks contained free plant sterols but no diosgenin or 25R-spirostan-3,5-diene. The other 3 compounds detected with Rf values of 0.51, 0.60 and 0.79 could not be identified since they did not co-run with any of the available standards. The overall pattern of the chromatogram was similar to that in the first chloroform extraction of the mevalonic acid incorporation experiment, (Figure 3.3.21(a))

ii)/

Sample

- I. Standard mixture.
2. Week 4, replicate I.
3. Week 4, replicate 2.
4. Week 4, replicate 3.
5. Week 5, replicate I.
6. Week 5, replicate 2.
7. Week 5, replicate 3.

Sample

8. Standard mixture.
9. Week 6, replicate I.
10. Week 6, replicate 2.
11. Week 6, replicate 3.
12. Week 7, replicate I.
13. Week 7, replicate 2.
14. Week 7, replicate 3.

○ = strong spot, ◌ = faint spot, v = violet, y = yellow, rb = reddish brown.

Figure 3.3.23(a). T.L.C. of the first chloroform extraction of suspension cultures of *D. composita* after incorporation of cholesterol, sprayed with anisaldehyde reagent.

ii) The second chloroform extraction

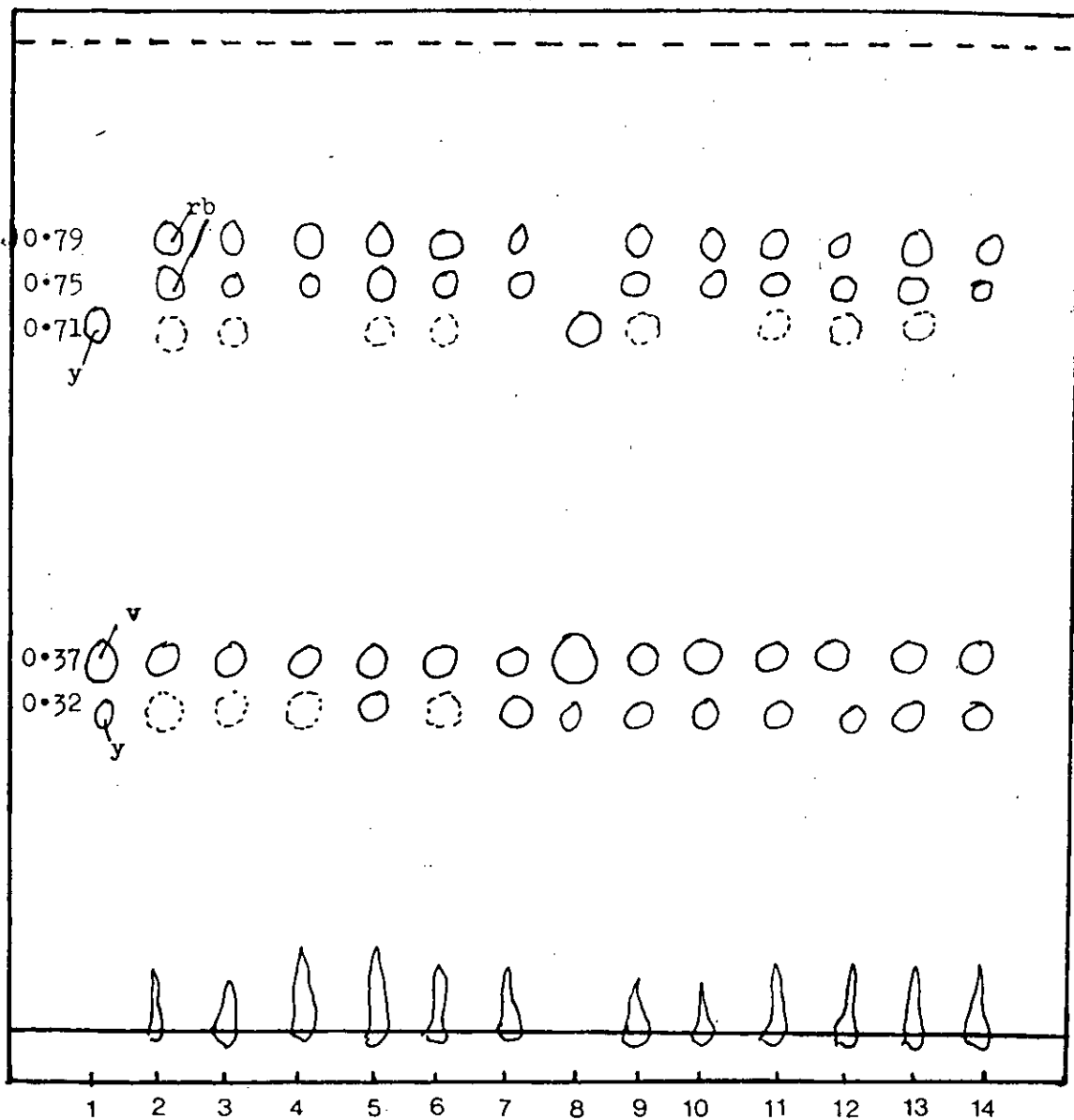
A diagram of the TLC of the samples from the second chloroform extraction of the suspension cultures of D. composita after being administered  $^{14}\text{C}$  cholesterol is shown in figure 3.3.23(b).

It can be seen from this that the mixture of steroid standards behaved identically to that described in the previous figure.

Four spots were detected in all the samples from the second chloroform extraction while 1 faint spot was detected in only 8 out of the 12 samples. The yellow spot with the Rf value of 0.37 co-ran with diosgenin while the violet spot with the Rf value of 0.37 co-ran with the sterol standard.

the faint yellow spot with the Rf value of 0.71 co-ran with 25R-spirostan-3,5-diene standard while the 2 reddish brown spots with Rf values of 0.79 and 0.75 did not co-run with any available standards, therefore, they could not be identified.

These results suggest that the overall pattern of the chromatogram, as in the first extraction above, was similar to that of its related chloroform extract in the mevalonic acid experiment (Figure 3.3.21 (b)), ie the cultures harvested at weeks 4,5,6 and 7 contained diosgenin and sterols with the amount of diosgenin in the week 4 samples comparatively lower than the rest of the experiment. Once again the presence of plant sterols in the second as well as the first chloroform extraction supports the suggestion that sterols were present in D. composita tissue as steryl glycosides as well as free sterols. The presence of trace amounts of 25R-spirostan-3,5-diene in 8 out of 12 samples suggested/

SampleSample

1. Standard mixture.

8. Standard mixture.

2. Week 4, replicate I.

9. Week 6, replicate I.

3. Week 4, replicate 2.

10. Week 6, replicate 2.

4. Week 4, replicate 3.

11. Week 6, replicate 3.

5. Week 5, replicate I.

12. Week 7, replicate I.

6. Week 5, replicate 2.

13. Week 7, replicate 2.

7. Week 5, replicate 3.

14. Week 7, replicate 3.

○ = strong spot, ◌ = faint spot, v = violet, y = yellow, rb = reddish brown.

Figure 3.3.23(b). T.L.C. of the second chloroform extraction of suspension cultures of *D. composita* after the incorporation of  $^{14}\text{C}$ -cholesterol, sprayed with anisaldehyde.

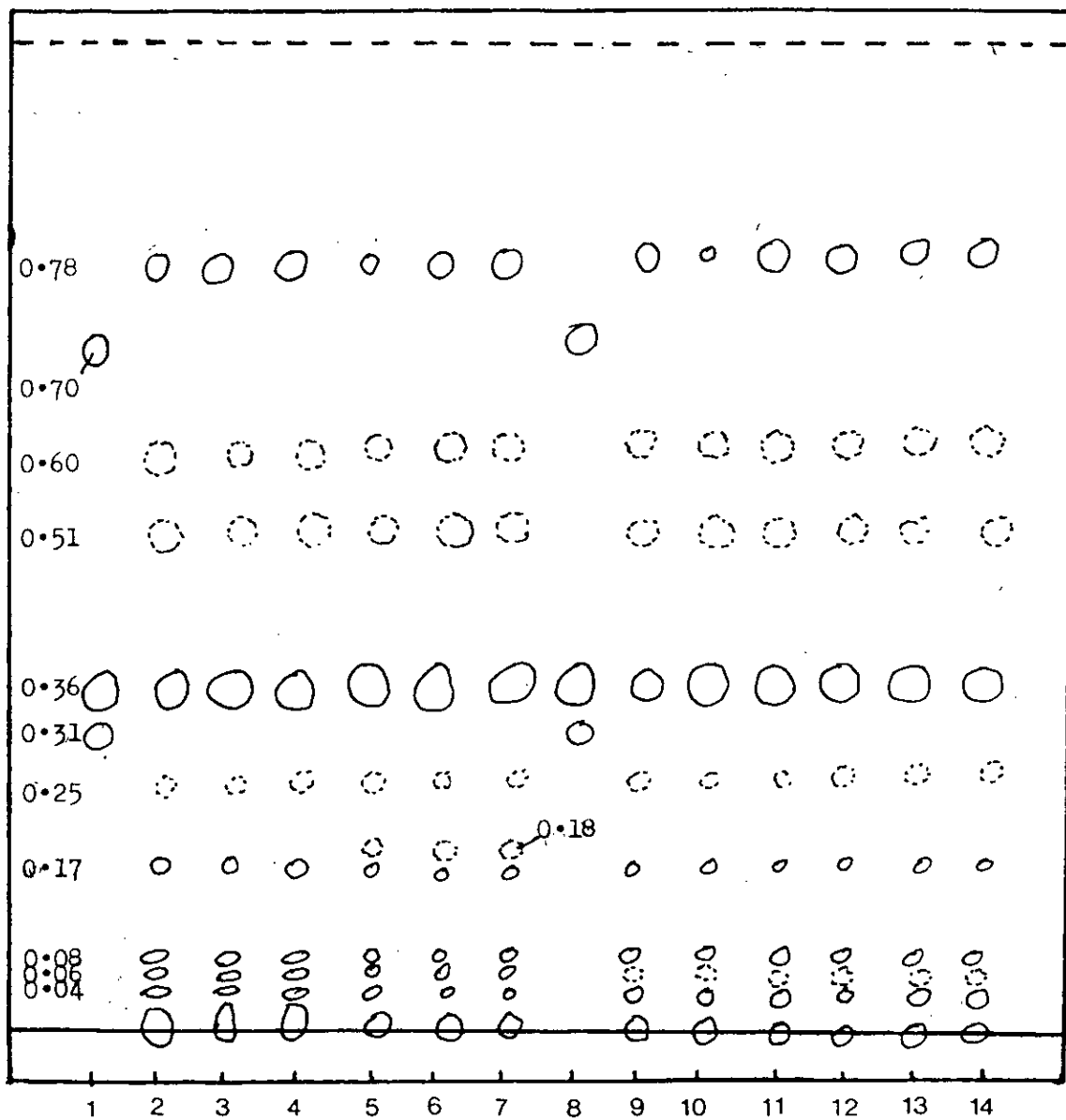
suggested that a similar level of dehydration of diosgenin occurred in this experiment as compared the mevalonic acid experiment.

2 b) AUTORADIOGRAPHY OF THE THIN LAYER CHROMATOGRAMS OF THE PLANT CELL CULTURE EXTRACTS AFTER  $^{14}\text{C}$  CHOLESTEROL INCORPORATION

i) The first chloroform extraction

A diagram of the autoradiograph of the first chloroform extraction of the suspension cultures of D. composita after being administered  $^{14}\text{C}$  cholesterol is shown in Figure 3.3.24(a).

It can be seen from this that 9 radioactive spots were detected in the samples from the cultures extracted at weeks 4, 5, 6 and 7 with the exception of the 3 samples at week 5 which had an extra spot. Of the 9 spots detected in all the samples, only the one with the Rf value of 0.36 coincided with any of the available standards, in this case the sterol standard. The Rf values of 3 of the 8 other spots were comparable with unknowns detected on TLC with anisaldehyde reagent (Figure 3.3.23 (a)). The radioactive spot at 0.78 was similar to the reddish brown spot at 0.79 in figure 3.3.23(a) while the other 2 radioactive spots coincided with the 2 violet spots with Rf values of 0.60 and 0.51. None of the remaining 5 spots with Rf values of 0.25, 0.17, 0.08, 0.06 and 0.04 or the extra spot in the week 5 samples with an Rf value of 0.18 could be matched with any spots on the TLC described in Figure 3.3.23. a Neither did they co-run with any of the available standards. The spot at 0.04 did however have a similar Rf value to a radioactive spot in the autoradiograph of the first chloroform extraction of the mevalonic acid experiment (Figure 3.3.22(a)) which like

Sample

1. Standard mixture.
2. Week 4, replicate 1.
3. Week 4, replicate 2.
4. Week 4, replicate 3.
5. Week 5, replicate 1.
6. Week 5, replicate 2.
7. Week 5, replicate 3.

Sample

8. Standard mixture.
9. Week 6, replicate 1.
10. Week 6, replicate 2.
11. Week 6, replicate 3.
12. Week 7, replicate 1.
13. Week 7, replicate 2.
14. Week 7, replicate 3.

○=strong spot, (○)=faint spot.

Figure 3.3.24(a). Autoradiograph of the first chloroform extraction of suspension cultures of D. composita after the incorporation of <sup>14</sup>C-cholesterol and T.L.C.

the above spot could not be identified.

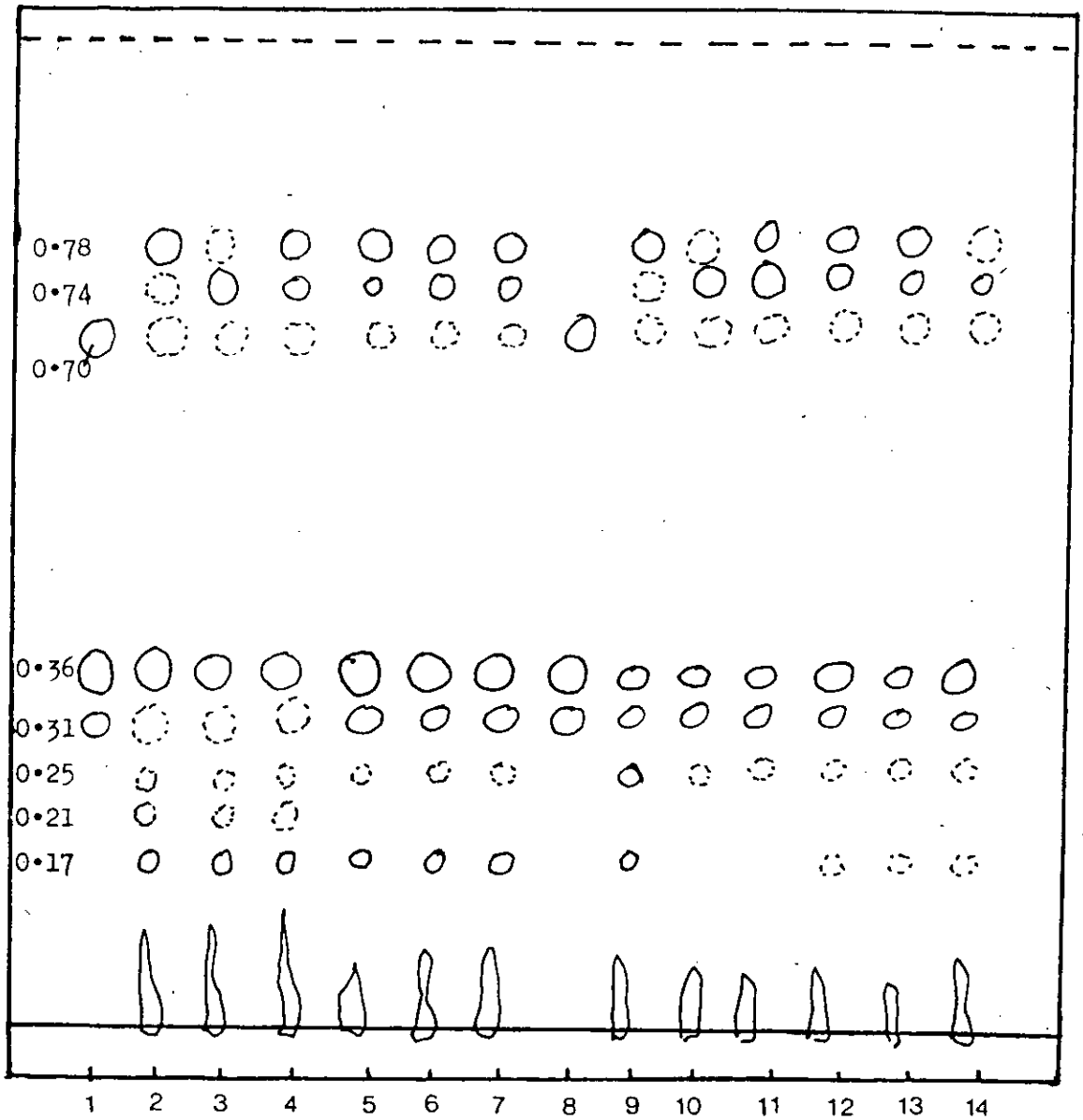
The above results suggest that as in the mevalonic acid experiment, the D. composita suspension cultures were synthesising free sterols during weeks 4,5,6 and 7 of their culture period, however, since this particular extraction removed any unincorporated  $^{14}\text{C}$  cholesterol and the TLC method did not separate it from other sterols then no distinction can be made between added  $^{14}\text{C}$  cholesterol and other sterols synthesised from it.

