THE REGULATION OF PLANT CELL GROWTH AND METABOLISM BY 2,4-DICHLOROPHENOXYACETIC ACID

By

Nicholas P Everett

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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

N. P. Everett

I certify that this statement is correct.

W. Cockell

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CONTENTS

PRELIMINARY DEFINITIONS

Two terms that appear regularly in this thesis are growth and hormone and as there is some controversy over their precise meaning I shall define my usage of them.

(a) Growth

The biological definition of growth given by Chambers Technical Dictionary (1966) is: "A change in the body of an organism and in the cells composing it, accompanied by cell division, by the utilisation of material, and, nearly always, by increase in the size and weight of the organism or of the part under consideration. Once growth has occurred, its results cannot be reversed." A contrasting view has been expressed by Thimann (1969) who states "It could be wished that biologists would not continue to speak of 'growth by cell division', for the division of cells does not itself cause enlargement. Growth is always by cell enlargement". I do not agree with Thimann and my own use of the term growth for an irreversible increase in quantity leads to the conclusion that growth can occur by either cell expansion or cell division. Greulach and Adams (1967) even consider cell expansion to be less important than cell division in whole plant growth as they state "At maturity, the cells composing a stem, root, or leaf of a plant have fairly well defined maximal sizes. Continued growth of a plant depends, therefore, on a continuous supply of new cells that in their turn become mature".

The methods used to follow growth may be either direct (cell counting, cell volume determination) or indirect (e.g. dry weight, total protein). The indirect methods measure parameters which may not increase irreversibly, and therefore do not themselves constitute growth, but when combined with direct methods give valuable information on growth-related processes. Indirect methods only measure true growth if cells are exhibiting 'balanced growth'.

(b) Hormone

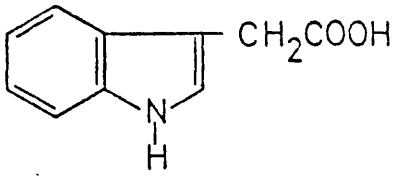
Etymologically, the word hormone means 'arousing to activity' but its use by animal physiologists has caused it to be associated with much more specific concepts. The main features of animal hormones are that they are produced in specialized glands and elicit a specific physiological response when translocated to responsive tissues in very small amounts. The chemical messengers of plants can be synthesized by unspecialized cells and differ from classical animal hormones in many other ways although, in the whole plant, they do usually cause a response at a site other than their place of origin. Because of these differences some authors (e.g. Trewavas, 1976) have preferred the terms "plant growth substance" or "plant growth regulator" to "plant hormone" to emphasise the differences between plant and animal hormones. Unfortunately these alternative terms suggest that auxins and cytokinins only regulate growth whereas in fact the 'plant growth regulators' may also act as developmental hormones to regulate differentiation and co-ordinate morphogenesis. If the differences in structure and action between and within plant and animal hormones are recognized I see no reason why the more convenient term 'hormone' should not be applied to auxins and cytokinins. In this thesis I also use the term 'hormone' to include synthetic compounds which appear to mimic the action of a naturally occurring auxin or cytokinin (e.g. 2,4-D as a synthetic auxin).

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Historical Background

Multicellular organisms develop by the related processes of growth and differentiation which cause quantitative and qualitative changes in either the whole or part of the organism. The highly ordered form of higher plants is indicative of the precise regulation maintained over both these processes. The concept that the co-ordination of these processes is controlled by hormones developed from the classical work of Charles Darwin in 1880. He noted that the site of perception of gravity, for geotropic responses, and light, for phototropic responses, was in the tip of roots and shoots but that the stimulus was transported basipetally to produce the growth responses behind the tip. The growth hormone isolated from oat coleoptile tips was given the name auxin (from the Greek auxein, to grow) and chemically identified as indole-3-acetic acid (IAA)(Fig.1).



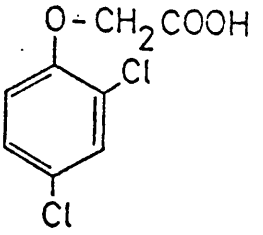


Fig.1 Indole-3-acetic acid (IAA) 2,4-dichlorophenoxyacetic acid (2,4-D)

Although other naturally occurring indole compounds may show auxin-like activity when applied to plants it is likely that this activity is due to their conversion to IAA. The simple structure of IAA led rapidly to the synthesis of many related compounds and testing them for growth promoting activity in bio-assays. The discovery of the auxin-like properties of synthetic phenoxy compounds, of which 2,4-dichlorophenoxyacetic acid (2,4-D) is generally the most active, gave information on the structural requirements

for auxin activity and provided a family of powerful herbicides. The importance of 2,4-D as a herbicide lies in the selectivity of its action in affecting broad-leaf plants much more adversely than narrow-leaved ones such as cereals and grasses.

2,4-D and Plant Tissue Cultures

The plant tissues used in bio-assays for auxins usually respond by exhibiting cell expansion (Thimann, 1969; Trewavas, 1976). However, auxins may also stimulate cell division as first shown in seedlings by Snow (1935) and in woody plants by Soding (1936). It is this cell-division stimulating property of auxins that is particularly exploited when they are used to induce and maintain the growth of tissue cultures (Gautheret, 1955). A tissue culture is initiated when an explant is surface-sterilized and incubated aseptically in a solution of salts, sugar, vitamins and hormones. The ensuing rapid unorganized cell division produce a mass of (callus) cells which are generally parenchymatous and/or meristematic in appearance. If the callus is then agitated in a liquid medium the tissue mass fragments, to give a suspension culture comprising small aggregates of cells.

The inability of auxin and nutrients alone to maintain cell division activity in some tissue cultures led to the identification and definition of another group of plant hormones, the cytokinins (Skoog et al 1965). Although other plant hormones exist most cultures can be maintained in active growth by supplying nutrients, an auxin and a cytokinin. If a culture ceases to grow when the auxin or cytokinin is omitted from the culture medium the cells are referred to as auxin- or cytokinin- dependent. Absolute dependency upon auxin or cytokinin for growth is difficult to determine as an increase in the level of one of the hormones in the culture medium may reduce or eliminate the requirement for the other (Einset 1977). Even so a loss of auxin dependence, without increasing the supply of cytokinin, is only observed when either organized structures are formed or the culture

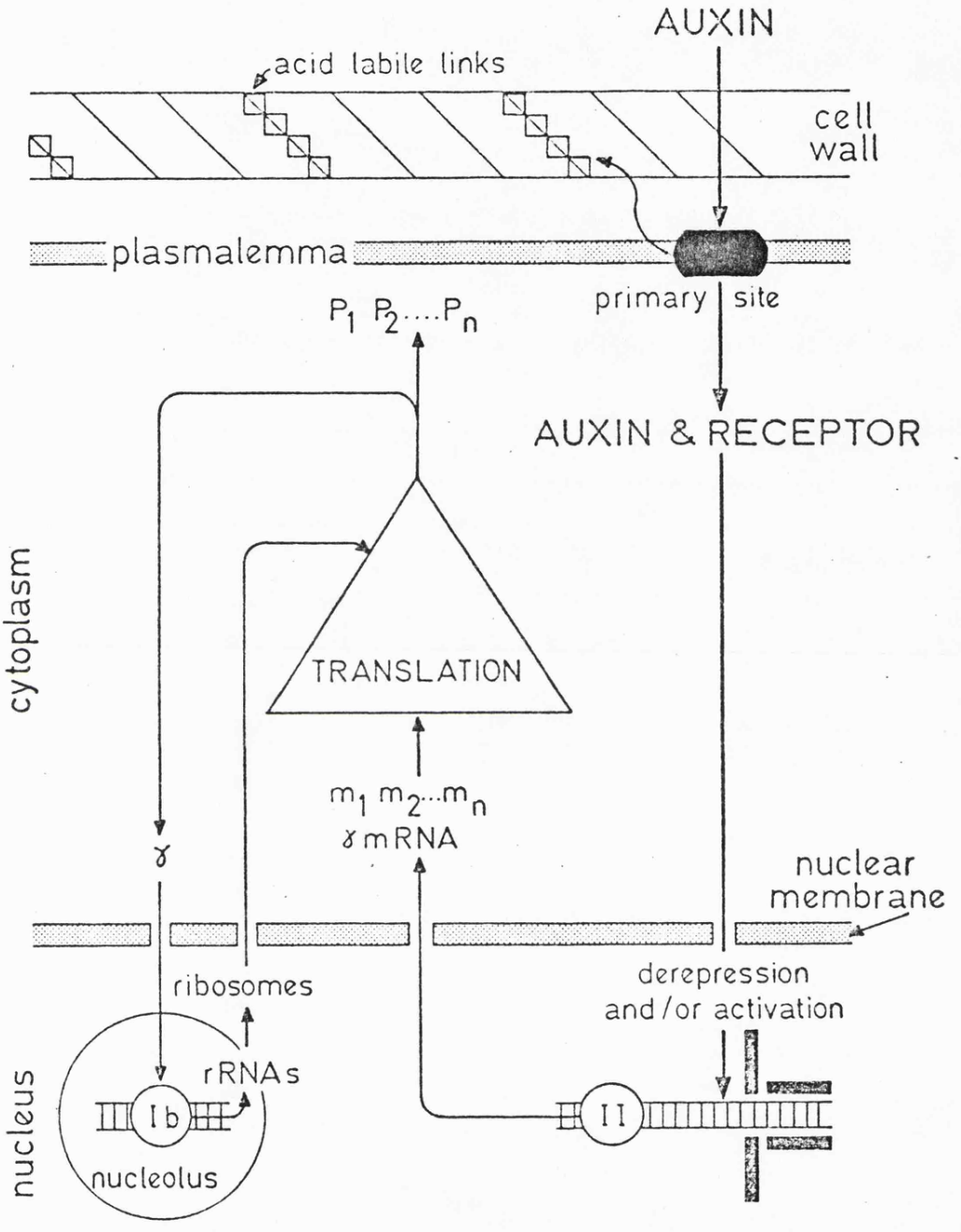
becomes habituated and then resembles tumor cells.

The development of organized structures in culture particularly shoots and somatic embryos, is generally suppressed by auxins (Street, 1976). Thus one consequence of omitting auxins from the culture medium may be the regeneration of plants that can be grown to maturity. The regeneration of plants in this way demonstrated the totipotency of plant cells (the retention of all of the genetic information of a cell during differentiation) and suggested that tissue cultures could be used for the rapid clonal propagation of selected varieties or individual plants (Street, 1976). The commercial realization of this potential depends on the ability to induce rapid growth in an explant in such a way that a high degree of regeneration of genetically identical plants may be subsequently achieved.

Work with cultures of herbaceous species has shown that 2,4-D and other auxins play a key role in the control of growth and differentiation in plant tissue and cell cultures. The lack of knowledge concerning how 2,4-D exerts its regulatory function has meant that attempts to culture species of agricultural importance have been empirical. This 'trial and error' approach has certainly been a major contributing factor to the slow rate of progress in the culture of recalcitrant species. Steward (1976) pinpoints the problem by saying that the need is "to obtain such knowledge that populations of somatic cells, not only of carrot but of any angiosperm, could routinely, and in high yield, be caused to develop into plants". The aim of the work described in this thesis was to contribute to the solution of this problem by elucidating the mechanisms by which 2,4-D exerts its control of growth in culture.

Mode of Action Studies

Auxins represent the class of plant growth regulators whose mechanism of action has been under the longest active investigation. The identification of auxins originated from their ability to promote cell



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J. Microscopic Biol. Cell (1976), 26, 139-150

FIG.1.2 A model mechanism of action of auxin on cell wall extensibility and gene expression in higher plants.

enlargement, a process that requires extension of the cell wall. The rapidity of elongation of responsive tissues after the application of auxin has attracted much attention because of its implications regarding the primary action of the hormone. Although cell elongation is one of the fastest responses to auxin this does not necessarily mean that it is indicative of the mechanism of longer term auxin responses such as cell division, differentiation and morphogenesis (Ray, 1974). If it was then this would certainly stretch the credibility of the gene activation hypothesis.

The apparent involvement of auxin in differentiation, a process which requires differential gene expression, has led to the expectation that the 'master reaction' (not necessarily the same as the primary action) of auxin should be at the level of replication and/or transcription (Everett, Wang and Street, 1978). There is some dispute as to whether the molecular entity that would exert such control is the auxin molecule itself. Thus it has been shown that auxin may (a) bind with nuclear proteins directly (Yasuda and Yamada, 1970; Roy and Biswas, 1977), (b) bind to a mediator protein, such that the auxin-mediator complex then migrates to its site of action in the nucleus (Hardin et al 1972; Ricard et al 1976) or (c) cause the release of a mediator compound which, once stimulated, does not require continued auxin association for its activity in the nucleus. These views are not necessarily mutually exclusive as shown by the model recently proposed by Ricard et al (1976). They propose that, following recognition at the plasmalemma, the auxin molecule is transported, with receptor protein, to the nucleus where the synthesis of heterogeneous RNA's, and the resulting mRNAs, is stimulated. Translation of these mRNA's in the cytoplasm produces specific proteins including the factor Υ which then penetrates the nucleus to cause enhanced synthesis of rRNA and subsequent amplification of protein synthesis (Fig.I.2). The view that primary recognition of auxin molecules occurs at the plasmalemma is currently favoured especially since the demonstrations that the plasmalemma appears to possess auxin-binding sites

('receptors') (Venis, 1977). This view may suggest that auxin transport and auxin action could be closely linked and would permit the rapid responses of cell wall extension to be a consequence of auxin perception.

The variation in responses that cells, derived from different regions of a plant, display in response to auxin application exemplifies the concept of target cells (Osbourne, 1977). Thus for a cell to elicit a particular hormonal response it must possess both the specific receptors and the necessary metabolic machinery to convert the primary signal into a metabolic response. Indeed in animal cells it is considered that changes in receptor number and affinity may be the major mechanism for the modulation of hormone action (Bradshaw and Frazier, 1977). A knowledge of the site(s) of hormone receptors is very important to the regulation of growth in culture as it tells us which pools of hormones are directly available for interaction with the receptors - do tissues respond directly to the concentration of hormones in the culture medium or via transport into some intracellular pool(s)? Once the identity of the "active" pool(s) is known, the in vivo nature of the receptor(s) can be investigated - do they represent on/off switches or can they generate concentration-dependent analogue signals? Tissue cultures are ideally suited to answering these questions, especially those in which growth reversibly arrests after the removal of exogenous hormone without displaying regeneration (which may confer hormone - independence).

The Concept of Determination in Cellular Differentiation

Studies in animal embryology led to the concept of pluripotency which was used to describe the multiple potency of the parts of the early egg cell. As the embryo develops, different regions of the embryo become progressively committed to particular fates. Analogous behaviour is observed in plants although botanists use the term totipotency rather than pluripotency (Steward and Moham Ram, 1961). Thus totipotency is used to

express the concept that "any diploid (somatic) plant tissue cell, in which irreversible differentiation had not proceeded, could, under the influence of appropriate stimuli, regain the ability to develop like the zygote and produce a new embryo plant", (Street, 1976). Irreversible differentiation is not considered to have occurred until after nuclear breakdown and hence the loss of the genome.

The process by which cells become progressively committed to particular fates is called determination and involves stable changes in phenotype which once induced, may persist in the absence of the factors that initiated the change and may be inherited by individual cells (Meins, 1975). Although a cell, or group of cells, may be determined to produce shoot or root meristems (amongst other possible fates) the expression of such determination may be influenced by the presence of factors that inhibit the development of such structures. This concept has profound effects on the interpretation of data relating to the hormonal regulation of differentiation. Thus the development of embryos from a culture of apparently 'dedifferentiated' carrot cells, after the removal of 2,4-D from the culture medium, does not necessarily mean that the absence of 2,4-D induces embryogenesis but may merely indicate that 2,4-D suppresses embryo development. Similarly the absence of observed embryogenesis in recalcitrant woody tissues such as sycamore may indicate that:-

(a) They have lost their genetic capability (totipotency).

(b) They are not genetically programmed (determined) to regenerate.

(c) The culture conditions do not permit a successful display of the determined program of development.

Although the present study was primarily directed towards characterizing the growth promoting properties of 2,4-D in culture, such investigations cannot easily be separated from attempts to regulate the differentiation of plant cells in culture.

MATERIALS and METHODS

1. GENERAL CULTURE TECHNIQUES

The general laboratory equipment and basic methodology used for tissue culture in Leicester have been previously described in detail (Street, 1977).

(a) Cleaning of glassware:

All glassware that came into contact with cultures or culture medium was initially cleaned with chromic acid (0.15M $Na_2 Cr_2 O_7$ in conc. sulphuric acid) and rinsed thoroughly with tap-water then glass-distilled water. Erlenmeyer flasks were routinely washed in a 'Hydrojet' washer (Heinicke, Hollywood, Florida) using BHC2008 detergent (Savilles Hydrological Corporation), rinsed in tap water then in two changes of glass-distilled water. They were finally dried in an oven at 100-130°C. All other glassware was cleaned with chromic acid and washed manually.

(b) Origins of cultures:

Most of the work reported in this thesis used sycamore cultures (Acer pseudoplatanus L., strain AM; Bayliss and Gould, 1974) as experimental material. The AM strain was isolated from a culture originally initiated from stem cambium by Lamport (1960) and supplied by Dr. D. H. Northcote of the Biochemistry Department, University of Cambridge. A new culture of sycamore was initiated from the petiole of a one year old seedling and denoted D_5 (see SECTION 2). An established suspension culture of Paul's Scarlet Rose was obtained from Dr. N. E. Robinson while at Leicester.

(c) Culture media:

Heller's medium, as modified by Stuart and Street (1969), was used for the maintenance of stock cultures of AM strain sycamore cells, its composition is shown in Table 1. The D_5 strain of sycamore was initiated and maintained using a modified half-strength Murashige and Skoog medium as shown in Table 2. Paul's scarlet rose cultures were maintained on

either MX_2 medium (Table 3) or the modified Heller's medium (Table 1). All media were prepared from concentrated stock solutions of analytical reagent (A.R.) grade chemicals stored at 4°C.

TABLE 1 Composition of Heller's medium for the maintenace of sycamore AM strain.

COMPONENT	FINAL CONCENTRATION IN MEDIUM (mg dm^{-3})	STOCK SOLUTIONS
KCl	750	x10
$NaNO_3$	600	x10
$Na\ H_2\ PO_4.2H_2O$	130	x10
$CaCl_2.2H_2O$	78	x10
$MgSO_4.7H_2O$	250	x10
$Zn\ SO_4.7H_2O$	1.0	x1000
H_3BO_3	1.0	x1000
$MnSO_4.4H_2O$	0.1	x1000
$Cu\ SO_4.5H_2O$	0.03	x1000
KI	0.01	x1000
$FeSO_4.7H_2O$	1.0	x1000
Na_2 EDTA	1.3	x1000
thiamine.HCl	1.0	x1000
pantothenic acid (Ca salt)	2.5	x1000
choline chloride	0.5	x1000
meso-inositol	100	Add as solids
Cysteine.HCl	10	Add as solids
sucrose	2.0×10^4	Add as solids
2,4-D	1.0	x100
kinetin	0.25	x100
NaOH	sufficient to adjust pH to 5.2 before autoclaving	
urea (added to medium after autoclaving)	200	commercially prepared. 40% w/v sterile solution.

TABLE 2 Composition of culture medium used for the initiation and maintenance of D_5 strain of sycamore.

COMPONENT	FINAL CONCENTRATION IN MEDIUM (mg dm^{-3})	STOCK SOLUTIONS
NH_4NO_3	800	x10
KNO_3	340	
$Ca(NO_3)_2.4H_2O$	980	
$MnSO_4.H_2O$	16.9	x20
$MgSO_4.7H_2O$	370	
KH_2PO_4	170	x10
KCl	65	
H_3BO_3	6.2	x200
$ZnSO_4.7H_2O$	8.6	
$Na_2MoO_4.2H_2O$	0.25	
KI	0.83	
$CuSO_4.5H_2O$	0.025	
$CoCl_2.6H_2O$	0.025	
$FeSO_4.7H_2O$	5.0	x200
Na_2 EDTA	6.5	
thiamine.HCl	5.0	x200
meso inositol	500	Added as solids
sucrose	3.0×10^4	
glutamic acid (Na salt)	1×10^3	
2,4-D	5.0	x20
kinetin	0.25	x100

pH adjusted to 5.8 before autoclaving.

TABLE 3 Composition of MX_2 medium for the maintenance of Pauls Scarlet rose cultures.

COMPONENT	FINAL CONCENTRATION IN MEDIUM (mg dm^{-3})	STOCK SOLUTIONS
KCl	750	x10
$NaNO_3$	850	x10
KH_2PO_4	140	x10
$CaCl_2.2H_2O$	78	x10
$MgSO_4.7H_2O$	250	x10
$ZnSO_4.7H_2O$	0.5	x1000
H_3BO_3	0.2	x1000
$MnSO_4.4H_2O$	1.0	x1000
$CuSO_4.5H_2O$	0.02	x1000
KI	0.1	x1000
$CoCl_2.6H_2O$	0.01	x1000
$Na_2 MoO_4.2H_2O$	0.02	x1000
$FeSO_4.7H_2O$	1.0	x1000
Na_2 EDTA	1.3	x1000
thiamine	0.5	x2000
pantothenic acid (Ca salt)	1.0	x1000
pyridoxine HCl	0.5	x1000
nicotinic acid	1.0	x1000
meso-inositol	100	Added as solid
sucrose	2.0×10^4	Added as solid
2,4-D	1.0	x100
kinetin	0.5	x50

pH adjusted to 6.0 before autoclaving.

(d) Maintenance of stock cultures:

For the maintenance of Am cultures 57cm^3 aliquots of medium were dispensed into 250cm^3 Erlenmeyer flasks closed with cotton wool plugs, and sterilized at 121°C (15psi) for 15 minutes. Sterilized media for stock cultures were stored for up to three weeks at 10°C although experimental media were always used within four days of preparation. Immediately before subculturing, 3cm^3 of diluted urea (1:100 dilution of 40% w/v filter-sterilized solution, Oxoid Ltd.) was added to each flask of medium, followed by 10cm^3 of 14-day suspension culture. D_5 and rose cultures were similarly subcultured by diluting 14-day cultures (10cm^3 or 5cm^3 respectively) with the appropriate medium (50cm^3). All flasks were sealed with a double layer of sterilized aluminium foil and were incubated on a horizontal orbital shaker (120rpm, 2.5inch throw; L. and H. Engineering Ltd.) in a controlled environment room at 25°C. The culture room was illuminated continuously with white light from fluorescent tubes (300 lux at flask level). All culture strains were also maintained as callus cultures, on medium solidified with agar (0.7% w/v Bacto-Agar, Difco Ltd.), to provide a secondary stock of material as an insurance against infection or technical failures.

(e) Sterility checks:

All cultures were regularly checked for contamination by micro-organisms. The tests applied were (a) culture appearance and odour, (b) examination by phase contrast microscopy (x1250), (c) low density plating in malt-extract agar (Oxoid Ltd.). On the rare occasions when contamination was detected the nature of the infection was usually clearly observable by phase contrast microscopy. Problems could arise in identifying yeasts as culture medium can contain starch grains of a similar appearance. If a low background yeast infection was suspected the malt-extract agar could be relied on to produce a definite answer as it is specifically formulated for the detection and isolation of yeasts and moulds.

Plate 1. The basic batch culture unit used for large scale suspensions of *Acer pseudoplatanus* L.

Key:

A = aerator

AO = air outlet

C = flat-flange lid clip

CW = cotton wool filter

F = 'Microflow' air filter

GC = glass, temperature control coil

IP = inoculation port

S = magnetic bar stirrer

SR = sample receiver

SWL = sterile water line

T = thermometer

TWC = temperature control water line

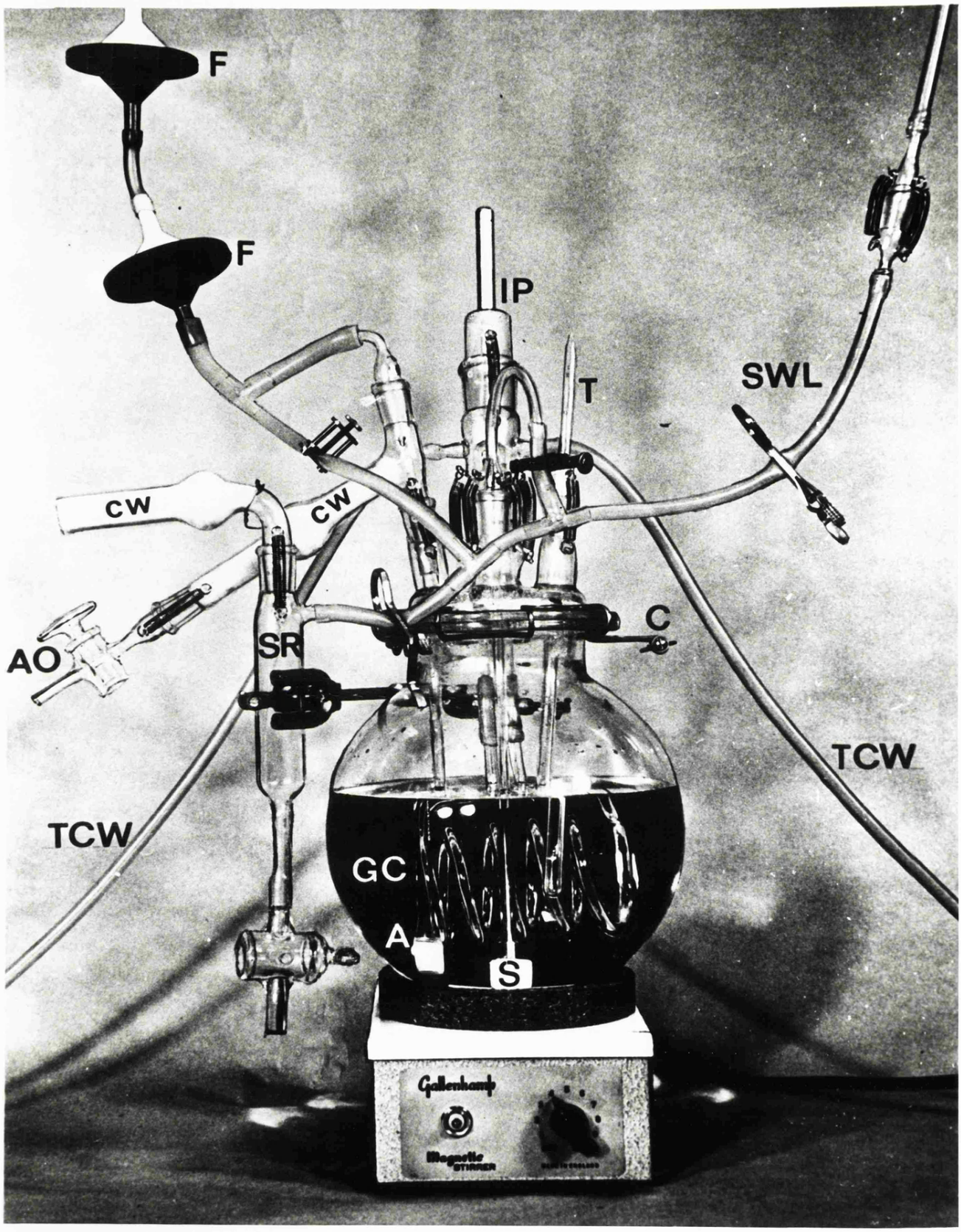
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Plate 2. The automatic sampling assembly

A. View of complete assembly.

Key:

AP = air pump

ES = empty solenoid valve

LD = latching device

NV = needle valve for automatic sample delivery

SO = sample outlet line from needle valve

SR = sample receiver (manual sampling)

SSL = sterile saline supply line

SVD = sample volume detector electrode

TCW = temperature controlled water inlet

TT = turntable

WS = wash solenoid valve

B. Sample volume detector.

E^- = electrodes

TC = teflon cones

C. Exploded view of stainless-steel valve unit.

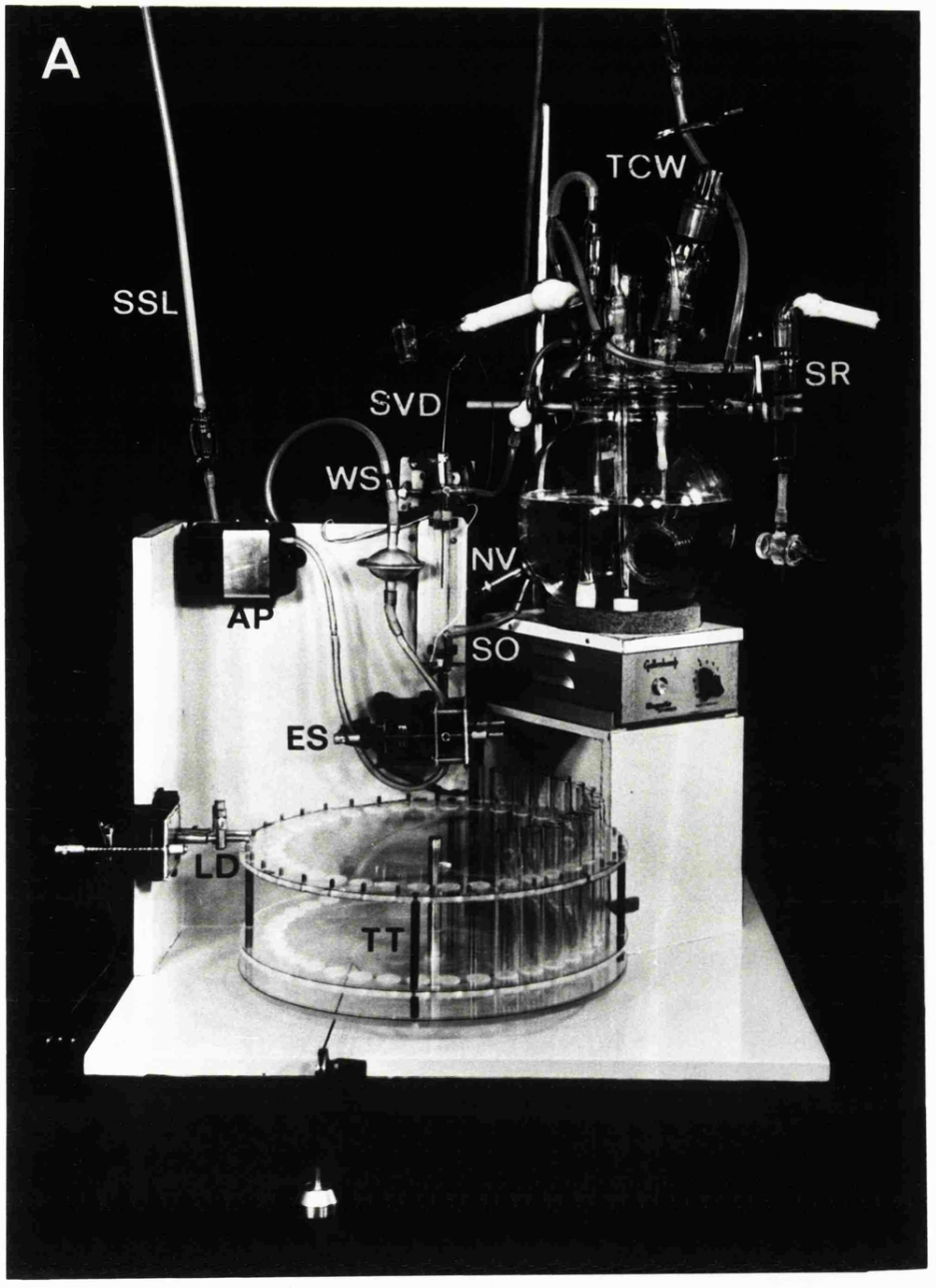
FC = flexible cable

R 1, 2, 3, 5 = silicon rubber 'O' rings

SO = sample outlet

SSL = sterile saline line

W1 = stainless steel washer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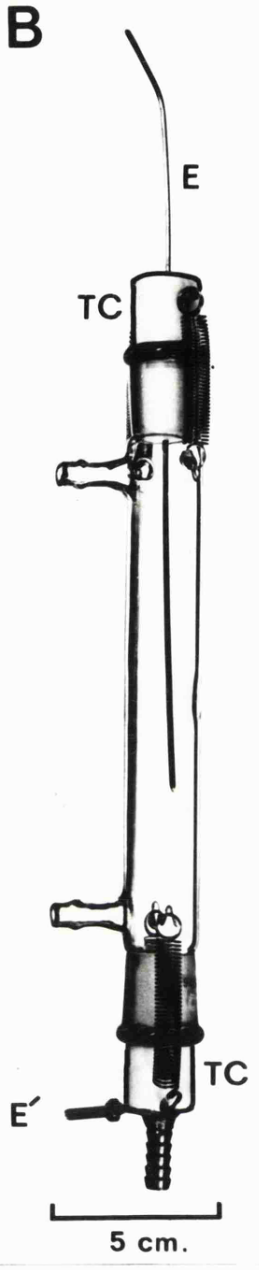
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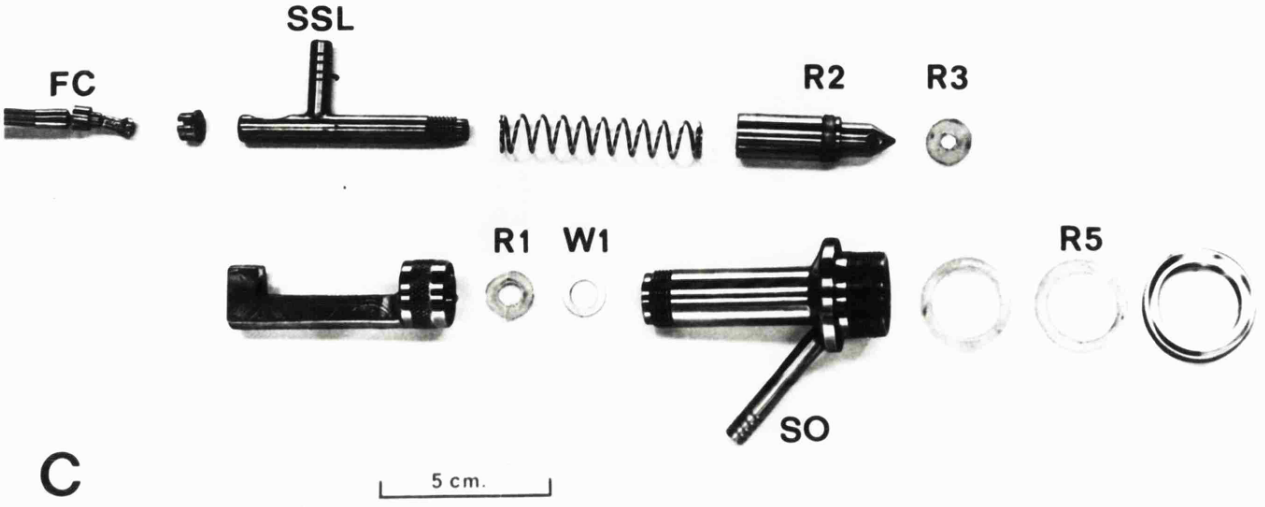
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W1
R5
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C

5 cm.

(f) Large-scale cultures:

The apparatus used to construct the vessels for large-scale cultures (culture volume 4 or 9 dm^3) has been previously described by Wilson et al (1971) and photographs of the two main designs, with and without automatic sampling, are shown in Plates 1 and 2. Effective sterilizing times at 121°C were found to be 35 minutes for vessels containing 4 dm^3 of liquid and 45 minutes for vessels containing 9 dm^3 of liquid. All culture vessels were inoculated with urea and cells inside a room which had been previously sterilized by UV irradiation and which was supplied with a laminar flow of sterile air (Street, 1977). Urea was added to cool medium, via an inoculation port, as a 40% filter-sterilized solution (0.5cm^3 per dm^3 of medium). Culture vessels were inoculated with cells by pouring the contents of 70cm^3 cultures through the inoculation port while an assistant flamed the inoculation port with a bunsen burner.

(g) Aseptic washing of cultures:

Cultures were washed free of extracellular growth regulators using the apparatus described by Shillito et al (1976). The apparatus was based on a 250cm^3 flask fitted with a glass sinter (G_2 grade) in a side-arm (Fig.1). Ancillary equipment permitted the aseptic removal of spent medium and the addition of fresh medium, via the sinter side-arm, by gentle vacuum. Routinely, 14-day stock cultures (70cm^3) were washed with three complete changes (50cm^3) of medium lacking one or all growth regulators and resuspended in the same medium (100cm^3). Aliquots of this washed culture were then used as inocula for experimental treatments.

(h) Plating techniques:

Sterile 10cm diameter polystyrene Petri dishes (Sterilin Ltd.) were used for all plating experiments. Standard media were solidifed with 0.7% w/v purified agar (Difco Laboratories) and if urea was normally present in the liquid medium this was replaced by sodium glutamate (1g dm^{-3}).

Suitably diluted suspension culture ($1cm^3$) was added to each dish using a graduated automatic pipette (A.R. Howell Ltd.). Molten agar medium ($9cm^3$), cooled to $40^{\circ}C$, was then added and the mixture swirled to ensure a uniform distribution of cellular aggregates. When the agar had solidified the plates were sealed with strips of Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan).

2. PARAMETERS FOR CULTURE ANALYSIS

(a) Cell number:

The cell counting technique was based on that of King et al (1974):

(i) Sample preparation (maceration)

A sample ($5cm^3$) from a well-mixed cell suspension was pipetted into an equal volume of 20% w/v chromium trioxide in a universal (McCartney-type) bottle. After 10 minutes at ambient temperature the bottle was placed in a water bath at $70^{\circ}C$ for 5 to 10 minutes, depending on the physiological state of the cells. After heating, the sample bottle was tightly closed with a screw cap and shaken vigorously on a flask-shaker (Baird and Tatlock Ltd.) for a duration (3 to 10 minutes) dependent on the fragility of the cells.

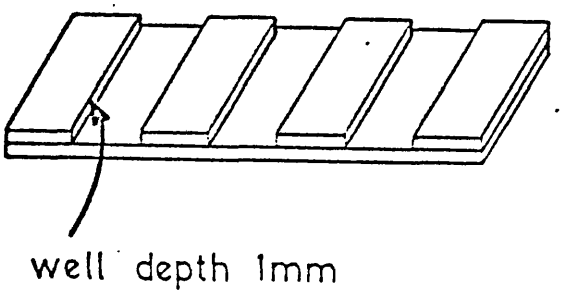


Fig.2. A cell counting slide.