Overall the main incorporation pattern of  $^{14}\text{C}$  label in the cholesterol experiment, like the mevalonic acid experiment remained fairly constant over the experimental period. It was qualitatively, however, very different containing not only the 5 main spots of the mevalonic acid experiment but 4 others.

ii) The second chloroform extraction

A diagram of the autoradiograph of the second chloroform extraction of the suspension cultures of D. composita after being administered  $^{14}\text{C}$  cholesterol is shown in Figure 3.3.24 (b).

It can be seen from this that 5 main radioactive spots were detected in all the samples from the cultures extracted at weeks 4,5,6 and 7. There were 3 other spots detected, various combinations of which were present in all the samples. Of the 5 main spots, the ones with the Rf values of 0.31, 0.36, 0.70 co-ran with the diosgenin, sterol and 25R-spirostan-3,5-diene standards respectively. The Rf values of the other 2 spots were comparable with unknowns detected on TLC with anisaldehyde reagent (Figure 3.3.23(b)). The radioactive



Sample

1. Standard mixture.
2. Week 4, replicate I.
3. Week 4, replicate 2.
4. Week 4, replicate 3.
5. Week 5, replicate I.
6. Week 5, replicate 2.
7. Week 5, replicate 3.

Sample

8. Standard mixture.
9. Week 6, replicate I.
10. Week 6, replicate 2.
11. Week 6, replicate 3.
12. Week 7, replicate I.
13. Week 7, replicate 2.
14. Week 7, replicate 3.

○ = strong spot, ⊙ = faint spot.

Figure 3.3.24(b). Autoradiograph of the second chloroform extraction of suspension cultures of D. composita after the incorporation of <sup>14</sup>C-cholesterol and T.L.C.

spots at 0.78 and 0.74 were both similar to the reddish brown spots at 0.79 and 0.75 in Figure 3.3.23(b). The 3 minor spots had Rf values of 0.25, 0.21 and 0.17. All 3 were present in week 4 samples while the first and third were present in the week 5 and 7 samples. In the week 6 samples the first and third were present in the first replicate sample while only the first was present in the other two replicates. None of these spots could be matched with any of the available standards or any spots from the TLC used to prepare the autoradiograph (Figure 3.3.23(b)). Therefore they could not be identified. The spots with Rf values of 0.25 and 0.7 do however match spots in the autoradiograph of the first chloroform extraction in the  $^{14}\text{C}$  cholesterol experiment (Figure 3.3.24(a)) which suggests whatever their nature they can exist as glycosides or free compounds.

These results suggest that the D. composita suspension cultures were synthesising steryl glycosides and diosgenin over the 4 week period of the experiment and while there seems to be little difference in the intensity of the sterol spots, those corresponding to diosgenin seem to show some increase from the beginning of the experiment to the end. This supports the TLC data shown in figure 3.3.23 (b).

The detection of the radioactive spot corresponding to 25<sub>R</sub>-spirostan -3,5-diene also supports data from figure 3.3.23(b) that suggests that some dehydration of diosgenin had taken place during the acid catalysed hydrolysis reaction. Over the period of the experiment, the pattern of  $^{14}\text{C}$  incorporation remained more or less the same and was very similar to the main pattern of  $^{14}\text{C}$  incorporation in the mevalonic/

mevalonic acid experiment except for the variations in the minor components.

The qualitative analysis of the  $^{14}\text{C}$  mevalonic acid and  $^{14}\text{C}$  cholesterol incorporation experiments has shown that these two precursors can be used to radiochemically label diosgenin and sterols in suspension cultures of D. composita. The exception to this being the problem in proving incorporation of  $^{14}\text{C}$  cholesterol into free sterols because of the difficulty in separating precursor from products by TLC. The experiments have also shown that the radiochemically labelled sterols formed are found in the culture tissue as free sterols and steryl glycosides.

The next part of this section describes the quantitative analysis of the incorporation studies to calculate how much of each precursor was incorporated into diosgenin and how much into sterols.

3) QUANTITATIVE ANALYSIS OF THE INCORPORATION OF  
RADIOLABELLED MEVALONIC ACID AND CHOLESTEROL  
INTO DIOSGENIN AND RELATED STEROIDS

---

3 a) THE INCORPORATION OF  $^{14}\text{C}$  MEVALONIC ACID  
INTO DIOSGENIN, STEROLS AND STERYL GLYCOSIDES

The results of the quantitative analysis of the incorporation of  $^{14}\text{C}$  mevalonic acid into sterols, steryl glycosides and diosgenin by suspension cultures of D. composita are shown in Tables 3.3.5, 3.3.6, and 3.3.7, respectively.

i) The incorporation of  $^{14}\text{C}$  mevalonic acid into free sterols

The amount of radiolabelled mevalonic acid incorporated into free sterols in suspension cultures of D. composita over 4 consecutive weeks is shown in Table 3.3.5. It can be seen from this that the level of incorporation remained relatively constant during weeks 4, 5 and 6 then increased markedly during the last week of the experiment. Almost 3 times as much  $^{14}\text{C}$  mevalonic acid was incorporated into free sterols during the seventh week as compared to week 6.

The percentages of the total added  $^{14}\text{C}$  mevalonic acid which was incorporated into free sterols during weeks 4, 5, 6 and 7 were 0.74, 0.56, 0.64 and 1.60% respectively. These values were calculated using the figures for the total dpm/extract rather than those corrected to dpm/g dry weight.

ii) The incorporation of  $^{14}\text{C}$ -mevalonic acid into steryl glycosides

The amount of radiolabelled mevalonic acid incorporated into steryl glycosides in suspension cultures of D. composita over 4 consecutive weeks is shown in Table 3.3.6. It can be seen from this that the level of incorporation remained

remained relatively constant during the first two weeks of the experiment. The incorporation then fell from an average value of 30,260 dpm to 18,233 dpm/g dry weight. The decrease only lasted one week however, and during the last week the incorporation rose to 64,253 dpm/g dry weight. This represented a 3.5-fold increase over the previous week's value and just over a 2 fold increase compared with the week before that.

The percentages of the total added  $^{14}\text{C}$ -mevalonic acid incorporated into steryl glycosides during weeks 4,5,6 and 7 were 1.82, 1.88, 1.04 and 2.24% respectively. These values were calculated as described above.

iii) The incorporation of  $^{14}\text{C}$ -mevalonic acid into diosgenin

The amount of radiolabelled mevalonic acid incorporated into diosgenin in suspension cultures of *D. composita* over 4 consecutive weeks is described in Table 3.3.7. It can be seen from this that the incorporation pattern for diosgenin was similar to that for steryl glycosides, The level of incorporation was relatively constant for the first two weeks then it decreased to 9113 dpm/g dry weight during the third week from an average value of 15,906 dpm/g dry weight. The level of incorporation then rose 4.6 fold to an average of 42,307 dpm during week 7.

The percentages of the total added  $^{14}\text{C}$ -mevalonic acid incorporated into diosgenin during weeks 4,5,6 and 7 were 0.76, 0.98, 0.52 and 2.26% respectively.

TABLE 3.3.5.

THE INCORPORATION OF  $^{14}\text{C}$  MEVALONIC ACID INTO STEROLS

DISINTEGRATIONS PER MINUTE PER GRAM DRY WEIGHT CELLS				
REPLICATE	WEEK 4	WEEK 5	WEEK 6	WEEK 7
1	8,222	6,128	11,206	35,202
2	10,483	7,602	12,792	28,970
3	15,988	13,305	9,636	25,256
$\bar{X} \pm \text{S.E.}$	11,564 $\pm$	9,012 $\pm$	11,211 $\pm$	29,809 $\pm$
	2,306	2,188	911	2,902

TABLE 3.3.6.

THE INCORPORATION OF  $^{14}\text{C}$  MEVALONIC ACID INTO STERYL GLYCOSIDES

DISINTEGRATIONS PER MINUTE PER GRAM DRY WEIGHT CELLS				
REPLICATE	WEEK 4	WEEK 5	WEEK 6	WEEK 7
1	26,197	29,607	25,956	83,469
2	35,497	37,132	17,460	63,893
3	23,546	24,040	11,283	45,392
$\bar{X} \pm \text{S.E.}$	28,413 $\pm$	30,260 $\pm$	18,233 $\pm$	64,253 $\pm$
	3,624	3,793	4,253	10,993

TABLE 3.3.7.

THE INCORPORATION OF  $^{14}\text{C}$  MEVALONIC ACID INTO DIOSGENIN

DISINTEGRATIONS PER MINUTE PER GRAM DRY WEIGHT CELLS				
REPLICATE	WEEK 4	WEEK 5	WEEK 6	WEEK 7
1	9,995	10,115	9,311	59,427
2	9,905	21,047	6,993	31,811
3	15,189	16,556	11,036	35,683
$\bar{X} \pm \text{S.E.}$	11,696 $\pm$	15,906 $\pm$	9,113 $\pm$	42,307 $\pm$
	1,746	3,172	1,171	8,632

3 b) THE INCORPORATION OF  $^{14}\text{C}$  CHOLESTEROL INTO DIOSGENIN AND STERYL GLYCOSIDES

The results of the quantitative analysis of the incorporation of  $^{14}\text{C}$  cholesterol into steryl glycosides and diosgenin by suspension cultures of D. composita are described in Tables 3.3.8; and 3.3.9. respectively.

i) The incorporation of  $^{14}\text{C}$  cholesterol into steryl glycosides

The amount of radiolabelled cholesterol incorporated into steryl glycosides in suspension cultures of D. composita over 4 consecutive weeks is shown in Table 3.3.8. It can be seen from this the level of incorporation during week 4 was an average 181,146 dpm. The level then rose during the following week to 361,047 dpm/g dry weight only to fall during week 6 to an average of 102,936 dpm. The fluctuations in the level of incorporation continue in week 7 when it rose 4 fold to an average of 420,645 dpm/g dry weight.

The percentages of the total added  $^{14}\text{C}$  mevalonic acid incorporated into steryl glycosides during weeks 4,5,6 and 7 were 6.08, 10.84, 2.66 and 12.05% respectively.

ii) The incorporation of  $^{14}\text{C}$  cholesterol into diosgenin

The amount of radiolabelled cholesterol incorporated into diosgenin in suspension cultures of D. composita over 4 consecutive weeks is shown in Table 3.3.9. It can be seen from this that the level of incorporation remained relatively constant during the first two weeks of the experiment/

TABLE 3.3.8.

THE INCORPORATION OF  $^{14}\text{C}$  CHOLESTEROL INTO STERYL GLYCOSIDES

DISINTEGRATIONS PER MINUTE PER GRAM DRY WEIGHT CELLS				
<u>REPLICATE</u>	<u>WEEK 4</u>	<u>WEEK 5</u>	<u>WEEK 6</u>	<u>WEEK 7</u>
1	228,335	377,713	104,694	497,813
2	122,767	499,364	97,266	377,774
3	192,336	206,064	106,849	404,348
$\bar{X} \pm \text{S.E.}$	181,146 <sup>±</sup>	361,047 <sup>±</sup>	102,936 <sup>±</sup>	420,645 <sup>±</sup>
	30,984	85,077	2,903	30,562

TABLE 3.3.9.

THE INCORPORATION OF  $^{14}\text{C}$  CHOLESTEROL INTO DIOSGENIN

DISINTEGRATIONS PER MINUTE PER GRAM DRY WEIGHT CELLS				
<u>REPLICATE</u>	<u>WEEK 4</u>	<u>WEEK 5</u>	<u>WEEK 6</u>	<u>WEEK 7</u>
1	54,333	39,022	26,282	25,724
2	40,054	60,452	25,338	29,131
3	47,862	32,049	36,212	29,547
$\bar{X} \pm \text{S.E.}$	47,416 <sup>±</sup>	43,841 <sup>±</sup>	29,277 <sup>±</sup>	28,134 <sup>±</sup>
	4,128	8,546	3,478	1,211

experiment around 45,000 dpm/g dry weight. The level then dropped slightly during week 6 to 29,277 dpm and then remained relatively constant till the end of the experiment.

The percentages of the total added  $^{14}\text{C}$  mevalonic acid incorporated into diosgenin during weeks 4, 5, 6 and 7 were 1.58, 1.32, 0.76 and 0.81 respectively.

3 c) THE DISTRIBUTION OF RADIOLABELLED PRECURSORS BETWEEN DIOSGENIN AND STEROL/STERYL GLYCOSIDES

A picture of the competition for common precursors may be obtained by comparison of the percentage of the total incorporated radioactive label detected in each class of steroid, ie sapogenin and sterol.

Such a comparison is described in Table 3.3.10. where it can be seen that in the  $^{14}\text{C}$ -mevalonic acid experiment the distribution of total incorporated label was approximately 25% into diosgenin and 75% into sterols/steryl glycosides and that this ratio remained constant during all four weeks of the experiment.

In comparison the distribution of total incorporated label was different in each week of the experiment. At the beginning of the experiment (week 4) the total incorporated label was distributed approximately 21% into diosgenin and 79% into steryl glycosides. During the fifth week, however, the ratio changed to 11:89 and during the third week it changed again to 22:78. The ratio changed once more during the last week of/

TABLE 3.3.10.

THE DISTRIBUTION OF TOTAL INCORPORATED  
RADIOLABELLED PRECURSORS BETWEEN DIOSGENIN  
AND STEROL/STERYL GLYCOSIDES

PERCENTAGE OF TOTAL INCORPORATED $^{14}\text{C}$ MEVALONIC ACID				
COMPOUND	WEEK 4	WEEK 5	WEEK 6	WEEK 7
DIOSGENIN	22.6	28.7	23.6	25.0
STEROL/STERYL GLYCOSIDES	77.4	71.3	76.4	75.0

PERCENTAGE OF TOTAL INCORPORATED $^{14}\text{C}$ CHOLESTEROL				
COMPOUND	WEEK 4	WEEK 5	WEEK 6	WEEK 7
DIOSGENIN	20.7	10.9	22.2	6.3
STERYL GLYCOSIDES	79.3	89.1	77.8	93.7

of the experiment (week 7) such that only 6% of the total incorporated label was detected in diosgenin and 94% was in steryl glycosides.

The main points to come out of the above experiments are as follows:

- 1 The incorporation pattern of  $^{14}\text{C}$ -mevalonic acid and  $^{14}\text{C}$ -cholesterol into diosgenin and sterols is quantitatively and qualitatively different.
- 2 The amount of incorporation of  $^{14}\text{C}$ -mevalonic acid into diosgenin and sterols remained more or less constant over the first 3 weeks of the experiment then it rose in both classes of steroid during the last week (week 7 of the culture period). The ratio however of the amount of radioactivity in each class to the total amount incorporated remained constant over the whole experiment.
- 3 The level of incorporation of  $^{14}\text{C}$ -cholesterol into diosgenin and sterols fluctuated over the first 3 weeks of the experiment then the incorporation into steryl glycosides increased during the last week of the experiment while that into diosgenin decreased. The ratio of the amount of radioactivity into diosgenin to the total amount incorporated fell to 6% during this time from a value of 22% in the previous week.

A great deal of information has been obtained from the experiments described in this section, however, while this has led to an understanding of the relationship between primary and secondary metabolism in D. composita suspension cultures, none of the experiments lead to any major increases in the yield of diosgenin.

The experiments described in the following section of the Chapter were designed to develop a rapid screening technique for the detection of cell lines yielding high levels of diosgenin.

S E C T I O N      4

THE DEVELOPMENT OF TECHNIQUES FOR  
THE SELECTION OF HIGH YIELDING CELL  
LINES OF D. COMPOSITA

The aim of this Section was to develop a simple and inexpensive technique to screen cell lines isolated from Dioscorea cultures to detect those which accumulated the highest levels of diosgenin. The strategy adopted was to exploit the ability of dioscin to haemolyse red blood cells.

Although a rapid technique was developed based on the comparison of the haemolytic activity of culture extracts with standard saponin solutions in multi-well microtitre plates, lack of time precluded its use within the experimental programme.

Although selection techniques may provide a means of identifying cell lines that produce commercially viable quantities of desired metabolites, the process itself will not become commercially viable unless the cell lines can be grown and continue to produce diosgenin at volumes required for an industrial process. This may be anything from hundreds to thousands of litres.

The experiments included in the following Section were designed to examine the ability of D.composita suspension cultures not only to grow in an 8l stirred fermentor vessel but to continue to accumulate diosgenin at least to the same level as was found in the flask experiments described in Section Three. Although an eight litre fermentor is not industrial scale, it should provide enough information to bridge the gap between laboratory and industry and indicate the feasibility of such a transfer.

S E C T I O N     5

GROWTH AND DIOSGENIN ACCUMULATION OF  
D. COMPOSITA BATCH SUSPENSION CULTURES  
IN MULTI-LITRE STIRRED FERMENTORS

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S E C T I O N    5    -    P A R T    I

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THE CONSTRUCTION OF THE 12 LITRE FERMENTOR  
VESSEL FOR THE LARGE SCALE GROWTH OF  
D. COMPOSITA SUSPENSION CULTURES

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The key step to the establishment of a process which exploits plant cell cultures to produce economically important secondary metabolites is the scaling up procedure from laboratory cultures of 50 - 500 ml to a commercial process in vessels of between 100 and 5000 l.

There are two methods which have been proposed for the commercial scale exploitation of plant cell culture techniques. The first is the culture in multi-litre fermentors, similar to those used in the microbial fermentation industry. In this process the plant cells are usually grown in batch but may be grown in continuous culture and the metabolite is extracted from the tissue or medium or both at the end of the process or in samples withdrawn at intervals.

The second technique is where the plant cell cultures are immobilised in a suitable matrix packed in a column and the culture medium is allowed to percolate through the bed of immobilised cells and the medium is continuously extracted to remove the desired metabolite. The advantages of such a system over the fermentor are the continuous nature of the process, the ease of extraction and the low biomass requirement. This method however can only be used when the plant cells release the desired metabolite into the medium or in instances when a chemical means can be used to alter the permeability of the cells to induce release.

It is generally accepted that the diosgenin containing glycosides such as dioscin are bound to intra-cellular membranes and are not released from cells in culture. This has been confirmed in this study and accordingly it was decided/

decided to study growth and diosgenin accumulation in multi-litre fermentor vessels rather than in an immobilised system. It was obvious however, that the size of vessels which will probably be used in industry, ie 100 - 5000 l were outside the scope of this project. It was decided, therefore, that a vessel of approximately 10 - 15 l would provide a good model system for bridging the gap between laboratory scale experiments and pilot/commercial processes.

The experiments described in the following section were designed to investigate the growth and diosgenin accumulation of D. composita suspension cultures grown in fermentor vessels with a working capacity of 12 l.

The first part of this section describes the construction of the fermentor vessel used in the following experiments, which was built in the departmental workshop. The second part describes three experiments, the first two of which were attempts to study the growth and diosgenin accumulation of D. composita suspension cultures at the end of ten weeks growth in a stirred fermentor vessel with a total volume of 8 litres. The aim of which was to provide a comparison with the flask experiments described in Section 3 of this Chapter. The third experiment was identical to the first except that growth and diosgenin accumulation were to be measured every 7 days over the 10 week culture period by aseptically removing samples for analysis.

The fermentor vessel used in the experiments described in Part 2 of this section was built in the departmental workshop. A diagram of the vessel is shown in Figure 3.5.1.

#### THE CONSTRUCTION OF THE VESSEL

The casing of the vessel consisted of Pyrex industrial glass piping which was 50 cm in length and had an internal diameter of 20 cm. The ends of the pipe were flanged to accommodate metal collars through which six bolts ran which provided a means of attaching and tightening down the end plates to the pipe. Each end plate was made of stainless steel plate 1cm in thickness. The bottom plate was not altered in any way, however, the top plate was machined in several places so that various ports and fittings could be added. The largest hole accommodated the bearing which housed the shaft of the stirrer paddles. The other main parts were the inoculation port which was 2cm in diameter and 3 smaller ports which housed the air inlet and outlet and a sampling tube.

#### AGITATION

Agitation of the cultures was by stirring paddles attached to a central shaft driven by an electric motor. The motor was permanently fixed to a metal frame onto which the fermentor vessel was placed. It fitted in such a way that the bottom end plate aligned with 2 metal guides which ensured the vessel was properly positioned under the motor. The motor was attached to the stirrer shaft by a piece of thick walled rubber tubing which acted as a flexible joint compensating for any small misalignment between motor and shaft. The speed of the motor was adjusted by a controller mounted on the front of the frame.

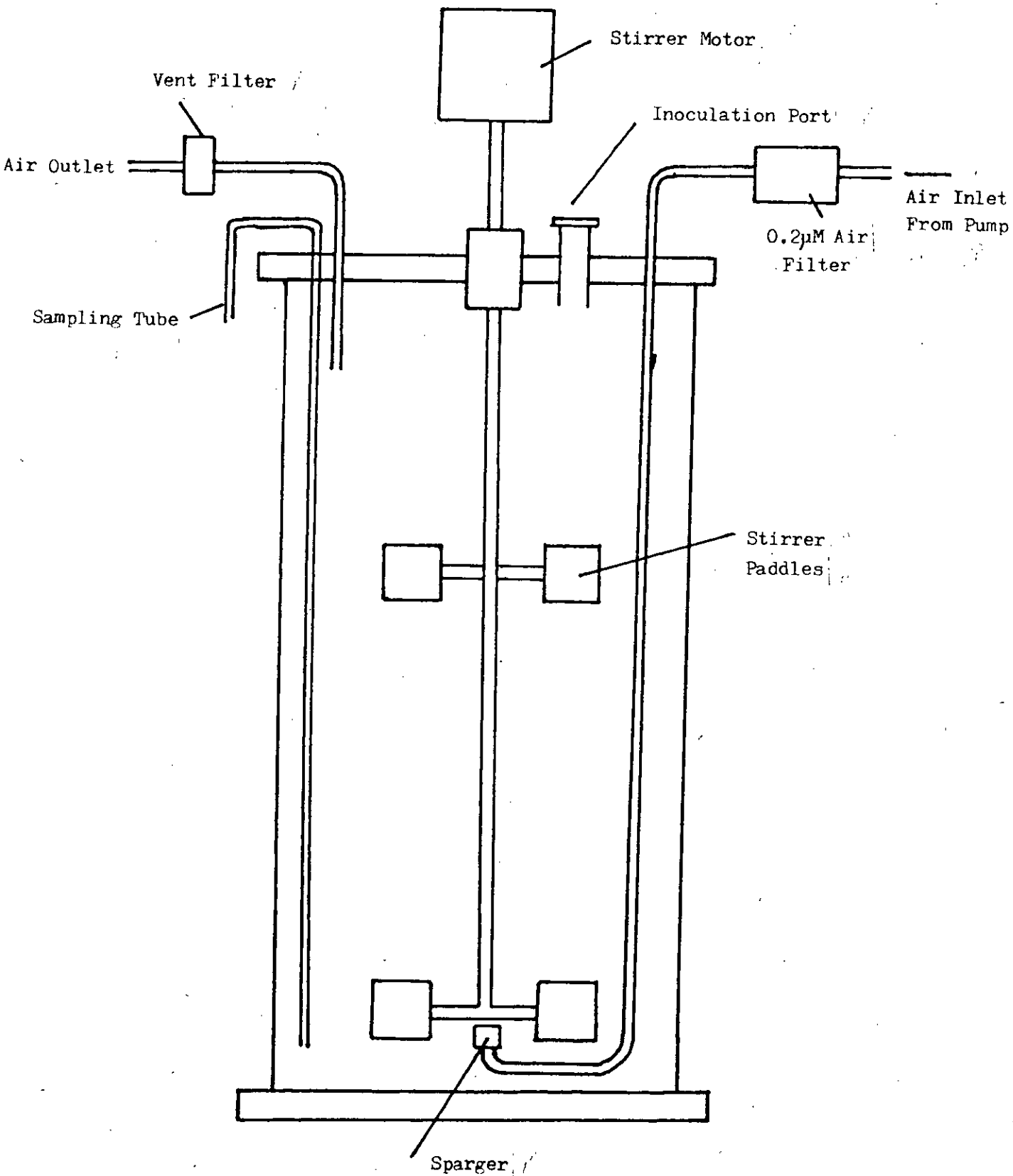


Figure 3.5.1. Fermentor vessel used for the multi-litre growth of *D. composita* suspension cultures.

### AERATION

The cultures were aerated by an electric pump, the air provided was passed through a sterile 0.2mm filter and down through a tube to the base of the vessel where it was delivered through a sparger. The air outlet was also fitted with a 0.2mm sterile filter which prevented back contamination when the vessel was cooling down after autoclaving. It also reduced vapour loss during the culture period since the inside face of the filter was hydrophobic.

### SAMPLING TISSUE

Sampling of the tissue during a culture period was carried out by blocking the air outlet tube. The resultant build up of pressure forced tissue up the sample tube where it was collected. The first 50 mls of culture collected at each sampling period was discarded since this represented the contents of the sample tube from the previous sampling period. The next 100 ml was collected, then the end of the sampling tube was sterilised using a portable gas burner (Camping Gaz Ltd) and then sealed with a fresh sterile rubber bung.

SECTION 5 - PART 2

GROWTH AND DIOSGENIN ACCUMULATION OF  
EIGHT LITRE SUSPENSION CULTURES OF  
D. COMPOSITA IN A FERMENTOR VESSEL

The aim of these experiments was to determine whether suspension cultures of D. composita could be grown in large volumes in the type of fermentor vessel described in Part 1 of this Section and to compare the growth and diosgenin accumulation under these conditions with the 60 ml flask experiments described in section 3.

The culture was inoculated by adding 6, 4 week old, 60 ml suspension cultures of D. composita to 7.64 l. of MS medium, containing 1 ml of antifoam agent (Sigma Chem Co Ltd) giving a total of 8 l. The culture was stirred at 100 rpm and aerated at 2 l/min, the intended culture period was 10 weeks.

#### RESULTS - FIRST FERMENTOR EXPERIMENT

There was no visible sign of growth in the culture after 1 week however, during the second week although there still seemed to be little growth, the culture medium began to turn brown as if the tissue was excreting substances into it. The tissue in comparison remained a creamy white colour. By the end of the third week, the culture did seem to be growing slowly, however, the culture medium was by this time dark brown, with the tissue a light brown colour. At the end of a month, the culture medium was black and the tissue was still a light brown colour with only a small noticeable increase in growth. Because of this lack of growth/

growth and the excretion into the culture medium of what seemed to be phenolic substances of the type associated with wound reactions and necrosis in plant tissue, it was decided to terminate the experiment.

The culture was harvested and cell viability, packed cell volume, fresh weight, dry weight and diosgenin accumulation were determined as described in section 2 of Chapter 2.

The culture medium was also analysed for diosgenin as described in Section 2 of Chapter 2. It was also analysed for signs of microbial contamination. This was carried out using 3 different tests:

- a) Gram's staining method was used on samples of the culture medium.
- b) Samples of the medium were plated out and incubated on Nutrient Agar Medium.
- c) Samples of the medium were plated out and incubated on Czapek Dox Medium.

The above tests were carried out as described in section 2 g of chapter 2.

The results of culture growth and diosgenin accumulation are presented in Table 3.5.1. from which it can be seen that over the four week period the packed cell volume of the culture increased from 1.6% to 5% which is a 3.1 fold increase. This is comparable with an approximate 3 fold increase over a similar period in the experiment with 60 ml flask suspension cultures of D. composita (Section 3 of the Results Chapter). The initial packed cell volume however in the flask experiment was approximately 11%, which is more than 7 times the value in the fermentor experiment.

TABLE 3.5.1

GROWTH AND DIOSGENIN ACCUMULATION OF AN 8 LITRE *D.composita* FERMENTOR CULTURE OVER A 4 WEEK PERIOD

	CELL VIABILITY (%)	PACKED CELL VOLUME (%)	FRESH WEIGHT		DRY WEIGHT		DIOSGENIN YIELD (mg)	
			TOTAL g	DENSITY g/l	TOTAL g	DENSITY g/l	TISSUE	MEDIUM
*INITIAL VALUES	90	1.6	36.00	4.50	3.00	0.38	-	-
FINAL VALUES	65	5.0	83.53	10.44	8.00	1.00	-	-

\* ESTIMATED VALUES FROM INOCULUM CULTURES

236.

Dry weight in the fermentor experiment increased 2.7 fold to a total of 8.00 g from an initial value of 3.00 g. This compares to an 8.9 fold increase in total dry weight of the flask experiment. If the dry weight of both systems are expressed in cell densities ie g dry weight/litre of culture then it can be seen from Table 3.5.1. that at inoculation the cell densities of the fermentor experiment was 0.38 g/l. This is more than two and a half times less then the equivalent value in the flask experiment. The final cell density in the fermentor experiment of 1.00 g/l is over eight and a half times less than the equivalent value in the flask experiment.