(ii) Counting

Macerated samples were diluted with water to give an estimated cell

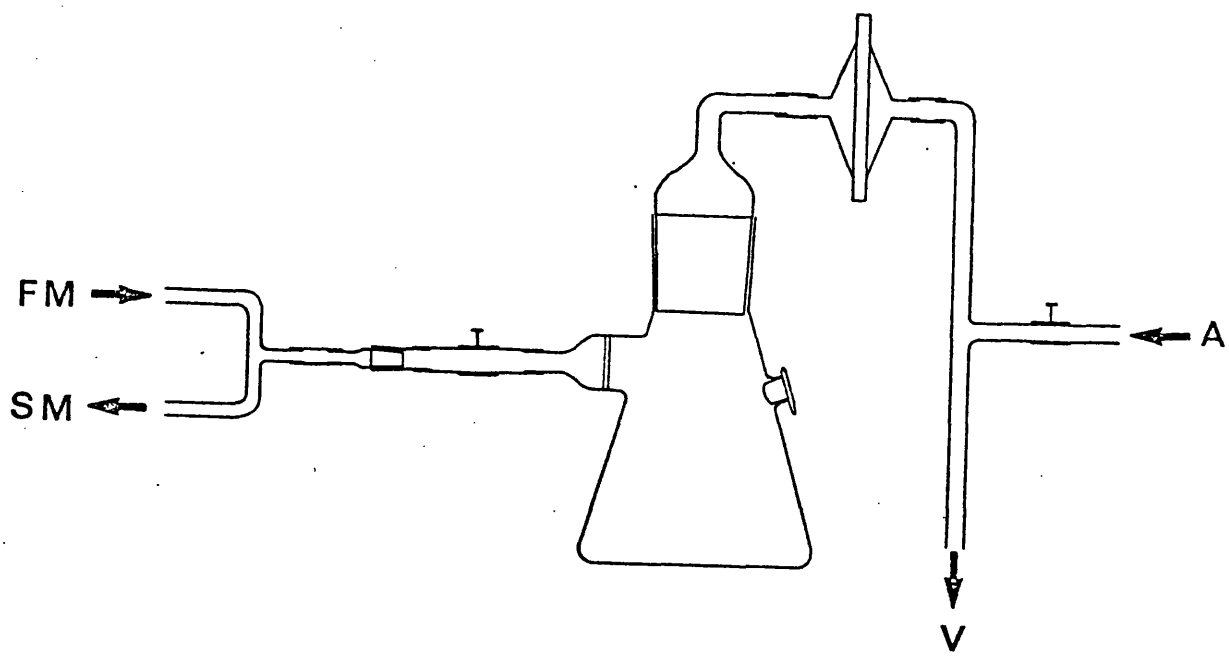


FIG.1. Basic design of flask for aseptically washing cultures. Spent medium (SM) or fresh medium (FM) leave or enter the flask via a side-arm containing a glass (G_2) sinter. Air (A) or Vacuum (V) are supplied to the flask via a sterilizing filter.

density of 5 to 15 x 10^3 cells cm^{-3} and pipetted into the wells of special counting slides (Fig.2). With a well-depth of 1.0mm the volume of each field of view in a Watson Microsystem 70 microscope at x 100 magnification was found to be 0.8mm^3. Estimations of the number of cells in each cm^3 of culture were made from cell counts of 100 random fields of view. The standard error of the mean was also calculated and if this exceeded 8% of the mean, the cell counting procedure was repeated. An analysis of the variance of cell number estimations within and between ten separate samples removed from a 4dm^3 culture indicated no significant difference (King, 1973). Although one sample was therefore considered sufficient statistically, duplicate samples were normally taken to allow for technical mishap or unpredictable changes in susceptibility to maceration during experimental treatments.

(b) Dry weight:

The cells from culture samples (10cm^3) were collected by vacuum filtration on discs of pre-dried, pre-weighed glass-fibre filter pads (Whatman GF/C, 25mm diameter) and washed with distilled water (3 x 5cm^3). After 16-24 hours incubation at 80°C the discs were cooled to room temperature inside a desiccator and reweighed. Dry weight was always determined from duplicate samples.

(c) Packed-cell volume:

A sample of culture (10cm^3 or 15cm^3) was centrifuged in a graduated polystyrene centrifuge tube at 1500g for 5 minutes. The volume of the pellet of cells was expressed as a percentage of the sample volume.

(d) Viability:

The vital stain fluorescein diacetate (FDA) was used in a manner similar to that described by Widholm (1972(a)):

A stock solution of fluorescein diacetate in acetone (5mg cm^{-3}) was stored in a dark-glass bottle at -20°C. One drop of this stock solution

was added to $10cm^3$ of culture and mixed thoroughly before an aliquot was transferred to a microscope slide. After 5 minutes at room temperature the percentage of cells showing fluorescence in ultra-violet light was estimated using a Zeiss microscope. For a cell to be counted as 'viable' by this method, it must contain active esterases and an intact plasmalemma to retain the fluorescent product.

These criteria do not necessarily mean that a cell is capable of further growth and as the scoring of aggregates is subjective, the results obtained from FDA staining should only be used as a guide to culture viability.

(e) Mitotic index:

Cells were fixed in formic acid (25% v/v) and, if necessary, stored at $4^{\circ}C$. Fixed cells were spread on a microscope slide, covered with propionic-orcein-HCl (1% w/v orcein in propionic acid : HCl (1M), 9:1) and warmed gently (ca. $60^{\circ}C$) for one minute. The cell-mass was squashed under a coverslip using thumb pressure only, before the coverslip was removed to replace the excess stain with acetic acid (25% v/v). The coverslip was then replaced and tapped gently to aid the formation of a monolayer of cells. If starch grains, which can obscure nuclei, were still present the slide was gently re-warmed until they dissolved. The slides were scanned (x 1000 magnification) until 1,000 nuclei had been examined and the mitotic index recorded as the percentage of cells containing mitotic figures.

(f) Estimation of total cellular protein:

Before solubilizing cellular protein with alkali, cells were washed to remove extracellular protein, extracted with alcohol to remove intracellular phenolic and amino acids, and acetone powdered. Solubilized protein was estimated by the method of Lowry et al (1951) using bovine serum albumen (BSA, sigma) as standard. The BSA standard was dissolved in alkali and incubated alongside samples during hydrolysis.

Method:

(i) Collect cells ($10cm^3$ of culture) on GF/C filter pad.
Wash with distilled water. $3 \times 5cm^3$
Transfer cells plus GF/C to test-tube, add 85% v/v methanol. $5cm^3$
Leave to stand at room temperature for 24 hours.
Filter through GF/C, collect filtrate.
Wash test-tube and cells with 85% v/v methanol. $3 \times 1cm^3$
Retain combined filtrate for phenolic acid and amino-N assays.
Wash cells on GF/C with acetone. $3 \times 1cm^3$
Transfer cells and GF/C to test-tube, vacuum desiccate, then store at minus $30^{\circ}C$.

(ii) Cover acetone-powdered cells with 1M NaOH. $5cm^3$
Incubate at $100^{\circ}C$ for 30 minutes.
Filter through GF/C, collect filtrate.
Wash test-tube and cells with 1M NaOH. $3 \times 1cm^3$
Make combined filtrate up to $10cm^3$ with M NaOH.

(iii) Filtrate from (ii), or standard. $0.2cm^3$
Alkaline copper reagent (2% w/v Na_2CO_3 in 0.1M NaOH plus 0.2% w/v NaK tartrate and 0.1% w/v $CuSO_4$). $3.0cm^3$
Mix and leave for 10 minutes at $25^{\circ}C$.
Add Folin-Ciocalteu reagent (BDH Ltd., diluted 1:1 with water immediately before use). $0.2cm^3$
Mix and leave for 30 minutes at $25^{\circ}C$.
Read absorbance at 700nm.
The calibration curve for BSA standard was not linear.

(g) Estimation of total soluble phenolics:

Cellular alcohol soluble phenolics were extracted as described in (f) above. The extracts were reduced to dryness in a stream of filtered air, dissolved in a small volume of absolute ethanol and made up to a known volume (2 to 10cm^3).

Method:

Sample or standard (up to 80μg gallic acid equivalents per cm^3).	0.5cm^3
Distilled water.	2.0cm^3
Folin Ciocalteu reagent (BDH Ltd.).	0.2cm^3
Mix and incubate at 25°C for 3 minutes.	
Add saturated aqueous $Na_2 CO_3$.	0.5cm^3
Mix and incubate at 25°C for 60 minutes.	
Read absorbance at 700nm.	

The calibration curve for gallic acid (3,4,5-trihydroxybenzoic acid) was linear.

(h) Estimation of total soluble amino-nitrogen:

Methanol extracts (see (f) above) were evaporated to dryness and made up to a known volume as for (g). Amino-nitrogen was estimated by a modification of the ninhydrin method of Yemm and Cocking (1955) using leucine as standard. The sensitivity of this assay varies for different amino-containing compounds and therefore gives only approximate total values.

Method:

Samples or standard (up to 0.2μmoles NH_2).	1.0cm^3
Sodium citrate buffer (0.2M, pH 4.75).	0.5cm^3
Ninhydrin reagent (5.2 x 10^{-2}M ninhydrin and 1.9 x 10^{-3}M ascorbic acid in 2-methoxyethanol).	1.2cm^3
Mix and incubate at 100°C for 20 minutes, (test-tubes stoppered with glass marbles to reduce evaporation).	

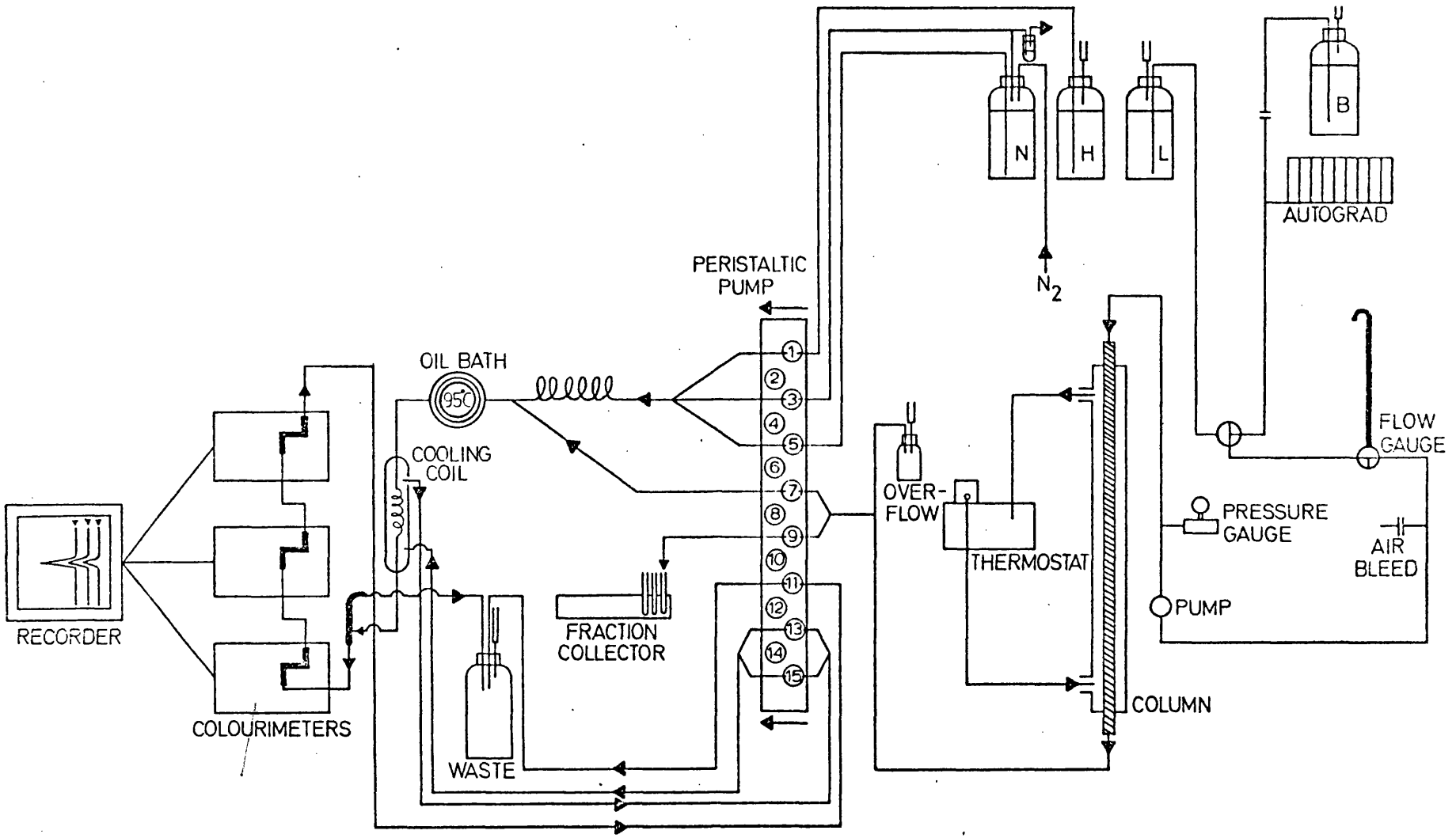
FIG.3. Flow diagram of Technicon Auto Analyzer as modified for the analysis of radioactive amino acids.

N = Ninhydrin reagent (0.037M Ninhydrin, 6.33M 2-Methoxyethanol in 0.87M acetate buffer, pH 5.48)

H = Hydrazine sulphate (2×10^{-3}M)

L = Lithium hydroxide (0.3M)

B = Buffer 1 (see Table 4)


N
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AUTOGRAD
N_2
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95°C
COOLING COIL
OVER-FLOW
THERMOSTAT
FLOW GAUGE
PRESSURE GAUGE
AIR BLEED
PUMP
COLUMN
RECORDER
COLOURIMETERS
FRACTION COLLECTOR
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Cool in iced water for 5 minutes.

Add 60% v/v ethanol in water. $3.0cm^3$

Mix and leave at $25^{\circ}C$ for 15 minutes.

Read absorbance at 570nm.

The calibration curve for leucine was linear.

(i) Amino acid analysis:

Methanol extracts (see (f) above) were evaporated to dryness using a rotary evaporator at $35^{\circ}C$, redissolved in a small volume ($0.5cm^3$) of absolute alcohol and made up to $3.0cm^3$ with distilled water. The extracts were stored in polyethylene scintillation vials at minus $30^{\circ}C$. Preliminary analyses used two dimensional paper chromatography to separate individual amino acids (first solvent: butanol 60% v/v, water 25% v/v, glacial acetic acid 15% v/v; second solvent: phenol 80% w/v, water 19.5% v/v, 0.880 ammonia 0.5% v/v) all other determinations used a Technicon Auto-Analyzer. The construction and operation of the Analyzer were based on instructions given by Boulter (1966) and the Technicon instruction manual (AAA-1, 1967) although various modifications were made to permit the analysis of radioactive samples (see MATERIALS and METHODS 3(a)). The flow diagram (Fig.3) illustrates the main features of the apparatus after modification. Initial analyses used the standard sodium citrate buffer system recommended by Technicon, but as soon as it became obvious that complete separation of threonine, serine, asparagine and glutamine were required, the lithium citrate buffer system recommended by Nunn and Vega (1968) was adopted. The technique for converting the ion-exchange resin (Tybe B chromobeads) was that proposed by Nunn and Vega (1968) except that, before repacking, the resin was washed in acetone as well as dichloromethane. Optimum separation of threonine, serine, asparagine, glutamic acid and glutamine was achieved by using a column temperature of $38^{\circ}C$ until glutamine eluted, and then switching to $60^{\circ}C$. Optimum sensitivity was maintained if starting buffer (pH 3.01) was passed through the column, at the end of each run, before

TABLE 4 Composition of buffers.

COMPONENT	BUFFER 1	BUFFER 2
Lithium citrate.$4H_2O$	19.74g dm^{-3}	28.20g dm^{-3}
Lithium chloride (anhydroun)	2.76g dm^{-3}	38.16g dm^{-3}
Thiodiglycol	0.1% v/v	-
Brij 35	0.25% w/v	0.25% w/v
Na_4 EDTA	0.1% w/v	0.1% w/v
Final lithium concentration	0.275M	1.20M
Final citrate concentration	0.070M	0.10M
Final pH*	3.010 ± 0.005	6.50 ± 0.02

*pH adjusted with 6M HCl.

TABLE 5 Composition of Autograd buffers.

CHAMBER NO.	BUFFER 1 (cm^3)	BUFFER 2 (cm^3)	Propan-2-ol (cm^3)
1	74.0	-	1.0
2	74.5	-	0.5
3	75.0	-	-
4	55.0	20.0	-
5	45.0	30.0	-
6,7,8,9	-	75.0	-

regenerating the resin with lithium hydroxide (0.3M).

Methods:

(i) Preparation of buffers.

The compositions of the buffers required for the preparation of the 'Autograd' are shown in Table 4. Lithium citrate, chloride and hydroxide and Brij 35 (polyoxyethylene lauryl ether) were obtained from BDH Chemicals Ltd., and thiodiglycol and pHix from Pierce Chemical Co. (via Koch-light Ltd.). Buffers were filtered through glass-fibre filters (Whatman GF/C) before the addition of Brij 35 and protected against microbial growth with pHix. The compositions of the Autograd buffers are shown in Table 5.

(ii) Manual sample loading.

Equilibrate column with starting buffer for 30 minutes.

Switch off pump, remove column head, remove excess buffer.

Layer sample and standard (up to 1.0cm^3) on to column.

Load sample and standard on to column by applying air pressure with a gas-syringe.

Release air pressure, fill column head with starting buffer.

Connect Autograd to pump inlet, and pump outlet to column head.

Start pump, recorder and fraction collector.

(iii) Manual shut-down and column regeneration.

When arginine had eluted, about 21 hours after sample loading, the column was regenerated in preparation for the next sample:

Disconnect Autograd and flush column with starting buffer.	30 mins.
Disconnect column from Auto Analyzer lines.	
Pump lithium hydroxide (0.3M) through column.	30 mins.
Flush column with starting buffer.	30 mins.
Connect column to Auto Analyzer lines.	
Equilibrate system with starting buffer.	30 mins.

The column is now ready to receive the next sample.

(j) Nitrate reductase:

The nitrate reductase activity of whole cells was estimated by the method of Ferrari et al (1971) as modified by Jones et al (1976). This method depends on the production of nitrite by tissues incubated anaerobically with nitrate. The nitrate is then determined by diazotising sulphanilamide with N-1-napthyl ethylene diamine (NED).

Method:

Collect cells (up to 10^6) on GF/C disc and wash with distilled water. $2 \times 5cm^3$

Transfer cells and GF/C to $25cm^3$ conical flask.

Add phosphate buffer (0.1M, pH 7.5). $0.3cm^3$

Flush flask with N_2 and equilibrate in water bath at 25^oC for 5 minutes.

Add substrate stock (0.2M KNO_3 in 2% propan-1-ol). $0.5cm^3$

Incubate in shaking water bath at 25^oC for 20 minutes.

Add sulphanilamide to stop reaction (6×10^{-3} sulphanilamide in 2M HCl). $1.0cm^3$

Mix.

Add NED (4×10^{-4}M N-1-napthyl ethylene diamine hydrochloride). $1.0cm^3$

Mix.

Add distilled water. $1.2cm^3$

Transfer mixture to centrifuge tube.

Read absorbance of clear supernatant at 540nm after 15 minutes at 25^oC.

Nitrite production was linear up to 30 minutes incubation and was proportional to the number of cells incubated.

(k) C_6/C_1 ratio:

For the determination of "C_6/C_1 ratio" (see SECTION 4.4) cells were collected on GF/C filter pads, washed with distilled water ($2 \times 5cm^3$) to

remove extracellular sugars, and transferred to a flask containing the standard Hellers medium lacking growth regulators, vitamins and sucrose ($5cm^3$). The flasks were sealed with rubber suba-seals and were fitted with centre-wells containing KOH (1.0M, $1.0cm^3$) to trap CO_2. After 5 minutes equilibration ^{14}C-glucose (C_1 or C_6 label) was added and the sealed flasks incubated on an orbital shaker for 30 minutes at 25°C. The incubation period was terminated by the addition of formic acid ($5cm^3$, 60% v/v) and $^{14}CO_2$ trapped in the KOH was determined by liquid scintillation counting using the dioxane-based scintillant.

3. THE USE OF RADIOISOTOPES

(a) General precautions:

The general precautions for the handling of radioisotopes were designed to contain any spillages (use of water-tight trays) and to avoid personal contact with ^{14}C either by touch or the inhalation of $^{14}CO_2$ (work in fume-cupboards). As the reaction between ninhydrin and amino acids involves decarboxylation (i.e. possible release of $^{14}CO_2$ from labelled amino acids) the amino acid analyser was modified (see Fig.3) so that all vents to the laboratory contained self-indicating soda-lime (10-14 mesh, Fisons Scientific Apparatus Ltd.).

(b) Radiochemicals:

All chemicals were obtained from the Radiochemical Centre, Amersham as either aqueous solutions or solids. The chemicals used were: [2-^{14}C] glycine (> 40mCi/mmole), 3-phospho [U-^{14}C] glyceric acid (100-150mCi/mmole) L-[U-^{14}C] serine (170mCi/mmole), D-[1-^{14}C] glucose (56.8mCi/mmole) and D-[6-^{14}C] glucose (52.8mCi/mmole).

(c) Liquid scintillation counting:

All samples for ^{14}C determination were in aqueous solution and the fractions from the amino acid analyser contained relatively high

concentrations of inorganic salts (see Tables 4 and 5). A Triton X-100-based scintillation cocktail was used for most samples since it can incorporate large quantities of inorganic salts in aqueous solution and yet maintain high counting efficiencies (see 'Sample preparation for liquid scintillation counting, Review 6,' The Radiochemical Centre, Amersham). The only samples for which the Triton X-100 scintillant was found to be unsuitable were those from C_6/C_1 ratio. As the alkalinity of these samples gave rise to high background counts (due to chemiluminescence) a dioxane-based scintillant was used for estimating $^{14}CO_2$ in KOH. All samples were counted in a Beckman LS-3100 liquid scintillation system fitted with refrigeration. Counting efficiency was determined for each sample using the external standards ratio method which had been previously calibrated with quenched standards. The counting efficiency of most samples was in the range 90-95%.

(i) Preparation of Triton X-100 scintillant.

PPO (4.0g, 2,5-diphenyloxazole, Fisons Scintillation Chemicals) was dissolved in toluene (1.0dm^3, AR grade, Fisons Ltd.), Triton X-100 (0.5dm^3, BDH Chemicals Ltd.) was then added and the solution was thoroughly mixed and stored in a dark glass bottle.

(ii) Preparation of Dioxan-based scintillant.

Napthalene (20g) and Butyl PBD (7g, 2-(4'-tert-butylphenyl)-5-(4"-biphenyl) - 1,3,4-oxadiazole, Fisons Ltd.) were dissolved in toluene (300cm^3) and made up to 1.0dm^3 with dioxane. The solution was thoroughly mixed and stored in a dark glass bottle.

(iii) Sample preparation.

Aqueous samples (1.0cm^3) were routinely mixed with 10cm^3 of scintillant to give a homogeneous solution. Fractions from the amino acid analyser were transferred to polyethylene scintillation vials using two aliquots of scintillant. The volumes of scintillant and/or water added were adjusted

to maintain homogeneous solutions. Using this technique the efficiency of sample transfer was found to be 99.3%. Before counting, sample vials were allowed to equilibrate in the scintillation counter (5°C, dark) for at least two hours to avoid errors due to chemiluminescence.

RESULTS

SECTION 1

2,4-D-STIMULATED GROWTH: EFFECTS OF LIGHT AND ENDOGENOUS PHENOLICS

INTRODUCTION

Sycamore (Acer pseudoplatanus L, Am strain) suspension cultures were adopted as initial experimental material because of the large amount of background information that was available, particularly from previous researchers in Professor Street's group at Leicester (Street, 1977). Particularly relevant to this project were the exploratory studies of Dr. P.J. King concerning 2,4-D utilization in continuous cultures of sycamore (King, 1976). During these studies he noticed an accumulation of phenolic compounds (phenolic acids, flavonoids and lignin) in cells receiving a low supply of 2,4-D. It was suggested that phenolic compounds might be responsible for the inhibition of growth observed in cultures deprived of 2,4-D and that such inhibition could mask, or prevent, the expression of other pathways under auxin control, including those involved in differentiation. Increases in the concentration of cinnamic acid derivatives of various degrees of polymerisation were recorded together with a ten-fold increase in extractable phenyl alanine ammonia lyase (PAL) activity. This enzyme is particularly significant since it catalyzes the formation of trans-cinnamic acid from phenylalanine and thus represents the point at which carbon skeletons are diverted into phenolic compounds.

Phillips and Henshaw (1977) have shown that 2,4-D can suppress the induction of phenolics biosynthesis in batch cultures of sycamore and similar observations have been made for other cultured tissues such as Paul's Scarlet Rose (Davies, 1972), Haplopappus (Constabel, Shyluk and Gamborg, 1971), Cassia (Shah, Subbaiah and Mehta, 1975) and Carrot (Sugano, Iwata and Nishi, 1975). Although the physiological role of phenolic substances is controversial, p-coumaric acid and some of its derivatives can certainly inhibit growth and carbohydrate metabolism when supplied exogenously either to excised whole plant tissues (Kefeli and Kutacek, 1978) or cultured (rose) cells (Danks et al, 1975). Thus the suggestions of King (1976) seemed to provide a reasonable preliminary working hypothesis: that

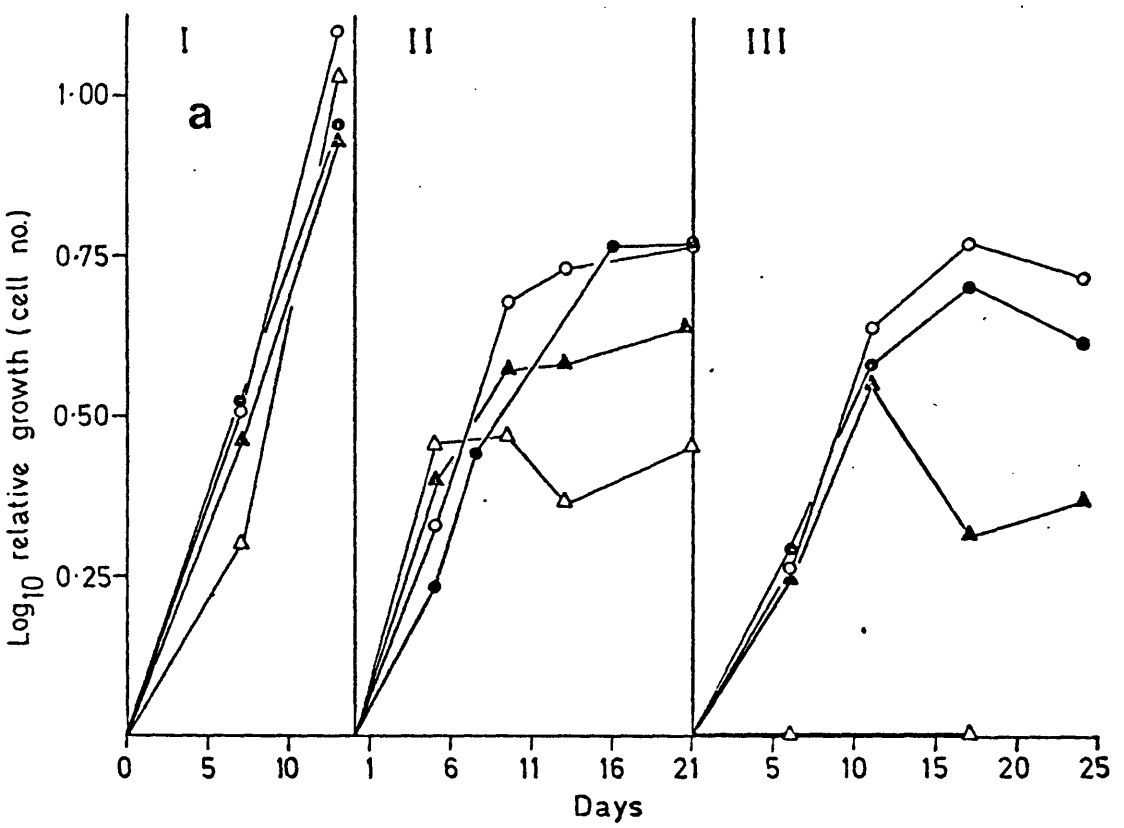
the inhibition of growth by phenolics prevents the expression of other 2,4-D-suppressed phenomena in tissues of a woody habit. This hypothesis would predict that a restriction of phenolics synthesis would permit continued growth in the absence of 2,4-D and perhaps lead to the expression of whatever path(s) of differentiation the cells were determined to follow. PAL activity and the synthesis and accumulation of many phenolic compounds have been shown to be stimulated by light both in whole plants and tissue cultures (Smith, Billett and Giles, 1977; Zaprometov, 1978). Thus the absence of light might be expected to reduce PAL activity to a low level, restrict the synthesis of phenolic compounds and permit prolonged growth in the absence of 2,4-D.

1.1 Effects of light on cells transferred to medium lacking 2,4-D

The stock suspension cultures of sycamore (Am strain) were usually maintained in Erlenmeyer flasks containing 70cm^3 of culture and incubated, between 14 day subcultures, on orbital shakers in a controlled environment room at 25°C. Fluorescent tubes provided the 'white light' illumination (300 lux) above the cultures. Although dark-room facilities were also available it was thought more desirable to incubate 'dark' cultures on the same orbital shaker as 'light' cultures to eliminate differences between orbital shakers and culture rooms. Dark-grown cultures were therefore individually wrapped in black polythene bags (as used for the exclusion of light from photographic paper). Subcultures during 'dark' treatments were carried out under diffuse (< 1 lux) green safelight (Ilford 909).

When stock Am strain sycamore suspension cultures, previously grown in the presence of 2,4-D (1mg dm^{-3}, 4.5 x 10^{-6}M), were inoculated into minus 2,4-D medium (10cm^3 into 60cm^3) growth continued into the second 14 day culture period (Fig.1.1). Only in the absence of white light did growth continue in the third culture period in minus-2,4-D medium. The working hypothesis (SECTION 1 INTRODUCTION) would predict that the light-grown cultures should have contained more growth-inhibitory phenolics than the

FIG.1.1 Growth of sycamore suspension cultures inoculated into minus-(Δ,▲) or plus- (O,●) 2,4-D media and incubated in the presence (open symbols) or absence (closed symbols) of light. Growth was measured as increase in cell number (a) or dry weight (b) relative to the inoculum during three successive 14-day culture periods.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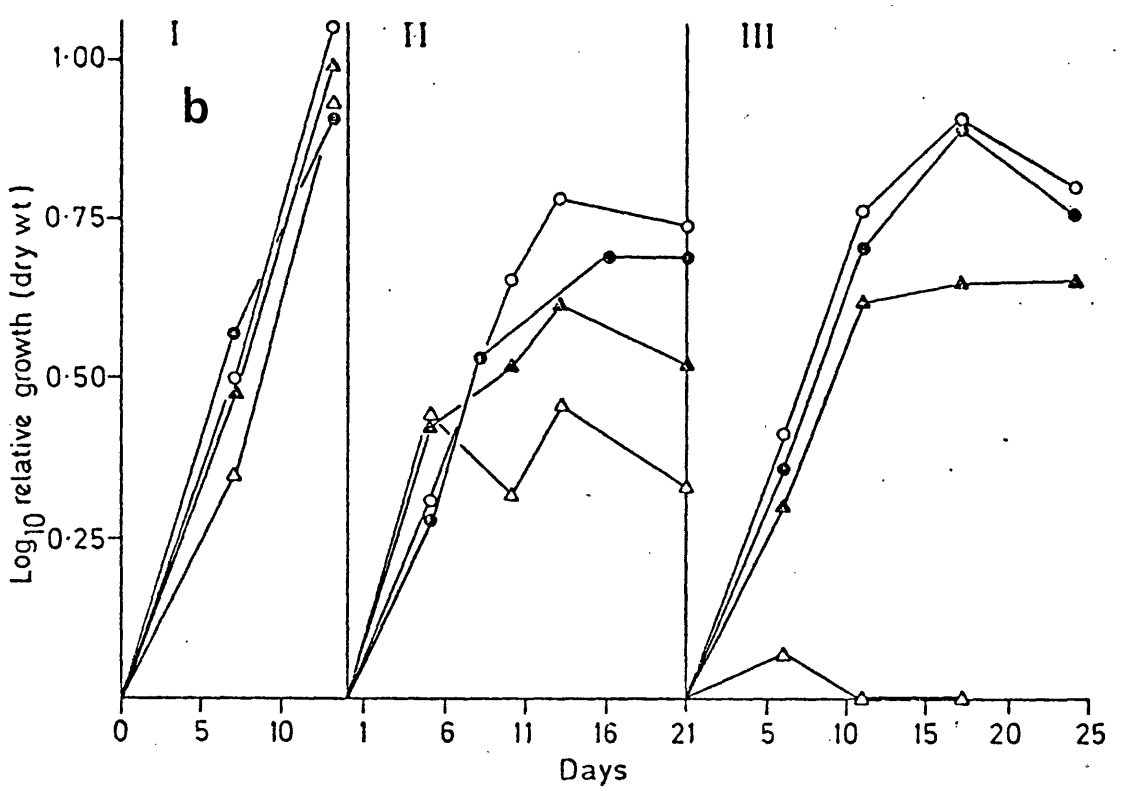
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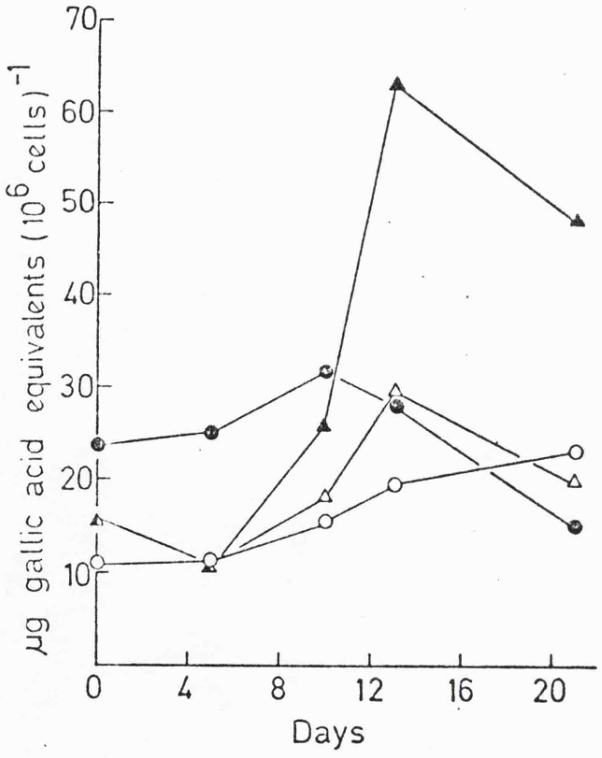


FIG.1.2 Cellular levels of soluble phenols in the cultures shown in Fig.1.1. Data relate to second passage, symbols as for Fig.1.1.

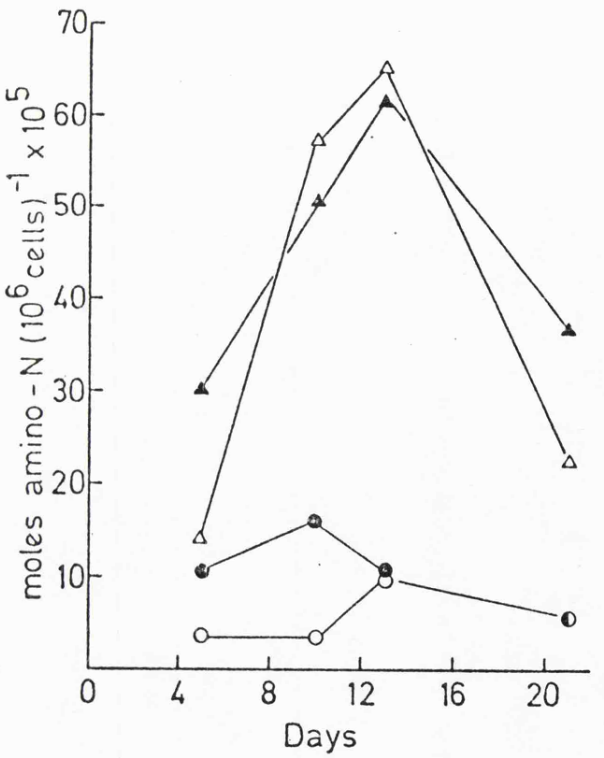


FIG.1.3 Cellular levels of soluble amino-nitrogen in the cultures shown in Fig.1.1. Data relate to second passage, symbols as for Fig.1.1.

dark-grown cultures, but Fig.1.2 shows that the reverse was true during the second 14 day growth period, i.e. when the growth inhibitory effects of 2,4-D depletion were beginning to manifest themselves. Indeed the levels of alcohol soluble phenolics of the light-grown minus-2,4-D cultures were not appreciably higher than those of the light-grown plus-2,4-D control cultures.