Fresh weight in the fermentor experiment increased 2.7 fold to a total of 8.00 g from an initial value of 3.00 g. This compares to an 8.9 fold increase in total dry weight of the flask experiment. If the dry weight of both systems are expressed in cell densities, ie g dry weight/litre of culture then it can be seen from Table 3.5.1. that at inoculation, the cell density of the fermentor experiment was 0.38 g/l. This is more than two and a half times less than the equivalent in the flask experiment. The final cell density in the fermentor experiment of 1.00g/l is over eight and a half times less than the equivalent value in the flask experiment.

Fresh weight in the fermentor experiment increased from 36.00g to a total of 83.53 g which is a 2.3 fold increase. Over a similar period in the flask experiment, total fresh weight increased by 9.0 fold. If fresh weight is considered in terms of cell densities, then the value in the fermentor experiment, at inoculation of 4.50 g/l was over 4 times less/

less than the equivalent figure in the flask experiment. Similarly the final cell density in the fermentor experiment of 10.44 g/l was over 16 times less than the equivalent value in the flask experiment.

Cell viability in the fermentor experiment dropped 25% over the four week culture period to 65% from an initial value of 90%.

It can also be seen from the results that no diosgenin was detected in either the tissue or the culture medium. This was what was found in the flask experiment after 4 weeks of culture.

The tests for microbial contamination of the culture all proved negative. No organism could be detected microscopically after samples of the culture medium were treated using Gram's staining method and neither was any microorganism detected on either nutrient agar medium or Czapek Dox medium after being inoculated with samples of the culture medium.

These results suggest that the poor growth of the culture could not be explained by either microbial contamination or by large losses in cell viability. The only feature which might explain this was the relatively low amount of tissue used in the inoculum compared with the flask experiment. In order to investigate whether the low density inoculum was responsible for the poor growth, the above experiment was repeated with a higher density of inoculum.

The second fermentor experiment was inoculated by adding 16, 4 week old, 120 ml suspension cultures of D. composita

D. composita to 6.08 l of MS medium (containing 1 ml of antifoam agent) giving a total volume of 8 l. The culture was stirred at 100 rpm, aerated at 2 l/min and grown for 10 weeks.

#### RESULTS - SECOND FERMENTOR EXPERIMENT

In contrast to the previous experiment, this culture grew well with no sign of the dark phenolic like excretions shown by the previous culture. It was therefore allowed to complete the planned 10 weeks of culture before being harvested.

After harvesting, cell viability, packed cell volume, fresh weight, dry weight and diosgenin accumulation in the tissue and culture medium were determined as described in Section 2 of Chapter 2.

The results of culture growth and diosgenin accumulation are shown in Table 3.5.2. from which it can be seen that over the 10 week period the packed cell volume of the culture increased from 8.4% to 30.5% which represents a 3.6 fold increase. This result is comparable with that found in the 60ml flask suspension culture experiment described in Section 3 of the results Chapter. The packed cell volume of the culture in the second fermentor experiment of 8.4% at inoculation was over 5 fold greater than that of the first fermentor experiment. This made it more comparable to the value of 11% at inoculation, in the flask experiment.

Dry weight in the fermentor culture increased by 4.2 fold to a total of 67.42 g from an initial value of 16.15 g. Although this represents a significantly smaller increase than/

TABLE 3.5.2

GROWTH AND DIOSGENIN ACCUMULATION OF AN 8 LITRE *D. composita* FERMENTOR CULTURE OVER A 10 WEEK PERIOD

	CELL VIABILITY (%)	PACKED CELL VOLUME (%)	FRESH WEIGHT		DRY WEIGHT		DIOSGENIN YIELD (mg)	
			TOTAL g	DENSITY g/l	TOTAL g	DENSITY g/l	TISSUE	MEDIUM
* INITIAL VALUES	87	8.4	339	42	16.15	2.02	-	-
FINAL VALUES	70	30.5	1341	168	67.42	8.43	24.95	-

\* ESTIMATED VALUES FROM INOCULUM CULTURES

240.

than the 7.5 fold increase seen in the flask experiment, it was 1.6 fold greater than the increase in the first fermentor experiment.

When dry weight values are expressed in terms of cell densities it can be seen from Table 3.5.2 that the final figure in the second fermentor experiment, at 8.43 g /l was 1.2 fold greater than the final density in the flask experiment and 8.4 fold greater than the final density in the first fermentor experiment. This was reflected in the inoculation densities where the value for the second fermentor experiment, at 2.02 g/l was 2.1 fold greater than the flask experiment value and 5.3 fold greater than the value in the first fermentor experiment.

Fresh weight in the second fermentor experiment increased from 339 g to a total of 1341 g which is just under a four fold increase which is a 1.7 fold improvement on the increase in the first fermentor experiment. It was however still smaller than the increase in the flask experiment which was 7.9 fold.

When the fresh weight values are re-expressed in terms of cell densities it can be seen that final value for the second fermentor experiment, at 168 g/l, was 1.1 fold greater than the final cell density in the flask experiment and 16.1 times greater than the value in the first fermentor experiment. As in the dry weight values, the above results are reflected in the cell densities of the inoculums. The value for the inoculum of the second fermentor experiment was/

was 42 g/l which was 2.2 times greater than in the flask experiment and 9.3 times greater than in the first fermentor experiment.

Cell viability dropped 17% from an initial value of 87% to 70% at the end of the ten week culture period. This was less than the first fermentor experiment where cell viability fell 25% over a 4 week period.

It can be seen from Table 3.5.2. that the total yield of diosgenin was 24.95 mg. Expressed as mg/g dry weight of culture this was 0.37 mg/g dry weight. No diosgenin was detectable in the culture medium. The yield of diosgenin in the second fermentor experiment was similar to that of the flask experiments. It was less than the average yield/g dry weight at the tenth week of culture in the first flask experiment but greater than that of the second.

This experiment confirmed that D. composita suspension cultures will grow in multi-litre batch in a stirred fermenter vessel. What the experiment did not clarify is whether by the end of the tenth week the fermentor culture was in a period of increasing, stationary or decreasing growth and whether the yield of diosgenin represents an accumulation profile which is increasing or decreasing.

To determine what the profiles of culture growth and diosgenin accumulation were in this culture system and to compare them with the flask data the above experiment was repeated.

In this case, however, growth and diosgenin accumulation were/

were to be determined every 7 days over a ten-week period using 100ml samples harvested from a fermentor culture.

The third fermentor experiment was inoculated by adding 16, 4-week old, 120ml suspension cultures of D.composita to 6.08ℓ of MS medium (containing 1ml of anti-foam agent) giving a total volume of 8ℓ. The culture was stirred at 100 r.p.m. and aerated at 2ℓ/min. The proposed culture period was 10 weeks with 100ml samples of culture harvested each week as described in part 1 of this Section.

#### RESULTS - THIRD FERMENTOR EXPERIMENT

As with the previous experiment, the culture grew well with no obvious sign of microbial contamination or phenolic like excretions and the first 100ml sample was harvested at day 7. The sample was used to determine cell viability, packed cell volume, fresh weight, dry weight and diosgenin accumulation, (in tissue and culture medium), as described in Section 2 of Chapter 2.

It became apparent about 3 days after the first sampling that the culture had become heavily contaminated with some micro-organism. The tissue turned brown, but there was no dark excretion as in the first experiment. The culture medium became extremely turbid and a thick scum formed round the inside face of the culture vessel especially at the surface of the culture medium. The culture was terminated at day 10 and using Gram's staining method and plating out of culture medium on Nutrient Agar and Czapek Dox media, the contaminant was

identified as a Gram negative bacterium.

The results from the fermentor experiments suggest that D.composita suspension cultures can be grown in stirred fermentor vessels up to volumes of 8l and that the growth is comparable to that found in flask culture. The results also suggest that the size of the inoculum plays a key role in the subsequent growth of the culture in that low amounts of inoculum (e.g. approximately 1% packed cell volume) will lead to poor growth while relatively high amounts of inoculum (approximately 8-9% packed cell volume) will lead to good growth.

The results not only showed that good growth could be obtained in a fermentor vessel, but that diosgenin accumulated in the cultures at levels comparable to that found in flask cultures of a similar age. Due to the loss of the third experiment, no detail could be gained on how the diosgenin accumulation profile compared to that of the flask culture.

DISCUSSIONTHE RELATIONSHIP BETWEEN GROWTH AND THE ACCUMULATION OF SECONDARY METABOLITES IN PLANT CELL CULTURES

The accumulation of secondary metabolites in plant cell cultures is a dynamic process and its relationship with culture growth may vary, (Tabata, 1977). The three most common patterns that may be observed are presented against a generalised growth profile of a plant suspension culture in Figure 4.1.1.

Pattern one is where the accumulation of the secondary metabolite takes place during the growth of the culture, i.e. during the exponential and linear growth phases. In pattern two although the accumulation begins during the mid to late part of the linear phase, the maximum accumulation extends into the stationary phase. In pattern three the accumulation does not start until growth has stopped completely and the entire accumulation occurs during the stationary phase.

Pattern three is the typical profile for the accumulation of secondary metabolites in microorganisms, while in plant cell cultures, the accumulation of secondary metabolites typically follows patterns two and three. This is in agreement with the growing body of evidence, (Yeoman et al., 1980, 1982) which suggests that in general there is an inverse relationship between growth and product accumulation.

The results described in this thesis which represent the first time the accumulation profile of the steroid diosgenin and the relationship to growth have been determined in cell cultures of D. composita, support the above view. Indeed the results described in parts one and two of section three of the results chapter show convincingly in two separate experiments that diosgenin accumulates in the cultures exclusively during the stationary phase (i.e. pattern three of Figure 4.1.1.). This shows that the accumulation of diosgenin is completely separated

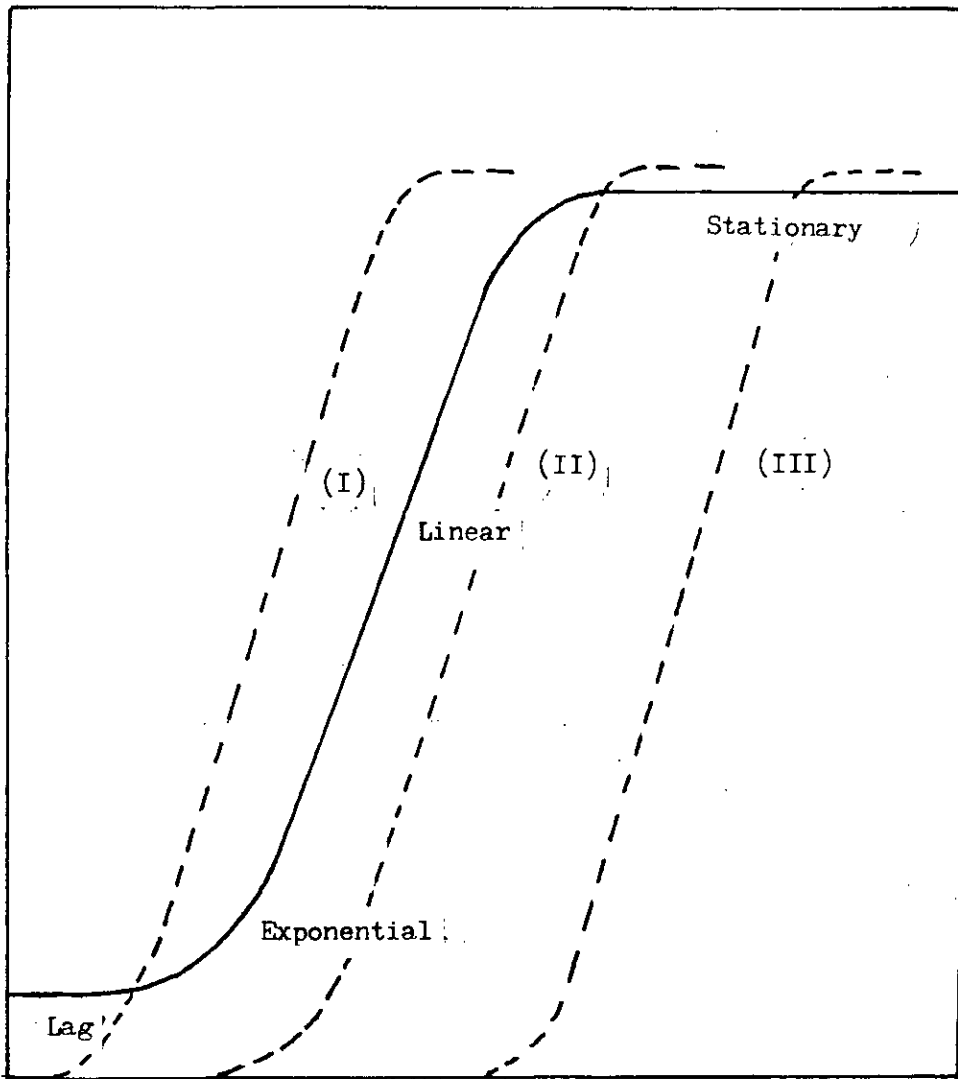


Figure 4.1.1. The relationship between three patterns (----) of secondary product accumulation (I, II and III) compared to a generalised growth profile of a plant cell suspension culture.

in time from all aspects of growth measured e.g. increases in cell number, P.C.V., dry weight and fresh weight.

This separation of growth and accumulation is further supported when the diosgenin accumulation data from the experiment in part one of section three of the results chapter is compared with the measured parameters of culture growth by means of scatter diagrams. Diosgenin accumulation is compared with cell number in Figure 4.1.2., with P.C.V. in figure 4.1.3., with dry weight in Figure 4.1.4. and fresh weight in Figure 4.1.5. It can be seen from these figures that the calculated correlation coefficients ( $r$ ) for each of the relationships shows that there are no positive correlations between diosgenin accumulation and any of the measured parameters of growth.

Similar results have been observed for diosgenin accumulation in the related species Dioscorea deltoidea, (Kaul et al., 1969; Tal and Goldberg, 1982; Taracanova et al., 1979). An inverse relationship between growth and diosgenin accumulation has also been reported in Momordica charantia, (Khanna and Mohan, 1973) and Solanum verbascifolium, (Jain and Sahoo, 1981).

It can be seen from the above results that the accumulation of diosgenin during the stationary phase of growth in batch suspension cultures of D. composita must be the result of an increase in the activity of the enzymes involved in the biosynthesis of diosgenin. Such increases in enzymatic activity prior to accumulation of secondary metabolites have been detected in several plant cell cultures. Sasse et al. (1982) have reported increased activity of tryptophan decarboxylase prior to the accumulation of serotonin in Peganum harmala, as have Knobloch et al. (1981b) prior to the accumulation of indole alkaloids in Catharanthus roseus cultures. Increases in the activity of phenylalanine ammonia-lyase have also been reported in a number of different cultures prior



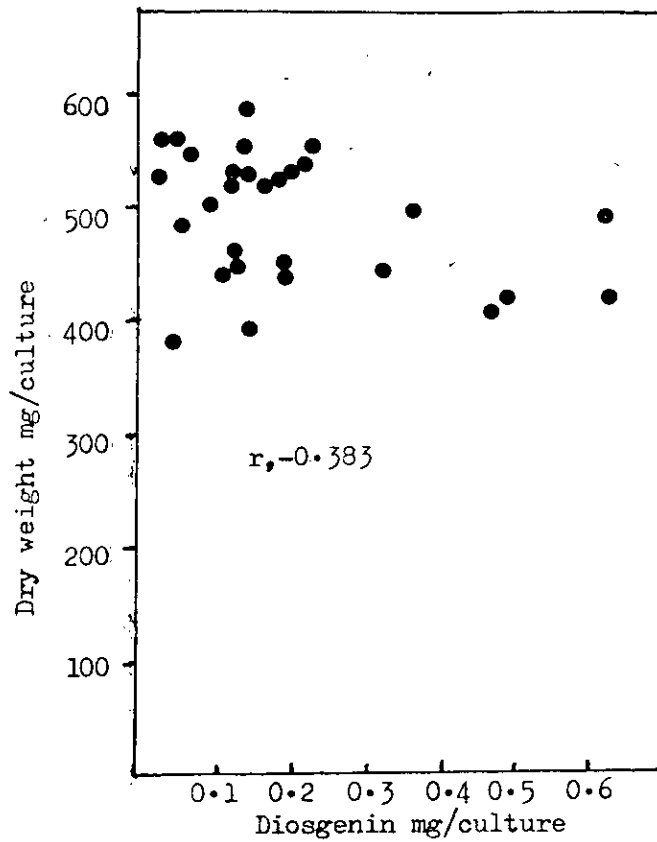


Figure 4.1.4. Scatter diagram of the correlation between dry weight and diosgenin accumulation in suspension cultures of D.composita.

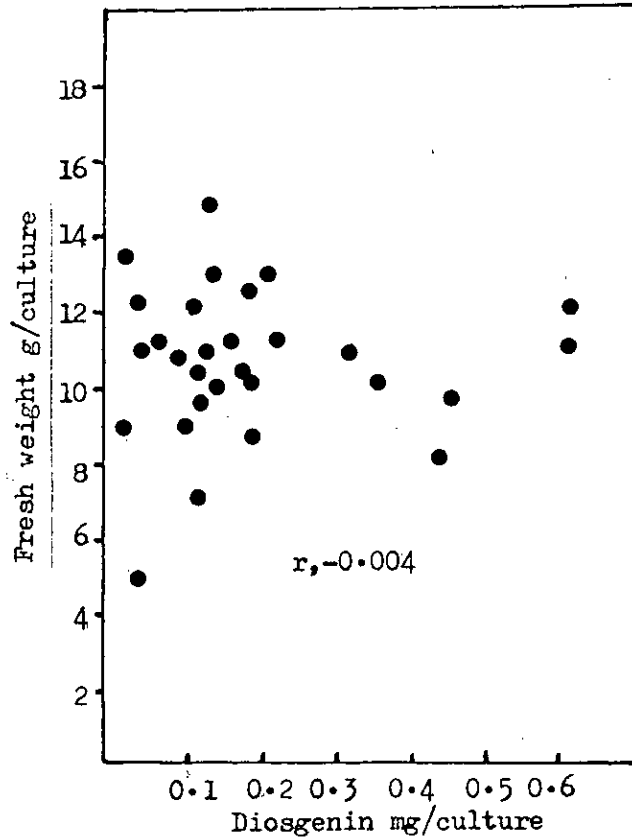


Figure 4.1.5. Scatter diagram of the correlation between fresh weight and diosgenin accumulation in suspension cultures of D.composita.

to the accumulation of a variety of phenolic compounds, (Berlin et al., 1982; Davies, 1972; Westcott and Henshaw, 1976; Hahlbrock and Wellmann, 1973). One question which arises from the above data is whether the increase in enzymatic activity is the result of the activation of an existing enzymatic pathway or the result of 'de-novo' synthesis of enzymes.

Evidence for the presence of enzymatic pathways prior to the accumulation of secondary metabolites in culture comes from work carried out on cell free extracts. Banthorpe and Barrow, (1983), have shown that although callus cultures of Rosa damascena cannot synthesise or accumulate monoterpenes, cell free extracts can sustain the biosynthesis of geraniol and nerol from  $^{14}\text{C}$ -isopentenyl pyrophosphate (IPP). Similar results have also been found for a variety of monoterpenes in cell free extracts prepared from cultures of Ocimum basilicum, Tanacetum vulgare, Rosmarinus officinalis and Lavendula spica, (Banthorpe, 1979, 1980 unpublished work quoted in Yeoman et al., 1982a). In a similar experiment at the Botany Department of the University of Edinburgh, Perez-Francis, (1980, unpublished data) showed that cell free extracts prepared from an actively growing culture of Solanum nigrum which was not accumulating hyoscyamine, could convert  $^{14}\text{C}$ -ornithine into the above-mentioned tropane alkaloid. McLauchlan et al., (1983), have shown that cell free extracts prepared from a cell line of Catharanthus roseus which does not accumulate vinblastine can catalyse the enzymatic conversion of anhydrovinblastine to vinblastine. The above evidence supports the view that for certain secondary metabolites, that enzymes involved in their biosynthesis can be shown to be present in cultures that are not accumulating them which suggests that any subsequent accumulation is the result of enzymatic activation rather than 'de novo' synthesis.

There is evidence in the literature that supports the alternative view that the accumulation of certain secondary metabolites can be the

result of 'de novo' synthesis of the enzymes involved in their synthesis. Kreuzaler et al, (1983) have presented evidence that suggests the induction of flavonoid accumulation in Petroselinum hortense by ultraviolet irradiation is the result of 'de-novo' synthesis of enzymes of flavonoid biosynthesis. Similarly, Lawton et al, (1983a, 1983b) have shown that the accumulation of the phytoalexin phaseolin induced by the addition of a fungal elicitor to Phaseolus vulgaris was also the result of 'de-novo' synthesis of enzymes in its biosynthetic pathway. The evidence from the literature suggests that the control of enzymatic activity which results in the accumulation of secondary metabolites may not be governed by a common regulatory mechanism. It may be 'enzyme activation' in one species and 'de-novo' synthesis in another or even a combination of the two in some species. In the absence of any direct experimental evidence it may be concluded therefore that the increase in enzymatic activity which resulted in the accumulation of diosgenin in the experiments described in the thesis, may have been due to either, or a combination of both, of the possible mechanisms mentioned above.

Although it was not possible to say which of the above mechanisms was responsible for the increase in enzymatic activity which led to the accumulation of diosgenin, possible changes in the cultural environment which may have triggered the increase have been investigated.

#### THE ROLE OF NUTRIENT FACTORS IN THE REGULATION OF SECONDARY

#### METABOLISM IN PLANT CELL CULTURES

Apart from a few cases where the accumulation of a secondary metabolite can be induced in plant cell culture by the addition of a fungal elicitor or by irradiation with ultraviolet light, it is changes in the nutrient medium which represent the largest and most common environmental change likely to influence culture growth and the accumulation

of secondary metabolites. Such changes have been implicated in the regulation of secondary metabolism in microorganisms, where the levels of inorganic phosphate, carbon and nitrogen in culture media which are optimal for growth, result in the inhibition of secondary metabolism. (Drew and Demain, 1977; Grootwassink and Gaucher, 1980).

Accumulation will only usually commence in these cases when one or a combination of the above nutrients is depleted from the culture medium.

The utilisation of inorganic phosphate, sucrose, ammonium and nitrate have been studied in batch suspension cultures of D. composita and their relationship to growth and diosgenin accumulation is discussed below.

#### Phosphate

The results presented in Figure 3.3.8a. show that phosphate was rapidly accumulated by the cells from the culture medium during the first seven days of culture and from Figure 3.3.6a it can be seen that the largest increase in cell number occurred in the seven days subsequent to the majority of the inorganic phosphate being removed from the medium. This suggests that the cultures accumulated high intracellular concentrations of phosphate prior to its utilisation for growth, rather than a slower absorption from the medium as it was required. A similar rapid accumulation of phosphate has been reported by Knobloch et al, (1981a) in tobacco cultures. After 14 days of culture phosphate was essentially depleted from the medium, this was also the time at which cell division came to a halt. It can be seen from Figures 3.3.8a-3.3.9b. that phosphate was the only nutrient, of the four measured, which was depleted at this time which suggests that it was probably this that was responsible for cell division coming to a halt. This implies of course that the intracellular level of phosphate, which was not measured in this experiment, was also depleted. Knobloch et al, (1981a) have

shown however that it is not necessary for the intracellular inorganic phosphate to be totally depleted to limit growth. They found that tobacco cultures which had depleted phosphate from the culture medium and in which cell division had ceased, still contained considerable levels of intracellular inorganic phosphate.

If, as the above results suggest, that the depletion of phosphate was responsible for halting cell division then could it also have been involved in the regulation of diosgenin accumulation? It can be seen from Figures 3.3.8a. that phosphate was depleted from the culture medium well before diosgenin began accumulating. If phosphate was involved in the regulation of diosgenin accumulation then the high levels of intracellular phosphate, which have been suggested were present at the beginning of the culture cycle, must have inhibited the synthesis of diosgenin. After some time, once the phosphate levels had been depleted below a certain critical point, then diosgenin synthesis would no longer be inhibited and accumulation would begin.

There is evidence in the literature which supports the idea that high levels of inorganic phosphate in culture media which are optimal for growth inhibit the accumulation of secondary metabolites not only in a wide range of microbial systems (Weinberg, 1974) but also in plant cell cultures. The transfer of cells from a medium optimal for growth to one free of phosphate leads to the accumulation of substantial amounts of indole alkaloids in cultures of Catharanthus roseus, (Knobloch and Berlin, 1980) and cinnamoyl putrescines in Nicotiana tabacum, (Knobloch and Berlin, 1981). It can be seen from Figure 3.3.8a, that there is approximately a four week interval between the start of the major phase of diosgenin accumulation and the depletion of phosphate from the culture medium. If the depletion of phosphate was the trigger for the start of the accumulation then one would have expected the lag between the two events to be much smaller. There are two possible

hypotheses which can be put forward to explain this. The first is that if phosphate is inhibiting some aspect of diosgenin biosynthesis then it is the intracellular concentration which will ultimately be important in a regulatory role and not the concentration in the culture medium. The interval between the depletion of inorganic phosphate from the culture medium and the start of diosgenin accumulation may be the time required for the intracellular inorganic phosphate concentration to fall below a certain point at which diosgenin biosynthesis is no longer inhibited and accumulation can begin. Knobloch et al (1981a) have in fact shown that the accumulation of cinnamoyl putrescines in tobacco cultures is inversely proportional to the intracellular inorganic phosphate concentration.

The second hypothesis is that it is not intracellular inorganic phosphate but intracellular ATP that inhibits diosgenin biosynthesis. The evidence for such a mode of regulation comes from studies carried out on a range of microorganisms where inorganic phosphate has been shown to be inhibitory to the production of chlortetracyclin, ergot alkaloids streptomycin and other antibiotics, (Weinberg, 1974). It has also been shown that inorganic phosphate represses the first enzyme in alkaloid biosynthesis in a Claviceps species (Krupinski et al, 1976). It has been suggested that the mechanism of phosphate inhibition in these secondary metabolic processes might involve regulation by energy charge, i.e. the relative intracellular levels of ATP, ADP and AMP, (Drew and Demain, 1977), with high ATP concentrations, caused by high levels of inorganic phosphate, leading to inhibition of secondary metabolism. There is evidence from work carried out on Escherichia coli which links high concentrations of inorganic phosphate in the culture medium with high intracellular ATP concentrations (Shechter et al, 1973). Correlations have also been reported between high intracellular ATP levels

and the inhibition of secondary metabolism in microorganisms e.g. inhibition of candicidin accumulation in Streptomyces griseus, (Martin and Demain, 1976).

The intracellular levels of ATP at any one time in a non-photosynthetic plant cell culture, such as the D.composita suspension cultures described in this thesis, is a balance between its production during the catabolism of sucrose by glycolysis and the TCA cycle and its utilisation in providing the energy for the primary metabolic processes of growth e.g. cell division. When one considers these various processes over the culture period described in section three, part two of the Results Chapter, an idea of the possible changes in the intracellular levels of ATP and their effects on diosgenin accumulation, may be gained.

It can be envisaged that over the first seven days of the culture period, as has already been mentioned, the high intracellular inorganic phosphate concentrations may have resulted in an initially high intracellular ATP concentration causing an inhibition of diosgenin synthesis. The period of culture growth which lasted to approximately the end of week four (Figures 3.3.6a-7b) will have been characterised by a rapid utilisation of ATP to provide energy for cell division, etc. The level of ATP however would probably remain relatively high during this period due to its replenishment via glycolysis and the TCA cycle, which would explain the continued inhibition of diosgenin synthesis during the growth phase. It can be seen from Figures 3.3.8b, that sucrose was being rapidly utilised during the growth phase and that by the end of week four only 5% of the original sucrose remained in the culture medium. During the two weeks between this point and the beginning of the major phase of diosgenin accumulation the level of sucrose fell further to just over 1% of the original value at a time when even though no active growth had occurred, some energy, via ATP, would be

required just to maintain cellular metabolism. It is possible therefore that the demand for ATP began to outstrip supply during this time because glycolysis and the TCA cycle may have been slowing down due to lack of substrate i.e. sucrose and of inorganic phosphate for the associated phosphorylation reactions. If this was the case then the intracellular ATP level could have fallen to a sufficiently low level by the end of the sixth week that the inhibition of diosgenin synthesis was lifted and accumulation began.

Although the above energy charge model was at first based on interpretation of the phosphate depletion data, it can be seen that towards the end, it was obvious that the depletion of sucrose as well as phosphate could be involved in determining the levels of ATP. The role of sucrose in the growth and diosgenin accumulation of D.composita suspension cultures is discussed below.

### Sucrose

The depletion of sucrose from the culture medium and its relationship to culture growth and diosgenin accumulation are presented in Figures 3.3.6a-7b

/3.3.8 It can be seen from these that although sucrose was rapidly utilised during the first four weeks of culture, by the time all aspects of growth had stopped increasing at the end of the fourth week, approximately 5% of the original amount of sucrose still remained in the culture medium. These results show that, unlike, phosphate, sucrose was never limiting with respect to growth. Even after the fourth week when the residual sucrose in the culture medium fell further to around 1% of the original value, the evidence suggests that sucrose was never totally exhausted. This is based on work carried out on cultures of Acer pseudoplatanus (King, 1977; Watson and Fowler, 1980) which showed that when growth in these systems was

carbohydrate limited, then a significant amount of cellular material (measured as dry weight) was lost after growth had ceased. It can be seen from Figure 3.3.7a. that no significant loss of dry weight was detected in the D.composita suspension cultures between the end of the growth phase and the termination of the experiment. If, as the results suggest, that the changes in the level of sucrose do not play any role in limiting the growth of the cultures, then is it involved in the regulation of diosgenin accumulation?

It can be seen from Figure 3.3.8b that sucrose, like phosphate, was almost depleted from the culture medium by the time diosgenin began accumulating, unlike phosphate however, the low point in its depletion coincided almost exactly with the start of the major phase of diosgenin accumulation at the end of the sixth week. This suggests that the two events may be linked. The possible hypothesis which may explain this, is that the high level of sucrose present in the culture medium during the first half of the culture period was inhibitory to diosgenin biosynthesis and that the accumulation of diosgenin only occurred once the level of sucrose had fallen below a certain value. This inhibition of secondary metabolism by rapidly utilised carbohydrates is very common in microbial culture where it is called carbon catabolite regulation, (Drew and Demain, 1977). A large number of carbohydrates have been implicated in such regulation including glucose, fructose, lactose, mannose, maltose and citrate, while sucrose and lactose in general have not. Although this type of regulation has never been reported in plant cell cultures, Tal et al, (1982), have shown that while sucrose, lactose, galactose and starch all promote the stationary phase accumulation of diosgenin in batch suspension cultures of Dioscorea deltoidea, glucose and fructose cause the yield of diosgenin to drop from over 1% of dry weight to 0.1% without causing large

increases in dry weight. Conclusive proof that this effect was due to carbon catabolite regulation would be obtained if diosgenin accumulation could be restored by feeding one of the inhibitory carbohydrates continuously in small amounts. This approach has been successfully applied to overcome carbon catabolite regulation in many industrial microbial fermentations (Demain, 1968). Balague and Wilson (1981) have adopted such an approach to produce evidence against carbon catabolite regulation in plant cell cultures. They found that if cultures of Catharanthus roseus were grown in a chemostat under steady state growth conditions, where sucrose was the limiting nutrient, then no secondary metabolite, in this case serpentine, was produced. In fact if such sucrose limited cultures were transferred to batch cultures containing high amounts of sucrose then serpentine was found to accumulate. Overall the above results seem to suggest that sucrose is not directly involved in the control of diosgenin accumulation via carbon catabolite regulation although they do not totally preclude the possibility.