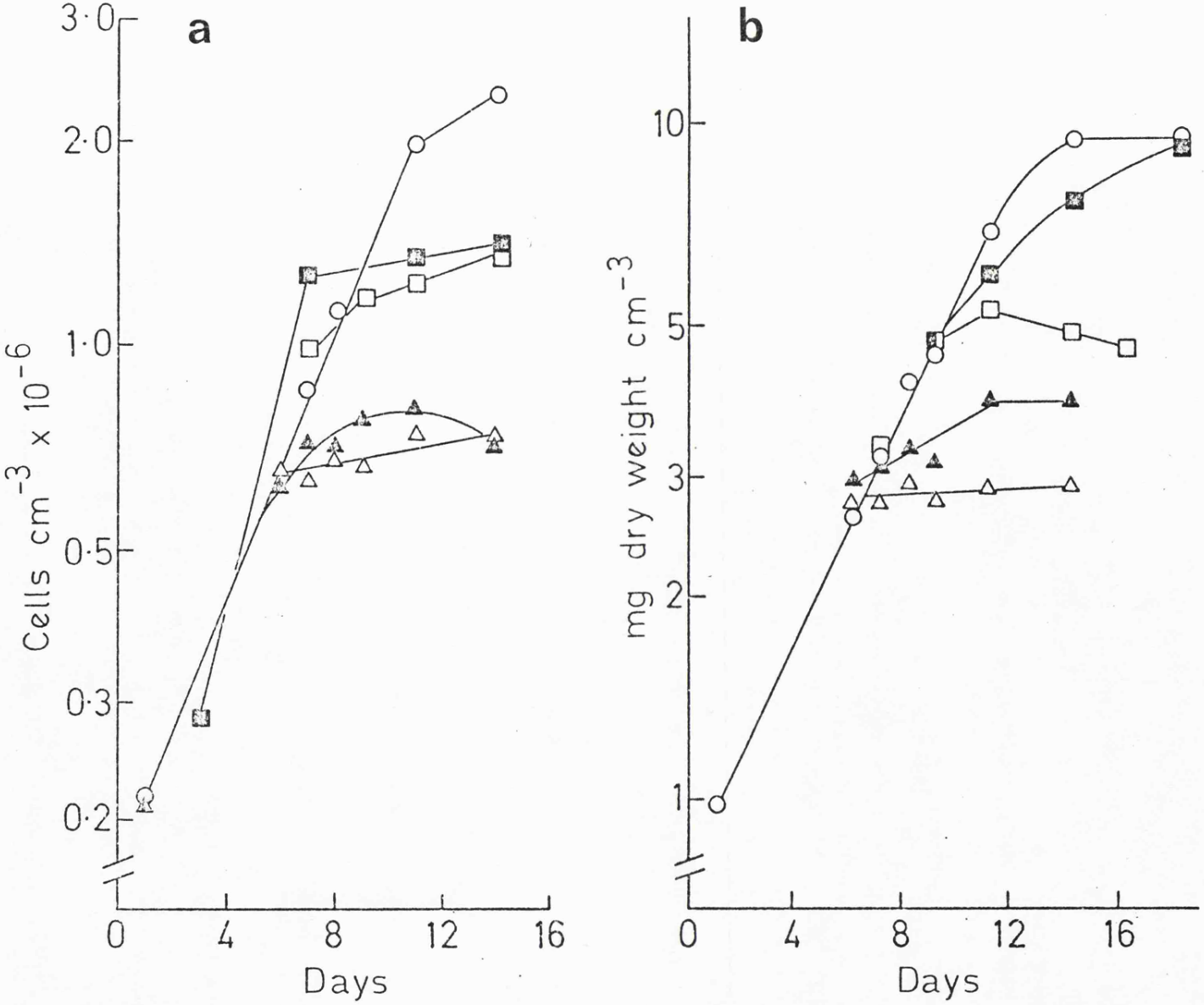
It was conceivable that, if the absence of light was restricting phenolics biosynthesis, the high soluble phenolics found in dark-grown cultures could have represented accumulated phenylalanine or tyrosine. Amino acid analysis of the alcoholic extracts, however, showed very low levels of both these amino acids so that they could not represent the accumulation of Folin-positive phenolics in dark-grown cultures. The analysis of these alcoholic extracts gave a lead to another consequence of 2,4-D withdrawal. Fig.1.3 shows that during the second, critical, growth period in the absence of 2,4-D sycamore cells accumulate soluble α-amino nitrogen (ninhydrin positive) irrespective of the presence or absence of light. The qualitative changes in amino acids that cause this response are discussed in detail in SECTION 4.

Although the working hypothesis had predicted the enhanced growth in dark-grown cultures, after 2,4-D withdrawal, there did not appear to be a simple relationship between this growth response and the levels of cellular alcohol soluble phenolics.

1.2. Effects of light during rapid 2,4-D withdrawal.

The experimental design described above appeared to be of only limited use for detailed studies of responses to 2,4-D depletion. The major problems were that (a) the cultures had to be monitored over two (at least) 14 day growth cycles and (b) the exact time when the extracellular supply of 2,4-D became growth limiting was not known. An improved method of 2,4-D withdrawal was developed (see MATERIALS and METHODS) by which cultures could

FIG.1.4. Influence of white light prior to, or at the time of, culturing sycamore cells in the absence of 2,4-D. Growth, after washing in minus-2,4-D medium, is expressed as change in either cell number (a) or dry weight (b). Before washing, cells were cultured for 14 days in plus-2,4-D medium in white light (○, △, ▲) or darkness (□, ■). After washing, cells were cultured in plus-2,4-D (○) or minus-2,4-D (all other symbols) in white light (open symbols) or darkness (closed symbols).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Cells cm^{-3} x 10^{-6}
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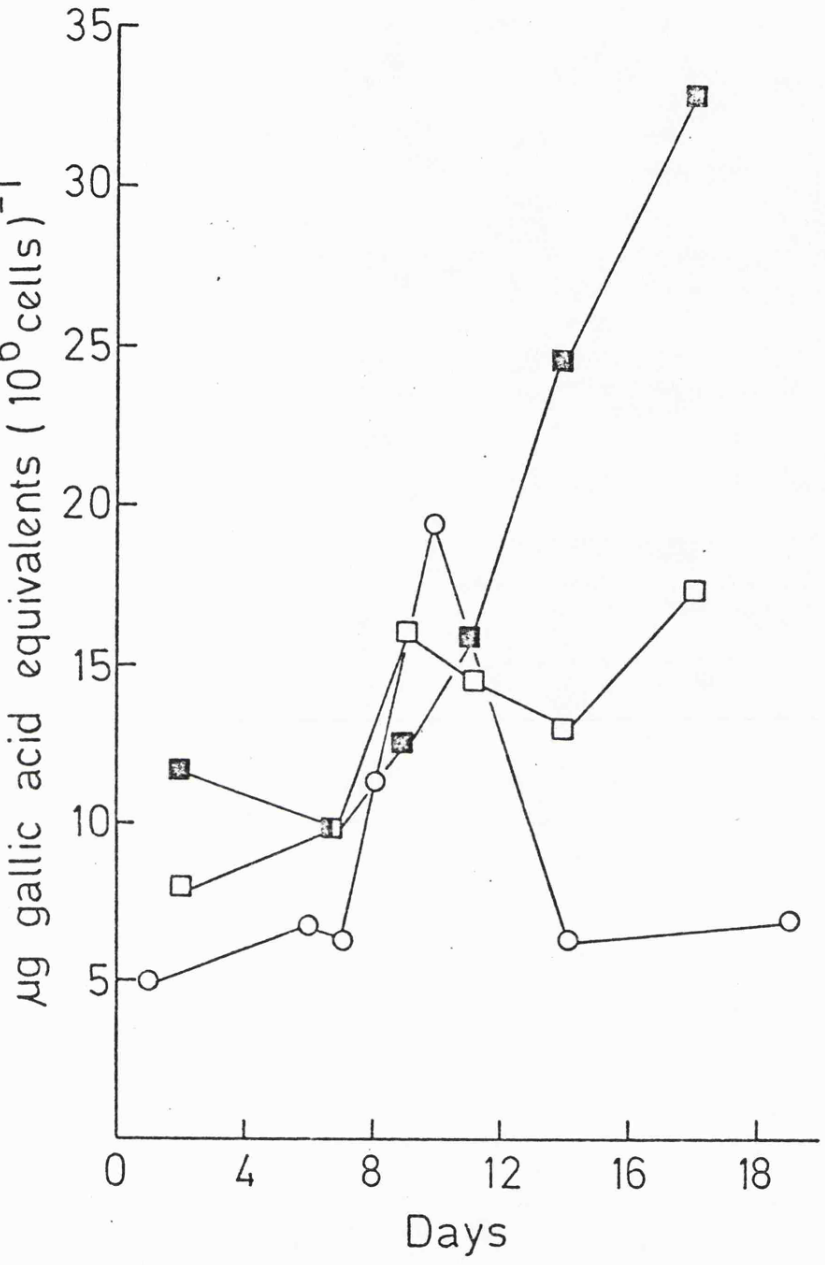


FIG.1.5 Cellular levels of soluble phenols in cultures exhibiting dark-enhanced growth. Symbols as for Fig.1.4.

be aseptically washed free of extracellular 2,4-D prior to the inoculation of experimental treatments.

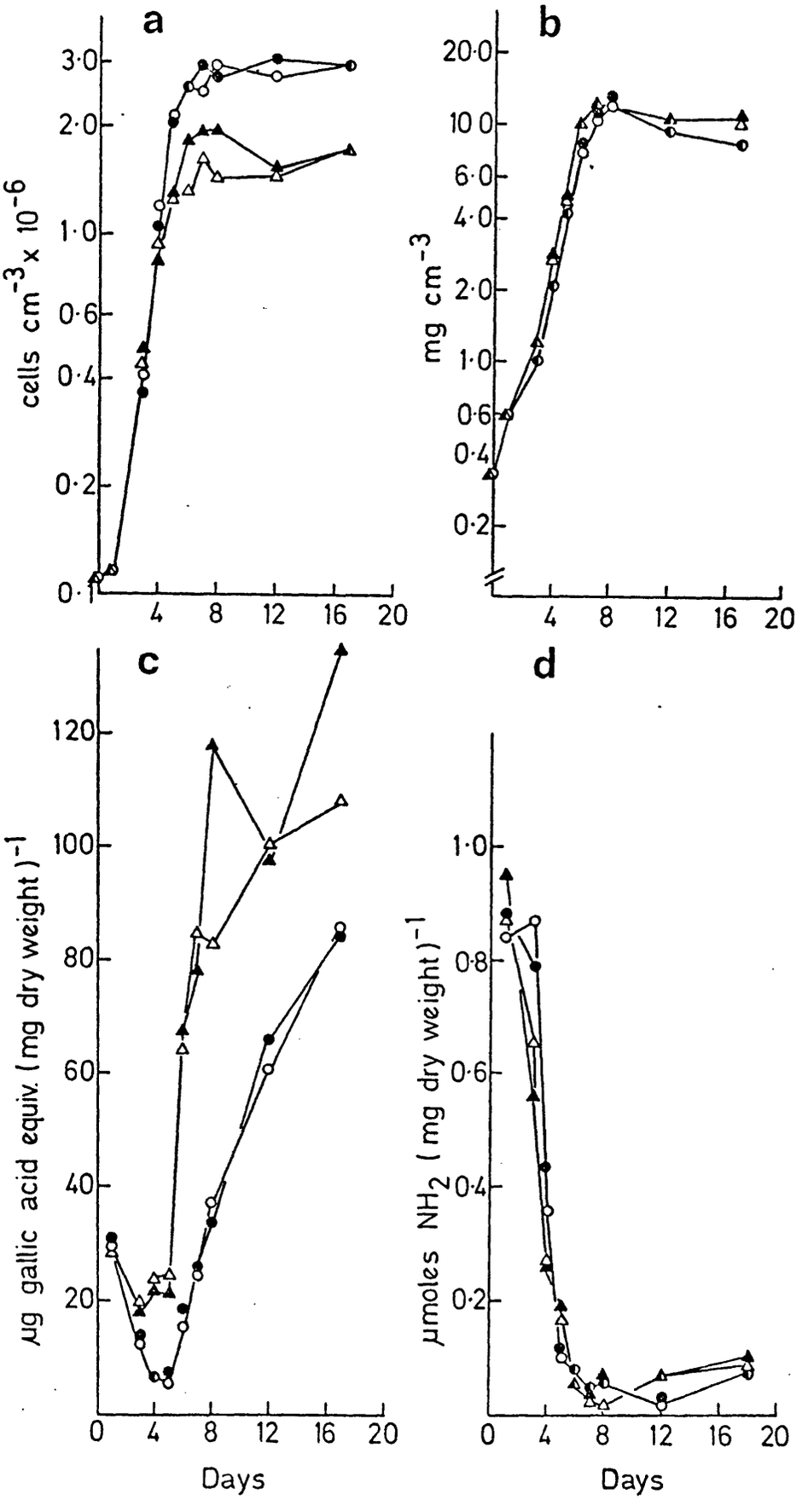
The growth of washed cultures is shown in Fig.1.4 in terms of cell number (a) or dry weight (b). If stock cultures were deprived of 2,4-D in this way and maintained in the light (-△-) growth arrested a few days after washing: within one culture period. If equivalent cultures were transferred to the dark at the time of 2,4-D withdrawal (-▲-) no substantial increase in cell number yield was observed although biomass (dry weight) yield was increased. If, however, the cultures were pre-treated in the dark prior to 2,4-D depletion enhanced growth, relative to light-grown controls, was observed irrespective of whether the cultures were subsequently incubated in the light (-□-) or the dark (-■-). The effects of sequential dark treatments on dry weight accumulation appeared to be cumulative such that the final dry weight yield of cultures maintained in the dark before and after 2,4-D depletion (-■-) was equivalent to that of the plus-2,4-D control (-○-). Continued accumulation of soluble phenolics (Fig.1.5) only occurred in the cells grown in the dark for two passages (-■-) and starved of 2,4-D i.e. those showing the most growth in the minus-2,4-D medium.

1.3. Effects of light on Paul's Scarlet rose cells.

To investigate whether the effects of light on the growth of 2,4-D depleted sycamore cells was a general phenomenon, Rosa cells were given treatments similar to those described in SECTION 1.2. Paul's scarlet rose cells were chosen for comparison because their general behaviour and 2,4-D requirement in suspension culture were similar to those of the sycamore cells. Established suspension cultures of Paul's scarlet rose were obtained from Dr. N. Robinson and were maintained using the culture medium denoted MX_2 (See MATERIALS and METHODS).

The responses of Paul's scarlet rose cells to washing in minus 2,4-D medium followed by incubation in minus-(-△-, -▲-) or plus-(1mg dm^{-3}; -○-, -●-)

FIG.1.6. Effects of 2,4-D and light on the cell number (a), dry weight (b), soluble phenols (c) and soluble amino-nitrogen (d) of Paul's Scarlet rose cultures. Stock cells were washed free of extracellular 2,4-D, inoculated into plus-2,4-D (○,●) or minus-2,4-D (△,▲) media and incubated in the presence (open symbols) or absence (closed symbols) of light.

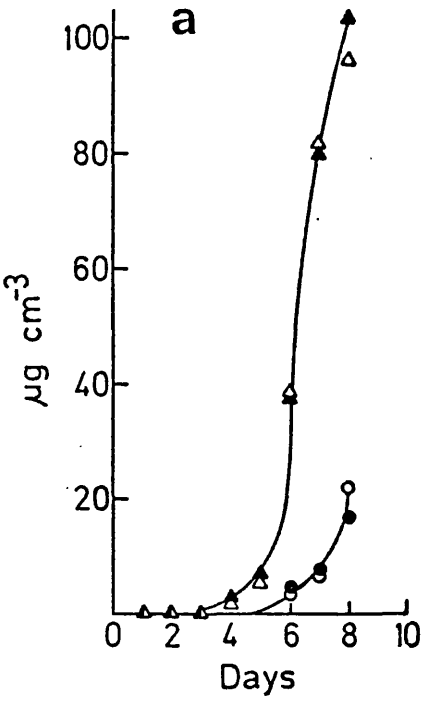

a
b
c
d
cells cm^{-3} x 10^{-6}
mg cm^{-3}
µg gallic acid equiv. (mg dry weight)$^{-1}$
µmoles NH$_2$ (mg dry weight)$^{-1}$
Days
Days

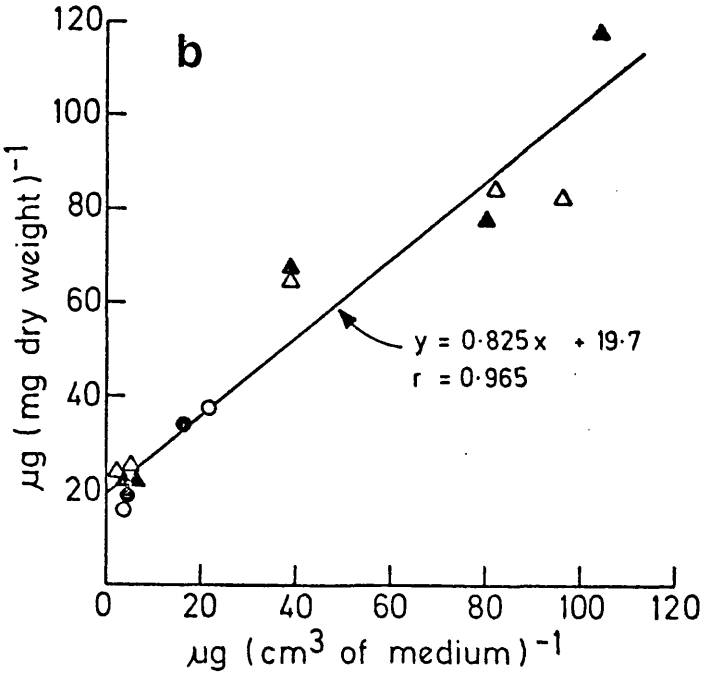
2,4-D medium in the light (open symbols) or dark (closed symbols) are shown in Fig.1.6. The final cell number yield in minus-2,4-D medium was approximately half that obtained in plus-2,4-D medium (Fig.1.6(a)) while the dry weight yields (Fig.1.6.(b)) were not significantly different. Although 2,4-D depletion caused premature accumulation of cellular soluble phenolics (Fig.1.6(c)) there was no effect on soluble amino-nitrogen (Fig.1.6(d) analogous to that observed in sycamore cultures. The absence of light appeared to have little effect on any of the parameters expressed in Fig.1.6.

If, as is generally thought, most cellular phenolics are compartmentalized in the vacuole these cellular phenolics might not be as physiologically active as those released into the culture medium. Danks et al (1975), for example, have shown that many phenolic compounds inhibit the growth of Paul's scarlet rose suspension cultures when present in the culture medium at concentrations of 10^{-4}M and above. The time-course of appearance of phenolics in the culture medium during this experiment (Fig.1.7) showed that when cell division arrested in minus-2,4-D media (day 6) the culture media contained 38μg gallic acid equivalents cm^{-3} (2×10^{-4}M) of Folin-positive phenolics. Although the identity of the spent medium phenolics is not known it appears that they may be present in concentrations sufficiently high to cause a physiological effect. A comparison of Fig.1.7(a) with Fig.1.6(c) suggests that spent medium phenolics are related to the level of cellular soluble phenolics and Fig.1.7(b) shows that this relationship appears to be linear ($r = 0.965$). Although this relationship could result from an equilibration of intracellular and extracellular phenolics a more plausible explanation is thought to be that the extracellular phenolics arise from cell lysis. The degree of lysis that would be required to account for this phenomenon would be in the range 3% to 5% of the total culture.

The results of this experiment suggested that the growth-promoting effects of darkness on minus-2,4-D cultures may have been peculiar to sycamore and could not therefore be used as a general method for prolonging growth in the absence of exogenous auxin. To be sure of this conclusion,

FIG.1.7. The release of soluble phenols into the culture medium of Paul's Scarlet rose cells (a) and their relationship to the cellular soluble phenols (b). Symbols as for Fig.1.6.


a
100
80
60
40
20
µg cm-3
0
2
4
6
8
10
Days


b
120
100
80
60
40
20
µg (mg dry weight)-1
y = 0·825x + 19·7
r = 0·965
0
20
40
60
80
100
120
µg (cm3 of medium)-1

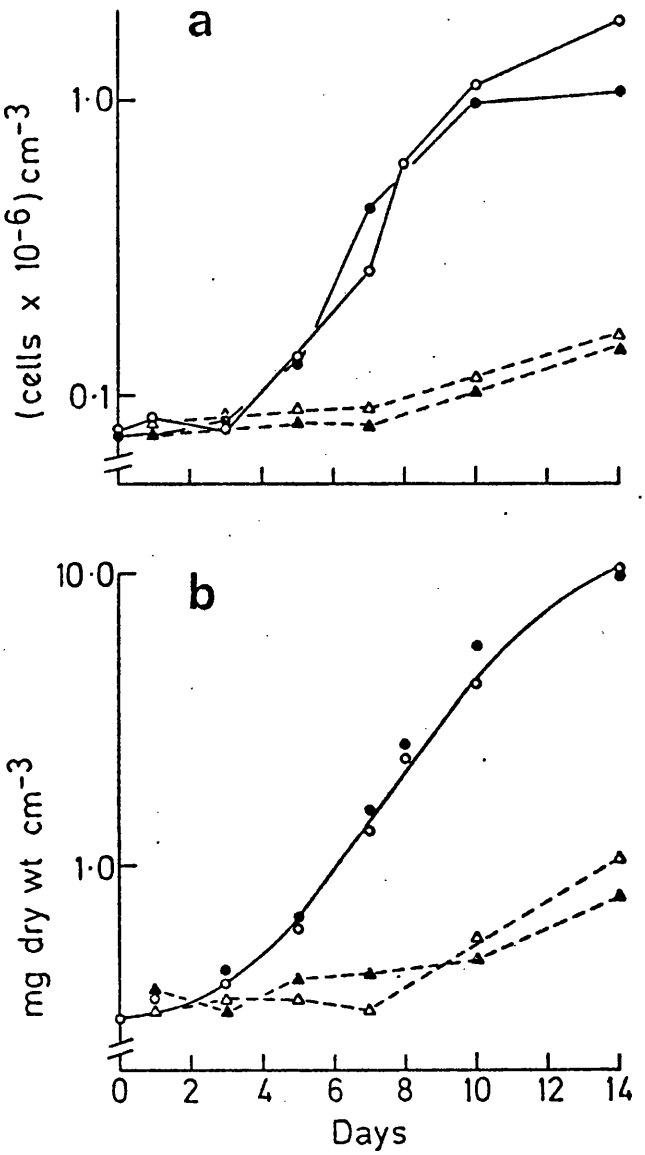


FIG.1.8. The effects of 2,4-D and light on cell number (a) and dry weight (b) of Paul's Scarlet rose cultures growing in the sycamore medium. Cells were washed free of extracellular 2,4-D, inoculated into plus-2,4-D (O, ●) or minus-2,4-D (△, ▲) media and incubated in the presence (open symbols) or absence (closed symbols) of light. The dark-treated cells had been incubated in the absence of light for four passages before washing.

however, it was necessary to test the effect of long dark pre-treatments before 2,4-D depletion. In order to eliminate the possibility that some component(s) of the MX_2 medium were preventing dark-enhanced growth the Paul's scarlet rose cells were also transferred to the standard sycamore medium and subcultured at regular 14 day intervals for 8 weeks. Throughout this 8 week period one set of cultures was maintained in the light and the other set in the dark. Cells from both sets of cultures were then washed free of extracellular 2,4-D and the light-grown cells inoculated into minus-2,4-D medium in the light while the dark-grown cultures were used to inoculate minus-2,4-D-dark, plus-2,4-D-dark and plus-2,4-D-light treatments.

The data shown in Fig.1.8 show that Paul's scarlet rose cells grow much slower in the sycamore medium than the MX_2 medium (Fig.1.6) and that even after 8 weeks dark pre-treatment there appeared to be no substantial difference between the growth of 2,4-D-depleted rose cells in the presence or absence of white light.

DISCUSSION

Although it has been shown that the absence of light aids the growth of sycamore cells during 2,4-D depletion, the precise mechanism of this effect is, at present, unknown. The results presented in this section show that it is not due to a reduction in the total soluble phenolics content of the cells, which may suggest that either PAL activity was not significantly reduced in the absence of light or that PAL activity and phenol polymerisation are closely linked. The increased levels of soluble phenolics in the dark-grown minus-2,4-D cultures suggest that further metabolism of the soluble phenolics, into alcohol insoluble compounds, may be light stimulated. When cells were dark-pre-treated before white light incubation in the absence of 2,4-D (Figs.1.4 and 1.5) the dark-induced effects on growth, but not phenolics accumulation, were expressed. Therefore it may be concluded that the total soluble phenolics of sycamore cells are not directly responsible

for either growth inhibition in the absence of 2,4-D or growth stimulation in dark-grown cultures.

The observation that a dark treatment prior to 2,4-D depletion is more effective in promoting growth than a dark treatment at the time of 2,4-D depletion suggests that either dark treatments are only fully effective in the presence of 2,4-D, or that a relatively long dark period is required to negate the effect of prolonged exposure to white light. Enhanced cell division in the absence of white light has also been observed in explants of Jerusalem artichoke (Helianthus tuberosus) tubers (Yeoman and Davidson, 1971). Data from this system suggest that 2,4-D must be present for light effects to be expressed (Yeoman and Davidson, 1971) that phytochrome-mediated responses contribute to the inhibitory effects of light (Davidson and Yeoman, 1974) and that prolonged darkness does not cause an appreciable decline in extractable PAL activity (James and Davidson, 1977). The results obtained for sycamore appear to be compatible with those obtained from Jerusalem artichoke although in the artichoke system these represented effects of light on the induction rather than maintenance of cell division.

There appears to be little direct evidence to indicate whether the growth-inhibitory effect of light on 2,4-D depleted sycamore cells includes either the light-dependent synthesis of a growth inhibitor e.g. xanthoxin (Thompson and Bruinsma, 1977) or the photo-oxidation of endogenous IAA. However, recent studies (Turnham 1978) have shown that the addition of the antioxidant DTT (dithiothreitol) to the culture medium of sycamore cultures can prolong the growth of 2,4-D depleted cells. In common with dark-enhanced growth a pre-treatment with DTT was much more effective than treatments after 2,4-D depletion. As DTT possesses 'auxin protector' properties (Stonier, 1971; Turnham, 1978) the similarities between DTT- and dark-enhanced growth may indicate that growth in the absence of 2,4-D depends, at least partly, on the maintenance of endogenous IAA. Davidson and Yeoman (1974) suggested that the presence of light caused the depletion of an essential metabolite below a critical level required for cell-division.

Furthermore they showed that exogenous phenylalanine could negate the inhibitory effects of light although it is not known whether the applied phenylalanine supplemented endogenous phenylalanine pools for protein synthesis (Phillips and Henshaw, 1977) or modified the synthesis of growth promoting ('auxin protectors'?) or growth inhibitory phenolic compounds. Although quantitative changes in total soluble phenolics are not related to growth inhibition in 2,4-D-depleted sycamore cells this does not preclude effects of qualitative changes in soluble phenolics.

The experiments with Paul's scarlet rose cultures show that dark-enhancement of the growth of 2,4-D- depleted cells is not a universal observation. Whatever the physiological role of intracellular phenolics the concentration of phenolics accumulated in the culture medium of 2,4-D depleted rose cells is sufficient to, at least partially, inhibit growth. Although incubation in the absence of white light does not reduce phenolic accumulation in rose cells another, more successful, approach was examined by a visiting research fellow, Dr.T.H.Lam. He showed (Lam and Street, 1977) that analogues of 2,4-D which lacked auxin activity (e.g. 2-chlorophenoxy isobutyric acid), as determined by standard bioassays, suppressed phenolic accumulation in Rosa damascena suspension cultures and permitted growth albeit at a low rate. It is unlikely that this growth promotion was due to low auxin activity as the same compounds behaved as anti-auxins with respect to carrot embryogenesis (Chandra, Lam and Street, 1978). Thus it appears that phenolic compounds can be inhibitory to growth and that the activity of chlorinated phenoxy acids (e.g. 2,4-D) as growth regulators may include effects on cellular metabolism not related to their activity as auxins.

SECTION 2

CONCENTRATION - DEPENDENT RESPONSES TO 2,4-D

INTRODUCTION

The relationships between the rate of cell expansion and the concentration of auxin supplied to plant tissues are well known and have been widely used as bio-assays for auxins (see Thimann, 1969). Much less information is available concerning the dose-response relationships of parameters related to cell division and biomass (dry weight) accumulation.

Leguay and Guern (1975), working with sycamore suspension cultures, showed that in the range of 2,4-D concentrations from 4×10^{-8}M to 4×10^{-6}M, the rate of cell division during the logarithmic phase was independent of the auxin (2,4-D) concentration while the maximum number of cell generations obtained was limited by the initial 2,4-D concentration. Similarly King (1976) showed that stepwise reduction of 2,4-D (from 4.5×10^{-6}M to 0.5×10^{-6}M) supplied to turbidostat cultures of sycamore had little effect on the rate of cell division while producing profound effects on cellular aggregation and phenolics metabolism. These observations suggest that 2,4-D regulates an on/off 'switch' such that cell division is initiated only when the concentration of 2,4-D at the site(s) of perception, and/or the intensity of a primary process initiated by 2,4-D, reaches a threshold value. The expression of some 'low-2,4-D' characteristics before the threshold for cell division promotion is reached (King 1976) suggests that more than one site or mechanism exists for the perception of 2,4-D. Clearly any metabolic responses which mediate auxin regulation of cell division should exhibit dose-response relationships similar to those observed for cell division.

It is commonly observed that the auxin requirement of cultures recently isolated from whole plant tissues is initially high but progressively declines to a more stable value which may, in some cases, even represent complete independence (see SECTION 3 and Meins, 1977). This behaviour provokes the question "Do recently isolated cells require a high

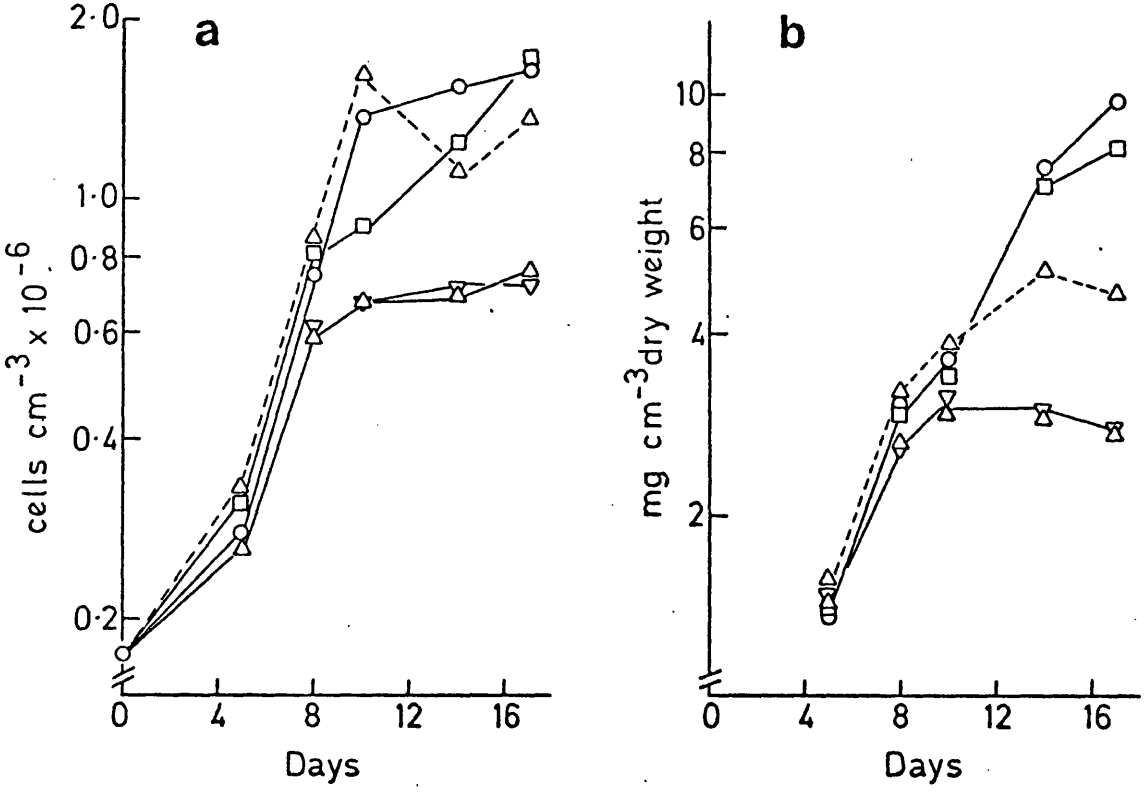
threshold concentration of auxin to stimulate cell division or does the high auxin-requirement of the culture result from a high rate of auxin inactivation?"

The aims of the experiments described in this section were (i) to compare the 2,4-D dose-response relationship of amino-nitrogen accumulation and growth in AM strain cultures and (ii) to compare the 2,4-D requirements of the AM strain with sycamore cells recently isolated from whole plant tissue.

2.1 The effects of initial 2,4-D and kinetin concentration on growth and the accumulation of amino-nitrogen in AM strain sycamore cells.

The results presented in SECTION 1 identified the accumulation of amino-nitrogen as a response to the interuption of 2,4-D supply to AM strain sycamore cells. As indicated in the introduction to this section, responses related to the regulation of cell division by auxin should exhibit similar dose-response relationships. To examine concentration-dependent responses to 2,4-D, AM strain sycamore cells (14 days from subculture) were washed free of extracellular 2,4-D and then inoculated into media containing either a range of 2,4-D concentrations and the usual concentration of kinetin ($0.25mg\ dm^{-3}$) or no 2,4-D and 'high kinetin' ($2.5mg\ dm^{-3}$). All treatments were incubated in white light (300 lux) at $25^{o}C$. The growth data (Fig.2.1) show that $0.05mg\ dm^{3}$ ($0.23 \times 10^{-6}M$) 2,4-D was below the threshold initial 2,4-D concentration required for growth promotion such that the patterns of accumulation of both cell number and dry weight were indistinguishable from the minus-2,4-D control. Cultures incubated in medium initially containing $0.20mg\ dm^{-3}$ 2,4-D achieved a final yield of cells and biomass similar to the control ($1.0mg\ dm^{-3}$ 2,4-D) although the patterns of growth differed. The results also show that the initial concentration of kinetin can have profound effects on 2,4-D-dependence for although $0.05mg\ dm^{-3}$ 2,4-D was

FIG.2.1 Influence of the initial 2,4-D and kinetin concentrations on the growth of sycamore cultures. Cells were washed in medium lacking both growth regulators and inoculated into media containing either 0.25mg dm^{-3} kinetin and a range of 2,4-D concentrations: 1.0mg dm^{-3} (O), 0.20mg dm^{-3} (□), 0.05mg dm^{-3} (▽), no 2,4-D (—△—) or 2.5mg dm^{-3} kinetin ('high kinetin') and no 2,4-D (-△-). Growth was determined by increase in cell number (a) or dry weight (b).


a
2·0
1·0
0·8
0·6
0·4
0·2
cells cm^{-3} x 10^{-6}
0
4
8
12
16
Days
b
10
8
6
4
2
mg cm^{-3} dry weight
0
4
8
12
16
Days

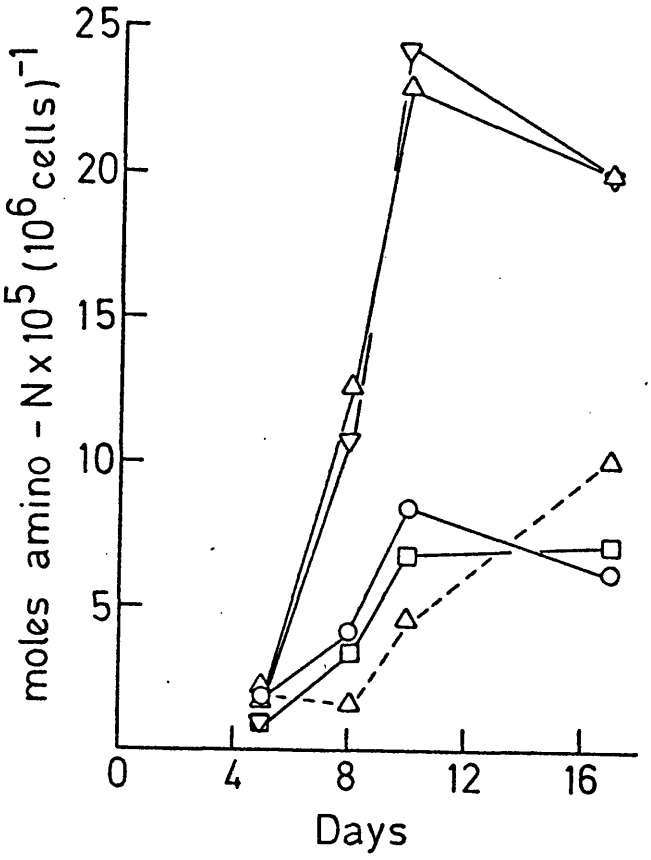


FIG.2.2 Influence of the initial 2,4-D and kinetin concentrations on the cellular alcohol-soluble amino-nitrogen content of sycamore cells. Symbols as for Fig.2.1.

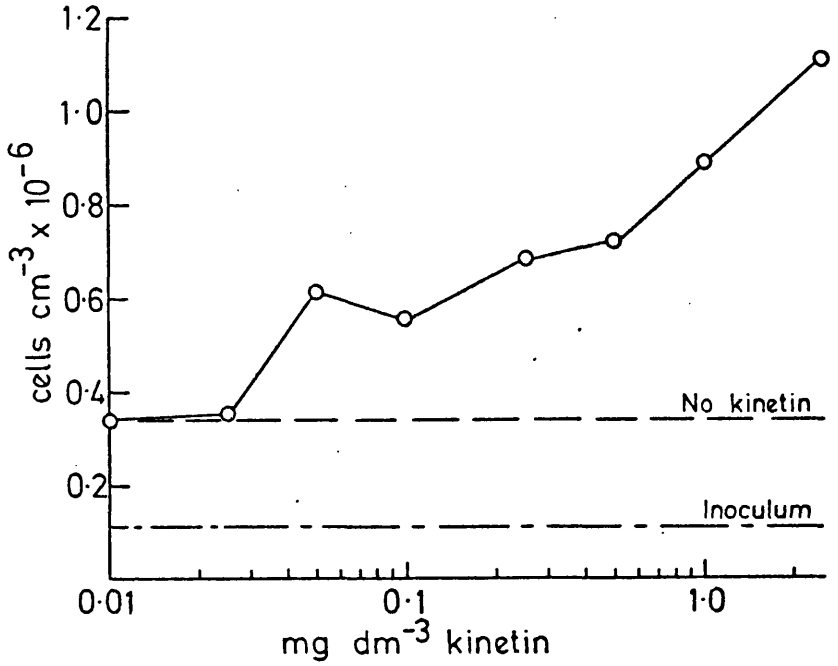


FIG.2.3 The effect of kinetin on the growth of sycamore cells in medium lacking 2,4-D. Stock cells were washed free of extracellular 2,4-D and kinetin and inoculated into media containing a range of initial kinetin concentrations but lacking 2,4-D. The cultures were harvested after 8 days incubation.

below the threshold initial 2,4-D concentration for growth promotion in the presence of 0.25mg dm^{-3} kinetin, 'high kinetin' (2.50mg dm^{-3}) could promote growth in the absence of 2,4-D.

The patterns of accumulation of soluble amino-nitrogen (Fig.2.2) appeared to correlate well with the growth data. Thus 0.05mg dm^{-3} 2,4-D, which did not promote growth, did not suppress the amino-nitrogen accumulation observed in minus-2,4-D cells; 0.20mg dm^{-3} 2,4-D and 'high kinetin' which did promote growth did suppress amino-nitrogen accumulation. The contributions of individual amino acids to the accumulation of total soluble amino-nitrogen are discussed in SECTION 4.