#### Ammonium and Nitrate

The results presented in Figures 3.3.9a. and 3.3.9b. show that both sources of nitrogen in the culture medium, ammonium and nitrate respectively, despite being rapidly utilised during the early part of the culture period, were never totally depleted. Substantial amounts of ammonium (approximately 5-10%) and nitrate (20-30%) could be detected in the culture medium from the end of the second week to the termination of the experiment. These results show that neither ammonium nor nitrate levels in the culture medium were ever limiting with respect to growth. The fact that the levels of each nutrient stayed more or less constant during the entire period of diosgenin accumulation also suggests that neither nutrient played any role in

initiating the accumulation.

This is in total contrast to the work of Phillips and Henshaw, (1977), who reported that the accumulation of phenolics in cultures of Acer pseudoplatanus was dependent on the depletion of inorganic nitrogen sources from the culture medium. The addition of a nitrogen source such as urea to the above cultures which were already accumulating phenolics caused an immediate inhibition of accumulation. Similar nitrogen sensitive accumulations of secondary metabolites have been reported in microbial systems, (Drew and Demain, 1977; Grootwassink, 1980). In general however it is nearly always those secondary metabolites which have nitrogenous precursors, such as amino acids and compete for these compounds with protein synthesis, that show sensitivity to the levels of inorganic nitrogen compounds. It is perhaps not surprising therefore that diosgenin synthesis which is an entirely carbon based process is not inhibited by these compounds. The work of Heble and Staba, (1980a) and Tal et al, (1982) suggests in fact that the presence of nitrate and ammonium is required for optimum diosgenin accumulation.

In the above discussion, hypotheses involving phosphate and sucrose in the regulation of diosgenin accumulation have been proposed. These involve the inhibition of diosgenin biosynthesis during the growth phase and a reverse of this inhibition during stationary phase once the concentrations of these nutrients drop below a critical level. Evidence for such regulation in other plant culture systems is sparse and suggests that a role for phosphate is the more likely of the two. Two hypotheses have been proposed for the action of phosphate, one involves inorganic phosphate in the direct inhibition of diosgenin biosynthesis, the other involves ATP as the inhibitory compound, with

inorganic phosphate indirectly involved in determining its intracellular level. In both these cases, more evidence is required that either substance can inhibit one or more of the enzymes of diosgenin biosynthesis.

The above discussion has so far concentrated on the possible direct effects that various nutrients can have on diosgenin accumulation. It can be seen however that inorganic phosphate also has an effect on culture growth and of the four nutrients studied was the only one which was shown to limit growth. To what extent, therefore do changes in growth rate affect diosgenin accumulation?

#### THE RELATIONSHIP BETWEEN GROWTH AND NUTRIENT FACTORS IN THE REGULATION OF SECONDARY METABOLISM IN PLANT CELL CULTURES

It can be seen from Figures 3.3.6a-7b that culture growth was finished approximately by the end of the fourth week, two weeks before the start of the major phase of diosgenin accumulation. This raises the question of whether the cessation in growth itself was the only prerequisite for the initiation of diosgenin accumulation and that changes in nutrient levels were only important in so far as they brought growth to a halt.

One of the techniques which has helped to clarify the relationship between growth rate, nutrient depletion and secondary metabolism is the use of chemostats (Street, 1977). Balague and Wilson (1981) have shown that a suspension culture of Catharanthus roseus grown in a chemostat under steady state growth conditions, where phosphate was the limiting nutrient, was still able to accumulate the indole alkaloid, serpentine. A similar culture under sucrose limiting conditions did not accumulate any serpentine. Similarly, Tal and Goldberg (1982) have shown that a chemostat culture of Dioscorea deltoidea grown

under steady state growth conditions can accumulate diosgenin, although the yield is less than that from a stationary phase batch suspension culture. Bu'lock (1975) has studied the secondary metabolism of the fungus Gibberella fugikoroï grown in a chemostat at three different growth rates using the nitrogen source as the limiting nutrient. He found, at the fastest growth rate, no secondary metabolites were produced, at a slower growth rate, the polyketide pigments, the bikaverins were produced and at the slowest growth rate when nitrogen limitation was at its most severe, the diterpenoid gibberellins are produced.

The above data while supporting the view that in general there is an inverse relationship between plant cell culture growth and secondary metabolite accumulation, suggest that it is not necessary for culture growth to come to a complete halt before accumulation will begin. It is the nutritional status of the culture, coupled to a slowing of the growth rate that is important. This brings us to the last proposed hypothesis for the regulation of diosgenin accumulation. It is one which is consistent with the above view that both nutrient status and growth rate are involved in the regulation of secondary metabolism.

This hypothesis which has been described by Yeoman et al, (1982), is based on kinetic effects rather than the inhibition or repression of enzymes and is based on the competition between two metabolic pathways, one primary and the other secondary, for a common precursor. In such a case where two pathways X and Y are competing for a common precursor, if the reactions of pathway X are much faster than pathway Y then most or all of the precursor will be utilised by pathway X. This will continue until reaction X slows down sufficiently or stops to allow precursors to accumulate to such a level that reaction Y can proceed. In the case of D.composita suspension cultures the common precursor is

acetyl CoA. The primary metabolic reactions which utilise it, i.e. reactions X, are fatty acid synthesis and sterol synthesis. The secondary metabolic reaction which competes with the primary metabolic reactions for acetyl CoA is diosgenin synthesis (reaction Y).

It can then be visualised that during the period of culture growth, described in Figures 3.3.6a-7b when primary metabolism is at its most active, that fatty acid synthesis and sterol synthesis will utilise most or all of the available acetyl CoA, leaving the enzymes of diosgenin synthesis inactive. Once growth has been stopped however by limiting levels of phosphate, it is likely that the demand for fatty acids and sterols will fall. Synthesis of these compounds however will probably not fall due to continued production of acetyl CoA from sucrose, which although low, is never limiting. The fall in the utilisation of fatty acids, coupled to their continued synthesis, will lead to a build-up of fatty acyl species. This will lead to the feedback inhibition of acetyl CoA carboxylase, the first enzyme of fatty acid synthesis, (Wakil and Barnes, 1971) which in turn will switch the utilisation of acetyl CoA to steroid synthesis, resulting in the increased accumulation of sterols and the start of the accumulation of diosgenin. This hypothesis clearly shows how a combined change of nutrient levels and growth can bring about a switch from primary metabolism to secondary metabolism without invoking repression/depression of enzymes.

Phillips and Henshaw(1977) have proposed a similar hypothesis for the regulation of secondary metabolism in cultures of Acer pseudoplatanus where protein synthesis and phenolic synthesis compete for the common precursor phenylalanine. They have shown by following the incorporation of radiolabelled phenylalanine into phenolics and protein that the switching of this precursor from primary to secondary metabolism

is controlled by the rate of protein synthesis. Evidence for a similar switching of a common precursor has been reported in cultures of Datura innoxia by Lindsey and Yeoman, (1983). They have shown that radiolabelled ornithine is preferentially incorporated into protein in fast growing cells of the above culture while it is preferentially incorporated into alkaloid in slow growing cells.

The kinetic hypothesis described above when applied to D.composita cultures predicts that after phosphate limitation has brought growth to a halt a subsequent build-up of fatty acid species will result in the feedback inhibition of fatty acid synthesis. It then predicts that the acetyl CoA which would normally be utilised by fatty acid synthesis will then be utilised by steroid synthesis resulting in the initiation of diosgenin synthesis and, since they share a common biosynthetic pathway with diosgenin, an increase in sterol synthesis. This competition for the acetyl CoA between diosgenin and sterol synthesis has been studied using radiolabelled precursors common to both types of steroid.

It can be seen from Tables 3.3.5., 3.3.6. and 3.3.7. that while the level of incorporation of  $^{14}\text{C}$ -mevalonic acid into sterols, steryl glycosides and diosgenin remained approximately similar during the fourth, fifth and sixth weeks of culture growth, the incorporation level for all three species rose substantially during the seventh week supporting the hypothesis that the accumulation of diosgenin is the result of a switching of a common precursor i.e. acetyl CoA from fatty acid synthesis to steroid synthesis. It can also be seen that the above incorporation pattern is very similar to the pattern of diosgenin accumulation shown in Figure 3.3.6a. It can be seen from Table 3.3.10. that the percentage distribution of the total incorporated  $^{14}\text{C}$ -mevalonic acid between diosgenin and the two classes of

sterol remained approximately the same during the four weeks of the experiment. This shows that the competition for available precursors between diosgenin and sterol synthesis did not change even during the period of increased incorporation and with threequarters of the total being utilised by sterol synthesis, this process represents one limiting factor in the accumulation of diosgenin.

It can be seen from Tables 3.3. 8. and 3.3. 9. that the results for the incorporation of  $^{14}\text{C}$ -cholesterol into diosgenin and steryl glycosides differ from the  $^{14}\text{C}$ -mevalonic acid results. The level of incorporation into steryl glycosides fluctuated over the four week period under study, while the incorporation into diosgenin dropped between the fourth week and the seventh week. These fluctuations are reflected in Table 3.3.10 where it can be seen that of the total incorporated  $^{14}\text{C}$ -cholesterol, the percentage incorporated into diosgenin varied between 6 and 22%. No conclusions can really be taken from this data except that it is puzzling that the level of incorporation of  $^{14}\text{C}$ -cholesterol into diosgenin actually fell at a time when diosgenin accumulation was beginning. It is especially so since cholesterol is a recognised precursor of diosgenin (Minotta, 1981) and is much closer in metabolic terms than mevalonic acid. The experiment would probably be more informative if the incorporation into the individual sterols and steryl glycosides could be determined and compared with diosgenin rather than just a total sterol value.

The results of the experiments described in parts one, two and six of section three of the Results Chapter clearly show how diosgenin accumulation in suspension cultures of D.composita occurs only during the stationary phase of growth. It is logical to assume therefore that with secondary metabolism effectively separated from the growth phase, which is the most active period of primary metabolism, that

the addition of precursors of diosgenin synthesis to stationary phase cultures would result in increased yields. The results of manipulating the metabolism of stationary phase cultures are discussed below.

#### THE SECONDARY METABOLISM OF STATIONARY PHASE PLANT CELL CULTURES

It can be seen from Figures 3.3.12a-b, 3.3.13a-b and Tables 3.3.11, 3.3.12, 3.3.13, and 3.3.14, that the addition of sucrose, 2,4-D and kinetin, singly and in combination to stationary phase cultures of D.composita resulted in a large increase in culture dry weight and a lower but still significant increase in fresh weight. The various treatments had no effect on cell number or packed cell volume. The above data also revealed that the increases in dry and fresh weight were entirely due to the effect of sucrose, neither plant growth substance, added singly or together had any effect on the parameters of growth that were measured. They also failed to modify the effect of the sucrose treatment when added singly or together. These results underline the evidence obtained from the callus initiation experiments, described in section one of Chapter three, i.e. that Dioscorea tissue seems to be relatively insensitive to exogenously applied plant growth substances when compared to most dicotyledonous species.

When one considers the effect of sucrose on the dry weight of stationary phase cultures described in figure 3.3.13a, then the increase cannot be explained in terms of increased cell division or cell expansion since no increases in cell number (Figure 3.3.12a, Table 3.3.11) or packed cell volume (Figure 3.3.12b, Table 3.3.12) were recorded. This leaves two possible explanations for the increase in dry weight. The first is that the sucrose was absorbed from the culture medium and used for cell wall synthesis which would make the increase, a true accretion of cellular dry matter. The second

explanation is that the sucrose was absorbed from the culture medium and stored either unchanged or converted to starch. Although both explanations are plausible, the fact that the addition of sucrose also caused an increase in fresh weight supports the former explanation. This is because thicker cell walls would allow more water to be trapped between the microfibrils whereas there is no reason to suggest that the storage of carbohydrate would in itself induce an increase in fresh weight.

If the addition of sucrose can cause an increase in the dry weight of stationary phase cultures of D.composita, what effect does it have on diosgenin accumulation? The results presented in Figures 3.3.14a-c and Tables 3.3.15, 3.3.16, and 3.3.17 show that neither the sucrose treatment nor any of the other treatments caused the yield of diosgenin to differ from the control value, regardless of whether it was calculated on a dry weight, culture or cell basis.

These results however, produce a paradox because of the doubling in culture dry weight in treatments 5-8 (Figure 3.3.12a) caused by the addition of sucrose. If the results and the statistical analysis are correct and there are no differences in the yield of diosgenin, on a dry weight basis, (Figure 3.3.14a, Table 3.3.15) between treatments 5-8 and 1-4, then the yield of diosgenin on a per cell and a per culture basis should increase in treatments 5-8. The same is true in reverse. If there are no differences in the yield of diosgenin on a per cell and per culture basis between treatments 5-8 and 1-4, then the yield of diosgenin on a dry weight basis should fall. It can be seen from the above data that any real differences are probably masked by the large errors and that the only way to overcome the contradictions in the data would be to repeat the experiment. Although it was not possible to repeat the whole of the experiment the effect of sucrose

on its own was studied as a part of the experiment described in section three/part four of the Results Chapter.

The data presented in Figures 3.3.16a-d confirms the results of the previous experiment that the addition of sucrose to stationary phase cultures of D.composita causes an approximate doubling of dry weight, a significant increase in fresh weight and has no effect on cell number or PCV. The effect of sucrose on the yield of diosgenin in stationary phase cultures is presented in Figures 3.3.17a-c. and Tables 3.3.22., 3.3.23. and 3.3.24. from which it can be seen that the addition of sucrose caused no increase in the yield of diosgenin on a per culture (Figure 3.3.17b, Table 3.3.23) or a per cell basis, (Figure 3.3.17c, Table 3.3.24). The yield on a dry weight basis however was almost halved which is consistent with the effects of sucrose on culture dry weight described above.

The above results show that the addition of sucrose to stationary phase suspension cultures of D.composita does not increase the yield of diosgenin above that of the control. Evidence supporting this observation comes from Heble and Staba (1980a) who reported that the transfer of stationary phase cultures of Dioscorea deltoidea to a medium lacking sucrose resulted in a doubling of the yield of diosgenin, compared to controls which had not been transferred. In contrast to the above observations there are several reports of the stimulatory effect of sucrose on the secondary metabolism of stationary phase cultures. Phillips and Henshaw (1977) reported that the addition of sucrose to stationary phase suspension cultures of Acer pseudoplatanus stimulated phenolic accumulation. Amorim et al (1977) reported a similar effect on phenolic accumulations in cultures of Paul's Scarlet Rose by adding glucose at the stationary phase.

Knobloch and Berlin (1980) have shown that if stationary phase cultures of Catharanthus roseus are transferred to a medium high in sucrose, the accumulation of indole alkaloids is stimulated. A similar effect has been reported to stimulate cinnamoyl putrescine accumulation in tobacco cultures (Knobloch and Berlin, 1981).

It can be seen from Figures 3.3.17.a-c. that while the addition of sucrose to stationary phase suspension cultures of D. composita did not cause any increase in the yield of diosgenin, neither was it inhibitory. Despite the fact that the sucrose was added at the end of week five, (stationary phase with respect to growth but before the main phase of diosgenin accumulation) the culture accumulated the same absolute amount of diosgenin as the control. This suggests the intracellular concentration of diosgenin in these cultures may be regulated by a feedback mechanism in which one or more enzymes in the biosynthetic pathway of diosgenin are inhibited by diosgenin itself if its intracellular concentration becomes too high. If such a mechanism is operating then any attempts to increase the yield of stationary phase cultures will fail unless the intracellular concentration of diosgenin can be reduced by excretion into the medium. If this cannot be done naturally, reducing the permeability of the cell membranes artificially may help. Diosgenin accumulates in cells as glycosides and there are no reports in the literature of the export of these into the nutrient medium of plant cell cultures. One possible way of inducing the export of diosgenin from plant cell cultures may be to select a mutant cell line that is incapable of glycosylating diosgenin. In this way the culture cells will be incapable of modifying the hydrophobic diosgenin into the relatively hydrophilic glycosides, such as dioscin and therefore may be forced to excrete them. Lindsey (1982) has shown that cultures of Capsicum frutescens freely export capsaicin into the culture medium when cultured appropriately.

Regardless of whether the yield of diosgenin is regulated by a feedback mechanism or not, the main effect of adding sucrose to stationary phase cultures of D. composita was to increase their dry weight. The most likely explanation for this was that the added sucrose was used for cell wall synthesis, i.e. primary metabolism. In an attempt to inhibit 'primary metabolism' and divert precursors to secondary metabolism abscisic acid was added along with sucrose to stationary phase suspension cultures of D. composita.

It can be seen from Figures 3.3.15a-16b and Tables 3.3.18., 3.3.19., 3.3.20. and 3.3.21. that abscisic acid (both concentrations) added singly or with sucrose to stationary phase cultures had no effect on cell number or packed cell volume. It can be seen, however, that both concentrations of abscisic acid when added with sucrose increased culture dry weight more than sucrose alone, although they had no effect when added without sucrose. Both concentrations of abscisic acid when added with sucrose increased culture fresh weight although in this case no more than sucrose did on its own, again abscisic acid had no effect on its own. These results suggest that contrary to expectations, the abscisic acid, at the two concentrations studied, interacted with sucrose to stimulate a primary metabolic process, i.e. cell wall synthesis, to a greater extent than sucrose on its own. This is assuming that the increase in dry weight is due to cell wall synthesis. These results are inexplicable in terms of the known effects of abscisic acid in the intact plant. Therefore it would be useful to repeat the experiment over a wider range of concentrations and to ascertain the nature of the induced increases in dry weight. If abscisic acid can induce changes in aspects of primary metabolism, can it alter secondary metabolism?

The effects of abscisic acid and sucrose on the accumulation of diosgenin are presented in figures 3.3.17.a-c and Tables 3.3.22., 3.3.23. and 3.3.24.

It can be seen from this that while sucrose itself has no effect on the yield of diosgenin per culture (Figure 3.3.17.b., Table 3.3.23.), it can be seen that abscisic acid at  $1 \times 10^{-5} \text{M}$  added along with sucrose can almost double the yield per culture. Unfortunately the results on a per cell basis (Figure 3.3.17.c., Table 3.3.24.) and on a dry weight basis (Figure 3.3.17.a., Table 3.3.22.) are confusing. If the addition of abscisic acid and sucrose does increase the yield of diosgenin on a per culture basis, because cell number does not increase, the yield of diosgenin on a per cell basis should increase over that of the control and the sucrose control. It can be seen from Figure 3.3.17.c., and Table 3.3.24. that the yield does rise on a per cell basis. However it is only statistically significant from the control (treatment 1) and not the sucrose control (treatment 2). Similar inconsistencies are apparent in the values for the yield of diosgenin on a dry weight basis. The results suggest that once again large experimental errors are masking possible differences between treatments. Because of this no conclusions can be reached regarding the effect of abscisic acid on secondary metabolism in stationary phase cultures of D. composita although the above results certainly justify further investigation.

It has been suggested that if the supply of the sugars involved in the glycosylation of diosgenin ever became limiting then any diosgenin not immediately converted to glycosides may be liable to degradation. The sugars involved, are glucose and rhamnose, while it is unlikely that glucose would ever be limiting, rhamnose is a relatively uncommon sugar, therefore the effects of adding it to stationary phase cultures of D. composita were studied.

It can be seen from Figures 3.3.18.a-b., 3.3.19.a-b. and Tables 3.3.18., 3.3.19., 3.3.20. and 3.3.21. that rhamnose and sucrose added singly and together produced some varied effects on growth. While it has already

mentioned that sucrose increases the dry and fresh weight of stationary phase cultures, rhamnose causes a twenty fold drop in cell number and a four fold drop in fresh weight while it had no effect on packed cell volume or dry weight. If sucrose is added along with rhamnose, the drop in cell number is reduced and effect on fresh weight is prevented. These results suggest that rhamnose at the concentrations used are toxic to the plant cell cultures which is a surprising effect for a simple sugar like rhamnose and is very difficult to explain. The results for the effects of rhamnose and sucrose on diosgenin accumulation (Figures 3.3.20.a-c., Tables 3.3.22., 3.3.23. and 3.3.24.) are consistent with the above mentioned effects on growth. The addition of rhamnose and rhamnose/sucrose had no effect on the yield of diosgenin on a per cell basis (Figure 3.3.20.c., Table 3.3.24.) while as a result of the drop in cell number, the yield on a per culture basis for both treatments fell (Figure 3.3.20.b., Table 3.3.23.). Consistent with the yield of diosgenin on a per culture basis falling and the culture dry weight remaining constant, the yield of diosgenin on a dry weight basis also fell. While the above results may be interesting and the effect of rhamnose worthy of further investigation, this approach does not show any promise as a means of increasing the yield of diosgenin in stationary phase cultures of D. composita.

One of the aims of the work described in this thesis was to study the regulation of growth and diosgenin production in cultures of Dioscorea composita with the aim of identifying ways of increasing the yield of diosgenin. The conclusions reached in this part of the study may be summarised as follows.

- a) Diosgenin accumulation in batch suspension cultures of D. composita occurs exclusively in the stationary phase and therefore is completely separated from all aspects of culture growth.
- b) A study on the depletion of the four main nutrients in the culture medium suggests that the growth of the above mentioned cultures is limited

by the levels of phosphate in the medium. Sucrose, ammonium and nitrate are never limiting with respect to growth.

c) The above study also suggests that the accumulation of diosgenin in batch suspension cultures of D. composita may be regulated by the combined action of changes in the nutrient status of the culture coupled to changes in the growth rate. A hypothesis based on the competition between primary and secondary metabolism has been proposed to explain the mechanism of this regulation. In this hypothesis, primary metabolic processes such as fatty acid synthesis preferentially utilise the common precursor acetyl CoA. On the slowing of growth fatty acid biosynthesis will be reduced, perhaps as a result of feedback inhibition, causing a diversion of acetyl CoA into diosgenin biosynthesis.

d) Studies on feeding sucrose to stationary phase batch suspension cultures of D. composita suggests that the intracellular level of diosgenin may be controlled by a feedback inhibition mechanism in which diosgenin itself controls the activity of its own biosynthetic pathway if the levels of the steroid become too high.

It can be seen that while the above studies have led to a better understanding of the relationship between primary and secondary metabolism in cultures of D. composita it has not led to the development of protocols which will increase the yield of diosgenin. Where does this leave the economic feasibility of producing diosgenin by plant cell culture?

#### THE COMMERCIAL PRODUCTION OF DIOSGENIN BY PLANT CELL CULTURE TECHNIQUES

One of the main factors which will determine whether plant cell culture becomes an economically viable process for the production of valuable natural products is yield. To gain some idea of the economic feasibility of producing diosgenin commercially by plant cell culture one can compare the yields of diosgenin from experiments described in this thesis with

other plant cell cultures and with the intact plant.

It can be seen from Figures 3.3.1., 3.3.6. and 3.3.17. that the three best average yields of diosgenin were 0.785, 0.867 and 0.876 mg/g dry weight respectively. These figures correspond to 0.08, 0.09 and 0.09% dry weight and compare favourably with the only other value in the literature for a culture of Dioscorea composita i.e. 0.06% dry weight (Mehta and Staba, 1970). The figures however do not compare favourably with other species of Dioscorea especially Dioscorea deltoidea where yields vary from 1.6% dry weight (Kaul et al., 1969) to 3.8% dry weight (Tal et al., 1982). The figures do even worse when you consider that the yield of diosgenin from the tuber of Dioscorea composita can be 13% dry weight, (Martin, 1969). These results suggest that unless the yield of diosgenin from the cultures of Dioscorea composita can approach those of the intact plant then the production of diosgenin by plant cell culture does not look feasible.

At one time, there were very few examples of plant cell culture which could accumulate a particular secondary metabolite in amounts similar to the intact plant from which it was raised. The numbers of such cultures today are numerous and rising all the time. Although there are several reasons for this one approach above all has contributed the most to the rising number of these cultures and this is the development of rapid screening techniques for the selection of high yielding cell lines. Such techniques have been used on a number of occasions to select high yielding cell lines from plant cell cultures which had previously only produced trace amounts of the desired metabolite, (Zenk et al., 1977; Ogino et al., 1978).

The development of a selection technique for the screening of Dioscorea composita cultures to detect cell lines yielding high levels of diosgenin is described in section four of the results chapter. The technique however

could be used to detect any compound which causes the haemolysis of blood cells.

Using the above technique, it is not unreasonable to suggest, taking the afore mentioned examples into account, that cell lines of D. composita yielding high levels of diosgenin could be selected from the low yielding cultures described in this thesis. If it is not possible to select cell lines of D. composita which accumulate diosgenin at levels close to the intact plant, i.e. 13% of dry weight, then it should be possible to do so using cultures of Dioscorea deltoidea. They have been consistently shown to contain between 1 and 3% dry weight of diosgenin, without selection. This would mean that only a 5-10 fold improvement would be necessary, compared to around a 100-fold improvement needed with D. composita.

Once a high-yielding cell line of D. composita or D. deltoidea has been selected that accumulates diosgenin to greater or equal levels than the intact plant there is still no guarantee that the process will be economically viable. This is because, if at this point, the cost of producing diosgenin by plant cell culture is more expensive than extraction from the intact plant, regardless of the yield, it will not be a feasible process, (Zenk, 1982). On top of this diosgenin has to compete against other naturally occurring steroids, as a feed stock for drug manufacture, e.g. hecogenin from sisal and sitosterol from soya bean, (Hardman, 1969). It is because of such economic arguments that it has been suggested that plant cell culture will only be a feasible proposition for the production of secondary metabolites which are of high value, with a large turnover, (Zenk, 1982, Fowler, 1981) e.g. pharmaceuticals and flavour compounds. A

number of figures have been proposed for the value above which it is worth considering production of a secondary metabolite by plant cell culture, the figures range from £17-1000/kg (Fowler, 1981). While it can be seen from the anti-tumour alkaloid, vinblastine, costing £9000/g is definitely worth investigating, diosgenin at £15-17/kg is at the bottom end of the scale.

Assuming one has overcome the problems of yield and economics and produced a cell line of D. composita which is considered acceptable for development into a commercial process, one more problem will have to be overcome. This is transferring a cell line, which produces high yields of diosgenin, on a flask scale in the laboratory, to an industrial scale and ensuring that it continues to grow and also continues to accumulate diosgenin in high yields.

In an attempt to investigate this problem the growth and diosgenin accumulation of a D. composita suspension culture was investigated in a 15 litre stirred fermentor vessel. It was decided that this would provide a good model system for testing the transfer of a culture process from laboratory scale experiments to industrial scale processes.

It can be seen from the data in Table 3.5.2. that the suspension cultures of D. composita that were used for all the flask experiments described in this thesis can be grown successfully as an eight litre stirred culture in a fermentor vessel and still retain the ability to accumulate diosgenin in amounts comparable to the flask cultures. Although the above experiment demonstrated that the cultures of D. composita could be grown in multi-litre stirred fermentors the data from the first experiment (Table 3.5.1.) shows that if the inoculum used to start the culture is too small then the tissue is liable to damage caused by the shearing action of the paddles. In this case the damage seemed to induce the excretion of large amounts of the polyphenolic substances associated with wound reactions.

In summary the above results suggest that it may be possible to:

- a) select a cell line of D. composita which will accumulate high levels of diosgenin using the techniques described in this thesis
- b) to successfully culture the cell line on an industrial scale retaining its synthetic ability.

Despite this, it is likely that the economic factors discussed above, will prevent plant cell culture techniques being used to produce diosgenin on a commercial scale.

Although plant cell culture may never be a commercial source of diosgenin, the results of the experiments described in parts 1-6 of section three of this thesis suggests that the production of diosgenin in batch suspension cultures of D. composita is a good model system to study the regulation of secondary metabolism.

#### FUTURE WORK

The results described in this thesis have indicated the importance of the interrelationship between the nutrient status of a plant cell culture and its growth rate in regulating secondary metabolism, they also suggest areas in which further work may be carried out.

- a) Work should be carried out to further investigate the effects of inorganic phosphate in regulating growth and diosgenin accumulation in suspension cultures of Dioscorea composita. Can altering the concentration of inorganic phosphate in the culture medium be used to manipulate the timing of when culture growth stops and thus the turning on of diosgenin accumulation? Will adding phosphate to a culture already accumulating diosgenin cause inhibition of production?
- b) The changes in intracellular ATP concentrations should be measured over a 10 week culture period and compared with changes in medium and intracellular inorganic phosphate concentrations and diosgenin accumulation to check for possible correlations.

- c) The effect of carbohydrate on diosgenin accumulation in stationary phase cultures should be investigated further. Do other sugars have the same effect as sucrose? Does adding them in small amounts over a period of time have the same effect as one large addition?
- d) Attempts should be made to repeat the work of Heble and Staba (1980a) in which the transfer of a stationary phase suspension culture of D. deltoidea into a carbohydrate free medium doubled the yield of diosgenin. If the experiment is successfully repeated, the source of the 'carbon' for the increased diosgenin synthesis should be traced to determine whether it is merely intracellular sugars such as glucose that are being used or the utilisation of some storage carbohydrate such as starch.
- e) Further experiments should be carried out using radiolabelled isotopes of mevalonic acid to study the competition for common precursors between fatty acid biosynthesis and diosgenin biosynthesis.
- f) Work should be carried out to identify the individual steps of diosgenin biosynthesis and the enzymes that catalyse them. Particular importance should be paid to the step at the junction with sterol biosynthesis. Once this was carried out real progress could be made on the mechanisms of the regulation of diosgenin biosynthesis.