The effects of kinetin on the growth of cells in the absence of 2,4-D was investigated in more detail to see whether the initial period of growth, after inoculation of cells into minus-2,4-D medium was due to either residual growth potential (e.g. intracellular 2,4-D) or the low level of kinetin (0.25mg dm^{-3}) present in the standard minus-2,4-D medium. Stock AM strain cells were washed in medium lacking both 2,4-D and kinetin and inoculated into media containing a range of initial kinetin concentrations but lacking 2,4-D. The cell number data, after 8 days incubation (Fig.2.3) show that although some growth is observed in the medium lacking both 2,4-D and kinetin, a substantial portion of the growth observed in the standard minus-2,4-D medium (0.25mg dm^{-3} kinetin) can be attributed to the presence of kinetin.

2.2 The 2,4-D requirement of a new culture strain of sycamore.

In order to investigate the 2,4-D requirement of sycamore cells recently isolated from whole plant material, callus was obtained from the petioles of a one year old sycamore seedling. The seed-stock had been obtained from Victoria Park, Leicester.

The culture medium used for the maintenance of AM strain cultures proved to be unsatisfactory for callus induction and a medium based on that

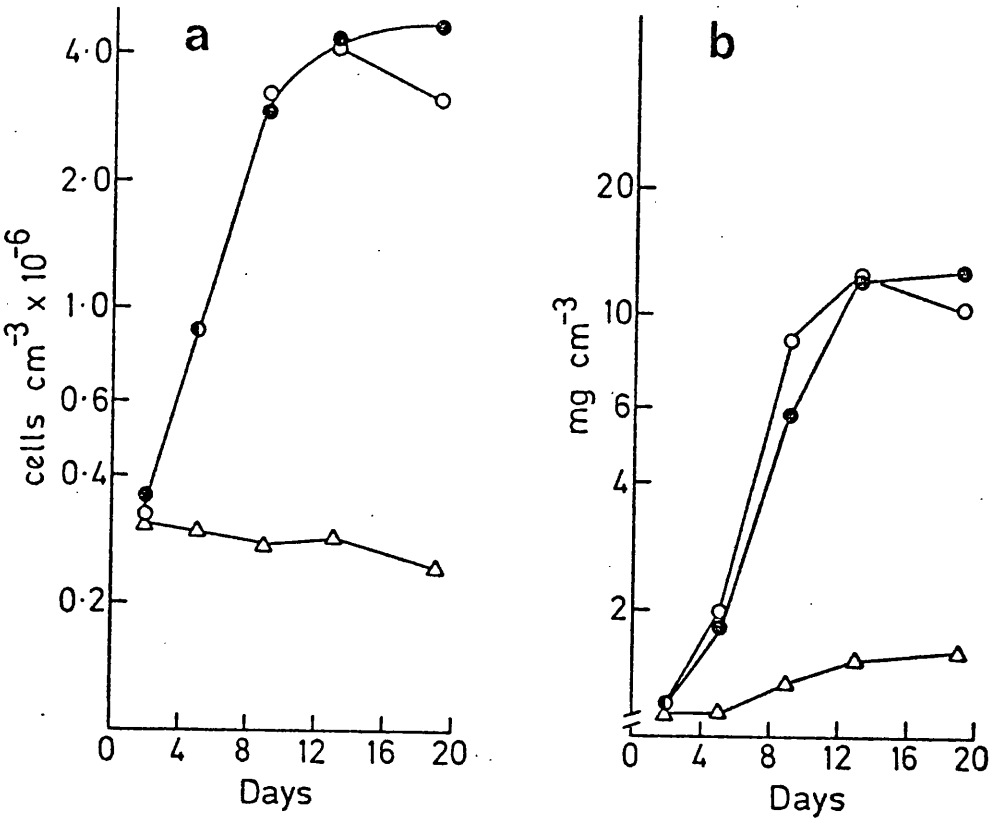


FIG.2.4 The growth, measured by cell number (a) or dry weight (b), of D_5 strain cultures in media containing 5mg dm^{-3} 2,4-D (●), 1mg dm^{-3} 2,4-D (O) or no 2,4-D (△).

of Murashige and Skoog (see MATERIALS and METHODS) was employed. This medium, supplemented with 2,4-D (5mg dm^{-3}, 22.5 x 10^{-6}M), kinetin (0.25mg dm^{-3}, 1.16 x 10^{-6}M), sodium glutamate (1g dm^{-3}, 5.35 x 10^{-3}M) and agar (0.7% w/v) induced and maintained vigorous callus growth. The primary callus was transferred to liquid medium to give a suspension culture, cloned by low density plating of small aggregates (see SECTION 3), and returned to liquid medium. This culture strain was denoted D_5.

At the same time as the initiation of the D_5 strain, an attempt was made to investigate the effect of using auxins other than 2,4-D to induce primary callus, on the subsequent auxin-requirement of the cultures. The alternative auxins tested were naphthalene acetic acid (NAA) and indole acetic acid (IAA). Although a range of initial concentrations of both compounds was tested, using the same basal medium as for the D_5 strain, all callus obtained was slow growing, compact and green. Because the lack of friability made such callus unsuitable for growth as suspension cultures and precluded cloning, this line of study was not pursued further.

A preliminary investigation of the 2,4-D requirement of D_5 cultures was carried out by inoculating stock cultures directly (without washing) into media containing 5mg dm^{-3}, 1mg dm^{-3} or no 2,4-D. The growth data (Fig.2.4) show that, in contrast to the AM strain (see SECTION 1), no increase in cell number was observed when D_5 cells were inoculated into medium lacking 2,4-D. Although 1mg dm^{-3} 2,4-D produced a final yield of cells and dry weight similar to that of the control (5mg dm^{-3} 2,4-D) the 1mg dm^{-3} 2,4-D cultures displayed increased aggregation and cell lysis at the end of the growth cycle.

The lack of cell division growth in the minus-2,4-D cultures may have been due to either a low carryover of physiologically active 2,4-D in the inoculum or a high threshold 2,4-D requirement for the stimulation of cell division. To distinguish between these two possibilities, stock D_5 cultures (20 days from subculturing in medium containing 5mg dm^{-3} 2,4-D) were washed

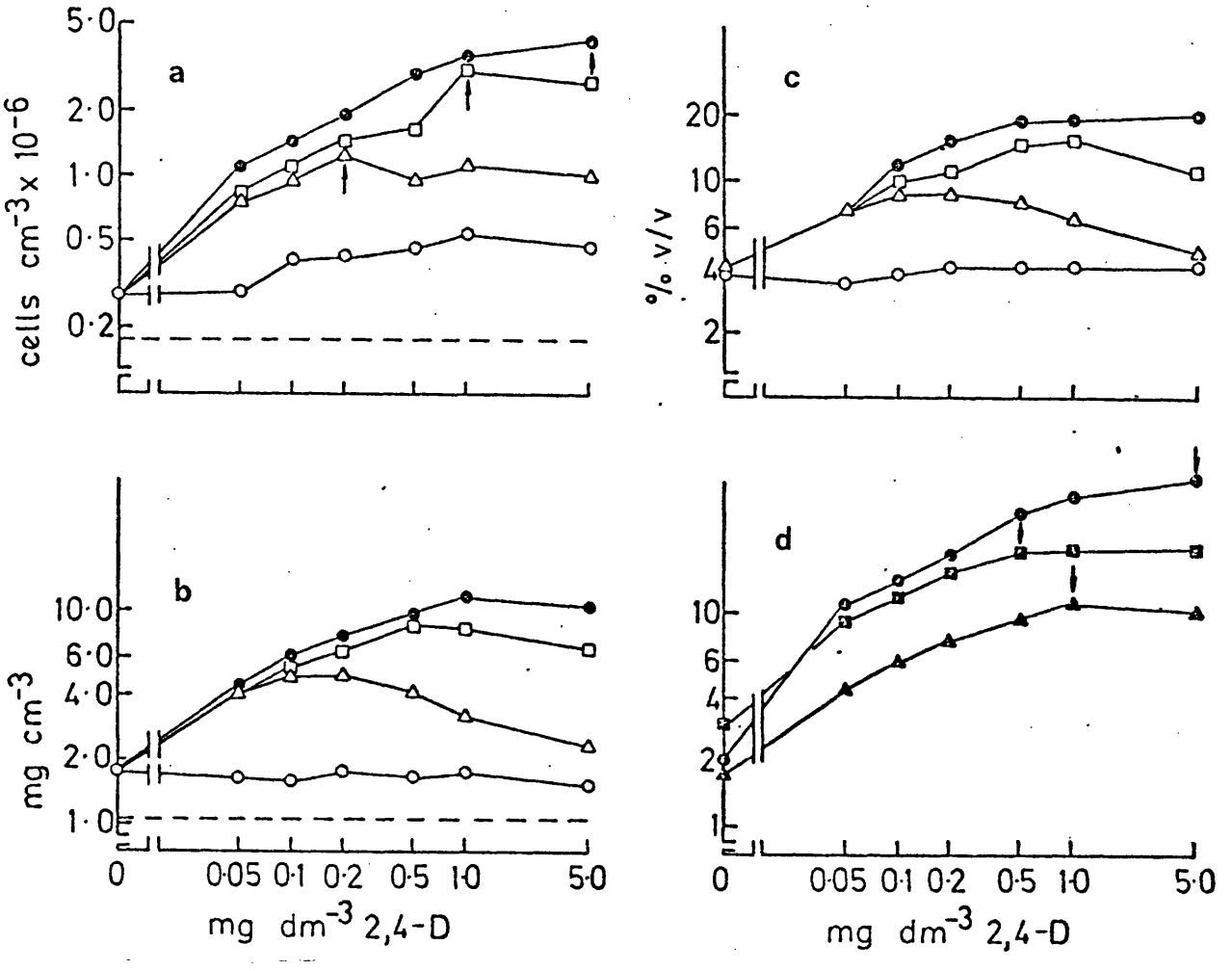


FIG.2.5 The relationship between the initial 2,4-D concentration and the growth of D_5 strain cultures as measured by cell number (a), dry weight (b) and packed cell volume (c). Stock cells were washed free of extracellular 2,4-D and inoculated into media containing a range of initial 2,4-D concentrations. Cultures were harvested after 6 (○), 10 (△), 14 (□) and 20 (◉) days incubation. The dotted lines in (a) and (b) indicate the inoculum density, and (d) shows the values for cell number (◉), dry weight (▲) and packed cell volume (■) on day 20.

in medium lacking 2,4-D and then cultured in media containing a range of initial 2,4-D concentrations below 5mg dm^{-3}. Culture growth was estimated as increase in cell number (Fig.2.5(a)), dry weight (Fig.2.5(b)) and packed cell volume (Fig.2.5(c)) when cultures were harvested after 6, 10, 14 and 20 days incubation. The data are presented in the form of dose-response curves where the $\log_{10}$ of each growth parameter is plotted against the $\log_{10}$ of the initial 2,4-D concentration. The results clearly show that the lowest concentration of 2,4-D tested (0.05mg dm^{-3}) produced an appreciable amount of growth although the medium lacking 2,4-D produced no growth. Thus the lack of growth in the previous experiment, where cells were inoculated into minus-2,4-D without washing, must have been due to a low carryover of active 2,4-D in the inoculum rather than a high threshold requirement of the cells. Although the dose-response curves for the different growth parameters are not indentical they do show, in general, that the higher 2,4-D concentrations were supra-optimal during the early stages of growth but that, as the culture period progressed, the position of optimum growth shifted to higher initial 2,4-D concentrations (as arrowed in Fig.2.5(a)). The different methods for assessing growth are compared, for day 20 samples, in Fig.2.5(d). This shows that although the dose-response relationships are similar for initial 2,4-D concentrations in the range 0.05mg dm^{-3} to 0.50mg dm^{-3}, the behaviour above this region is dependent on the growth parameter under consideration. Thus the initial 2,4-D concentrations above which the final growth yield of packed cell volume, dry weight and cell number does not increase are 0.5, 1.0 and 5.0mg dm^{-3} respectively (indicated by arrows in Fig.2.5(d)).

DISCUSSION

The results of this section show that cultures which, when grown in batch culture, require high initial 2,4-D concentrations to ensure that

growth is not 2,4-D-limited, do not necessarily contain cells that have a high 2,4-D requirement. The facts that (1) D_5 cells respond to low 2,4-D concentrations (2) 20-day D_5 cultures do not grow if inoculated, without washing, into medium lacking 2,4-D and (3) the initial 2,4-D concentration of the medium producing optimum growth changes throughout the growth cycle, all indicate that the high 2,4-D requirement of D_5 cultures is due to rapid inactivation of 2,4-D rather than a high threshold requirement for growth promotion. Thus the relationship between final growth yield and 2,4-D concentration is a function of the duration of the period in which the actual (not initial) 2,4-D concentration is between two limits: above the threshold for growth promotion but below concentrations that are growth-inhibitory. Thus when the 2,4-D requirement of established cultures shifts to lower values this probably represents a shift in the rate of 2,4-D inactivation. These conclusions are consistent with the studies of Leguay and Guern (1977) who considered that the utilization of 2,4-D molecules by Acer cells is governed mainly by a glucosylation process which produces biologically inactive derivatives. They showed (1977) that 2,3,5-tri iodobenzoic acid (TIBA), by inhibiting 2,4-D glucosylation, has a sparing effect on 2,4-D molecules and that TIBA treatments increased growth yields. Leguay and Guern (1977) have also shown that 2,4-D molecules were still present outside and inside the cells when cell division stopped due to a specific deprivation of auxin molecules. Therefore although the concentration of free 2,4-D molecules must have been low in a 20-day inoculum of D_5 cells, it need not have been zero.

The observation of differential optimum initial 2,4-D concentrations for growth measured by cell number, dry weight and packed cell volume suggest that cells responding to a declining supply of 2,4-D would show a sequence of responses such that cell division would arrest before dry weight accumulation which would precede the arrest of cell expansion. This behaviour is generally observed as King (1976) showed that, when the 2,4-D

supply to a chemostat culture of AM strain cells was terminated, cell number declined before dry weight, packed cell volume or total protein and Nishi et al (1977) have reported that 2,4-D depletion of carrot cells resulted in an increase in mean cell size.

The effects of cytokinin on auxin-dependent growth are likely to be complex. Lau and Yang (1973) have shown that cytokinins may alter the metabolism of auxins so that perhaps the effect of cytokinins could be compared with that of TIBA in sparing auxin molecules from inactivation (Leguay and Guern, 1977). If this is the case then the continued growth of sycamore cells in the absence of exogenous auxin, but in the presence of 'high kinetin', could be due to the maintenance of pools of endogenous auxin(s). Whether or not this hypothesis is correct the accumulation of soluble amino-nitrogen by AM strain sycamore cells appeared to be closely related to the regulation of growth and thus justified further investigation (see SECTION 4).

SECTION 3

2,4-D-INDEPENDENT GROWTH

INTRODUCTION

Mechanisms by which cells cease to respond to a particular hormone are of obvious importance in studies of the hormonal regulation of growth. Plant tissues usually require an exogenous supply of auxin for continued growth in culture (Gautheret, 1955) and are therefore referred to as auxin-dependent. Cultures which continue to grow in the absence of exogenous auxin may arise spontaneously or by selection and can be referred to as auxin-independent. Such auxin- independence may arise by one or more of the following mechanisms:-

(i) increase in endogenous auxin to a level that does not require supplementation with exogenous auxin,

(ii) increase in the level of a substance which can act as an alternative to auxin (eg. another hormone),

(iii) alterations in the sensitivity and/or location of receptors,

(iv) the development of alternative metabolic pathways which by-pass auxin-sensitive processes.

A study of auxin-independent cells is therefore likely to provide information on the nature of auxin action. Furthermore, reversion from the auxin-independent to the auxin-dependent state may allow investigation of epigenetic control during the determination phase of cell differentiation (Meins and Binns, 1977(a), 1977(b)). These considerations then invoke the concept of permissive, as opposed to inductive, regulation of differentiation. Metabolic variations associated with auxin-independence may be conveniently detected as changes in sensitivity to compounds which inhibit specific pathways. Such metabolic variations may not be directly linked to primary auxin action(s) but should provide a basic framework of information concerned with the regulation of growth and metabolism by auxin.

3.1 The isolation of 2,4-D-independent cells.

Lescure (1967) has reported the appearance of auxin-independent sycamore cells following mutagen treatment, at a frequency of ca.10^{-6}. If auxin-independent cells are present, at a low level, in the auxin-dependent population it follows that their frequency of isolation, in the absence of exogenous auxin (i.e. 2,4-D), is likely to be sensitive to the loss of viability of the auxin-dependent cells. For this reason 2,4-D-independent cells were isolated from the Am strain by plating in semi-solid media to allow spatial separation of cellular aggregates. Various additions were made to the basal medium (Table 3.1) in an attempt to maximize the frequency of isolation.

TABLE 3.1 The composition of media used for the isolation of 2,4-D-independent sycamore cells.

MEDIUM * CODE	KINETIN mg dm^{-3}	CYSTEINE-HCl mg dm^{-3}	Na.GLUTAMATE g dm^{-3}	POLYVINYL-PYRROLIDONE % w/v
A	0.25	10	-	-
B	0.25	10	5	-
C	0.25	100	-	-
D	2.50	10	-	-
E	0.25	10	-	1.0
F	2.50	10	-	1.0

* Basal medium as in MATERIALS and METHODS TABLE 1.

Glutamate-(B) and cysteine-(C) enriched media were designed to assist low density growth (Street, 1977). 'High kinetin' (D,F) has been shown (Simpkins, Collin and Street, 1970) to partially relieve the auxin-requirement of sycamore cells while stimulating the production of phenolic compounds. Polyvinyl-pyrrolidone (M.W.700,000, BDH Cehmicals Ltd.) was therefore added to some media (E,F) to adsorb toxic phenolics released from lysing 2,4-D-independent cells.

Stock Am cells (day 14) were washed free of extracellular 2,4-D and plated at a density of ca. 1 x 10^6 cells per plate. Of the ten plates of each treatment inoculated five were incubated in diffuse white light (300 lux) and five in darkness. Light-grown plates were observed after 1, 3, 9 and 14 weeks incubation and growth was recorded on shadowgraphs. One dark-grown plate from each treatment was shadowgraphed after 3 and 9 weeks incubation, marked to record the exposure to light, and returned to the dark. An initial period of growth was observed after 2,4-D depletion as also seen is suspension culture (SECTION 1). There appeared to be little difference between treatments, after 3 weeks incubation, except for a slight inhibition of growth (colony size) on the media enriched with glutamate (B) or cysteine (C). By week 9 the growth of all colonies appeared to have ceased thus providing little evidence for the existence of 2,4-D-independent cells. All plates (light and dark) were examined after 14 weeks incubation. Only in the plates containing medium 'B' were there any differences from the week 9 shadowgraphs. On this glutamate-supplemented medium 4 colonies were continuing to grow on light-incubated plates and 25 colonies on dark-incubated plates. All 29 colonies continued to grow when subcultured on fresh semi-solid 'B' medium and were therefore classified as 2,4-D independent.

3.2 The stability of 2,4-D-independence.

A number of studies have been made of the stability of hormone-independence in tissue culture (Syono and Furuya, 1974(a) 1974(b); Sacristan and Wendt-Galletilli, 1971; Meins and Binns, 1977(a), 1977(b)). Although some of the authors discussed the problem of back-selection, their experimental material almost invariably comprised primary explants and uncloned callus cultures.

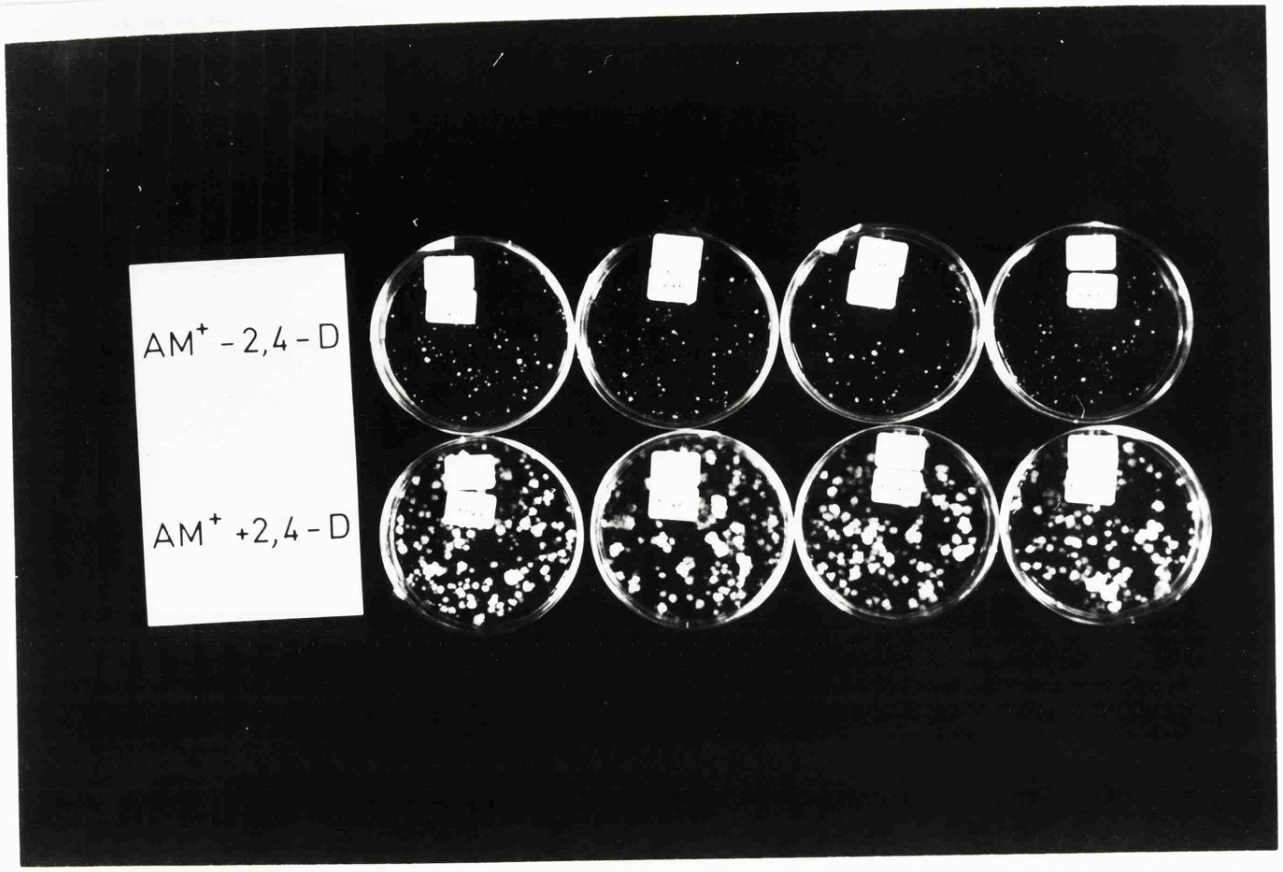
It has been generally assumed, in the past, that hormone-independence is the result of tissues gaining the capacity to synthesize sufficient of a

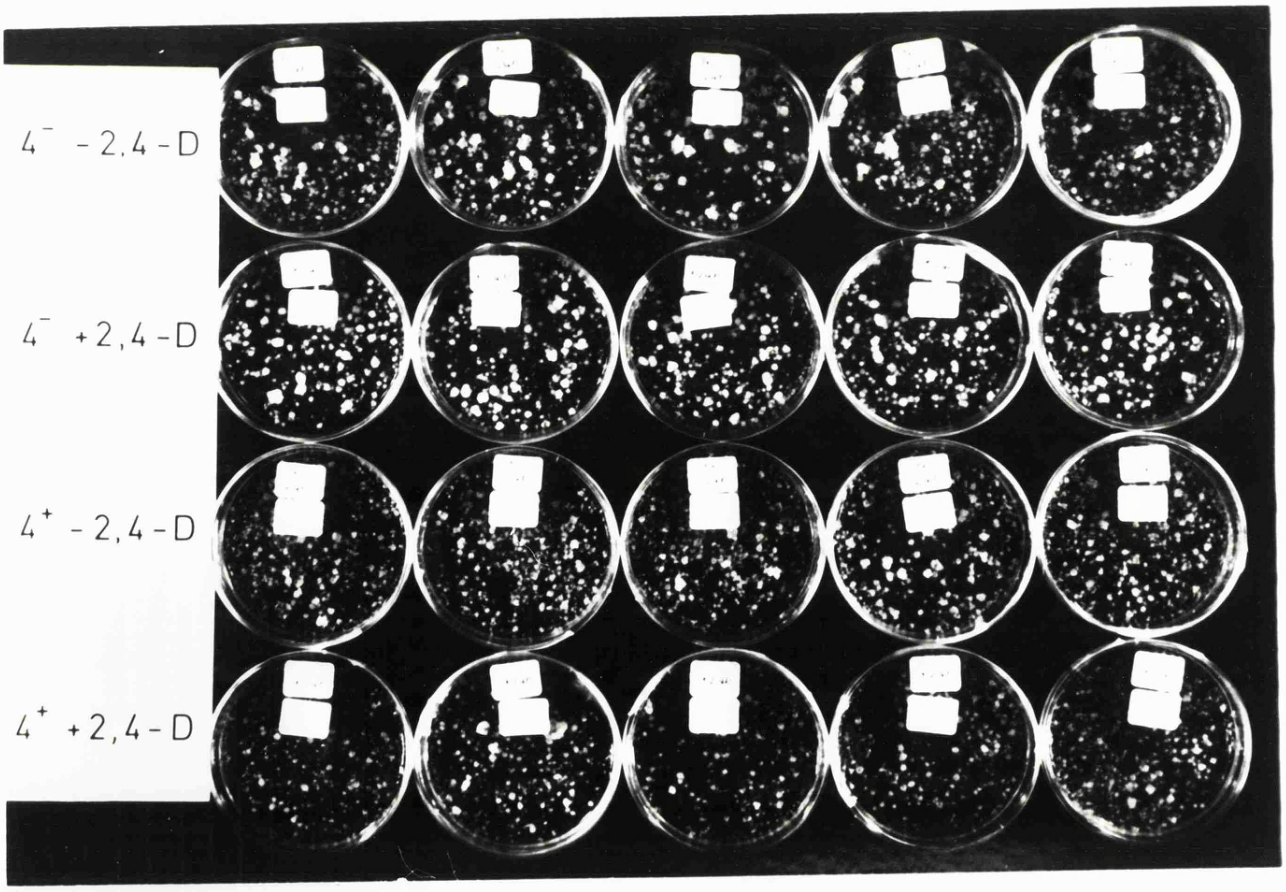
particular factor for the maintenance of growth (Everett, Wang and Street, 1978). It follows that cells which are themselves auxin-dependent could possibly be maintained in active growth, in the absence of applied auxin, by receiving auxin from adjacent auxin-independent cells in a mixed culture. Apparent reversions of such a mixed culture to auxin-dependence, following exposure to auxin, could therefore be due to back-selection. To avoid such problems it was considered essential to use only cloned material from the 2,4-D-independent strains of sycamore. Since cloning requires low density plating of small aggregates of cells (a practical approximation to single cells) only strains producing suspension cultures of small cellular aggregates (< 1mm diameter), when incubated in the light, were maintained for cloning (Table 3.2).

TABLE 3.2 Culture history of 2,4-D-independent strains.

CULTURE CODE	MAINTAINED AS CALLUS CULTURE	MAINTAINED IN SUSPENSION CULTURE IN DARK	IN LIGHT	CLONED
IL_2	+			
IL_3	+	+		
ID_1	+	+		
ID_2	+	+	+	
ID_4	+	+	+	+
ID_5	+	+	+	+
ID_7	+	+		
ID_8	+			
ID_9	+	+	+	
ID_{10}	+	+	+	+
ID_{13}	+			
ID_{14}	+			
ID_{19}	+	+		
ID_{20}	+			
ID_{21}	+	+	+	+
ID_{24}	+	+	+	+

Plate 3.1 The effect of 2,4-D on the plating efficiencies of Am(top) and ID4(bottom) cells. Stock cells which had been grown in the presence($Am^+,4^+$) or absence(4^-) of 2,4-D were washed and plated in plus- or minus-2,4-D medium.


AM+ - 2,4 - D
AM+ +2,4 - D


4− - 2,4 - D
4− +2,4 - D
4+ - 2,4 - D
4+ +2,4 - D

Cloning was achieved by low density plating of cell suspensions from which large aggregates (> 20 cells) had been removed by sedimentation. The resulting clonal colonies were returned to suspension culture and three strains: ID_4, ID_{10} and ID_{21} were used to examine the stability of 2,4-D-independence in sycamore cells. The 2,4-D-independence of all strains was stable in the absence of 2,4-D, as shown by the lack of gross changes in growth rate during the period from isolation to cloning (6 months).

Due to the large number of growth measurements that would be required to identify partial reversion to 2,4-D-dependence in suspension culture, an experiment was devised to make use of the plating technique previously used for selection and cloning. The three independent strains (ID_4, ID_{10}, ID_{21}) were incubated in both the absence and presence (1 mg dm^{-3}) of 2,4-D for 14 days. Aliquots ($10cm^3$) of each treatment were then washed (2 x $50cm^3$) and resuspended ($100cm^3$) in medium lacking 2,4-D before being plated ($1cm^3$ in $10cm^3$) in minus- and plus- 2,4-D (1 mg dm^{-3}) media. The number of aggregates on each plate was determined microscopically, immediately after plating, to permit the subsequent estimation of the plating efficiencies. For this purpose coincidental aggregates (within ca. 0.5mm of each other) were counted as a single colony-generating unit. The plates were re-examined (five replicates per treatment) after 35 days incubation (light, 25°C) and growth was expressed as the mean number of colonies per plate achieving a diameter greater than 1mm (Table 3.3). The variation within treatments is expressed numerically as the standard error of the mean and photographically in Plate 3.1 which also shows the extremes of response observed. As the absolute plating efficiency varies between different culture strains, 2,4-D-induced changes in the sensitivity to 2,4-D are most easily observed by deriving a '2,4-D sensitivity factor', D, such that:

$$D = \frac{\text{plating efficiency in minus - 2,4-D medium}}{\text{plating efficiency in plus - 2,4-D medium}}$$

then,

D^+ = the sensitivity to 2,4-D after an exposure to 2,4-D

and

D^- = the sensitivity to 2,4-D without a previous exposure to 2,4-D

Therefore it follows that D = 1 for cells that are insensitive to 2,4-D and D = 0 for cells that show no growth in the absence of 2,4-D.

TABLE 3.3 Effects of 2,4-D on 2,4-D-independent strains.

EXPERIMENTAL CODE	2,4-D EXPOSURE Before plating	2,4-D EXPOSURE On plates	PLATING DENSITY (aggregates per plate)	GROWTH YIELD (colonies>1mm per plate ± S.E.M.)	PLATING EFFICIENCY = $\frac{\text{growth yield}}{\text{plating density}} \times 100\%$
4^- - 2,4-D	-	-	1060	361.4 ± 34.0	34.1
4^- + 2,4-D	-	+	1060	374.5 ± 16.6	35.3
4^+ - 2,4-D	+	-	1080	411.2 ± 31.6	38.1
4^+ + 2,4-D	+	+	1080	387.4 ± 34.2	35.9
10^- - 2,4-D	-	-	390	37.8 ± 3.4	9.7
10^- + 2,4-D	-	+	390	40.2 ± 3.9	10.3
10^+ - 2,4-D	+	-	575	176.2 ± 5.3	30.6
10^+ + 2,4-D	+	+	575	261.8 ± 5.3	45.5
21^- - 2,4-D	-	-	330	25.2 ± 2.4	7.6
21^- + 2,4-D	-	+	330	25.4 ± 1.8	7.7
21^+ - 2,4-D	+	-	765	92.0 ± 4.2	12.0
21^+ + 2,4-D	+	+	765	157.8 ± 7.9	20.6
Am^+ - 2,4-D	+	-	575	96.6 ± 6.1	16.8
Am^+ + 2,4-D	+	+	575	162.2 ± 12.1	28.2

The results from Table 3.3 are expressed in terms of D in Table 3.4. The values of D^- clearly show that the three 2,4-D-independent strains are insensitive to the presence of 2,4-D on plates. The values of D^+ show that, while strain ID_4 retains its insensitivity, strains ID_{10} and ID_{21} develop a sensitivity to 2,4-D after a brief exposure to 2,4-D.

TABLE 3.4 2,4-D-induced sensitivity to 2,4-D.

CULTURE STRAIN	D^-	D^+
ID_4	0.97	1.06
ID_{10}	0.94	0.67
ID_{21}	0.99	0.58
Am	--	0.60

It is important to note that, although 2,4-D-dependent, the Am strain gives a value for D^+ of 0.60 due to the delay in growth cessation following 2,4-D withdrawal (see Section 1). A comparison of D values is therefore only of limited value for studying reversion to 2,4-D dependence as a low D value may result from either reduced growth in the absence of 2,4-D (2,4-D-dependence) or more efficient growth in the presence of 2,4-D (higher plating efficiency). This problem of data interpretation arises from the low overall plating efficiencies observed in Table 3.3, which are probably a consequence of washing the inocula. Such washing is bound to remove conditioning factors which are released into the culture medium and are essential for efficient growth at low density or on plates (Street, 1977). The results of this experiment should perhaps be considered as a measure of the ability of the cells to grow in a minimal medium.

3.3 The role of cytokinin in 2,4-D-independent growth.

High initial concentrations of kinetin can, at least partially, relieve the 2,4-D-dependence of Am strain cells (SECTION 2). It follows that 2,4-D-independence could be a result of increased sensitivity to cytokinin especially when one considers the apparent reciprocity of auxin- and cytokinin- dependence in tobacco cultures (Einset, 1977). Syōno and Furuya (1972) considered that kinetin was required to maintain the endogenous auxin in callus cultures so that kinetin could modify 2,4-D-dependence via its effect on endogenous auxin metabolism. Cells gaining 2,4-D-independence

by virtue of metabolic by-passes to auxin sensitive pathways could therefore be predicted to be both 2,4-D- and kinetin- independent. This is not, of course, the only route by which such double-habituation could occur (see INTRODUCTION).

The kinetin-dependence of 2,4-D-independent strains ID_4, ID_5, ID_{10} and ID_{24} was determined, after washing in medium lacking both growth regulators, by culturing each strain in minus- and plus- kinetin (0.25 mg dm^{-3}) media lacking 2,4-D. Growth as estimated by dry weight measurements was determined at the end of 21 days incubation when each treatment was subcultured into the same treatment medium. Growth, after 14 days of this second passage, was then used to determine kinetin-dependence in terms of the factor 'K' where

$$K = \frac{\text{Relative growth in absence of kinetin}}{\text{Relative growth in presence of kinetin}}$$

$$= \frac{R^-}{R^+}$$

and where

$$\text{Relative growth} = \frac{\text{Final dry weight - initial dry weight}}{\text{initial dry weight}}$$

$$= \frac{W - Wo}{Wo}$$

The 21 day incubation period of the first passage represented the normal culture period used for the 2,4-D-independent strains to permit all strains, including the slower growing ones, to be maintained on the same subculture regime. However, strain ID_4 grows as fast as the Am strain (14 day subculture regime). The kinetin-dependence of ID_4 was determined, therefore, using cells maintained on both 21 day and 14 day subculture regimes. The growth at the end of the first passage was not expressed in terms of K due to the possible complication of transient growth in response to intracellular kinetin. The duration of the second passage was set at 14 days to permit comparison of growth rates rather than final yields. The results (Table 3.5) show that strains ID_4 and ID_5 may be considered kinetin-independent, strain ID_{10} kinetin-dependent, and strain ID_{24} partially dependent on kinetin.

TABLE 3.5 The cytokinin requirements of 2,4-D-independent strains.

EXPERIMENTAL CODE		DRY WEIGHT (W, mg cm^{-3}) Passage 1	Passage 2	$R = \frac{W - Wo}{Wo}$	$K = \frac{R^-}{R^+}$
$ID_{4/14}$*	- kinetin	8.21	9.22	5.29	0.95
$ID_{4/14}$	+ kinetin	8.67	10.16	5.56	-
$ID_{4/21}$†	- kinetin	8.16	8.55	4.87	0.88
$ID_{4/21}$	+ kinetin	7.97	9.30	5.54	-
ID_5	- kinetin	5.22	5.29	4.68	1.26
ID_5	+ kinetin	6.38	5.37	3.71	-
ID_{10}	- kinetin	0.91	0.18	0.10	0.02
ID_{10}	+ kinetin	4.67	5.33	5.39	-
ID_{24}	- kinetin	2.11	0.79	1.10	0.29
ID_{24}	+ kinetin	6.40	5.47	3.79	-

*Strain ID_4 maintained on a 14 day subculture regime.

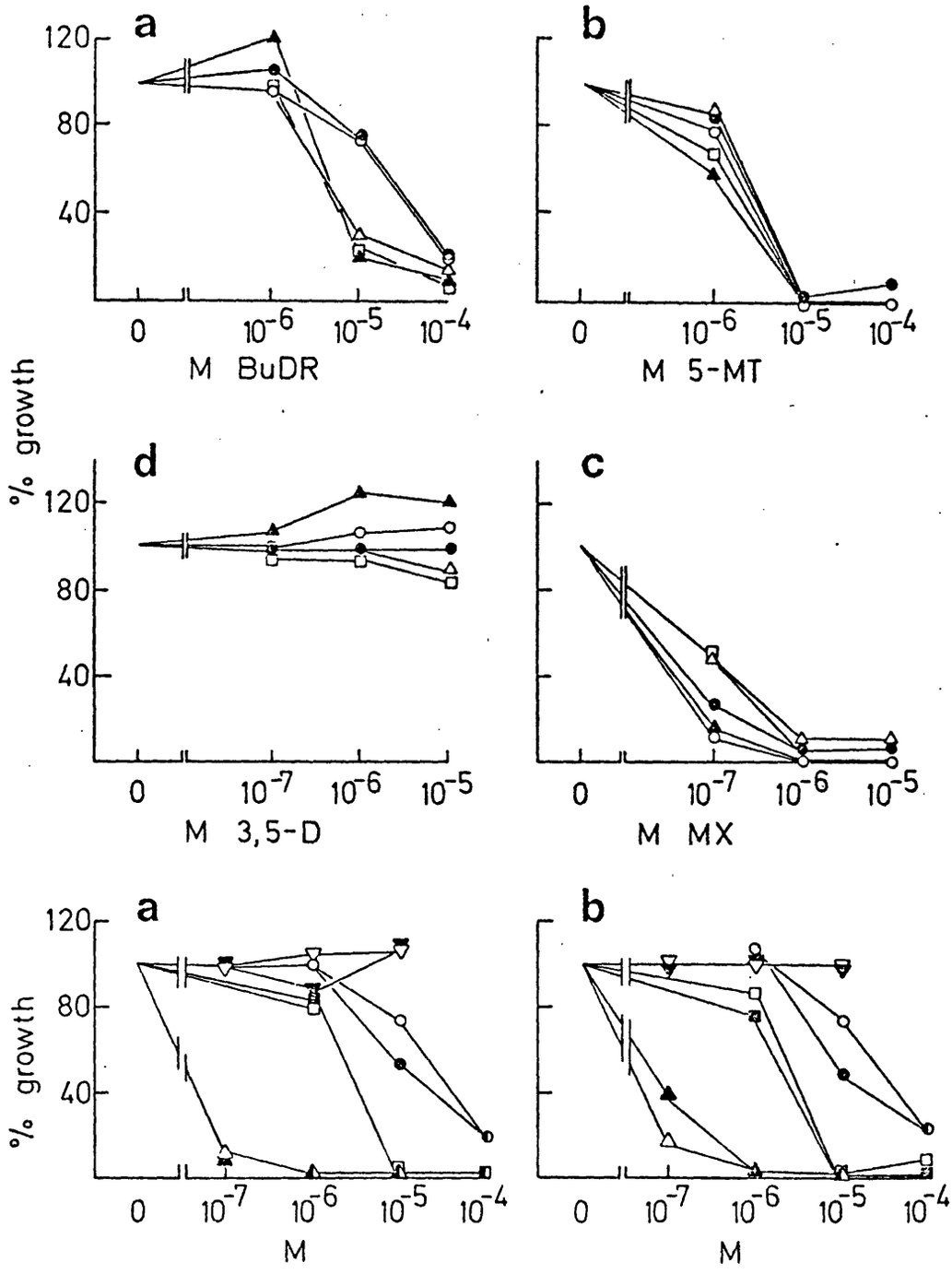
†Strain ID_4 maintained on a 21 day subculture regime.

3.4 2,4-D-independence and the resistance to anti-metabolites.

Meins (1976) has suggested that the thymidine analogue, 5-bromodeoxyuridine (BUdR), is a specific inhibitor of cytokinin-habituation in tobacco cell cultures. Vyskot et al (1977) suggested a link between BUdR resistance and the expression of genes for hormone biosynthesis. Thus they consider that the disposition of a cell for transient BUdR tolerance requires at least active expression of genes for cytokinin biosynthesis and that the tolerance becomes permanent when the auxin system is turned on. In contrast, Kandra and Maliga (1977) showed that BUdR resistance and cytokinin habituation were not linked characters in a BUdR-resistant tobacco mutant and suggested that cytokinin habituation and BUdR resistance are independent phenomena. Resistant to BUdR is considered to be a consequence of alterations in the metabolism of thymidine nucleotides (Ohyama, 1976;

FIG.3.1 The sensitivities of 2,4-D-independent cell lines ID_4 (●), ID_5 (△), ID_{10} (▲) and ID_{24} (□) and the 2,4-D-dependent Am strain (○) to 5-bromodeoxyuridine (a), 5-methyl tryptophan (b), methotrexate (c) and 3,5-dichlorophenoxyacetic acid. Growth was assessed as dry weight.

FIG.3.2 A comparison of the effects of 5-bromodeoxyuridine (○,●), methotrexate (△,▲), 5-methyl tryptophan (□,■) and 3,5-dichlorophenoxyacetic acid (▽,▼) on growth by increase in dry weight (open symbols) or cell number (closed symbols) for the 2,4-D-dependent Am strain (a) and the 2,4-D-and kinetin-independent ID_4 cell line (b).


a
b
d
c
a
b
120
80
40
% growth
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
M BuDR
M 5-MT
M 3,5-D
M MX
M

Davidson and Kaufman, 1977).

5-methyl-tryptophan (5-MT) is a tryptophan analogue which can prevent cell growth by inhibiting anthranilate synthetase thus starving the cells of tryptophan (Widholm, 1972b). Widholm (1977) observed a high incidence of 2,4-D-independence in cultures originally selected for their resistance to 5-MT and proposed that the accumulation of tryptophan can confer auxin-independence in addition to 5-MT resistance.

Methotrexate (MX, a gift from Lederle Laboratories Ltd.) inhibition of dihydrofolate reductase depletes reduced folate pools necessary for the metabolic transfer of 1-carbon units in a variety of biochemical reactions (Bleyer, 1977). Methotrexate is widely used in cancer chemotherapy although cells can become resistant to the drug by selective multiplication of dihydrofolate reductase genes (Alt et al, 1978; Shields, 1978).

Chandra, Lam and Street (1978) have shown that 3,5-dichlorophenoxy-acetic acid (3,5-D), an analogue of 2,4-D and IAA, can behave as an anti-auxin with respect to carrot embryogenesis and may therefore be useful to identify changes in receptor sensitivity.

Each of the above mentioned anti-metabolites was dissolved in distilled water, filter sterilized (Millipore filter, 0.22μm porosity), and added to autoclaved media to give three initial concentrations covering a two decade range. The medium used for the Am strain was that used for the 2,4-D-independent strains (basal medium shown in MATERIALS and METHODS TABLE 1 containing kinetin (0.25 mg dm^{-3}) and sodium glutamate (1 g dm^{-3}), pH 5.80) except that 2,4-D (1 mg dm^{-3}) was added before autoclaving. Cultures were harvested after 14 days (Am and ID_4) or 21 days incubation (ID_5, ID_{10}, ID_{24}) and growth was estimated as dry weight (all strains) and cell number (Am, ID_4). Cell viability was estimated by the fluorescein diacetate method (Widholm, 1972; Zilkah and Gressell, 1978). The results are expressed in Figs. 3.1 and 3.2 as growth, after subtraction of the initial inoculum, relative to the control (= 100).

Fig. 3.1(a) shows that, rather than showing resistance to BUdR, most

of the 2,4-D-independent strains are more sensitive to 10^{-5}M BUdR than the Am strain. Additionally ID_4, which is kinetin-independent, shows a sensitivity to BUdR almost identical to the Am strain while ID_5, which is also kinetin-independent, is as sensitive to BUdR as the kinetin-dependent strains ID_{10} and ID_{24}. Thus there appears to be no correlation between resistance to BUdR and auxin- or cytokinin- independence.

The growth of all strains tested was totally inhibited by 10^{-5}M 5-MT (Fig. 3.1(b)). Slight differences in sensitivity to 10^{-6}M 5-MT were observed such that ID_4 and ID_5 were less sensitive and ID_{10} and ID_{24} were more sensitive than the parent Am strain. If, as Widholm (1977) suggests, resistance to 5-MT is accompanied by tryptophan accumulation then the lack of resistance to 5-MT indicates that strains ID_4, ID_5, ID_{10} and ID_{24} do not gain 2,4-D-independence by the accumulation of tryptophan.

Methotrexate (MX) was the most inhibitory agent tested (Fig. 3.1(c)) and partially inhibited the growth of all strains at a concentration of 10^{-7}M. Ohyama (1976) showed that a BUdR-resistant strain of soybean could withstand concentrations of aminopterin (a folate antagonist similar to MX) 10^3 times those required to irreversibly inhibit the growth of wild-type cells. It might be expected, then, that the patterns of sensitivities to MX would be similar to those observed for BUdR (Fig. 3.1(a). This is clearly not the case as ID_5 and ID_{24}, which are the least sensitive to MX, are two of the strains most sensitive to BUdR. Also, as ID_5 and ID_{24} differ widely in their requirements for kinetin, there appears to be no correlation between cytokinin-independence and the sensitivity to folate antagonists.

3,5-D does not antagonize the growth promoting effects of 2,4-D on Am strain cells (Fig. 3.1(d)) and has little or no effect on the growth of strains ID_4, ID_5 and ID_{24}. The growth of strain ID_{10} is considerably promoted by 3,5-D (25% at 10^{-6}M) and if this growth promotion was to be explained as weak auxin activity of 3,5-D then the complete absence of any response by ID_4 would become more interesting.

The data expressed in Fig. 3.1 relate to growth inhibition as measured by dry weight accumulation. It was conceivable, however, that some of the analogues used could inhibit cell division without reducing biomass accumulation. The cell number data (Fig. 3.2) for strains Am and ID_4 show little difference from the dry weight data, except perhaps for BUdR, although even in this case the responses of the Am and ID_4 strains appeared identical. Similarly the assessment of viability (Table 3.6) using the fluorescein diacetate method, showed very little difference between strains except for Am cells arrested by 5-MT which appeared to retain viability better than the other strains receiving the same treatment.

TABLE 3.6 Effects of anti-metabolites on cell viability (%)

TREATMENT		Am	ID_4	ID_5	ID_{10}	ID_{24}
Control		100	100	100	100	100
BUdR	10^{-6}M	100	100	100	100	100
	10^{-5}M	100	100	100	90	95
	10^{-4}M	100	100	90	90	90
5-MT	10^{-6}M	100	100	95	100	100
	10^{-5}M	100	100	70W	80	90
	10^{-4}M	100	60	50W	40	50
MX	10^{-7}M	100	100	90	90	100
	10^{-6}M	60	90	65	90	95
	10^{-5}M	70	90W	70	70	90
3,5-D	10^{-7}M	100	100	100	100	100
	10^{-6}M	100	100	100	100	100
	10^{-5}M	100	100	100	100	100

W denotes weak fluorescence.

DISCUSSION

The low frequency of isolation of 2,4-D-independent cells from the parent Am strain (1 to 5 x 10^{-6}) may suggest a procedure of selection rather than adaptation. The slow appearance of independent colonies and the beneficial effects of glutamate and darkness could then be seen as being related to growth from low initial densities (Shillito, 1978) rather than to the induction of 2,4-D-independent growth. If a process of adaptation is involved then it would need to be relatively slow (cf. substrate-induced nitrate reductase) such that the low frequency of isolation would then be a consequence of the death of many cells, deprived of active growth, before adaptation permitted re-growth.

It is not possible, from the data presented here, to discuss the reversion from hormone-independent to hormone-dependent growth in sycamore cells. Nevertheless, it has been shown that sensitivity to 2,4-D can be induced in some cells by a brief exposure to 2,4-D. The criterion used for determining this sensitivity to 2,4-D was the ability to grow on a non-conditioned medium: precisely the conditions applied to explants during callus induction. Thus, if such sensitivity to 2,4-D increases with increasing exposure time, it may contribute to the development of 2,4-D-dependence during callus induction.

The culture strains selected for further investigation displayed a range of requirements for kinetin from total dependence to total independence. The responses of these strains to anti-metabolites indicate that resistance to BUdR, 5-MT or MX are phenomena which are not pre-requisites for auxin- or cytokinin- independence in cultured sycamore cells. Thus the general lack of resistance to these compounds indicates that the development of auxin- or cytokinin- independence does not require gross changes in thymidine, tryptophan or folic acid metabolism. The apparent lack of anti-auxin action of 3,5-D in these experiments, measuring growth rather than differentiation (Chandra, Lam and Street, 1978), suggests that

the promotion of growth and the suppression of differentiation by 2,4-D may involve separate mechanisms.

SECTION 4

EFFECTS OF 2,4-D ON AMINO ACID METABOLISM

INTRODUCTION

The results described in SECTIONS 1 and 2 showed that when Am strain sycamore cells were deprived of 2,4-D alcohol-soluble amino-containing compounds accumulated in the cells. Furthermore treatments with 2,4-D or kinetin which maintained active growth also suppressed the accumulation of amino-nitrogen. Thus the accumulation of amino-nitrogen appeared to possess characteristics consistent with it being closely related to the regulation of growth in cultured sycamore cells.

The general aims of the experiments described in this section were (1) to identify the changes in the levels of individual amino acids following 2,4-D withdrawal, (2) to decide whether these changes were initiated before or after the cessation of growth and (3) to identify the areas of metabolism which may contribute to the hormonal regulation of growth in culture.

In this thesis the total free amino acids which can be extracted from tissues with 85% v/v methanol will be referred to as "the soluble amino acid pool". The use of this term is not intended to suggest that these amino acids are derived from a single metabolic pool.

4.1 Relationships between initial 2,4-D and kinetin concentrations and the soluble amino acid pool.

The experiment described in SECTION 2.1 provided amino acid extracts from cells supplied with sub-optimal initial 2,4-D concentrations in the presence of the standard (0.25mg dm^{-3}) or 'high' (2.5mg dm^{-3}) initial concentrations of kinetin. Separation and analysis of the amino acid constituents of the extracts (Table 4.1) showed that cells which had been arrested by 2,4-D depletion contained amino acid pools enriched with serine but depleted of glutamic acid. If the minus-2,4-D medium was enriched with kinetin, however, an amino acid profile similar to that of plus-2,4-D

Kinetin (mg dm^{-3})	0·25	0·25	0·25	2·50
2,4-D (mg dm^{-3})	1·0	0·2	None	None
Amino acid	%[a]	%	%	%
Aspartic acid	3·4	7·0	0·6	12·8
Threonine	4·4	3·2	0·6	4·5
Serine	11·4	24·2	90·8	33·0
Asparagine	—[b]	—	1·5	1·8
Glutamic acid	47·3	37·6	0·7	22·0
Glutamine	—	1·0	0·6	3·9
Proline	2·3	3·5	1·5	—
Glycine	1·1	1·6	0·3	0·7
Alanine	11·2	14·2	0·9	7·2
Valine	2·8	2·3	0·6	3·4
Cysteine	—	—	—	—
Methionine	—	—	—	—
Isoleucine	2·0	0·9	0·2	2·9
Leucine	4·6	1·7	0·5	5·3
Tyrosine	0·4	—	—	—
Phenylalanine	2·1	—	—	—
γ-Aminobutyric acid	2·7	—	—	—
Lysine	2·1	1·0	0·3	1·7
Histidine	2·2	1·6	0·5	0·7
Arginine	—	1·0	0·2	—

[a] Molar % of amino acids detected.
[b] Denotes < 0·1%.

TABLE 4.1 Influence of initial 2,4-D and kinetin concentrations on the alcohol-soluble amino acid pool of sycamore cells. Amino acids were extracted from cells after 10 days incubation in each medium. (see SECTION 2, Figs.2.1 and 2.2).

controls appeared to be maintained. Thus 'high kinetin' counteracted not only the effects of 2,4-D depletion on growth and the accumulation of total soluble amino-nitrogen but also the distribution of reduced nitrogen within the soluble amino acid pool.

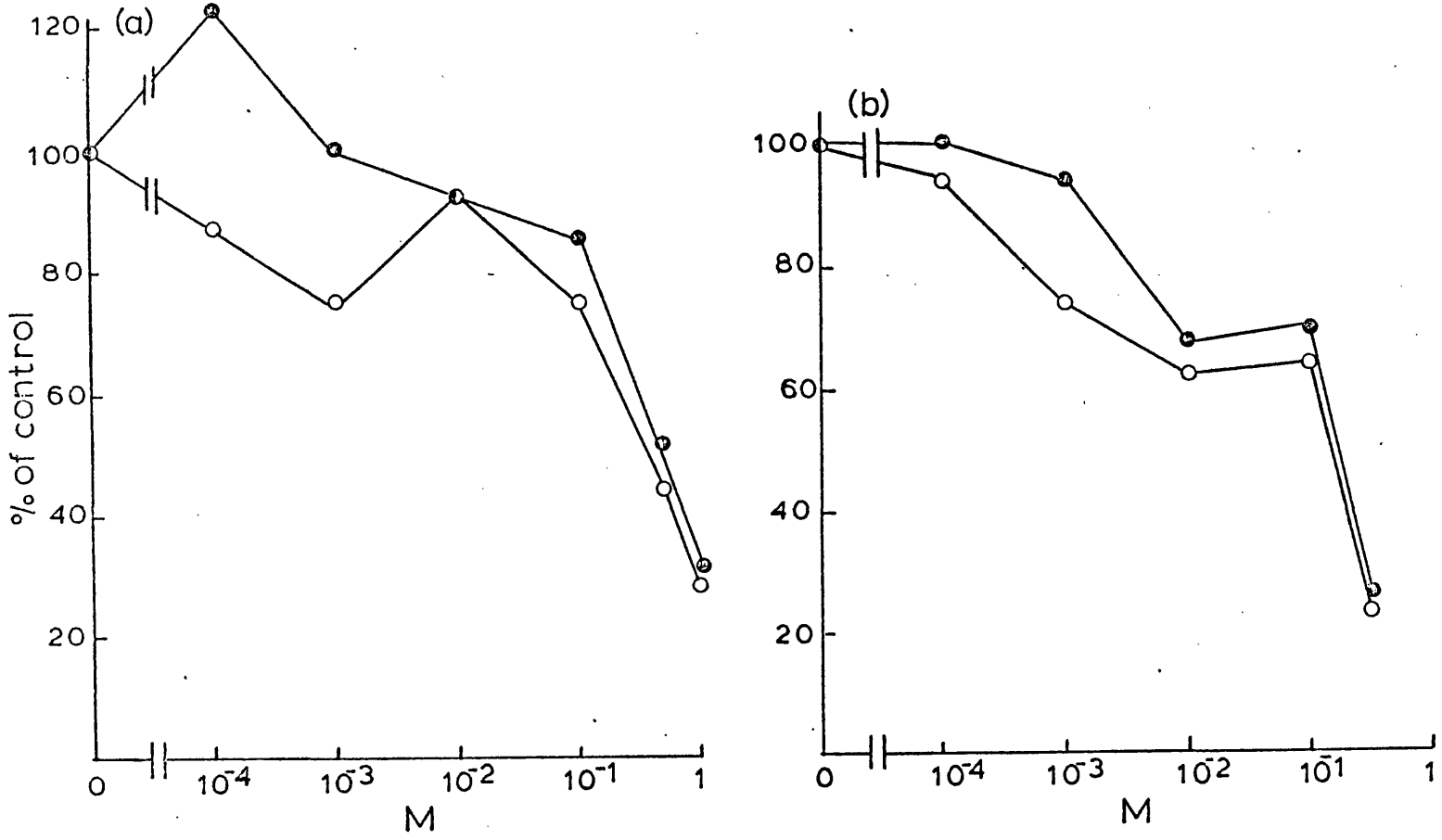
It seemed unlikely that the accumulation of serine was a consquence of inhibited protein synthesis as the amino acids usually associated with nitrogen storage and translocation are asparagine, glutamine, aspartic acid, glutamic acid, arginine and alanine. These preliminary results suggested, therefore, that changes in amino acid metabolism could be related to the cause of growth-arrest in the absence of 2,4-D.

4.2 Effects of exogenous amino acids on growth.

The results in Table 4.1 showed that the major qualitative changes in the soluble amino acid pool following 2,4-D depletion were an increase in serine and a decline in glutamate. If either of these changes was directly responsible for growth inhibition, it would be expected that either (a) elevated intracellular levels of serine would inhibit the growth of cells in the standard plus-2,4-D medium or (b) supplementation of intracellular pools of glutamic acid would prolong the growth of cells in minus-2,4-D medium.

The growth-inhibitory nature of serine was investigated by incubating stock Am strain cells in the standard (plus-2,4-D) culture medium supplemented with either serine or glycine. It was thought that exogenous serine might not duplicate exactly the effects of endogenous serine due to possible transport into the vacuole without prior equilibration with metabolically active serine pools. The toxicity of glycine, which is rapidly converted to serine (Wang and Burris, 1963; Sinha and Cossins, 1964), was investigated to minimize such complications. Fig.4.1 shows the final cell numbers and dry weights, as percentages of the control, after

FIG.4.1 The effects of glycine (a) and serine (b) on the growth of Am strain sycamore cultures. Growth was assessed as increase in cell number (O) and dry weight (●) after 14 days incubation.


(a)
120
100
80
60
40
20
% of control
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
M
(b)
100
80
60
40
20
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
M

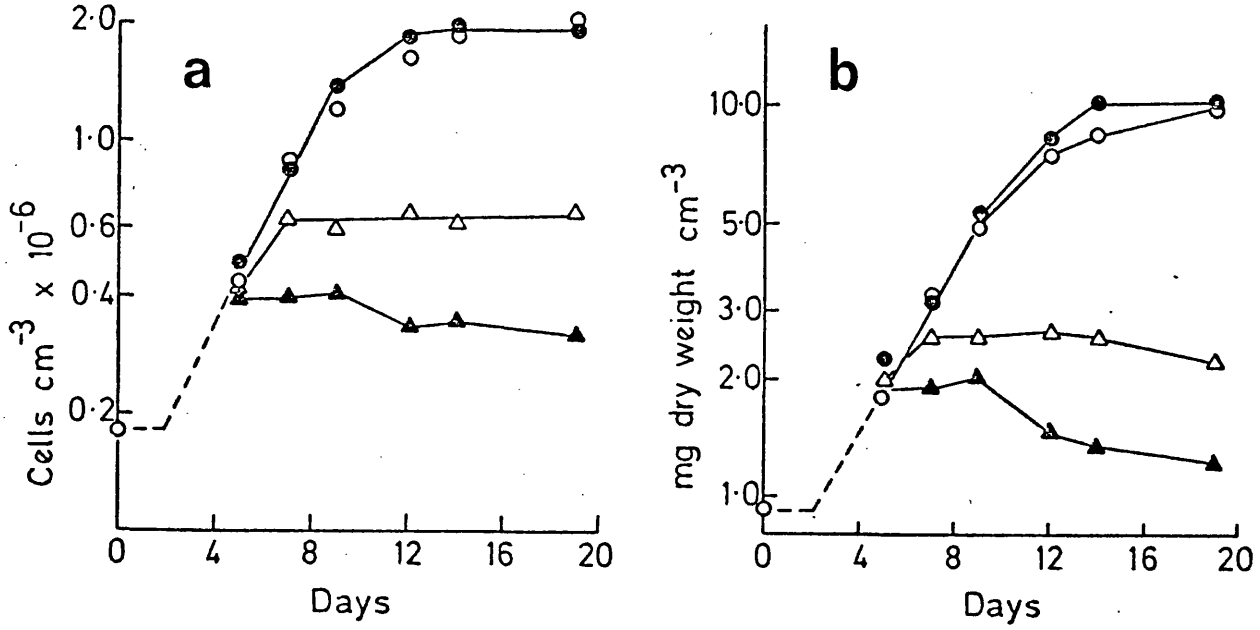


FIG.4.2 The effects of glutamate on the growth of sycamore cultures in the presence and absence of 2,4-D. Stock cells were washed free of extracellular 2,4-D and inoculated into plus-2,4-D (○,●) or minus-2,4-D (△,▲) media in the presence (closed symbols) or absence (open symbols) of 5.35 x 10^{-3}M glutamate. Growth was assessed as increase in cell number (a) and dry weight (b).

incubation in a range of concentrations of glycine (a) or serine (b) for 14 days. As neither glycine nor serine caused appreciable growth inhibition until molar initial concentrations were approached it is unlikely that the growth inhibition following 2,4-D starvation is a consequence of serine accumulation.

The effect of exogenous glutamic acid on growth was investigated by incubating stock Am strain cells, previously washed free of extracellular 2,4-D, in medium minus- or plus-2,4-D (1mg dm^{-3}) and minus- or plus-sodium glutamate (1g dm^{-3}, 5.35×10^{-3}M). The results shown in Fig.4.2 show that, rather than promoting growth, exogenous glutamate reduced the final growth yield in minus-2,4-D medium irrespective of whether growth was expressed in terms of cell number or dry weight. Thus it is unlikely that growth arrests, in the absence of 2,4-D, because of a limited supply of glutamic acid. However, the fact that glutamate did not inhibit the growth of cells in plus-2,4-D medium supported the hypothesis that changes in amino acid metabolism were related to the cessation of growth in the absence of 2,4-D. These data also supported the observations in SECTION 3.1 that glutamate, when incorporated into minus-2,4-D medium, suppressed the growth of 2,4-D-dependent cells on plates.

4.3 Kinetics of changes in amino acid metabolism following 2,4-D withdrawal

Once a series of responses has been identified the next major obstacle to understanding regulatory functions is distinguishing between cause and effect. When this can be achieved, experiments can be designed to examine events progressively closer to the primary site of action of the original stimulus. The complexity of rationalizing a large number of related observations, such as changes within the soluble amino acid pool, is indicated in Fig.4.3. This shows some of the possible relationships between a primary stimulus (X), a metabolic response (M), and a growth response (G).

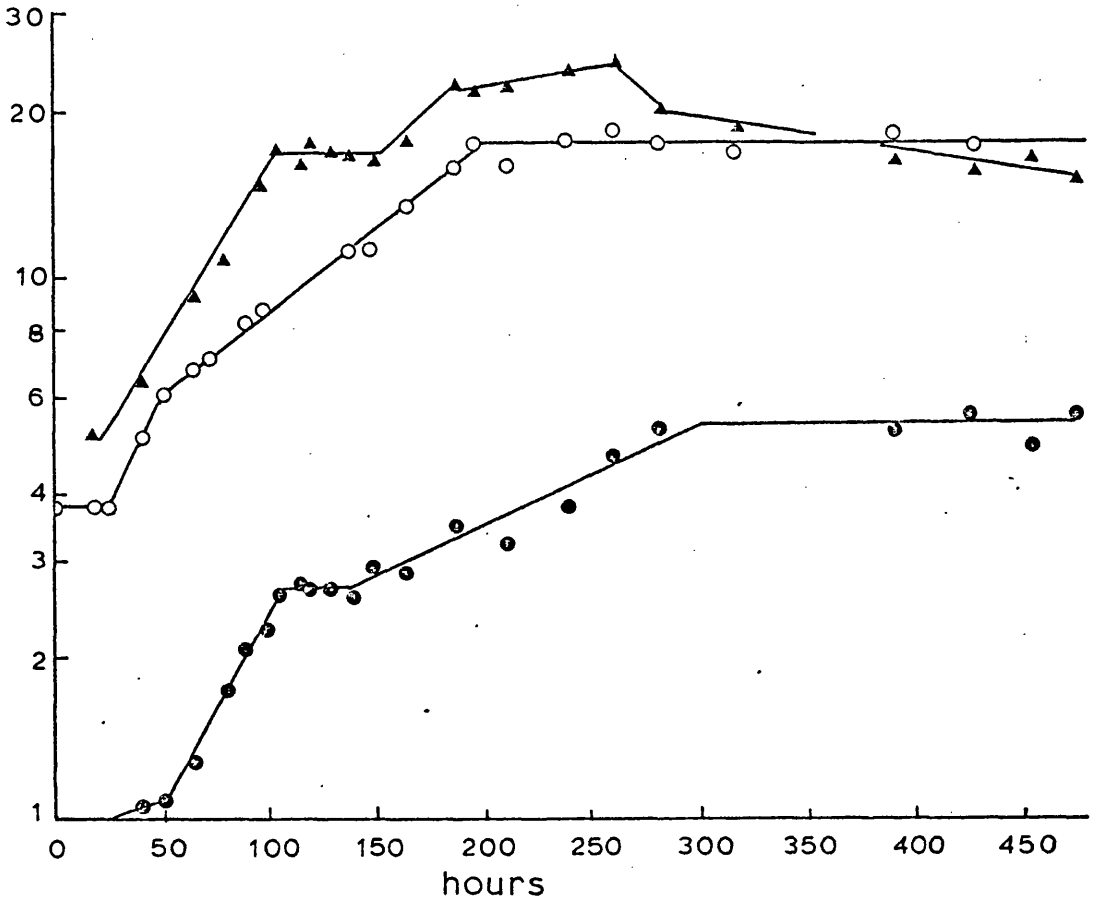


FIG.4.4 The growth of washed sycamore cells in a $4dm^3$ culture, lacking 2,4-D, as measured by cell number (●), dry weight (O) and total protein (▲).

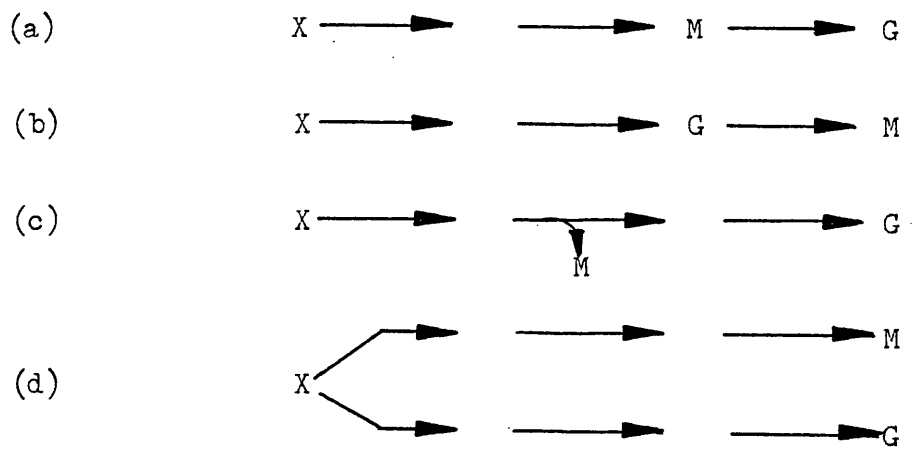


FIG.4.3 Some possible arrangements of two responses (M,G) to a primary stimulus (X).

Although the number of possible combinations will increase as the number of observed responses increases some simple experiments can eliminate many of the possibilities. If a number of responses are arranged in a single causal sequence (Fig.4.3(a), (b)) the relationships between the responses may be identified by the order in which they are expressed. The results from SECTION 4.2 suggest that neither serine accumulation nor glutamate depletion could be represented by the sequence shown in Fig.4.3(a).

The kinetics of changes in the soluble amino acid pool were investigated by inoculating a batch culture, containing $4dm^3$ of minus-2,4-D medium, with stock cells previously washed free of extracellular 2,4-D. The initial values of growth parameters were: 1.0×10^5 cells cm^{-3}, 0.38mg dry weight cm^{-3}, 50µg total protein cm^{-3}. The subsequent growth, (Fig.4.4) differed from the growth observed in $70cm^3$ batch cultures following 2,4-D withdrawal (e.g. Fig.4.2). Instead of a sharp transition between rapid growth and cessation of growth the cell number data showed an intermediate period of slow growth (doubling time, td = 260 hours) which was reflected in total protein but not dry weight. The reasons for the difference in behaviour are not entirely clear as growth rates in the large batch cultures are usually very similar to those obtained in $70cm^3$ batch cultures. This particular culture, however, was maintained with new ancillary equipment and

when subsequently checked the light intensity at the surface of the culture vessel was found to be about four times higher than in the culture room (for $70cm^3$ cultures). Certainly when a similar experiment was carried out using lower light intensities (SECTION 4.5) the growth following 2,4-D withdrawal resembled that of $70cm^3$ cultures. It seems possible then that light effects may be seen not only between darkness and low light (SECTION 1) but also between low and high light intensities.

As previously stated, the aim of this experiment was to identify the temporal relationships between 2,4-D withdrawal, changes in amino acid metabolism and growth. The total amino nitrogen (ninhydrin positive) content of alcoholic extracts of the cells was estimated to facilitate efficient use of the amino acid analyser. A comparison of total soluble amino-nitrogen and total soluble amino acid-nitrogen (Fig.4.5) shows that the values obtained for total amino acids are consistently lower than the values estimated for total amino-nitrogen. This discrepancy could arise either from the presence of amino acids that generated more colour per mole with ninhydrin than the leucine standard or the presence of compounds not detected by the amino acid analyser. Very few amino acids generate more colour per mole with ninhydrin that leucine (Table 4.2) and if total soluble amino-N is plotted against total amino acid N (Fig.4.6) the slope of the line is very close to unity (slope = 1.012, r = 0.996). The intercept on the y-axis suggests the presence of compounds in the alcoholic extract not included in the value of total amino acid-N. As ammonia was found to contribute less than 20% of this intercept value the remainder must be due to compounds more basic than arginine that would not be eluted from the Auto Analyzer column by the final buffer (pH6.50). The high correlation coefficient (0.996) for the straight-line fit to the data in Fig.4.6 shows that the level of these compounds did not alter significantly during the experiment. For this reason these unidentified compounds were not studied further.

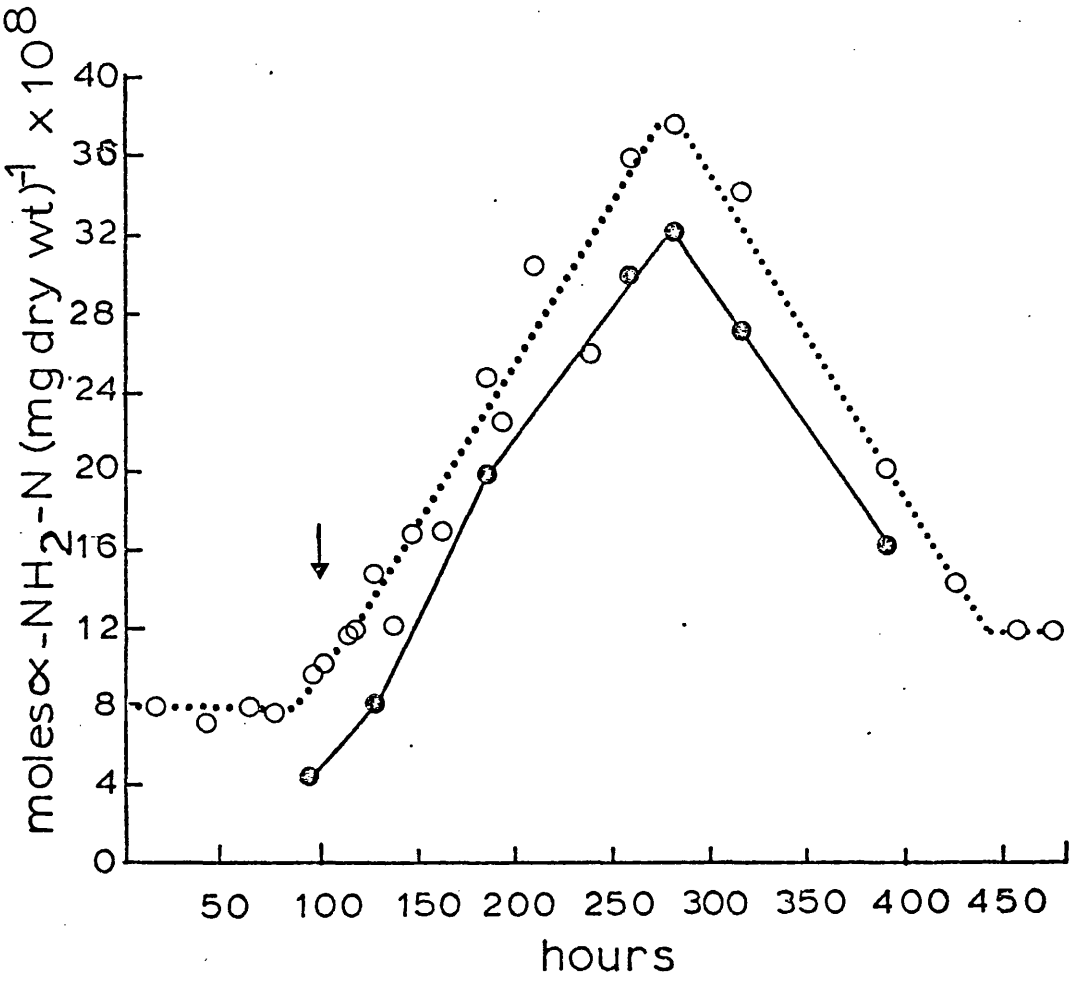


FIG.4.5 Changes in the cellular levels of total amino-N (O) and amino acid-N (●) for the culture shown in Fig.4.4. The arrow marks the time of the change in rate of cell number increase.

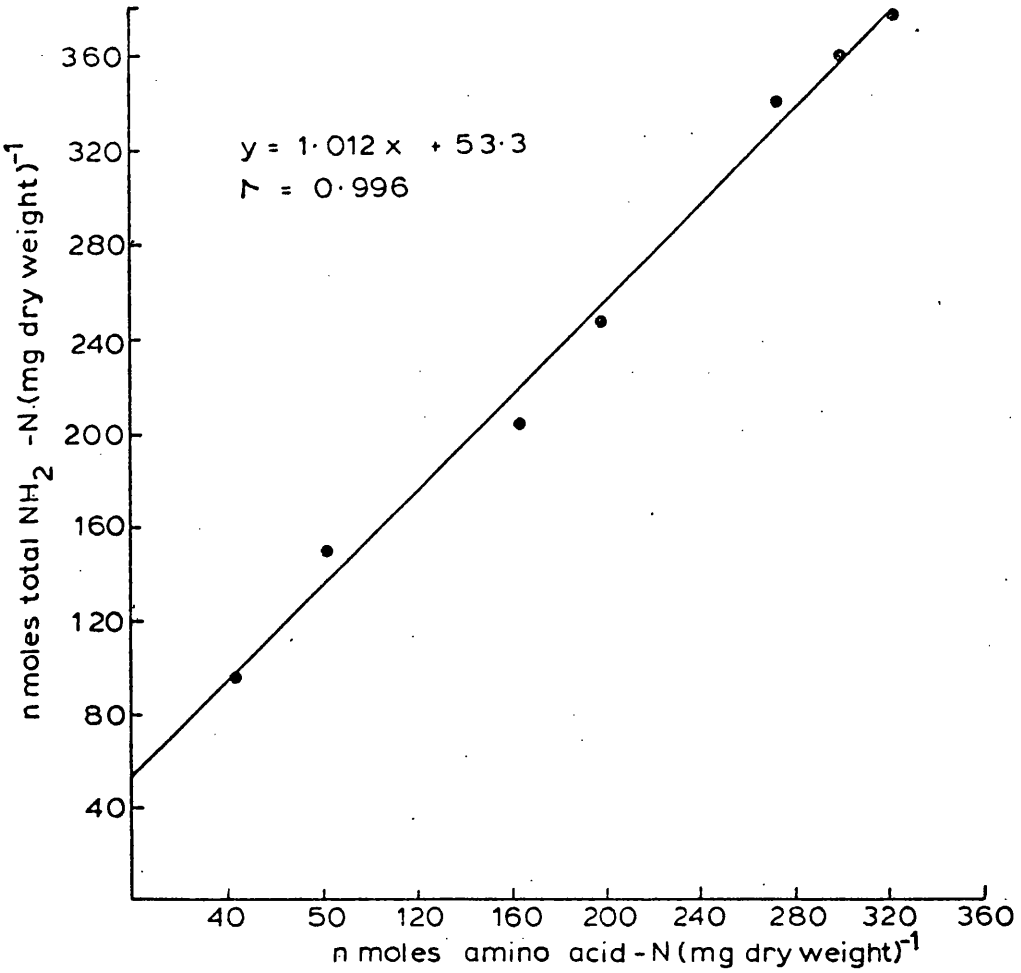


FIG.4.6 Plot of total amino-N against total amino acid-N for cells accumulating total amino-N after 2,4-D withdrawal (from Fig.4.5)

TABLE 4.2 The relative intensities of the reaction between amino acids and ninhydrin.