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## APPENDIX

TABLE 3.3.11

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECT OF SUCROSE AND PLANT GROWTH SUBSTANCES ON CELL NUMBER IN STATIONARY PHASE SUSPENSION CULTURES OF *D.composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio F	
				Calculated	Tabulated
Treatments	7.70119	7	1.10017	2.82	2.36
Blocks	0.80705	4	0.20176	0.51	2.71
Error	10.91806	28	0.38993	-	-
(total)	19.42630	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS

Treatment Number	4	1	8	2
Q	4.63	4.49	4.32	4.13
SSR	1.29297	1.25388	1.20640	1.15334
$\bar{X}$	2.54275	2.66180	3.21385	2.25296
Treatment Number	7	3	5	6
Q	3.86	3.50	2.90	
SSR	1.07794	0.97741	0.30985	
$\bar{X}$	3.37915	3.45558	3.79759	3.82432

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGEST THAT THE ONLY DIFFERENCE WHICH IS STATISTICALLY SIGNIFICANT IS THAT BETWEEN TREATMENT 4 and 5

TABLE 3.3.12.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECT OF SUCROSE AND PLANT GROWTH SUBSTANCES ON PACKED CELL VOLUME IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio F	
				Calculated	Tabulated
Treatments	0.04095	7	0.00585	0.58	2.36
Blocks	0.01329	4	0.00332	0.33	2.71
Error	0.28221	28	0.01008	-	-
(Total)	0.33645	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS DOES NOT EQUAL OR EXCEED THE TABULATED VALUE WHICH SUGGESTS THERE ARE NO SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

TABLE 3.3.13.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE AND PLANT GROWTH SUBSTANCES ON DRY WEIGHT IN STATIONARY, PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance ratio F	
				Calculated	Tabulated
Treatments	2.2530	7	0.3214	60.64	2.36
Blocks	0.0613	4	0.0153	2.89	2.71
Error	0.1489	28	0.0053	-	-
(Total)	2.4632	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THAT THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS

Treatment Number	4	2	1	3
Q	4.63	4.49	4.32	4.13
SSR	0.1509	0.1464	0.1408	0.1346
$\bar{X}$	0.4785	0.5151	0.5242	0.5434
Treatment Number	5	6	8	7
Q	3.86	3.50	2.90	
SSR	0.1258	0.1141	0.0945	
$\bar{X}$	0.9580	0.9598	1.0100	1.0204

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THE ONLY DIFFERENCES WHICH ARE STATISTICALLY SIGNIFICANT ARE THOSE BETWEEN TREATMENT 1 and 5,6,7 and 8, BETWEEN 2 and 5,6,7 and 8, BETWEEN 3 and 5,6,7 and 8 AND BETWEEN 4 and 5,6,7 and 8.

TABLE 3.3.14.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE AND PLANT GROWTH SUBSTANCES ON FRESH WEIGHT IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio F	
				Calculated	Tabulated
Treatments	174.2976	7	24.8997	9.16	2.36
Blocks	32.8900	4	8.2225	3.02	2.71
Error	76.1124	28	2.7183	-	-
(total)	283,3000	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS

Treatment Number	2	1	3	4
Q	4.63	4.49	4.32	4.13
SSR	3.4137	3.3105	3.1851	3.0450
$\bar{X}$	13.6768	13.6802	13.9303	14.2599
Treatment Number	6	5	8	7
Q	3.86	3.50	2.90	
SSR	2.8460	2.5805	2.1382	
$\bar{X}$	16.9109	17.0072	17.8614	19.4143

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THE ONLY DIFFERENCES WHICH ARE STATISTICALLY SIGNIFICANT ARE THOSE BETWEEN TREATMENT 1 and 5,6,7 and 8, BETWEEN TREATMENT 2 and 5,6,7 and 8, BETWEEN TREATMENT 3 AND 5,6,7 and 8 AND BETWEEN TREATMENT 4 and 5,6,7 and 8.

TABLE 3.3.15.

THE ANALYSIS OF VARIANCE FOR THE EFFECTS OF SUCROSE AND PLANT GROWTH SUBSTANCES ON DIOSGENIN ACCUMULATION /G DRY WEIGHT IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	Variation Ratio (F)	
				Calculated	Tabulated
Treatments	300394	7	42913	2.11	2.36
Blocks	50583	4	12646	0.62	2.71
Error	568789	28	20314	-	-
(Total)	919766	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS DOES NOT EQUAL OR EXCEED THE TABULATED VALUE WHICH SUGGESTS THERE ARE NO SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS

TABLE 3.3.16.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECT OF SUCROSE AND PLANT GROWTH SUBSTANCES ON DIOSGENIN ACCUMULATION/CULTURE IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variation Ratio (F) Calculated Tabul'ed	
Treatments	156912	7	22416	2.39	2.36
Blocks	54746	4	13686	1.46	2.71
Error	262188	28	9364	-	-
(Total)	473846	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS JUST EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE MAY BE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS

Treatment Number	1	4	3	2
Q	4.63	4.49	4.32	4.13
SSR	200	194	187	179
$\bar{X}$	237	267	294	324
Treatment Number	5	6	7	8
Q	3.86	3.50	2.90	
SSR	167	151	126	
$\bar{X}$	364	382	410	417

IN SPITE OF THE CALCULATED VARIANCE RATIO SUGGESTING THAT THERE WERE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SHOWS THAT NONE OF THE DIFFERENCES BETWEEN TREATMENT MEANS ARE STATISTICALLY SIGNIFICANT.

TABLE 3.3.17.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECT OF SUCROSE AND PLANT GROWTH SUBSTANCES ON DIOSGENIN ACCUMULATION/CELL IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio F	
				Calculated	Tabulated
Treatments	228.3	7	32.6	1.68	2.36
Blocks	39.2	4	9.8	0.51	2.71
Error	543.2	28	19.4	-	-
(Total)	810.7	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS IS LOWER THAN THE TABULATED VALUE WHICH SUGGESTS THERE ARE NO SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

TABLE 3.3.18.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON CELL NUMBER IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance ratio F	
				Calculated	Tabulated.
Treatments	115.01010	7	16.43001	24.20	2.36
Blocks	4.23485	4	1.05871	1.56	2.71
Error	19.00665	28	0.67881	-	-
(Total)	138.25160	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THAT THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

Treatment Number	3*	4*	5	2
Q	4.63	4.49	4.32	4.13
SSR	1.70597	1.65438	1.59175	1.52174
$\bar{X}$	0.25036	2.12229	4.37087	4.61817
Treatment Number	6	1	4	3
Q	3.86	3.50	2.90	
SSR	1.42226	1.28961	1.06853	
$\bar{X}$	4.73356	5.01734	5.22506	5.28155

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THERE ARE NO STATISTICALLY SIGNIFICANT DIFFERENCES WITHIN THE ABSCISIC ACID TREATMENTS BUT TREATMENTS 3\* and 4\* FROM THE RHAMNOSE EXPERIMENT DIFFERED SIGNIFICANTLY FROM BOTH THE DISTILLED WATER CONTROL (TREATMENT 1) AND THE SUCROSE CONTROL (TREATMENT 2). TREATMENTS 3\* and 4\* ALSO DIFFERED SIGNIFICANTLY FROM EACH OTHER.

TREATMENTS MARKED \* REPRESENT THE RHAMNOSE TREATMENTS LISTED IN TABLE 3.3.4. WHILE THE OTHERS REPRESENT THE ABSCISIC ACID TREATMENTS LISTED IN TABLE 3.3.3.

TABLE 3.3.19.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON PACKED CELL VOLUME IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance ratio (F)	
				calculated	tabulated
Treatments	0.11326	7	0.01618	1.45	2.36
Blocks	0.04240	4	0.01060	0.95	2.71
Error	0.31158	28	0.01113	-	-
(Total)	0.38244	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS DOES NOT EXCEED OR EQUAL THE TABULATED VALUE WHICH SUGGESTS THAT THERE ARE NO SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS

TABLE 3.3.20.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON DRY WEIGHT IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio F	
				Calculated	Tabulated
Treatments	4.2412	7	0.6059	116.52	2.36
Blocks	0.0342	4	0.0086	1.65	2.71
Error	0.1471	28	0.0052	-	-
(Total)	4.4225	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THAT THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

Treatment Number	1	4	3	3*
Q	4.63	4.49	4.32	4.13
SSR	0.1491	0.1446	0.1391	0.1330
$\bar{X}$	0.3938	0.4068	0.4103	0.4304
Treatment Number	2	4*	6	5
Q	3.86	3.50	2.90	
SSR	0.1243	0.1127	0.0934	
$\bar{X}$	0.9584	1.0761	1.0844	1.1058

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THERE IS A STATISTICALLY SIGNIFICANT DIFFERENCE BETWEEN THE DISTILLED WATER CONTROL AND THE SUCROSE CONTROL. IN THE ABSCISIC ACID EXPERIMENT, BOTH TREATMENTS 5 and 6 DIFFER SIGNIFICANTLY FROM BOTH CONTROLS AND TREATMENTS 3 and 4. IN THE RHAMNOSE EXPERIMENT TREATMENT 4\* DIFFERS SIGNIFICANTLY FROM BOTH CONTROLS AND TREATMENT 3\* WHILE TREATMENT 3\* DIFFERS SIGNIFICANTLY FROM THE SUCROSE CONTROL.

TREATMENTS MARKED \* REPRESENT THE RHAMNOSE TREATMENTS LISTED IN TABLE 3.3.4, WHILE THE OTHERS REPRESENT THE ABSCISIC ACID TREATMENTS LISTED IN TABLE 3.3.3.

TABLE 3.3.21.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON FRESH WEIGHT IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance Ratio F	
				Calculated	Tabulated
Treatments	1235.3522	7	176.5503	58.07	2.36
Blocks	5.4368	4	1.3592	0.41	2.71
Error	92.4258	28	3.3009	-	-
(Total)	1333.7148	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

Treatment Number	3*	4	3	4*
Q	4.63	4.49	4.32	4.13
SSR	3.7619	3.6481	3.5100	3.3556
$\bar{X}$	3.8876	14.3036	15.1610	15.2827
Treatment Number	1	6	2	5
Q	3.86	3.50	2.90	
SSR	3.1362	2.8438	2.3562	
$\bar{X}$	15.7279	20.6459	21.2201	22.9005

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THERE IS A STATISTICALLY SIGNIFICANT DIFFERENCE BETWEEN THE DISTILLED WATER CONTROL AND THE SUCROSE CONTROL. IN THE ABSCISIC ACID EXPERIMENT BOTH TREATMENTS 5 and 6 DIFFER SIGNIFICANTLY FROM THE DISTILLED WATER CONTROL AND TREATMENTS 3 and 4. TREATMENTS 3 and 4 ALSO DIFFER SIGNIFICANTLY FROM THE SUCROSE CONTROL. IN THE RHAMNOSE EXPERIMENT TREATMENT 3\* DIFFERS SIGNIFICANTLY FROM BOTH CONTROLS AND TREATMENT 4\*, TREATMENT 4\* DIFFERS FROM THE SUCROSE CONTROL.

TREATMENTS MARKED \* REPRESENT THE RHAMNOSE TREATMENTS LISTED IN TABLE 3.3.4, WHILE THE OTHERS ARE THE ABSCISIC ACID TREATMENTS LISTED IN TABLE 3.3.3.

TABLE 3.3.22.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON DIOSGENIN YIELD /G DRY WEIGHT IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance ratio F	
				Calculated	Tabulated
Treatments	2346444	7	335206	9.44	2.36
Blocks	94752	4	23688	0.67	2.71
Error	994325	28	35512	-	-
(Total)	3435521	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

Treatment Number	3*	4*	6	2
Q	4.63	4.49	4.32	4.13
SSR	390	378	364	348
$\bar{X}$	59	165	444	490
Treatment Number	4	5	3	1
Q	3.86	3.50	2.90	
SSR	352	295	244	
$\bar{X}$	526	547	649	876

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THERE IS A STATISTICAL SIGNIFICANT DIFFERENCE BETWEEN THE FOLLOWING TREATMENTS: DISTILLED WATER CONTROL (TREATMENT 1) AND TREATMENTS 2,5,6 IN THE ABSCISIC ACID EXPERIMENT. IN THE RHAMNOSE EXPERIMENT TREATMENT 3\* DIFFERED SIGNIFICANTLY FROM BOTH CONTROLS AS DID TREATMENT 4\*. TREATMENT 3\* and 4\* ALSO DIFFERED SIGNIFICANTLY BETWEEN THEMSELVES.

TREATMENTS MARKED \* REPRESENT THE RHAMNOSE TREATMENTS LISTED IN TABLE 3.3.4, WHILE THE OTHERS ARE THE ABSCISIC ACID TREATMENTS LISTED IN TABLE 3.3.3.

TABLE 3.3.23.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON DIOSGENIN YIELD/CULTURE IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance ratio F	
				Calculated	Tabulated
Treatment	1215937	7	173705	15.40	2.36
Blocks	43766	4	10942	0.97	2.71
Error	315831	28	11280	-	-
(Total)	1575534	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

Treatment Number	3*	4*	4	3
Q	4.63	4.49	4.32	4.13
SSR	220	213	205	196
$\bar{X}$	25	175	210	263
Treatment Number	1	2	6	5
Q	3.86	3.50	2.90	
SSR	183	166	138	
$\bar{X}$	347	450	473	601

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT IN THE ABSCISIC ACID EXPERIMENT THAT TREATMENTS 2, 5 and 6 ARE ALL DIFFERING SIGNIFICANTLY FROM BOTH TREATMENT 3 and 4. TREATMENT 5 ALONE DIFFERS SIGNIFICANTLY FROM TREATMENT 1. IN THE RHAMNOSE EXPERIMENT TREATMENT 3\* DIFFERS SIGNIFICANTLY FROM TREATMENT 1, 2 and 4\* WHILE TREATMENT 4\* DIFFERS FROM THE SUCROSE CONTROL (TREATMENT 2)

TREATMENTS MARKED \* REPRESENT RHAMNOSE TREATMENTS LISTED IN TABLE 3.3.4. WHILE THE OTHERS ARE ABSCISIC ACID TREATMENTS LISTED IN TABLE 3.3.3.

TABLE 3.3.24.

THE ANALYSIS OF VARIANCE DATA FOR THE EFFECTS OF SUCROSE, ABSCISIC ACID AND RHAMNOSE ON DIOSGENIN YIELD/CELL IN STATIONARY PHASE SUSPENSION CULTURES OF *D. composita*

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	Variance ratio F	
				Calculated	Tabulated
Treatment	1049.13	7	149.87	4.57	2.36
Blocks	158.66	4	39.66	1.21	2.71
Error	918.59	28	32.81	-	-
(Total)	2126.38	39	-	-	-

THE CALCULATED VALUE FOR THE VARIANCE RATIO (F) OF THE TREATMENTS EXCEEDS THE TABULATED VALUE WHICH SUGGESTS THERE ARE SIGNIFICANT DIFFERENCES BETWEEN TREATMENT MEANS.

Treatment Number	4	3	1	4*
Q	4.63	4.49	4.32	4.13
SSR	11.85	11.49	11.06	10.57
$\bar{X}$	6.68	8.33	11.88	13.72
Treatment Number	3*	2	6	5
Q	3.86	3.50	2.90	
SSR	9.88	8.96	7.42	
$\bar{X}$	17.00	17.06	18.50	23.12

THE MULTIPLE RANGE TEST USING SHORTEST SIGNIFICANT RANGES SUGGESTS THAT THE ONLY STATISTICALLY SIGNIFICANT DIFFERENCES ARE IN THE ABSCISIC ACID EXPERIMENT AND THAT THEY ARE BETWEEN TREATMENT 5 AND TREATMENTS 1 and 3 AND BETWEEN TREATMENT 4 and TREATMENTS 5 and 6.

TREATMENTS MARKED \* REPRESENT RHAMNOSE TREATMENTS AS LISTED IN TABLE 3.3.4, WHILE THE OTHERS ARE ABSCISIC ACID TREATMENTS AS LISTED IN TABLE 3.3.3.