AMINO ACID	*RELATIVE INTENSITY
Aspartic acid	1.016
Threonine	1.021
Serine	1.009
Asparagine	0.702
Glutamic acid	1.163
Glutamine	0.926
Proline	1.511
Glycine	1.190
Alanine	0.921
α Amino butyric acid	0.990
Valine	0.875
Cysteine	0.689
Methionine	1.144
Isoleucine	1.113
Leucine	1.140
Norleucine	1.000
Tyrosine	1.138
Phenylalanine	1.196
γ Amino butyric acid	0.287
Lysine	1.229
Histidine	1.206
Arginine	1.226

* Abosrbance mole^{-1} compared with norleucine and measured at 570nm, except for proline which was measured at 440nm.

TABLE 4.3 Changes in the soluble amino acid pool of Acer cells following 2,4-D withdrawal.

AMINO ACID	* 96		128		186		260		281		317		390	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Aspartic acid	6.82	15.49	15.40	18.69	15.58	7.83	8.77	2.91	7.17	2.23	4.76	1.74	-	
Threonine	1.29	2.93	2.60	3.18	8.18	4.11	11.86	3.94	14.68	4.56	15.72	5.75	9.20	5.60
Serine	6.24	14.18	12.39	15.04	70.67	35.51	169.59	56.36	193.30	60.00	151.71	55.48	55.70	33.88
Asparagine	-		-		4.35	2.19	33.55	11.15	46.39	14.40	57.06	20.87	70.34	42.78
Glutamic acid	19.33	43.91	32.49	39.44	28.43	14.28	14.21	4.72	11.04	3.42	3.59	1.31	-	
Glutamine	3.20	7.27	5.06	6.14	19.41	9.75	11.78	3.92	11.19	3.47	13.35	4.88	8.17	4.97
Proline	-		-		6.59	3.31	7.90	2.63	-		-		-	
Glycine	0.54	1.23	0.64	0.78	1.34	0.67	1.48	0.49	1.82	0.57	1.53	0.56	0.62	0.38
Alanine	3.68	8.36	8.80	10.68	21.37	10.74	8.63	2.87	8.03	2.49	5.69	2.08	5.10	3.10
Valine	0.92	2.09	1.77	2.15	7.49	3.76	9.48	3.15	9.97	3.09	5.27	1.93	1.29	0.79
Cysteine	-		-		-		-		-		-		-	
Methionine	-		-		-		-		-		-		-	
Isoleucine	0.41	0.93	0.59	0.72	1.72	0.86	4.89	1.63	5.32	1.65	3.77	1.38	0.84	0.51
Leucine	0.78	1.77	0.97	1.18	5.24	2.63	9.37	3.11	7.28	2.26	3.20	1.17	0.73	0.44
Tyrosine	-		-		-		-		-		-		2.73	1.66
Phenylalanine	-		-		-		-		-		-		0.73	0.44
Lycine	0.81	1.84	0.52	0.63	1.60	0.80	1.78	0.59	-		2.34	0.86	1.97	1.20
Histidine	-		0.54	0.66	4.63	2.33	7.62	2.53	6.01	1.87	5.45	1.99	3.45	2.10
Arginine	-		-		-		-		-		-		3.56	2.17
∝-Amino butyric acid	-		0.59	0.72	0.51	0.26	-		-		-		-	
ɣ-Amino butyric acid	-		-		1.92	0.97	-		-		-		-	
TOTAL	44.02		82.38		199.03		300.91		322.20		273.44		164.43	

* hours post 2,4-D withdrawal

a nmoles $(\text{mg dry weight})^{-1}$

b molar % of amino acids detected

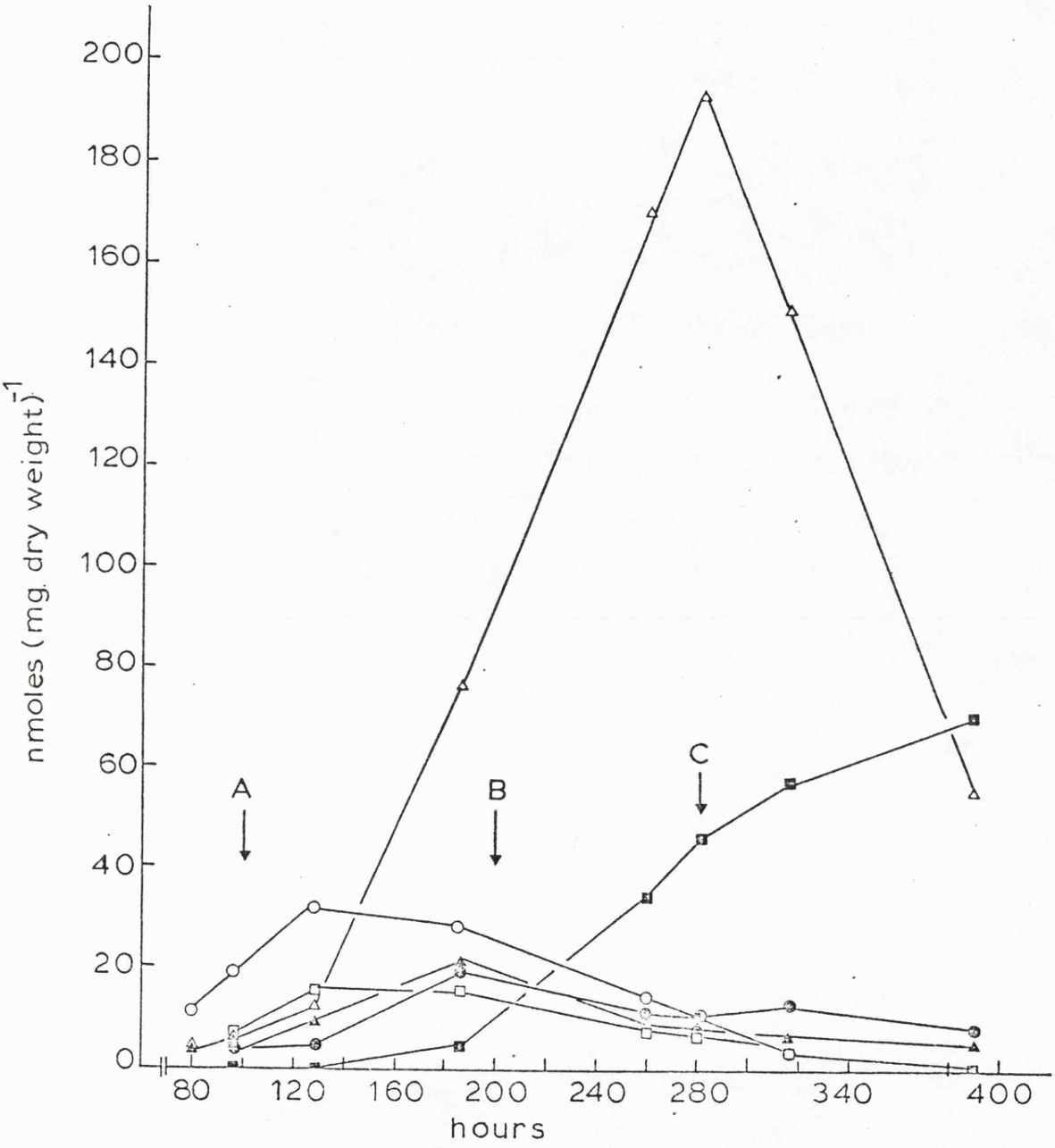


FIG.4.7 The cellular levels of soluble serine (△), glutamic acid (○), glutamine (●), aspartic acid (□), asparagine (■) and alanine (▲) found in sycamore cells after 2,4-D depletion. The arrows mark the time of (A) the change in the rate of cell division, (B) the cessation of dry weight accumulation and (C) the cessation of cell number accumulation (from Fig.4.4).

The changes in the levels of individual amino acids following 2,4-D depletion are shown in Table 4.3, but to facilitate visual interpretation the changes in the levels of the major amino acids are also represented graphically in Fig.4.7. The most notable features were:

(a) Serine accumulation began after a reduction in the rate of cell division but before growth finally arrested.

(b) Glutamate and aspartate declined slowly while the corresponding amides, normally absent in control cells, (Table 4.1), became detectable and eventually represented a major proportion of the total soluble amino acid pool.

As amino acids are, in general, metabolic intermediates which may be readily interconverted a change in the activity of a single enzyme may bring about many changes in the composition of the soluble amino acid pool. For this reason it may be useful to compare effects on groups of, rather than individual, amino acids. Tables 4.4 and 4.5 show the results of Table 4.3 with the amino acids regrouped to show either synthetic routes or end products of their metabolism. Clearly there is no simple relationship between the synthesis and degradation of amino acids sensitive to 2,4-D starvation. It may be interesting to note that sycamore cells supplied with urea as sole nitrogen source (King, J., 1976) possess soluble amino pools comprising large amounts of asparagine and very little glutamate. The standard medium used for sycamore cultures contains both nitrate and urea but nitrate appears to be used preferentially (Young, 1973). It therefore appeared feasible that some of the changes in amino acid metabolism following 2,4-D withdrawal may have been due to a loss of nitrate assimilation, forcing the cells to use urea as sole nitrogen source. The results of SECTION 4.2 have shown that growth, in the absence of 2,4-D, is not promoted by glutamic acid although this amino acid can serve as a sole nitrogen source in the presence of 2,4-D (Jessup and Fowler, 1976(a)). Thus any changes in amino acid metabolism which resulted from a switch in

TABLE 4.4 Amino acids grouped to show synthetic routes.

GLUTAMATE	ASPARTATE	PYRUVATE	†PEP	*G-3-P
Glutamate (-)	Aspartate (±)	Alanine (+)	Phenylalanine	Serine
Glutamine (+)	Asparagine (++)	Leucine (+)	Tyrosine	Glycine (+
Proline (±)	Lysine (±)	Valine (+)	Tryptophan	Cysteine
Arginine	Methionine	(Isoleucine)(+)		
	Threonine (+)			
	(Isoleucine)(+)			

† phospho-enol pyruvate

* glycerate-3-phosphate

Symbols in brackets denote increase (+) or decrease (-) in the levels of free amino acid after 2,4-D depletion.

TABLE 4.5 Amino acids grouped to show end products of metabolism.

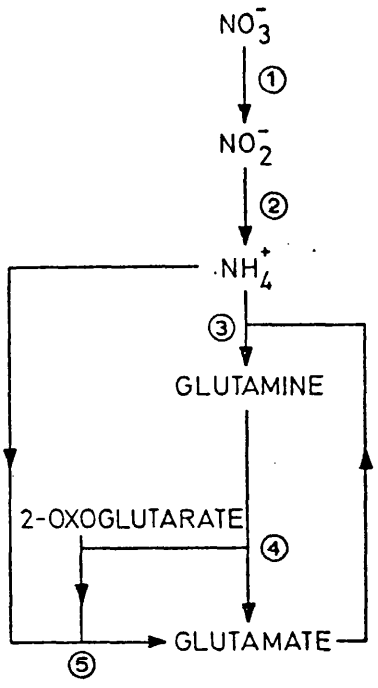
OXALOACETATE + PYRUVATE	2-OXOGLUTARATE	SUCCINYL CoA	ACETYL CoA + ACETYLOACETYL CoA
Aspartate (+)	Glutamate (-)	Methionine	Lysine (±)
Asparagine (++)	Glutamine (+)	Threonine (+)	Isoleucine (±)
Alanine (+)	Proline (±)	Isoleucine (+)	Leucine (+)
Serine (+++)	Arginine	Valine (+)	Tryptophan
Glycine (+)	Histidine (+)		Phenylalanine
Cysteine			Tyrosine

nitrogen assimilation needed to be identified so that they did not interfere with the identification of events more closely related to the regulation of growth.

4.4 Effects of 2,4-D on nitrate assimilation and glucose oxidation.

The activity of nitrate reductase, which catalyzes the first step in nitrate assimilation (Fig.4.8), appears to control the input of reduced nitrogen, from nitrate, in whole plants (Beevers and Hageman, 1969). Therefore a reduction in the ability to utilize the nitrogen of nitrate (as proposed above) might be expected to be reflected in the activity of nitrate reductase. The demands of nitrate assimilation for supplies of reducing power may result in a close interrelationship between nitrate assimilation and carbohydrate metabolism (Sarkissian and Fowler, 1974). A reduced flux of nitrate into amino acids could therefore result from either a loss of active assimilatory enzymes or a restricted supply of reducing power. For this reason the effect of 2,4-D on nitrate reductase activity was investigated using the *in vivo* method of Ferrari *et al* (1971), as modified for suspension cultures by Jones *et al* (1976), rather than an *in vitro* method involving enzyme extraction and the supply of exogenous reducing power.

The interrelationship between carbohydrate and nitrogen metabolism may also result from effects of nitrogen source on carbohydrate metabolism (Jessup and Fowler, 1976(a); 1976(b)). Other observations particularly relevant to this study are that the contribution of the pentose phosphate pathway for carbohydrate oxidation is modified by the degree of cellular differentiation (Beevers 1961; Fowler and ap Rees, 1970; Fowler, 1975) is sensitive to 2,4-D (Humphreys and Dugger, 1957(a); 1957(b)) and that cells capable of showing a positive growth response to auxin also show increased respiration in response to the addition of auxin (Beevers, 1961).



① NITRATE REDUCTASE
② NITRITE REDUCTASE
③ GLUTAMINE SYNTHETASE (GS)
④ GLUTAMATE SYNTHASE (GOGAT)
⑤ GLUTAMATE DEHYDROGENASE (GDH)

FIG.4.8 Pathways of nitrate assimilation.

Valuable information regarding the importance of the pentose phosphate pathway in the respiratory breakdown of glucose can be obtained by comparing the radioactivity of the CO_2 produced from equal samples of tissue, one supplied with [1-^{14}C]-glucose and the other with [6-^{14}C]-glucose. This comparison gives rise to the "C_6/C_1 ratio" introduced initially by Bloom and Stetten (1953) as a means of demonstrating the participation of the pentose phosphate pathway in animal tissues. The important difference in this respect between the two main pathways of glucose catabolism is that C_1 is the first carbon to appear as CO_2 from the pentose phosphate pathway, and is unaccompanied by C_6. The first carbon atoms to appear from the Embden-Meyerhof-Parnas (EMP) sequence are C_3 and C_4, however, and C_1 and C_6 eventually appear indistinguishably. Drainage of some tricarboxylic acid cycle intermediates into cellular constituents may cause the EMP contribution to be underestimated to an extent equal to the amounts of C_1 and C_6 retained. Although it may be difficult, therefore, to assign exact values to the relative contributions of the EMP and pentose phosphate pathways, the determination of "C_6/C_1 ratios" is an extremely useful preliminary approach to the comparison of carbohydrate metabolism in similar cells receiving different experimental treatments.

The effects of 2,4-D-depletion on nitrate assimilation and glucose catabolism were observed from determinations of *in vivo* nitrate reductase activity and "C_6/C_1 ratio" using cells washed free of extracellular 2,4-D and incubated, as 70cm^3 batch cultures, in the standard medium minus- or plus-2,4-D (1mg dm^{-3}).

The data shown in Fig.4.9 show that differences in nitrate reductase activity, between plus- and minus-2,4-D cultures, became apparent after 80 hours incubation. Irrespective of whether activity is expressed per cm^3 of culture (Fig.4.9(a)) or per cell (Fig.4.9(b)) the patterns of activity are such that the nitrate reductase activity of 2,4-D-starved cells initially reached a higher value than the control but then decayed away more rapidly.

FIG.4.9 The effects of 2,4-D-depletion on nitrate reductase activity. Stock cells were washed free of extracellular 2,4-D and inoculated into plus- (●) or minus-2,4-D (○) media and incubated as 70cm^3 batch cultures. Nitrate reductase activity is expressed per cm^3 of culture (a) and per 10^6 cells (b).

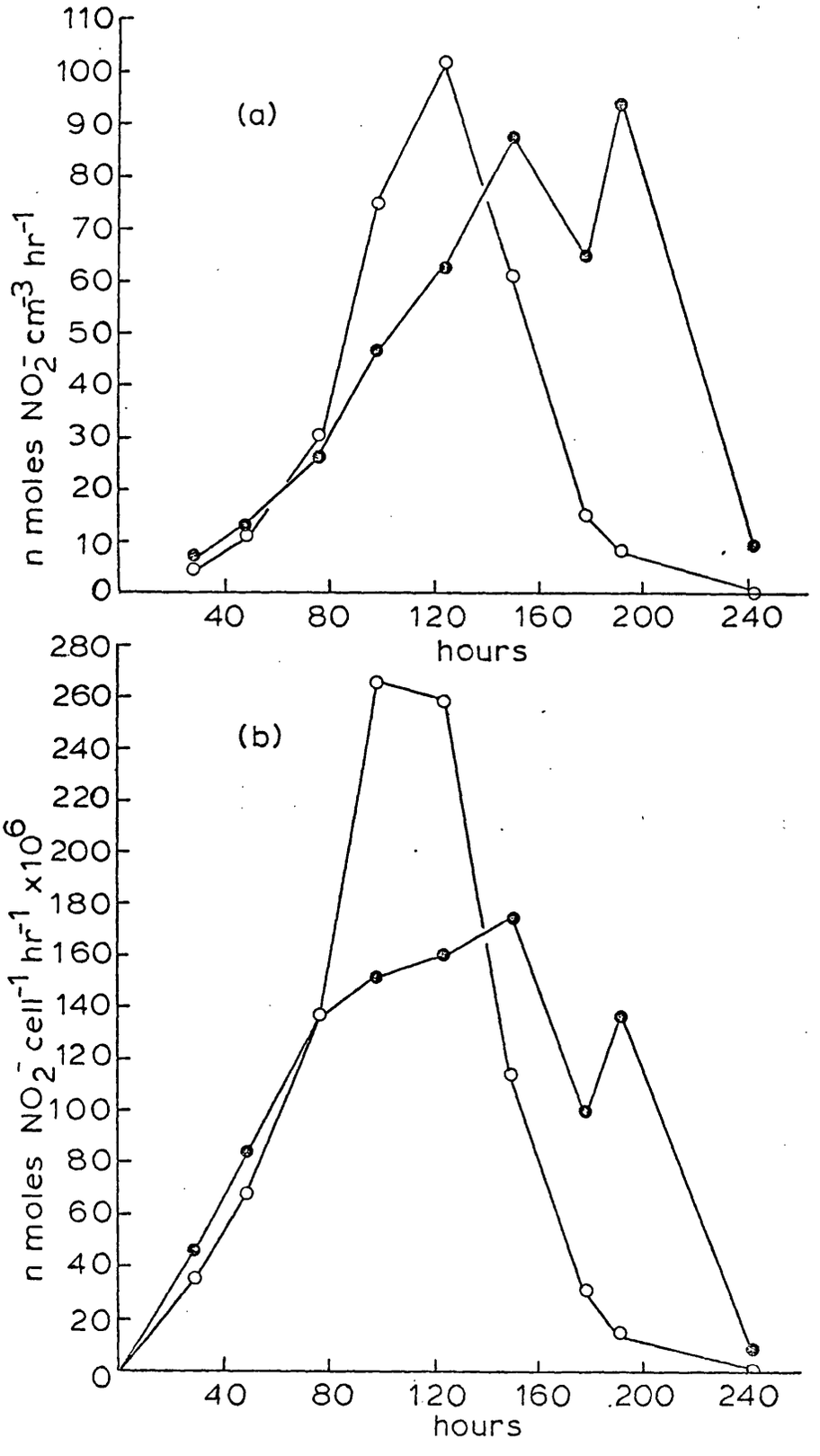
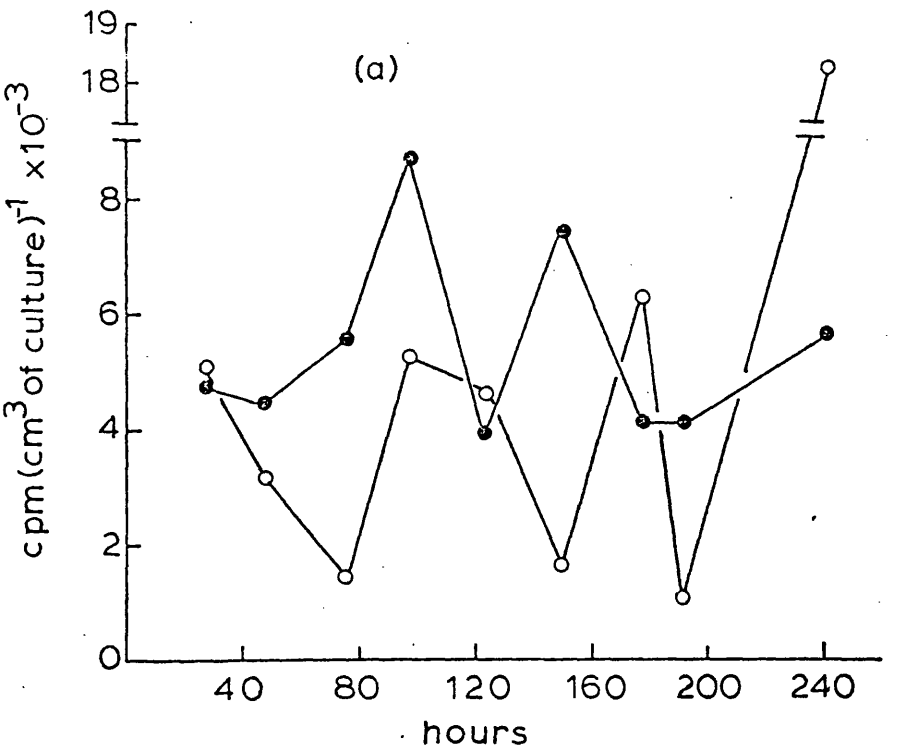

(a)
n moles NO_2^- cm^{-3} hr^{-1}
110
100
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
240
hours
(b)
n moles NO_2^- $cell^{-1}$ hr^{-1} $x10^6$
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
40
80
120
160
200
240
hours

FIG.4.10 The effects of 2,4-D-depletion on the release of $^{14}CO_2$ from [1-^{14}C]-glucose (a) and [6-^{14}C]-glucose (b). Symbols as for Fig.4.9.


(a)
19
18
8
6
4
2
0
cpm (cm3 of culture)-1 x10-3
40
80
120
160
200
240
hours

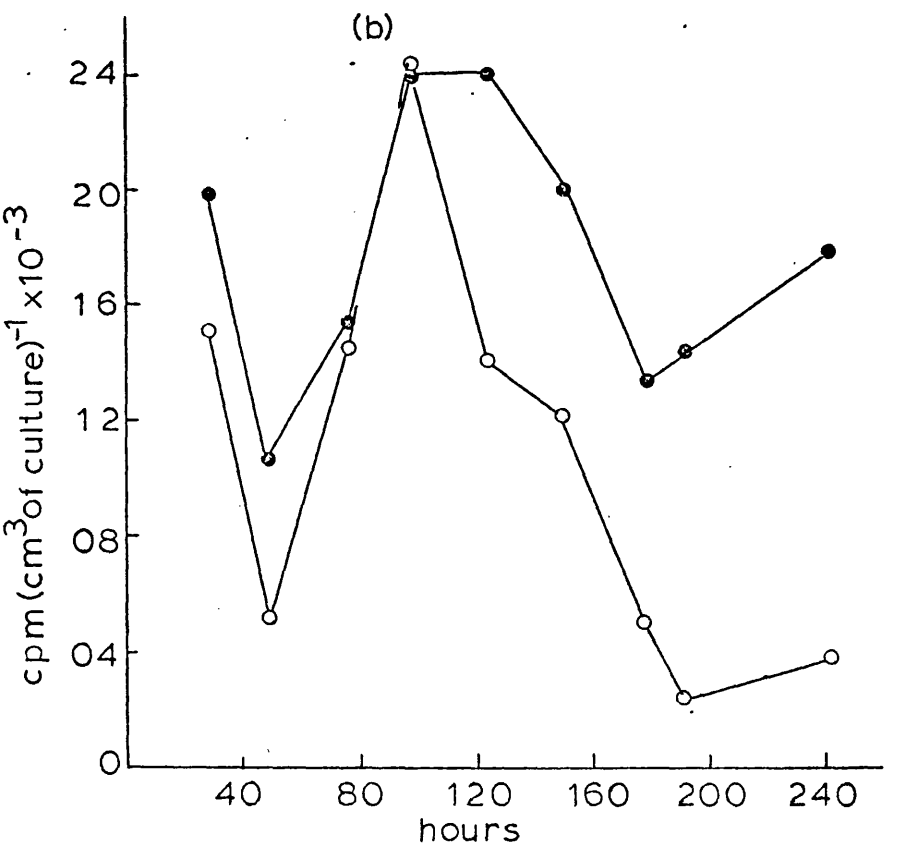
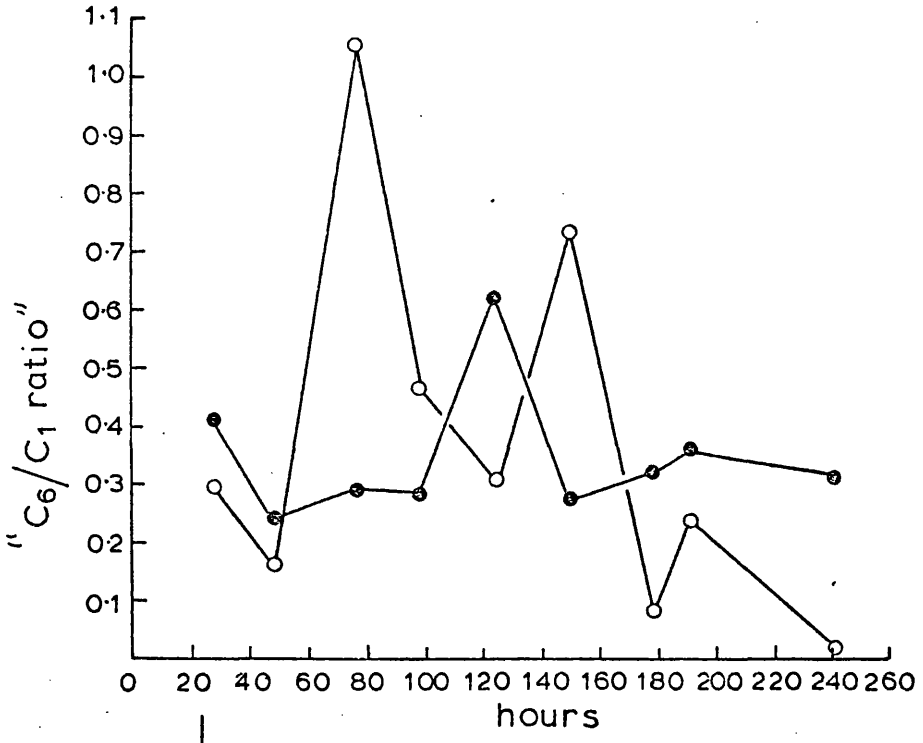
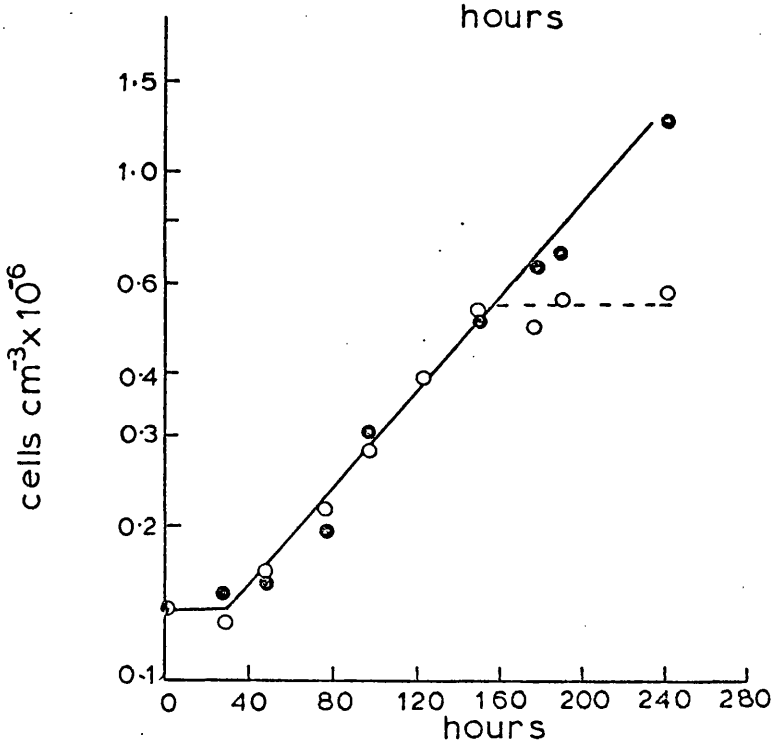

(b)
24
20
16
12
08
04
0
cpm (cm3 of culture)-1 x10-3
40
80
120
160
200
240
hours

FIG.4.11 The effects of 2,4-D-depletion on glucose oxidation as measured by C_6/C_1 ratio. Symbols as for Fig.4.12.

FIG.4.12 The growth of cultures used to determine the changes in nitrate reductase activity (Fig.4.9) and pathways of glucose oxidation (Figs.4.10 and 4.11) in washed cells incubated in plus-(●) or minus-2,4-D (O) media.


"C6/C1 ratio"
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0 20 40 60 80 100 120 140 160 180 200 220 240 260
hours


cells cm⁻³ x 10⁻⁶
1·5
1·0
0·6
0·4
0·3
0·2
0·1
0 40 80 120 160 200 240 280
hours

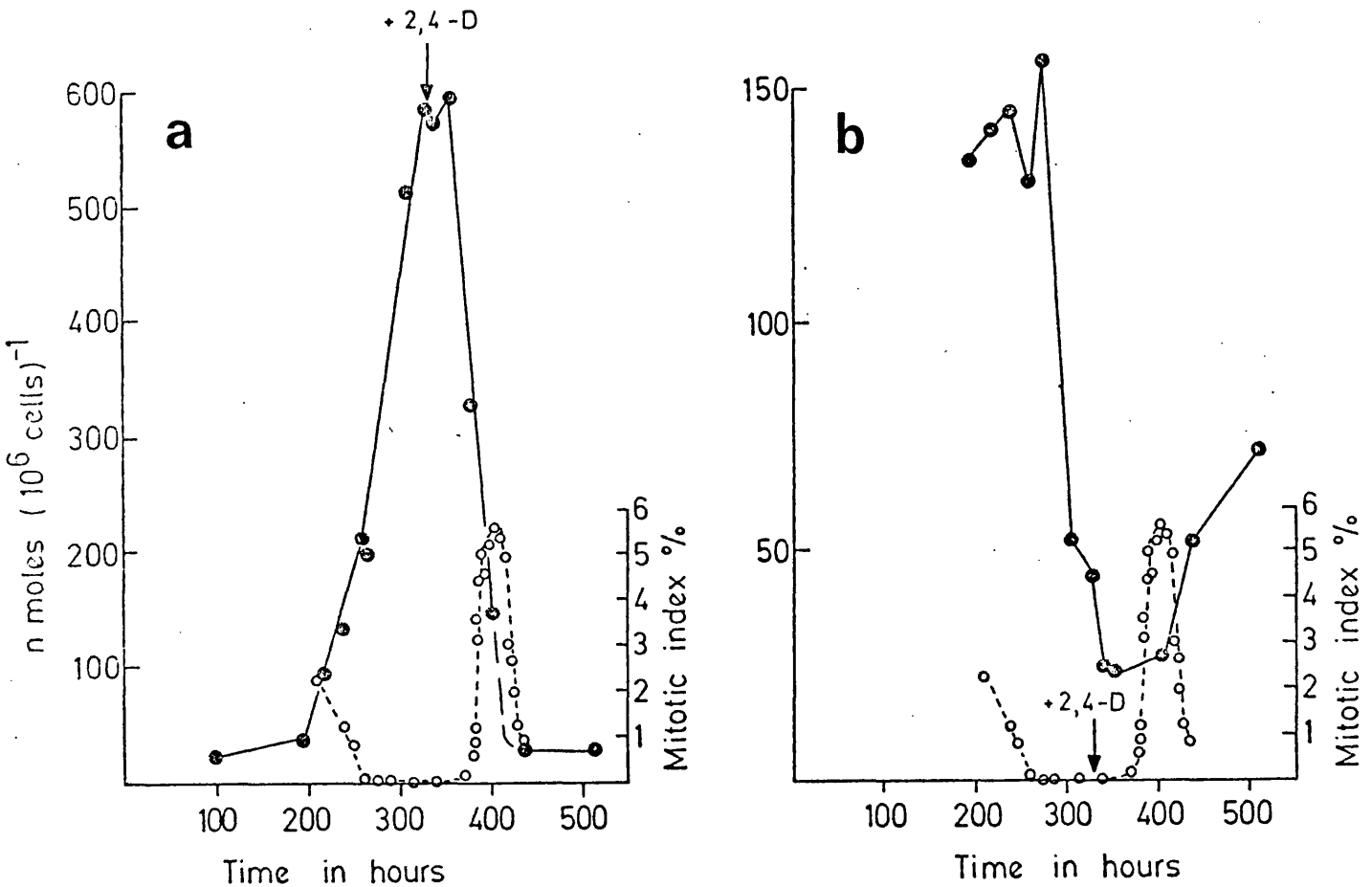
Complex changes throughout the growth cycle were detected for the $^{14}CO_2$ released from [1-^{14}C]-glucose (Fig.4.10(a)) or [6-^{14}C]-glucose (Fig.4.10(b)). These changes were not due to differences in uptake of label from the incubation medium and, if it can be assumed that most of the ^{14}C in the incubation medium was still in the form of glucose, uptake represented less than 35% of the supplied label. Calculation of the "C_6/C_1 ratio" (Fig.4.11) gave a relatively constant value for plus-2,4-D cells but varied widely between unity and zero for minus-2,4-D cells. These variations of "C_6/C_1 ratio" and nitrate reductase activity began well before cell division arrested (Fig.4.12) and therefore suggest that the cells, detect and respond to, the absence of 2,4-D well before effects on growth are expressed. As appreciable nitrate reductase activity was present when cell division arrested, it is unlikely that this arrest, or early changes in amino acid metabolism, are due to a lack of reduced nitrogen.

4.5 The kinetics and reversibility of serine accumulation and glutamate depletion.

If some of the growth following 2,4-D withdrawal is due to intracellular auxin (see SECTION 2) the time separating the detection of subthreshold auxin levels and observed responses is difficult to determine. An alternative approach is to observe the delay between the readdition of 2,4-D, to 2,4-D-starved cells, and the disappearance of minus-2,4-D characteristics.

The first part of this experiment not only provided cells exhibiting responses characteristic of 2,4-D-depletion, but also served as a repeat of the experiment described in SECTION 4 which may have been complicated by unusual growth characteristics. Similarly the regrowth period, after the addition of 2,4-D, was used to study not only the reversibility of serine accumulation and glutamate depletion but also to determine whether regrowth proceeded synchronously (see SECTION 5.2).

FIG.4.13 The effects of 2,4-D withdrawal and re-addition on the soluble pools of serine (a) and glutamic acid (b) of sycamore cells in a $4dm^3$ batch culture. Stock cells were washed free of extracellular 2,4-D and inoculated into minus-2,4-D medium. The arrows indicate the time of addition of 2,4-D and the mitotic index data (O) show when cell division became arrested and was re-initiated.


+ 2,4 -D
a
600
500
400
300
200
100
n moles (10^6 cells)$^{-1}$
100 200 300 400 500
Time in hours
6 5 4 3 2 1
Mitotic index %
b
150
100
50
+2,4-D
100 200 300 400 500
Time in hours
6 5 4 3 2 1
Mitotic index %

A large batch culture vessel (MATERIALS and METHODS Plate 2), containing 9dm^3 of minus-2,4-D medium was inoculated with cells previously washed free of extracellular 2,4-D. To ensure an adequate supply of nutrients for regrowth, after the addition of 2,4-D, the culture medium initially contained twice the usual concentration of inorganic salts and vitamins and 3% w/v sucrose (usually 2% w/v). The initial cell density was 5×10^4 cells cm^{-3} and growth was followed by total protein, cell number and mitotic index determinations, the results of which are fully described in SECTION 5.2. For the purpose of this experiment the growth data are only required to indicate when cell division activity ceased and was re-initiated, therefore only relevant portions of the mitotic index data are included in Fig.4.13. The increase in cell number of this culture ceased abruptly as in the 70cm^3 cultures (e.g. Fig.1.4) but in contrast with the 4dm^3 culture described in SECTION 4.3. Thus the different growth pattern observed in Fig.4.4 was not due to any inherent difference between shake-flask and fermenter cultures.

The intracellular levels of free serine and glutamate were determined using a Technicon Auto Analyzer and are expressed on a per cell basis in Fig.4.13. However, as the cell number did not increase between 250 hours and 380 hours, the same pattern of changes would be observed for serine and glutamate per unit volume of culture. Thus there was depletion or consumption of glutamate and not just a subdivision of a constant amount into an increasing number of cells. The data presented in Fig.4.13 show that serine accumulation preceded, while glutamate depletion followed, the cessation of mitotic activity observed after 2,4-D depletion. Similarly the addition of 2,4-D appeared to rapidly halt the accumulation of serine and after a lag of about 24 hours the level of soluble serine began to rapidly decline to standard plus-2,4-D levels. Glutamate was much slower to return to plus-2,4-D levels than serine. It can be seen from Fig.4.7 that serine accumulation is transient even without the addition of 2,4-D so

that it could be argued that the halt in serine accumulation may have been coincidental with, but not caused by, the addition of 2,4-D. This explanation is thought to be unlikely as serine accumulation continued for 150 hours in the culture shown in Fig.4.7 but for 125 hours when 2,4-D was added (Fig.4.13). Also without 2,4-D addition the rate of decay was exactly equal to the rate of accumulation but when 2,4-D was added the rate of decay was almost twice (x 1.99) the rate of accumulation.

The results of this experiment may have been more decisive if 2,4-D had been added earlier but the reasons for delaying the 2,4-D addition until 70 hours after cell division arrested are described in SECTION 5.2. Certainly any future experiments concerned with the kinetics of 2,4-D action on serine accumulation should either include a parallel control culture to show how long serine accumulation would have preceded without 2,4-D addition or should be designed so that 2,4-D is added immediately the mitotic index declines to zero. The need for such precautions arises from the slow rate of analysis of amino acid extracts on the Auto Analyzer (one sample every 24 hours) and the necessary devotion of experimental time to cell number and mitotic index determinations.

Despite the limitations of this experiment the results do clearly show that changes in the intracellular levels of serine precede changes in glutamate levels both before and after the addition of 2,4-D to 2,4-D-starved sycamore cells. Also, as 2,4-D promotes the return of soluble serine to normal plus-2,4-D levels before the promotion of cell division, changes in the metabolism of this amino acid may be indicative of the final metabolic state, achieved in the absence of 2,4-D, that is not conducive to growth. Certainly, of all the changes in amino acid depletion observed after 2,4-D depletion, serine metabolism appeared to be the most promising candidate for a more detailed investigation.

4.6 Effects of 2,4-D on serine biosynthesis.

In common with all metabolic intermediates an accumulation of serine could result from either increased synthesis or decreased utilization or a combination of both. Three general biosynthetic routes exist for serine (Miflin and Lea 1977) although two of the pathways are very similar and both derive the carbon skeleton of serine from 3-phosphoglyceric acid:

(i) 3-phosphoglycerate $\rightleftharpoons$ glycerate $\rightleftharpoons$ hydroxypyruvate $\rightleftharpoons$ serine

(ii) 3-phosphoglycerate $\rightarrow$ phosphohydroxypyruvate $\rightarrow$ phosphoserine $\rightarrow$ serine

(iii) glycine + N^5N^{10}-methylenetetrahydrofolate $\rightleftharpoons$ serine + tetrahydrofolate

Hydrolysis of 3-phosphogylceric acid yields glyceric acid. The latter, with a NADP-requiring enzyme, provides hydroxypyruvic acid, which may be transaminated in a reaction catalyzed by alanine-serine transaminase or other transaminases to form serine (i). Alternatively, 3-phosphoglyceric acid may be oxidised by 3-phosphoglyceric acid dehydrogenase to give phosphohydroxypyruvate which yields 3-phosphoserine after transamination with glutamate acid. Finally hydrolysis by serine phosphatase yields serine. This pathway (ii) is suggested to be the major route of serine synthesis in animals and microorganisms (White, Handler and Smith), is present in plants (Slaughter and Davies 1968) and may be regulated by the inhibition of 3-phosphoglyceric acid dehydrogenase and serine phosphatase by serine.

The reaction catalyzed by serine hydroxymethyl transferase (E.C.2.1.2.1) (iii) is considered to be a major route of glycine synthesis in animals (Yudkin and Offord 1973). However, the reaction proceeds very slowly to give glycine in wheat leaves (Wang and Burris 1963, 1965) while the reverse reaction, yielding serine, proceeds rapidly. Similarly the interconversion appears to favour serine formation in non-green plant tissues such as castor bean (Ricinus communis L. var. zanzibarensis) endosperm, carrot (Daucus carota) storage tissue (Sinha and Cossins, 1964) and tobacco

(Nicotiana rustica) roots (Prather and Sisler 1972) both in vivo and in vitro (Cossins and Sinha, 1966). Carbon atoms from glycine not only enter serine directly (iii) but also via the generation of the methylene folate carbon (Foo and Cossins, 1978):

(iv) Glycine + H_4PteGlu + NAD $\rightleftharpoons$ 5,10-CH_2H_4PteGlu + CO_2 + NH_3 + NADH

In this reaction the methylene folate carbon is derived from C-2 of glycine while C-1 is eliminated as CO_2. Changes in serine biosynthesis may therefore be most conveniently detected by observing the metabolism of exogenous ^{14}C-labelled 3-phosphoglyceric acid and glycine (C_2-label).

To provide a source of 2,4-D-depleted cells actively accumulating serine, stock Am cells were washed free of extracellular 2,4-D and inoculated at a density of 0.136 x 10^6 cells cm^{-3} into minus-and plus-2,4-D media. The subsequent growth closely resembled that shown in Fig.4.12 such that after 206 hours incubation the cell densities of minus- and plus-2,4-D treatments were 0.668 x 10^6 cells cm^{-3} and 0.965 x 10^6 cells cm^{-3} respectively. Interpolation suggested that cell division in minus 2,4-D cultures arrested after 160 hours incubation. After 206 hours incubation aliquots ($15cm^3$) of minus- and plus-2,4-D cultures were transferred to $100cm^3$ conical flasks, fitted with a centre-well containing 10% w/v KOH ($0.5cm^3$) to trap CO_2, and sealed with rubber suba seals. Labelled 3-phosphoglyceric acid (1µCi, [U-^{14}C]-PGA,) or glycine (1µCi, [2-^{14}C]-glycine) were added at the specific activities supplied by Amersham. The cultures were incubated on an orbital shaker at 25°C for up to three hours and individual flasks harvested after 30, 90, and 180 minutes. The cells from $10cm^3$ of culture were collected on GF/C filter pads and rapidly washed (? x $5cm^3$) with distilled water. After transfer to pyrex test tubes the cells were killed with hot 85% v/v methanol ($5cm^3$) then, after 5 minutes, cooled in iced water and extracted for 48 hours at 4°C.

The radioactivity of the alcoholic extracts and culture medium was determined using the toluene/Triton X-100 scintillation fluid while a

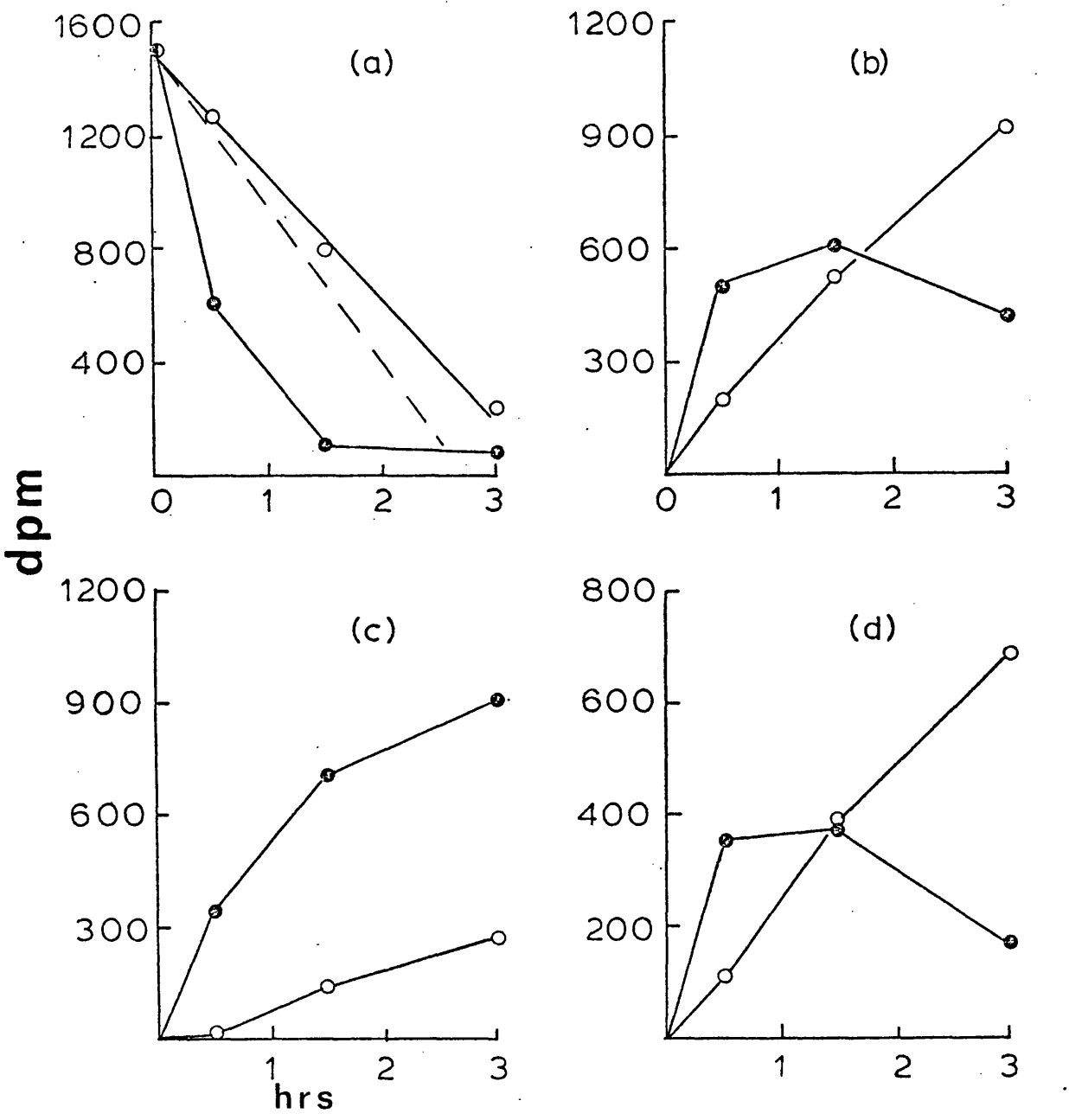


FIG.4.14 The utilization of [2-^{14}C]-glycine by cells growing in the presence of 2,4-D (●) or arrested by the absence of 2,4-D (○). The radioactivities from 10cm^3 of culture found in the culture medium (a), alcohol-soluble compounds (b), alcohol-insoluble compounds (c), and serine (d) are shown for samples taken during a 3-hour incubation period.

TABLE 4.6 The metabolism of [2-^{14}C]-glycine into alcohol-soluble compounds by cells growing in the presence of 2,4-D or arrested by the absence of 2,4-D.

TABLE 4.6 The metabolism of [2-^{14}C]-glycine.

	PLUS-2,4-D						MINUS-2,4-D					
	*30		90		180		30		90		180	
	a	b	a	b	a	b	a	b	a	b	a	b
Acid/neutral fraction	23.8	4.7	53.6	8.9	77.3	18.2	3.0	1.4	16.9	3.2	44.6	4.8
Aspartic acid	6.0	1.2	22.8	3.8	41.7	9.8	-	-	2.0	0.4	2.0	0.2
Serine	358.3	70.7	373.7	61.9	168.4	39.6	111.0	53.1	388.6	74.1	678.9	73.7
Asparagine	3.0	0.6	1.0	0.2	7.9	1.9	-	-	-	-	-	-
Glutamic acid	10.4	2.1	35.7	5.9	19.8	4.6	-	-	-	-	6.9	0.8
Glycine	29.8	5.9	11.9	2.0	5.0	1.2	75.3	36.0	82.2	15.7	67.4	7.3
Alanine	-	-	2.0	0.3	2.4	0.6	-	-	-	-	4.0	0.4
Citrulline	-	-	-	-	5.0	1.2	-	-	-	-	-	-
Cysteine	-	-	2.0	0.3	3.0	0.7	-	-	-	-	-	-
γ-Amino butyrate	15.9	3.1	23.8	3.9	29.8	7.0	1.0	0.5	6.0	1.1	4.0	0.4
Ethanolamine	-	-	-	-	9.9	2.3	-	-	-	-	-	-
Lysine	-	-	-	-	8.4	2.0	-	-	-	-	-	-
Histidine	-	-	-	-	6.0	1.4	-	-	-	-	-	-
Arginine	-	-	-	-	24.8	5.8	-	-	-	-	-	-
Basic fraction	60	11.8	78	12.9	16	3.8	18.7	8.9	28.9	5.5	113.2	12.3
Total alcohol-soluble	507		604		425		209		525		921	
Total uptake	849		1310		1329		228		673		1190	

* Incubation time, minutes.

a dpm from 10cm^3 of culture x 10^{-3}.

b % of alcohol soluble activity.

dioxan-based cocktail was used to determine $^{14}CO_2$ absorbed in KOH during the incubation period. As very little $^{14}CO_2$ was released (< 300cpm, 0.013%) from any of the treatments it was assumed that the difference between the radioactivity detected in the alcoholic extracts and the amount missing from the culture medium represented label incorporated into alcohol-insoluble cellular constituents. Serine was separated from other alcohol-soluble compounds using a modified amino acid analyzer (see MATERIALS and METHODS). The radioactivity found in the culture medium (a), alcohol-soluble compounds (b), alcohol-insoluble compounds (c), and serine (d) is shown in Fig.4.14. Clearly the uptake of glycine by control (plus-2,4-D) cells is much more rapid than that of cells deprived of 2,4-D, even if the difference in cell density (broken line in (d)) is allowed for. These differences in uptake were found to be reflected in both the total alcohol-soluble pool (a) and serine (d), the major labelled compound extracted by 85% methanol. The low rate of incorporation into alcohol-insoluble compounds in the minus-2,4-D cells is unlikely to be simply due to differences in glycine uptake but may represent either decreased utilization of serine or comparable utilization (to the plus-2,4-D cells) from a low specific activity pool. The distribution of ^{14}C within the alcoholic extracts (Table 4.6) shows that the conversion of glycine to serine proceeds efficiently in both plus- and minus-2,4-D cells such that more than 70% of the alcohol-soluble label may be found in serine. The similar percentage conversions of glycine to serine suggest that serine synthesis, by this route, is not enhanced in the absence of 2,4-D. This conclusion would not be valid if the appearance of label in serine was restricted by the rate of uptake of ^{14}C-glycine. The percentage label remaining in glycine in minus-2,4-D cells suggests that this did not occur. The significance of the decreased incorporation of label into other amino acids in minus-2,4-D cells cannot be assessed without knowing whether they were derived from serine or via other routes (e.g. directly from glycine).

TABLE 4.7 The metabolism of [U-^{14}C]-3-phosphoglyceric acid into alcohol-soluble compounds by cells growing in the presence of 2,4-D or arrested by the absence of 2,4-D.

TABLE 4.7 The metabolism of [U-^{14}C]-3phosphoglyceric acid.

	PLUS-2,4-D				MINUS-2,4-D			
	*90		180		90		180	
	a	b	a	b	a	b	a	b
Acid fractions	21.8	36.8	44.6	42.4	6.4	35.8	19.8	35.3
Aspartic acid	4.4	7.5	6.9	6.6	0.6	3.1	3.0	5.4
Serine	-	-	-	-	-	-	3.0	5.4
Glutamic acid	18.9	32.0	33.7	32.1	0.6	3.1	6.4	11.5
Glutamine	-	-	-	-	-	-	6.0	10.7
Proline	-	-	-	-	-	-	0.6	1.0
Alanine	6.0	10.2	5.0	4.8	5.0	27.8	7.9	14.1
Valine	-	-	-	-	-	-	0.6	1.0
Basic fraction	8.0	13.5	14.9	14.2	5.4	30.2	8.8	15.7
Total alcohol-soluble	59.1		105.0		18.0		56.0	

* Incubation time, minutes.

a dpm per 10cm^3 of culture x 10^{-3}.

b % of alcohol soluble activity.

The rate of uptake of phosphoglyceric acid (PGA) by both plus- and minus-2,4-D cells was found to be slow and could not be accurately determined from the activity remaining in the spent medium. Due to the low radioactivity present in the cells only 90 minute and 180 minute samples were analysed for ^{14}C-labelled amino acids. Table 4.7 shows that PGA proved to be a poor precursor of serine in both plus- and minus-2,4-D cells. If PGA entered the cells intact then this would indicate that little endogenous serine was directly PGA-derived. However as exogenous PGA might be expected to encounter phosphatases during uptake perhaps it would be safer to conclude that little serine is glyceric acid-derived. Despite the lack of label in serine, other amino acids did become labelled with glycerate-derived carbon; notably glutamate, alanine and aspartate. The labelling of these amino acids is consistent with the metabolism of PGA by the EMP pathway to give pyruvate which may then act as the precursor for alanine or enter the TCA cycle where ^{14}C- -oxoglutarate would yield labelled glutamate, and ^{14}C-oxaloacetate would give rise to labelled aspartate. Alternatively pyruvate could be converted directly to oxaloacetate by pyruvate carboxylase which satisfies the indispensible need for replenishing TCA intermediates during active growth. Although these labelling experiments were primarily designed to study serine biosynthesis, the reduced incorporation of ^{14}C from both glycine and PGA into glutamate in minus-2,4-D cells may indicate that the net depletion of glutamate (SECTIONS 4.1, 4.2, 4.3) was a result of reduced synthesis.

The results of feeding ^{14}C-labelled precursors of serine gave little indication of any stimulation of synthesis in minus-2,4-D cells, i.e. those actively accumulating serine. If extracellular glycine enters an endogenous pool before being used for serine synthesis the results presented here would suggest that this is the major route of serine biosynthesis in cultured sycamore cells. The resulting hypothesis must then be that serine accumulation, after 2,4-D withdrawal, is the result of reduced serine

utilization. Even so, as the appearance of label in serine may have been restricted by the intracellular availability of ^{14}C-glycine additional information was required before this hypothesis could be accepted.

4.7 Studies of serine utilization by pulse-labelling

The most convenient way of testing the hypothesis of reduced serine metabolism in the absence of 2,4-D appeared to be by pulse-labelling plus- and minus-2,4-D cells with labelled serine. The rate of loss of label from the alcohol soluble (serine) pool, after removing extracellular ^{14}C-serine, should then be a measure of the rate of serine utilization. This method relies rather heavily on a number of assumptions that may or may not be valid for the cells in question. Firstly it must be assumed that most of the serine extracted by 85% methanol is in a single metabolic pool and secondly that this pool represents metabolically available, rather than stored, serine. If these assumptions are valid then the specific activity of serine may be calculated to correct for effects of pool size on serine turnover. Because of these assumptions it could only be safely assumed that reduced serine utilization had been demonstrated if the rate of utilization in minus-2,4-D cells approached zero.

As in the previous experiment a source of 2,4-D-starved cells actively accumulating serine was required. The cells used for this experiment were those described in SECTION 4.4, 242 hours after washing in minus-2,4-D medium, when the cell densitied in plus- and minus-2,4-D media were 1.250×10^6 and 0.581×10^6 cells cm^{-3} respectively (Fig.4.12). The growth data suggested that cell division arrested after about 160 hours, therefore serine should still have been accumulating at 242 hours (see SECTIONS 4.3 and 4.5).

Pulse labelling was achieved by incubating plus- or minus-2,4-D cultures ($60cm^3$) with 2.5μCi of L-[U-^{14}C]-serine (170mCi/mmol) for 30

minutes in wash-flasks fitted with a glass-sinter side-arm (see MATERIALS and METHODS Fig.1). The use of these flasks facilitated rapid and complete washing of the cultures with the minimum of handling. After 30 minutes incubation on an orbital shaker at 25°C the radioactive medium was separated from the cells by applying gentle vacuum to the wash-flask. The cells were washed ($50cm^3$) and resuspended ($60cm^3$) in medium obtained from equivalent cultures that had not received any serine addition. The use of fresh (i.e. unconditioned) medium for washing was avoided to minimize the effects of the washing procedure itself on the cells. Samples ($5cm^3$) of plus- and minus-2,4-D cultures were taken at regular intervals after the removal of label, washed (2 x $5cm^3$) with distilled water and killed in 85% v/v methanol in water ($5cm^3$). After the extraction of soluble amino acids (see MATERIALS and METHODS) the cells were extracted with 3% w/v sodium dodecyl sulphate (SDS) ($4cm^3$) for 30 minutes at 100°C, with mixing. After cooling to room temperature the SDS extracts were filtered through GF/C filter pads and made up to $10cm^3$. The radioactivity of the SDS extracts was determined using the toluene/Triton X-100 scintillation cocktail. The radioactivities found in alcohol- or SDS-soluble compounds after pulse labelling with L-[U-^{14}C]-serine are shown in Table 4.8. These show that, at the end of the labelling period (t = o), the 2,4-D-starved cells contained approximately twice the amount of alcohol-soluble label found in control (plus-2,4-D) cells. Once the external supply of ^{14}C-serine was removed, the alcohol-soluble label declined steadily in control cells but remained relatively constant in minus-2,4-D cells. Fractionation of alcohol-soluble compounds, using the amino acid analyser, showed that almost all the alcohol-soluble label of minus-2,4-D cells was confined to serine while a significant proportion of the alcohol-soluble label of control cells was not accounted for by serine.

TABLE 4.8 The results of pulse-labelling cells growing in the presence of 2,4-D or arrested by the absence of 2,4-D with [U-^{14}C]-serine. The labelling period (30 minutes) was terminated by washing at time t=0.

TABLE 4.8 The metabolism of [U-^{14}C]-serine.

	*t (min)	ALCOHOL-SOLUBLE LABEL (dpm x 10^{-3})				SDS-SOLUBLE LABEL (dpm x 10^{-3})	
		total (10^6 cells)$^{-1}$		serine (10^6 cells)$^{-1}$			
PLUS-2,4-D	10	273.5	43.8	202.2	32.4	177.6	28.4
	25	227.4	36.4	147.8	23.7	171.4	27.4
	40	192.9	30.9	119.4	19.1	164.3	26.3
	70	150.0	24.0	88.0	14.1	172.2	27.6
MINUS-2,4-D	15	234.1	80.6	233.4	80.3	26.6	9.2
	40	231.9	79.8	219.2	75.5	11.7	4.0
	60	250.4	86.2	233.8	80.5	15.4	5.3
	90	238.4	82.1	232.8	80.1	17.2	5.9

*Time after the removal of extracellular ^{14}C-serine.

The data obtained for minus-2,4-D cells could have resulted from either a low rate of serine utilization (compared to control cells) or a large endogenous (i.e. low specific activity) serine pool. The separation of amino acids on the Auto Analyzer permitted the determination not only of ^{14}C-serine but also the total (labelled and unlabelled) serine extracted by alcohol. If the total soluble serine and the labelled serine were derived from a single metabolic pool then the specific activity of serine in minus-2,4-D cells would have been thirty times lower than that of plus-2,4-D cells. If the absolute rates of serine utilization in plus- and minus-2,4-D cells were the same then the difference in specific activities would predict the drop in activity of serine from minus-2,4-D cells to be in the order of 1.5 x 10^3dpm during the 'chase' period. Although one cannot be sure of the degree of possible error in the measurements shown in Table 4.8 it seems possible that the rate of serine utilization in minus-

2,4-D cells was lower than that of plus-2,4-D cells.

An alternative method for determining the rate of utilization of a compound is to measure the rate of appearance of label in the metabolic products of that compound during a pulse-chase experiment. The SDS extracts were intended to fulfil this purpose as SDS is extremely efficient for extracting total protein (Weber and Osbourne, 1975) and, when used hot, will also extract nucleic acids and some polysaccharides (Fry, 1978). However, the data given in Table 4.8 show that although radioactivity was present in the SDS-soluble fraction of control cells this activity did not increase during the chase period, when activity was continuously lost from intracellular serine. These results show that serine was being metabolized into SDS-insoluble compounds (very little label was released as $^{14}CO_2$) and that the activity in the SDS-soluble fraction represented either a steady-state level of intermediates or compounds that only received label during serine uptake and not after the serine had equilibrated with intracellular pools. The latter suggestion may not be as unlikely as it initially sounds as similar behaviour has been observed for the incorporation of extracellular serine into protein in hamster kidney (BHK 21) cells (Allen and Moskowitz, 1978). If incorporation into the SDS-soluble fraction did occur almost directly from the culture medium then the low activity of this fraction in minus-2,4-D cells must reflect a reduced rate of serine utilization. Alternatively if exogenous serine equilibrates with the endogenous pool(s) of serine prior to incorporation in the SDS-soluble fraction one could not propose that the specific activity of metabolically available serine in minus-2,4-D cells was thirty times lower than that of plus-2,4-D cells. Thus although individual results are not conclusive the most likely explanation for the results obtained from the pulse-chase experiment is that the rate of serine utilization in 2,4-D-starved sycamore cells is lower than that of control cells.

DISCUSSION

The experiments described in this SECTION were all directed towards identifying the nature and cause of the changes in amino acid metabolism observed during 2,4-D-starvation. It has been shown that changes in the level of the single amino acid, serine are almost entirely responsible for the accumulation of soluble amino-nitrogen in 2,4-D-starved sycamore cells (SECTIONS 1 and 2). Less drastic changes in the soluble amino acid pool, notably glutamate depletion, are also observed following 2,4-D withdrawal and appear to be related to a change in the pattern of nitrogen assimilation. This change in nitrogen assimilation is not caused by a loss of nitrate reductase activity but may be due to a reduced flux of carbon skeletons into glutamate. The eventual appearance of substantial amounts of asparagine should not be surprising, therefore, as this amino acid is readily synthesized under conditions of excess reduced-nitrogen (Miflin and Lea 1977)

Neither serine accumulation nor glutamate depletion directly cause the cessation of growth in the absence of 2,4-D, but the kinetics and reversibility of serine accumulation suggest that this response could be related to the cessation of growth. Labelling studies showed that glycine is a much more efficient precursor of serine than is phosphoglyceric acid and that the efficiency of this conversion appeared to be similar in both plus- and minus-2,4-D cells. The results of pulse-labelling cells with ^{14}C serine were not conclusive but were consistent with a reduced rate of serine utilization in 2,4-D-starved cells. Thus, considering all the labelling data, the most likely cause of serine accumulation following 2,4-D withdrawal is that serine utilization is inhibited while serine biosynthesis proceeds unhindered. The significance of this behaviour with respect to growth, cannot be assessed here as the identities of the products synthesized from serine in control cells are not known, except that they

are not soluble in alcohol or SDS.

The problems encountered in the labelling experiments show that any future experiments designed to either repeat the pulse-labelling experiment or identify the products of serine utilization should use mitotic index, rather than cell number, estimations to determine when cell division activity ceased after 2,4-D withdrawal. This would permit labelling experiments to be executed using plus- and minus-2,4-D cells which contained similar endogenous pools of serine.

SECTION 5

2,4-D AND THE CELL CYCLE

INTRODUCTION

An important consideration in studies of auxin action relates to whether their cell division stimulating properties result from a direct impact on the cell cycle or an indirect effect via growth promotion. The term 'cell cycle' is used to describe the series of metabolic and structural events which occur in the interval separating successive divisions of a cell into two daughter cells. The simple classical model (Fig.5.1) comprises a cycle in which the time between successive nuclear divisions (mitoses) is subdivided into G1, S (the period of DNA synthesis) and G2.

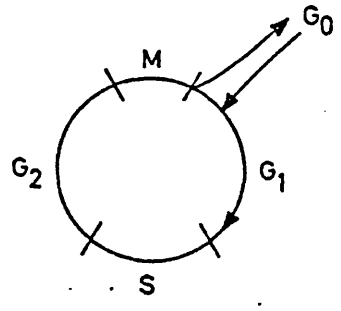
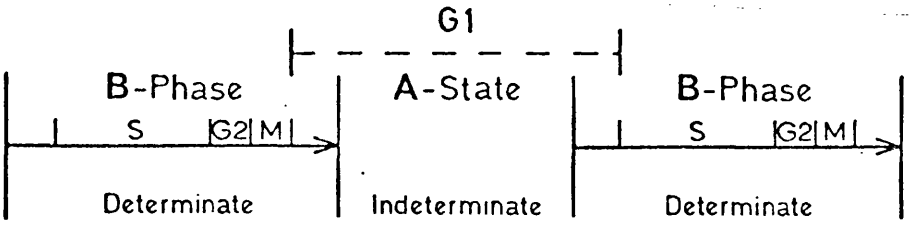


Fig.5.1 The classical cell cycle.

This simple cell cycle model suggests a series of consecutive events requiring a causal relationship between one event and the next. When such a model is applied to slowly proliferating cells the existence of an additional fundamental state is invoked. This state, generally called G0, can be of indefinite duration and represents cells that are not committed to nuclear division. Many modifications to the basic model have been made (Nagl, 1974; Peaud-Lenoel, 1977) which make some allowances for unconnected or non-consecutive pathways within the cell cycle. An alternative model, described by Smith and Martin (1973), proposes that the intermitotic period is composed of a B-phase (Fig.5.2) which is deterministic and includes S,

G2, M and part of G1, and an A-state which cells enter some time after mitosis. Cells then leave the A-state, to initiate DNA replication and cell division (B-phase), with a constant, time-independent transition probability. Initiation of cell replication processes is thus random, in the sense that radioactive decay is random. This model, if valid for plant cells, would require a re-assessment of the concept of G0 and, in the absence of entrainment, would predict a rapid loss of synchrony.



Redrawn from Smith, J. A. and Martin, L.
Proc. Nat. Acad. Sci. USA (1973) 70, 4, 1263-1267

Fig. 5.2 The G1 transition probability model.

Studies of the hormonal regulation of events in the cell cycle may be facilitated by the use of populations of cells dividing synchronously. Some confusion seems to exist in the nomenclature associated with synchrony. Scherbaum (1964), for instance, suggested that cultures obtained by selection should be termed synchronous while those induced to divide synchronously should be termed synchronized cultures. The term synchronized has been used very loosely when applied to plant cells to include cells progressing through the cell cycle in unison (Komamine et al, 1978), cells exhibiting periodic bursts of mitotic activity (Constabel et al, 1977) and even multicellular bodies at a similar stage of development, such as globular embryos (Komamine et al, 1978). In the following work the term 'synchronous' has been reserved for cell populations exhibiting nuclear (cell cycle) synchrony as opposed to periodic bursts of (synchronized)

growth.

The approach used in this study to investigate auxin regulation of events in the cell cycle was to assume that withdrawal of 2,4-D from 2,4-D-dependent cells would give rise to a population of cells arrested immediately prior to any 2,4-D requiring stage. Such an arrested population might be expected to exhibit synchronous growth once the 2,4-D supply was re-established. As many metabolic processes are indirectly required for continued progress through the cell cycle extremely critical data interpretation is required to distinguish between synchronous and synchronized growth and, more particularly, to determine the degree of synchrony.

5.1 Regrowth following prolonged 2,4-D starvation

The 4 dm^3 culture described in section 4.3 provided a source of cells, arrested by 2,4-D depletion, which were used to determine the reversibility of such growth inhibition. Fresh medium (2 dm^3) containing 2,4-D (2 mg dm^{-3}) was added to the 2 dm^3 of arrested culture that remained 200 hours after the increase in cell number had ceased. Fig.5.3 shows that before growth, by dry weight or cell number accumulation, was resumed the total cellular protein in the culture showed transient accumulation which increased in amplitude on each oscillation (14%, 36%, 250%). During the third pulse of protein accumulation, about 150 hours after the addition of 2,4-D, cell number and dry weight began to increase. The pattern of increase in cell number could be interpreted as exponential growth (linear semi-log plot) with a population doubling time (td) of 116 hours. The periodicity of the protein data, however, casts some doubt on the validity of assuming that cell number increases followed a simple exponential relationship. From the limited data available it was certainly feasible that increase in cell number was discontinuous and could even have been compatible with partial synchrony (Fig.5.3, broken line). The accumulation of biomass (dry weight) showed considerable deviations from a simple

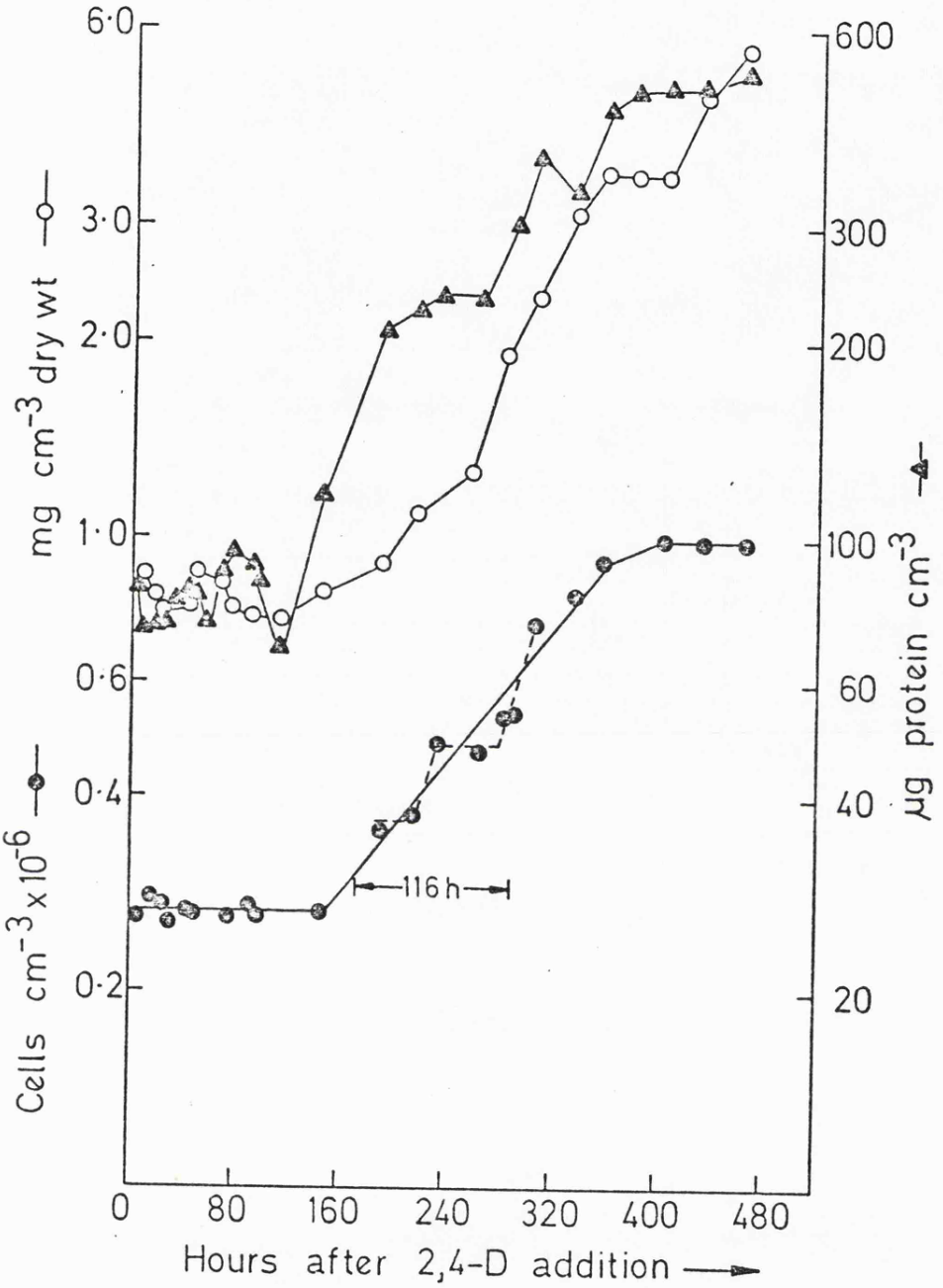


FIG.5.3 Growth as measured by increase in cell number (●), dry weight (○) and total protein (▲) for sycamore cells previously arrested by 2,4-D starvation. Fresh medium containing 2,4-D was added at time 0.

exponential increase although the data presented here do not permit a more detailed interpretation to be made.

It can be concluded that, although growth inhibition following 2,4-D starvation is reversible, the subsequent regrowth is not unaffected by the preceding starvation.

5.2 Regrowth following a short 2,4-D starvation

The experiment described above was designed to investigate the reversibility of growth inhibition following 2,4-D starvation. The apparent periodicity of a number of parameters during the regrowth period suggested that a more detailed investigation was required to decide whether or not the periodicity was a consequence of synchronous regrowth. Although Gould (1975) had reported that 2,4-D starved sycamore cells did not appear to arrest at a particular stage of the cell cycle Nishi et al (1977) reported partial synchronization of a carrot cell culture by auxin deprivation.

A number of modifications were made to the experimental design described in section 5.1 to facilitate analysis of growth in terms of induced synchrony. The culture vessel employed was one of 9 dm^3 capacity, to permit frequent sampling over a long period, and was fitted with the automatic sampling assembly (see MATERIALS and METHODS) to permit regular 'round-the-clock' sampling. The automatic samples of culture were dispensed into an equal volume of 60% v/v formic acid to fix the cells for subsequent mitotic index determination. Such automatic sampling is invaluable when one operator is studying a synchronous culture as this permits short rest-periods without missing rapid changes in mitotic index. Samples for cell number, total protein and amino acid analyses were removed from the culture manually.

A criticism that could be made of the experiment described in section 5.1 is that metabolic perturbations during the regrowth period may have resulted from the addition of fresh medium rather than 2,4-D. If

2,4-D alone was to be added to the culture it was thought desirable that the initial culture medium should be nutrient-enriched to avoid nutrient limitation during the early stages of regrowth. The culture medium therefore initially contained twice the usual concentrations of inorganic salts and vitamins and 3% w/v sucrose (usually 2% w/v).

The exact time of 2,4-D addition to cells arrested by 2,4-D depletion was also thought to be important. If the 2,4-D addition was delayed too long then some of the characteristics of regrowth could result from the deprivation of active growth rather than 2,4-D. This consideration was particularly important as this culture was also being used to study the reversibility of changes in amino acid metabolism during the starvation period (see section 4.4). If the 2,4-D addition followed immediately after cell division arrest then recently formed daughter cells could not progress through the cell cycle to a point distant from G1 (eg. a G2 block) to contribute to synchronous regrowth. A suitable compromise was considered to be one in which the addition of 2,4-D was delayed until approximately one population doubling time after the mitotic index declined to zero.

Am strain sycamore cells (14 days from subculture) were washed free of extracellular 2,4-D and inoculated into 9 dm^3 of minus-2,4-D medium to give an initial cell density of 5×10^4 cells cm^{-3}. After an initial lag period, exponential growth (td = 62 hours) continued for about 190 hours (Fig.5.4) to give a population of arrested cells at a density of 0.4×10^6 cells cm^{-3}. 2,4-D was added as a concentrated aqueous solution (100 mg dm^{-3}), 70 hours after the mitotic index declined to zero, to give an initial concentration of 1 mg dm^{-3} (4.5×10^{-6}M). Mitotic figures began to appear in the culture 40 hours after the addition of 2,4-D and rose to give a maximum mitotic index of 5.6% 34 hours later. Subsequent changes in the mitotic index took the form of periodic oscillations. Despite prolonged mitotic activity only a 38% increase in cell number was observed during the first mitotic wave (66 hours duration). As previously observed (Fig.5.3), the rate of increase in cell number was low (td = 150 hours) when compared

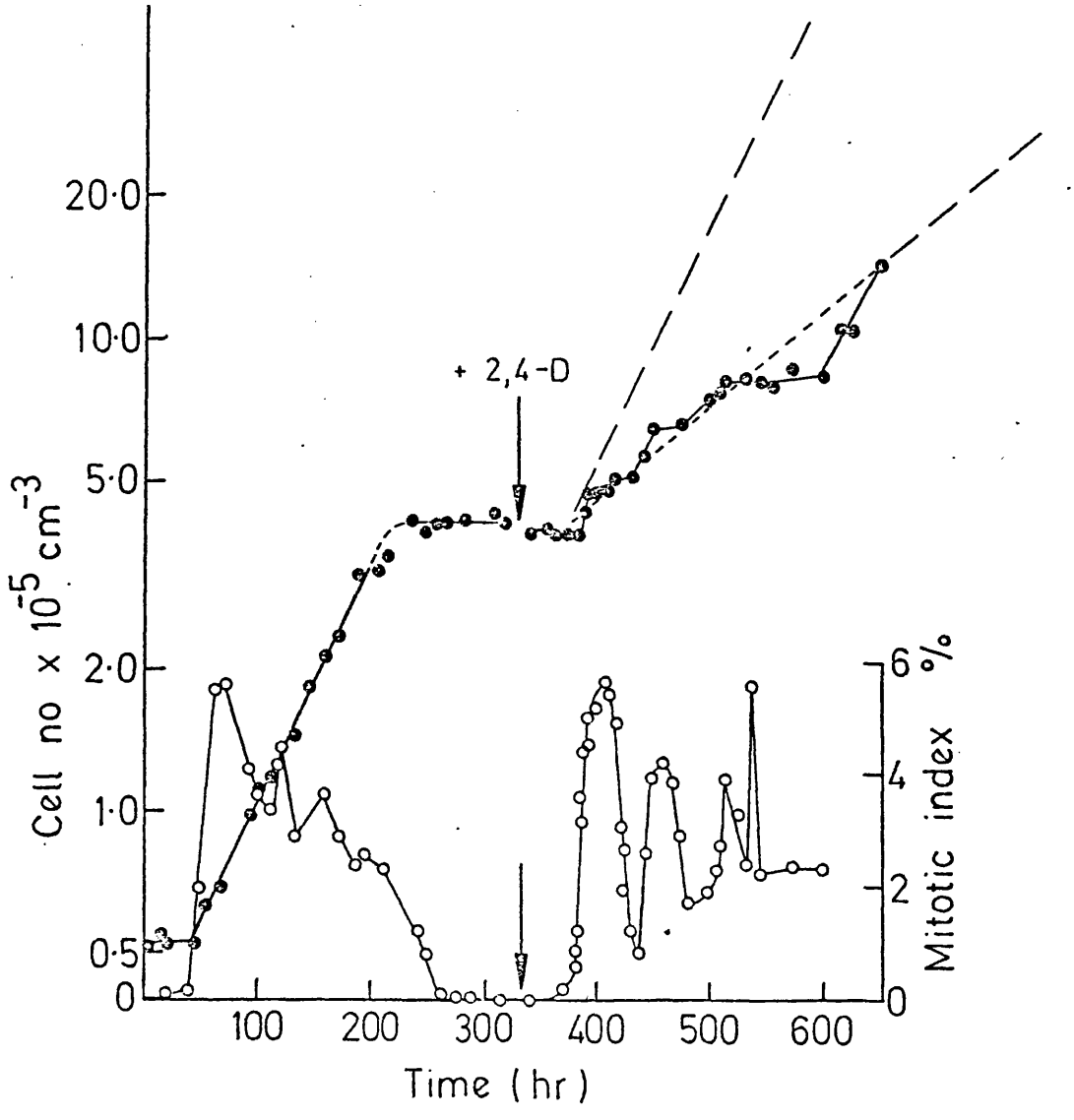


FIG.5.4 The effect of a short 2,4-D starvation on cell division activity in sycamore cells. Washed cells were inoculated into minus-2,4-D medium at time zero, and the arrow marks the time of addition of 2,4-D. Cell division activity was measured by mitotic index (O) and increase in cell number (●). The dotted lines provide a comparison of the mean growth rates before and after the 2,4-D starvation.

with the same culture before growth became arrested (td = 62 hours). This slow growth again made precise interpretation of the cell number data difficult although the suggested periodic bursts of cell division were now supported by periodic fluctuations in mitotic index.

Oscillations in mitotic index alone have often been considered to be evidence for the induction of synchrony (Jouanneau, 1971; Constabel et al, 1977). Changes in growth rate, or more particulary cell cycle time, may also have profound effects on the mitotic index of a population (King and Street, 1977). Before interpreting the experimental data in detail, therefore, it may be useful to consider the theoretical extremes in behaviour that might be expected. The alternative models of growth to be considered are:-

(a) Exponential asynchronous growth

(b) Exponential synchronous growth (no decay of synchrony with time)

(c) Periodic bursts of exponential asynchronous growth

Fig.5.5 shows the changes in cell number (A) and mitotic index (B) that would be predicted for such idealized systems. As experimental systems rarely exhibit ideal behaviour intermediate growth patterns might also be expected.

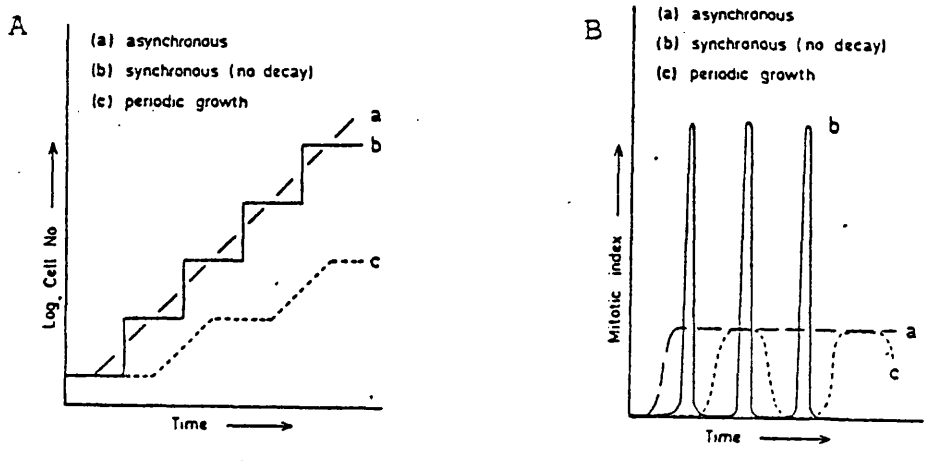


Fig.5.5 Model growth patterns.

The onset of exponential asynchronous growth should be marked by a rise in mitotic index to a value which remains constant during growth at a constant rate (linear semi-logarithmic plot of cell number against time).

The absolute value of the mitotic index during such asynchronous exponential growth is a reflection of the duration of mitosis relative to the mean cell cycle time.

If growth, at the same rate, proceeded synchronously the cell number should increase abruptly and double at each step. Similarly the mitotic index should rise rapidly, before the rise in cell number and, for perfect synchrony should fall to zero before the step in cell number.

Waves of mitosis and discontinuous increases in cell number would also result from periodic bursts of growth. During the periods of growth the cell number would increase exponentially and the mitotic index would approach the value obtained for continuous asynchronous growth. The main characteristics of periodic, as opposed to synchronous, growth are that the rate of cell number increase during each 'step' will approximate to the asynchronous exponential growth rate, and the broad wave of mitotic index will peak at a value similar to the value observed for continuous growth.

Now that the theoretical extremes in behaviour have been described it appears that regrowth following 2,4-D starvation (Fig.5.4) most closely resembles periodic growth rather than synchrony. This suggests that some metabolic system had been made to oscillate in such a way that entry into at least mitosis (M) and cell division was periodically inhibited.

It is well known that cells which have stopped proliferating characteristically do so in G1 or G2 but not S or M (Van't Hof and Kovacs, 1972; Van't Hof et al, 1973). Therefore unless cells are suddenly and totally arrested, S and M will be completed to give an arrested population enriched with cells in G2 and G1. This behaviour has been observed for cells arrested by carbohydrate starvation (Van't Hof and Kovacs, 1972; Van't Hof et al, 1973; Gould, 1975), phosphate starvation (Gould, 1975), nitrate starvation (Gould, 1975), 2,4-D starvation (Gould, 1975) and supra-optimal auxin (Scadeng and MacLeod, 1977). Growth from populations of arrested or quiescent cells in which all stages of the cell cycle are not represented might be expected to exhibit at least partial synchrony. This

is the basis of the synchronous growth observed when explants of Jerusalem artichoke (Helianthus tuberosus) enter culture (Aitchison et al, 1977; Yeoman et al, 1977) and when sycamore cells are supplied with a complete medium following nitrate starvation (King et al, 1973; King et al, 1974). The degree of synchrony will depend on the distribution of cells between and within G1 and G2. Thus the cell population represented in Fig.5.6 (A) and (B), although representing similar distributions between G1 and G2, would give rise to very different degrees of synchrony. Even the G1-arrested population in 5.6 (B) may not give rise to appreciable synchrony if the position of arrest preceded an A-state with a low transition probability (Smith and Martin, 1973).

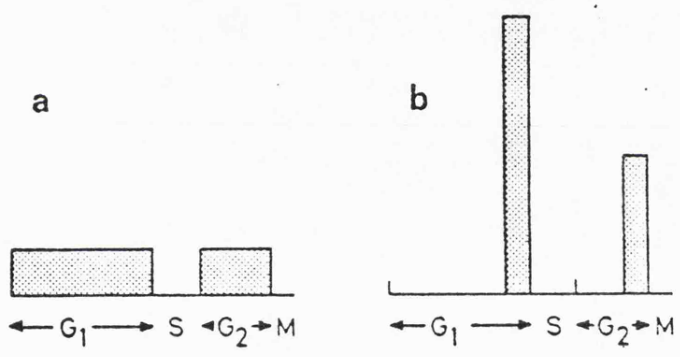


Fig.5.6 Hypothetical frequency profiles of G1/G2 arrested cell populations.

The complete absence of mitotic figures in sycamore cells starved of 2,4-D shows that arrest is not indiscriminate. However, the degree of partial synchrony following 2,4-D starvation would be very difficult to determine when superimposed on periodic growth.

The mitotic index (MI) during steady state or perfectly synchronous growth can be related to the duration of mitosis (tm) and the mean cell cycle time (T) by the expression:-

$$MI = \frac{tm}{T}$$

which, for asynchronous exponential growth becomes:-

$$MI = \frac{tm.\ ln2}{T}$$ (see Smith and Dendy, 1962)

If tm and T remained constant before and after 2,4-D starvation then the relationship between MI and increase in cell number should be independent of whether exponential growth occurred continuously or in periodic bursts. A numerical value for this relationship was obtained by integrating the changes in MI with time and expressing the result per doubling of the cell population ($\int_n^{2n}$ (MI)dt). When this procedure was applied to the data in Fig.5.4 the values of $\int_n^{2n}$ (MI)dt obtained for the first and second mitotic peaks after 2,4-D addition were 200% and 150% greater than the value for growth before the addition of 2,4-D. This control value for $\int_n^{2n}$ (MI) dt was obtained from the entire growth period before 2,4-D was added and agreed well with values obtained from batch cultures using the standard plus 2,4-D medium. The initial period of growth of a culture is often characterized by a transiently high mitotic index, however even if this period of growth is used as a control the value of $\int_n^{2n}$ (MI) dt only increases by 5%.

The absolute value of MI is sensitive to the fraction of dividing cells in a population so that the presence of dead or non-cycling cells would give a low value of MI. This culture showed >90% viability throughout the experiment and if the increase in cell number during the first mitotic wave after 2,4-D addition represented one doubling of the cycling population (i.e. partial synchrony) $\int_n^{2n}$ (MI) dt increases to 250% of the control. This makes this interpretation very unlikely as the period between mitotic peaks (52 hours) is less than the normal population doubling time. The apparently single waves of MI are thought to have resulted from a frequency profile similar to that in Fig.5.6 (a) such that any degree of fine structure (double peaks) was not resolved.

It is conceivable that a high value of $\int_n^{2n}$ (MI) dt could result from a failure or disruption of cytokinesis. The data shown in Fig.5.4 would then represent a high proportion of the arrested population entering mitosis

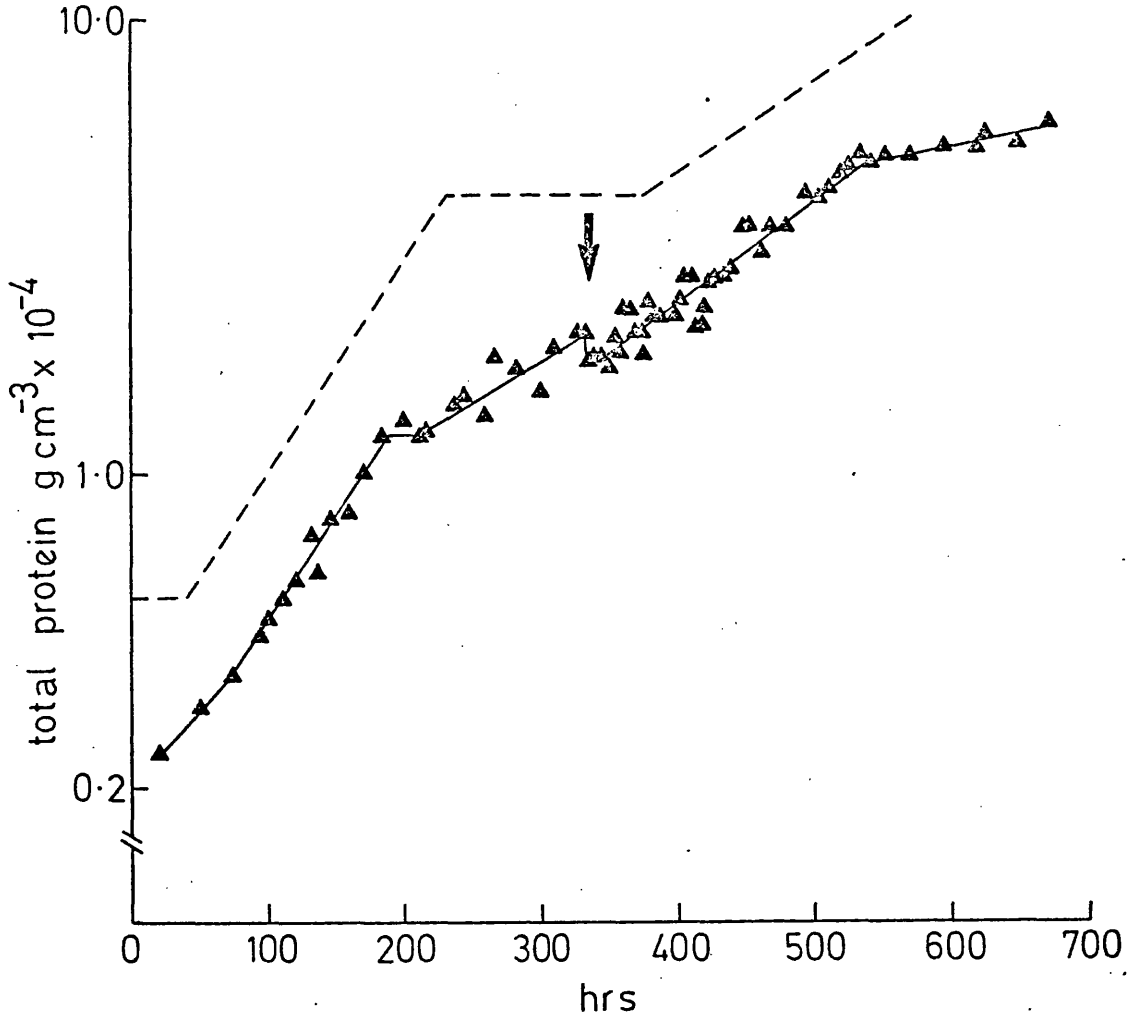


FIG. 5.5 Changes in the rate of accumulation of total cellular protein for the culture described in Fig. 5.4. The arrow marks the time of addition of 2,4-D and the dotted line indicates the changes in the mean rate of cell number accumulation.

with partial synchrony followed by 50% of the population failing to divide into daughter cells. No evidence for multinucleate cells was observed in this culture although this could be accounted for by rapid nuclear fusion. Although this interpretation is thought to be unlikely it cannot be completely discounted without measurements of the DNA per cell.

After considering the alternatives the most reasonable explanation for the data shown in Fig.5.4 would appear to be that the addition of 2,4-D following 2,4-D starvation causes periodic stimulation of growth and that, during the periods of growth, the cell cycle is disturbed in such a way that mitosis occupies a greater proportion of the total cycle time than in cells growing without a previous 2,4-D starvation.

Changes in total cellular protein for the culture described in Fig.5.4 are shown in Fig.5.5. These data indicate that the rate of protein accumulation parallels the rate of increase in cell number although the patterns of changes for both parameters are complex. The deviations of protein accumulation from exponential growth show little signs of the oscillations observed in Fig.5.3 which may therefore have been due to the prolonged absence of growth rather than 2,4-D. It may be of interest to note that the slow rate of protein accumulation during the regrowth period was similar to the rate established before cell division activity ceased.

DISCUSSION

The aim of the work described in this section was to resolve certain disparities in the information regarding auxin regulation of the cell division cycle. The two classes of apparently contradictory information were that, (i) Auxin deprivation could induce synchrony in carrot cells (Nishi et al, 1977)

(ii) NAA deprivation of tobacco cells (Gamburg and Osharova, 1973) and 2,4-D deprivation of sycamore (Gould, 1975) caused cells to arrest in both G1 and G2.

Regrowth from a population of cells representing more than one phase of the cell cycle would only be expected to exhibit very limited synchrony The data presented here suggest that 2,4-D starvation of sycamore cells does not induce synchrony and are therefore consistent with arrest in more than one phase of the cell cycle.

The concept of 'control points' in the cell cycle, such as the 'principal control points' (P.C.P.'s) proposed by Van't Hof and Kovacs (1972), suggests that at various stages of the cell cycle it is determined whether or not cells are going to proliferate. If P.C.P.'s have a precise regulatory function it might be expected that the control could be exerted by specific regulatory molecules, such as hormones. However, in addition to P.C.P's, it might be expected that a number of processes, usually associated with a particular phase of the cell cycle, would have exacting metabolic requirements without possessing a precise regulatory function. Thus if, for example, protein synthesis was blocked it might be expected that cells would arrest at points in the cell cycle which require 'de novo' protein synthesis. When protein synthesis was not totally inhibited, cells would not arrest, so that these points should not be referred to as 'control points' but perhaps 'sensitive points' of the cell cycle. More specifically the positions of cells arrested by auxin deprivation do not necessarily indicate cell cycle control points that respond to auxin, they may merely be 'sensitive points' that require many aspects of basic primary metabolism to be functioning.

The relationship between carbohydrate nutrition and cell division has been studied in considerable detail by Van't Hof and co-workers (Van't Hof, 1973). They have shown that carbohydrate starvation of root meristems causes potentially proliferative cells to arrest in G1 and G2 and that the proportions in G1 and G2 show inter-species variations. If auxin deprivation caused carbohydrate starvation the degree of arrest in G1 or G2 would be predicted to vary between species. Such an indirect effect of auxin on progress through the cell cycle could then provide an

explanation for why sycamore and tobacco cells arrest in both G1 and G2 and do not exhibit synchrony whereas carrot cells apparently arrest in G1 and do exhibit synchronous regrowth following auxin starvation.

The slow periodic growth following 2,4-D starvation suggests that some metabolic process, essential for proliferation, had been severely disrupted and that when the 2,4-D supply was re-established the system oscillated. It may be worth noting that the activity of a number of enzymes of carbohydrate metabolism can display rhythmic oscillations when perturbed (Fowler and Clifton, 1975) and that a 'control point' sensitive to carbohydrate, but not nitrate starvation, exists at or near mitosis such that the S-G2-M sequence is slowed down by glucose limitation (Gould, 1975).

A relationship between auxin and mitosis has been suggested by Nishinari and Yamaki (1976, 1978) who obtained tobacco cells showing partial synchrony by a complex sequence of treatments. They subsequently observed an increase in endogenous free IAA coincident with a wave of mitosis (Nishinari and Yamaki, 1976) which followed a wave of IAA-synthesizing enzyme activity (Nishinari and Yamaki, 1978). They suggested a causal relationship between the enhanced IAA-synthesizing enzyme activity and cell division. Although such a causal relationship may exist Mitchison (1977) has pointed out that, as some enzyme systems are fairly easily perturbed, such oscillations in enzyme activity may be a consequence of the synchronizing procedure rather than a feature of normal cell cycles.

If, as Nishinari and Yamaki (1976) suggest, increases in endogenous auxin are causally related to mitotic processes then the effect of 2,4-D starvation on $\int_n^{2n}$ (MI) dt may be due to an extension of the duration of mitosis. The results presented here suggest that the cell-division stimulating properties of exogenous auxin are not due to the activation of a cell cycle control point to commit cells to division. Auxin may therefore act more indirectly by priming the cells so that they are prepared for, and capable of division.

GENERAL DISCUSSION

GENERAL DISCUSSION

At the beginning of this century Haberlandt (1902) first proposed that the culture of isolated vegetative cells from higher plants would facilitate studies of the factors affecting or controlling cell division and differentiation.

The refinement of culture techniques has provided many experimental systems for hormonal and nutritional studies and has led to some appreciation of the factors regulating morphogenesis in vitro. In addition to providing a powerful tool for basic research, tissue culture has also found commercial applications, one of which (clonal propagation) was predicted by Haberlandt (1902) when he said "I believe, in conclusion ,that I am not making too bold a prediction if I point to the possibility that, in this way, one could successfully cultivate artificial embryos from vegetative cells". Somatic embryogenesis has now been observed in many species (Kohlenbach, 1978) and the efforts aimed at achieving this goal have helped to produce a catalogue of responses to a wide range of hormone treatments. Thus attempts to achieve a practical goal can assist basic hormone research while a deeper understanding of hormone action would undoubtedly be invaluable to attempts to manipulate plant cell growth and development.

The studies described in this thesis have been focused on the mechanisms by which 2,4-D promotes growth, particularly cell division, in plant tissue and cell cultures.

The origins of 2,4-D-dependence

The induction of callus growth from an explant involves both the induction of growth and the loss of the structural characteristics associated with differentiation ("dedifferentiation"). As growth may be a prerequisite for dedifferentiation, or dedifferentiation may be a prerequisite for continued proliferation, the 2,4-D requirement for callus

induction could be related to either or both of these processes. Thus once a dedifferentiated culture, such as a sycamore cell suspension culture, has been established, the 2,4-D requirement for continued growth could mean either that 2,4-D was actively stimulating growth or that 2,4-D was suppressing a pathway of differentiation which, if expressed, could cause the cessation of growth. An example of differentiation which does not require cell division and results in the death of the differentiating cell is wound vessel member formation,(E Wangerman, personal communication, see also Gresshoff, 1978). This example may be of particular relevance if one considers auxin-induced callus formation to be an extension of the early stages of the wound response (see Aitchison et al, 1977).

The working hypothesis employed at the beginning of this thesis was that the cessation of growth following 2,4-D-withdrawal was due to the toxic effects of enhanced production of phenolic compounds, which may have represented partial differentiation (King, 1976). 2,4-D certainly can suppress the production of phenolic compounds by cultured cells (see SECTION 1 - INTRODUCTION) but release of this suppression is not the cause of growth cessation after 2,4-D withdrawal in sycamore cell suspension cultures. Irrespective of whether some preliminary stages of differentiation are involved in the 2,4-D-starvation-induced cessation of growth, 2,4-D can promote growth without causing dedifferentiation as indicated by the growth-promoting activity of 2,4-D in the classical auxin bio-assays. Chandra et al (1978) showed that some analogues of 2,4-D, which are inactive in auxin bio-assays, could antagonize the effects of 2,4-D on embryogenesis but not growth (see SECTION 3). Thus, and perhaps rather surprisingly, the effects of 2,4-D on growth and differentiation may be mediated by different regulatory mechanisms (different receptors?).

The growth-promoting properties of 2,4-D under culture conditions may be due, at least partially, to a 'conditioning' effect on cells such that they are better able to grow on relatively inadequate culture media. Thus

Gibbs and Dougall (1965) found that plating of single tobacco cells on agar medium required preculture in high 2,4-D media to give high plating efficiency and Gresshoff (1978) found that soybean callus maintained on White's (a nutritionally inadequate medium) or B_5 (nutritionally rich) medium required different levels of 2,4-D and kinetin for optimal growth: on White's 10μM 2,4-D and 1μM kinetin, and on B_5 0.5μM 2,4-D and no kinetin. Similarly the results presented in SECTION 3 show that 2,4-D can increase the plating efficiencies of 2,4-D-independent sycamore cells on non-conditioned media (washed inocula) and that, in common with the observations of Gibbs and Dougall (1965), this effect is only seen if the cells are cultured in the presence of 2,4-D before plating. These observations suggest that the 2,4-D-dependence observed in culture may represent not only the suppression of differentiation and activation of cell division but also modifications to cellular metabolism which permit growth in culture.

The perception of 2,4-D

Until hormone perception in plant cells is fully comprehended we cannot be sure which pools of a particular hormone, and its metabolites, are available for interaction with particular organelles, receptors and the action of other hormones.

King (1976) attempted to identify the "active pool" of 2,4-D in sycamore cells by terminating the supply of 2,4-D to a chemostat, previously fed with ^{14}C-2,4-D, and following the decline in activity in various pools. Under these conditions growth responses appeared to correlate with the decline in the concentration of 2,4-D in the culture medium, rather than to the concentration in cellular fractions. King (1976) concluded that the cessation of growth following 2,4-D withdrawal probably resulted from a failure of extracellular 2,4-D to satisfy effector sites on the cell wall or plasmalemma. However, as the fractionation of cellular pools was relatively crude, the active fraction could also have been a minor

intracellular pool which was rapidly affected by changes in external 2,4-D supply. This latter view is favoured by Leguay and Guern (1977) and co-workers who have shown that when the growth of sycamore cells is 2,4-D limited some free 2,4-D remains in the culture medium but, if the uptake of this extracellular 2,4-D is promoted by changes in extracellular and intracellular pH (Kurkdjian and Leguay, 1978), cell division activity is temporarily restored. Similarly Nishi et al (1977) showed that addition of IAA to carrot cells washed fee of extracellular 2,4-D promoted the export of intracellular 2,4-D into the culture medium and accelerated the arrest of growth. These observations suggest that cells respond to the 2,4-D contained in an intracellular pool rather than directly to the 2,4-D concentration in the culture medium.

Another, and more widely studied, approach to the localisation of the site(s) of auxin perception has been the isolation of proteins which bind auxin molecules and may therefore represent the specific receptors (see Kende and Gardner, 1977; Venis, 1977). If the widely reported plasmalemma-associated auxin binding sites are in contact with the 'active pool' which has been proposed on the basis of physiological data then we must conclude that they must be able to be directed inwards towards cytoplasmic 2,4-D molecules. The little autoradiographic evidence available (Veen, 1966) is consistent with a cytoplasmic auxin pool being located adjacent to the plasmalemma but insufficient evidence is currently available to decide whether individual auxin-binding proteins are receptors, transport carriers or both.

We are now faced with the question: "How do these concepts influence our interpretation of responses to applied 2,4-D?" If the active pool was the only cytoplasmic pool of 2,4-D then the active concentration of 2,4-D at any instant would be a balance between the net rate of uptake of 2,4-D and its rate of inactivation. Examples of the inactive metabolites are the glucosides and glucosyl esters of hydroxylated derivatives (Feung et al 1975; Bristol et al 1977) which appear to be compartmentalized in the vacuole

(Makoveichuk et al 1978). Under conditions of constant rates of uptake and metabolism of 2,4-D, a steady state concentration of 2,4-D would be achieved in the active pool. However if the rate of uptake of 2,4-D was increased, by increasing the extracellular 2,4-D concentration (Rubery 1977), then a higher steady state concentration of 2,4-D in the active pool would be established. Clearly this behaviour would permit responses to be related to the extracellular concentration without the receptors being located on the cell surface. If we now consider cells with a higher capacity for 2,4-D inactivation, they would obviously require a higher rate of uptake (and therefore a higher external concentration of 2,4-D) to maintain the same steady state 2,4-D concentration in the active pool. This behaviour would permit cells to apparently exhibit different sensitivities to 2,4-D while possessing receptors with identical binding characteristics.

Most tissue cultures are maintained as batch cultures and therefore receive a transient, rather than continuous, supply of 2,4-D. Under these conditions a high rate of 2,4-D inactivation would influence not only the concentration of 2,4-D in the active pool at any instant but also the duration of responses resulting from a particular pulse of 2,4-D. This behaviour is clearly illustrated by the D_5 cultures (SECTION 2) which require high initial 2,4-D concentrations to maintain growth throughout the culture period but contain cells which respond to low extracellular concentrations of 2,4-D.

The mechanisms of 2,4-D action

Ample evidence has accumulated over many years to show that the treatment of plant tissues with 2,4-D or other auxins can have profound effects on RNA metabolism (see Jacobson, 1977). These effects include both increased template availability for DNA transcription and a massive synthesis of new ribosomes (Teissere et al 1975). The various observations from many groups of workers can, and have been, incorporated into unifying

models of auxin action (Ricard et al, 1976) which propose that the initial "master reaction" is the derepression of particular sets of genes. Following transcription and translation a corresponding set of proteins should appear. The model of Ricard et al (1976; see also GENERAL INTRODUCTION) requires that some of these auxin-induced proteins are regulatory proteins which modulate nucleolar RNA polymerase activity, and thus promote r RNA synthesis. It is likely however that this set of auxin-induced proteins would also comprise modified structural proteins and an array of enzymes associated with both the promotion of growth and dedifferentiation. Clearly the diversification of auxin-induced responses could originate from the "master reaction" itself in such a way that the knowledge that this "master reaction" is the regulation of transcription does not increase our understanding of how a physiological process, such as cell division, is regulated by 2,4-D.

Van't Hof and Kovacs (1972) have proposed that continued cell division activity relies on the successful negotiation of 'principal control points' (PCP's) in the cell cycle (see SECTION 5). If the cell cycle of plant cells includes an 'A-state' (Smith and Martin, 1973) then a modulation of the transition probability would not only change the mean generation time, but could also give rise to a population of cells which appeared to be arrested (or non-cycling, G_0) but in which all cells still had an equal finite probability of escaping the 'A-state' (and hence were still cycling).

Insufficient evidence is available to decide whether or not an A-state exists in plant cell cycles The observations of Webster (1979), that the differences between sister-cell cycle times in root meristems are relatively small, suggest that the transition probabilities of any A-states must be high in meristematic cells such that the average residence time in the A-state is short. The cultural restraints which preclude the attainment of maximum growth rates in plant cell cultures appear to primarily cause an extension of G_1 (Bayliss, 1975). If, as proposed in the model (Smith and Martin, 1973), the G_1 transition probability is the major means of regulation of growth rate then the temperature-dependent variations of G_1 in

cultured plant cells (Gould, 1977) show that the transition probability may possess a wide range of values.

The results presented in this thesis suggest that 2,4-D does not regulate cell division by direct interaction with PCP's, as cells arrested by 2,4-D depletion do not arrest at discrete points (as opposed to regions) of the cell cycle. The on/off dose-response relationship between 2,4-D and cell division (SECTION 2) suggests that 2,4-D does not regulate the transition probability of an A-state unless the only available values for the transition probability are extreme (very high and very low). In the latter case the A-state would resemble a PCP. Thus irrespective of the model one chooses to consider the conclusion must be that 2,4-D does not regulate cell division by any direct interaction with cell cycle controls.

We are now faced with the question "What are the mechanisms by which 2,4-D indirectly promotes cell division?" The effects of 2,4-D starvation appeared to be able to cause an extension of the duration of mitosis, (SECTION 5) a phenomenon also observed in carbohydrate limited sycamore cells but not in those limited by either nitrate or phosphate (Gould, 1973). Other evidence for the disruption of carbohydrate metabolism by 2,4-D starvation includes the effects on the respiratory breakdown of glucose (SECTION 4.4) and on the availability of TCA (Krebs) cycle intermediates for the synthesis of amino acids (SECTION 4.6). These results are entirely consistent with the experiments of Van Hove and Carlier (1968) who, from analyses of various steps in glycolysis and the TCA cycle using metabolic inhibitors, concluded that a step between pyruvic acid and oxalosuccinic acid was stimulated by auxin.

These observations point towards the mitochondrion as a site of processes which are sensitive to auxin regulation (but not necessarily auxin molecules). More direct evidence is provided by the observations that mitochonria isolated from auxin-treated soybean (Glycine max) exhibited enhanced size and protein synthesizing capacity (Key et al, 1960; Baxter and Hanson, 1968). However, as the processes regulating the structural and functional integrity

of mitochondria are not well charaterized, the significance of these observations cannot be assessed.

Although effects of auxin on respiration were reported as early as 1933 (Bonner, 1933) there has been little recent progress in this field. The most probable reason for this apparent lack of interest is that the use of metabolic inhibitors showed that this effect was unlikely to be the 'master reaction' regulating growth (Higgins and Jacobson, 1978). The considerable delay between effects of 2,4-D depletion on respiration and growth (SECTION 4.4) in sycamore cells also suggest that these two processes are not closely linked. However perhaps it is time to discard the naive view that auxins promote growth by regulating a single metabolic process and to re-examine some of the more complex responses to auxins.

The effects of 2,4-D on amino acid metabolism are potentially complex although in this thesis (SECTION 4) the effects on serine metabolism have been the prime concern as the soluble pool of this amino acid showed the greatest change following 2,4-D withdrawal. The accumulation of soluble serine appeared to be a consequence of decreased utilization rather than increased synthesis. Protein synthesis did not appear to represent the major demand on serine pools in control cells so that it is unlikely that a reduced demand from this process could have been responsible for the observed accumulation.

Another important drain on serine pools might be expected to be the conversion of serine to phosphatides for lipid synthesis. Although lipid synthesis has been shown to be sensitive to chlorinated benzoic acid herbicides (Muslih and Linscott, 1977) it would be expected that most lipids would be extracted by SDS which did not extract the labelled product of ^{14}C-serine metabolism (see SECTION 4 and Maddy and Dunn, 1976). Also it has been shown (Willemot and Boll, 1967) that only a small proportion of exogenously supplied serine is incorporated into lipids in cultured tomato roots.

If proteins and lipids can be discounted as major products of serine

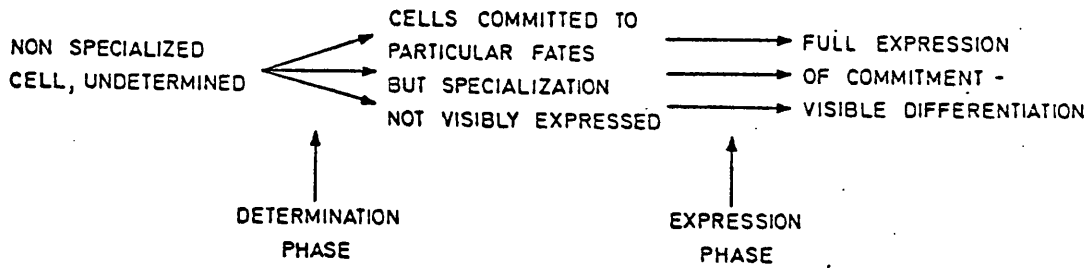


FIG.1 The two-phase concept of cellular differentation.

metabolism then it would appear that serine is being used as a source of carbon skeletons for some aspect of carbohydrate metabolism. If this is the case the metabolic route concerned must be highly directed towards the synthesis of high molecular weight (cell wall?) polysaccharides as very little label from serine reappeared as either organic acids or amino acids other than serine (alcohol-soluble pool). Clearly much more work is required to determine the significance of serine accumulation as a response to 2,4-D depletion. Even if such responses are eventually found not to be directly related to the regulation of growth their elucidation should further our knowledge of plant cell metabolism and could be extremely useful to attempts to direct the metabolism of cultured cell plants towards the synthesis or modification of particular metabolites (Zenk, 1978).

Prospects for the manipulation of plant cell development in culture

Early results from model (i.e. successful) systems, such as tobacco callus, generated the opinion that plant hormones could induce different patterns of differentiation in culture. However in many cases the results are probably more consistent with the hormone treatments favouring the expression of one type of differentiation while suppressing the expression of others. Thus if differentiation is considered to be a two-phase process (Fig.1) one may conclude that although plant hormones can suppress or permit, and under permissive conditions modify, morphogenesis or cytodifferentiation (Street, 1978) there is very little evidence to support a view that simple hormone treatments can switch cells from one determined state to another (see also INTRODUCTION). Consequently it should not be surprising that many tissue cultures do not exhibit regeneration (production of shoots or somatic embryos) even if many hormone combinations (to produce permissive conditions) are tested. Even if a particular treatment succeeds in switching cells from one development fate to another there is no reason why a long delay should not separate a change in the determined state from the expression of

that change. Indeed a specific sequence of treatments may be required for the successful induction and expression of a particular developmental program (Higgins and Jacobson, 1978).

It is now clear that, to fully understand the regulation of differentiation, we need to be able to identify the factors which can influence the determination phase. In culture these factors probably include: source of explant, nutritional and hormonal status of the culture medium, temperature and light regimes, type of culture (callus or suspended cells) and frequency of subculture. The loss of hormone-dependence in culture (habituation) is considered to be under epigenetic control (Meins, 1977) and to be an example of a change in determined states. If this view is correct then the effects of glutamate and light on the isolation of 2,4-D-independent sycamore cells (SECTION 3) could be considered to be examples of effects of environmental factors on determination. To assess the relative importance of individual factors we need to be able to identify when a change in determined state has occurred. Recent studies by Khavkin et al (1979) suggest that immunochemical techniques may be useful in this respect. These authors detected embryonal antigens in callus and suspension cultures of Zea mays L., irrespective of their tissue origin. These embryonal antigens were distinct both from the meristematic antigens found in intact seedlings and callus cultures, and from organ-specific antigens found only in intact plants. Khavkin et al (1979) suggest that these embryonal antigens are "proliferation proteins" produced by cells that have not undergone any determination and therefore lack any tissue or organ specificity. The disappearance of such proteins could clearly be used as a marker for the determination event in this system.

In conclusion it is obvious that a full understanding of the effects of 2,4-D on higher plant cells requires further research, not only direct studies of its mode of action but also studies of the basic mechanisms regulating cell division, cell expansion, cell polarity and the determination and co-ordination of differentiation.

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THE REGULATION OF PLANT CELL GROWTH AND METABOLISM BY 2,4-DICHLOROPHENOXYACETIC ACID

N P EVERETT

The growth-promoting effects of 2,4-dichlorophenoxyacetic acid (2,4-D) were studied using cell suspension cultures of sycamore (Acer pseudoplatanus L.) Omission of 2,4-D from the standard culture medium caused a cessation of growth after an initial period which was dependent on the rate of 2,4-D inactivation, the level of kinetin in the culture medium and the presence or absence of light. Substantial changes in the metabolism of amino acids, particularly serine and glutamic acid, which accompanied the cessation of cell division were studied as possible markers of the final metabolic state, achieved in the absence of 2,4-D, which was not conducive to growth. Clonal 2,4-D-independent cell lines which varied in their requirements for kinetin were isolated from the 2,4-D-dependent cultures. The responses of these cell lines to anti-metabolites indicated that resistance to 5-bromodeoxyuridine, 5-methyltryptophan or methotrexate are phenomena which are not prerequisites for auxin- or cytokinin-independence in cultured sycamore cells. A temporary 2,4-D starvation did not induce appreciable synchrony in 2,4-D-dependent cultures which suggests that 2,4-D does not promote cell division by activating a specific cell cycle control point to commit cells to division. 2,4-D appears to act more indirectly by priming cells so that they are prepared for, and capable of division.