THE MOLECULAR MODE OF ACTION OF ABSCISIC ACID IN THE INDUCTION OF DORMANCY

by

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I declare that this thesis was composed by myself and that the work presented herein, unless otherwise stated, is my own.

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ABBREVIATIONS

ABA	abscisic acid
AcOH	acetic acid
ara	arabinose
BuOH	butanol
Butyl-PBD	2-(4′-tert-butylphenyl)-5-(4″-biphenyl)-1,3-4-oxadiazole
cDNA	copy deoxyribonucleic acid
Ci	curie
cpm	counts per minute
D	dextro-rotatory
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EtOAc	ethyl acetate
GA_1	gibberellin A_1
GA_3	gibberellic acid
gal	galactose
galA	galacturonic acid
glc	glucose
glcA	glucuronic acid
H/2	half-strength Hutners medium
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
IAA	indoleacetic acid
K_D	dissociation constant
L	laevo-rotatory
MES	morpholinoethane sulphonic acid
mRNA	messenger ribonucleic acid

NAA	naphthaleneacetic acid
NAD	β-nicotinamide adenine dinucleotide
NDP	nucleoside diphosphate
O.D.	optical density
poly (A)	polymer of AMP
PPO	2,5-diphenyloxazole
p.s.i.	pounds per square inch
RNA	ribonucleic acid
r.p.m.	revolutions per minute
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloride
UDP	uridine diphosphate
UV	ultra-violet
W	watt
xyl	xylose

ABSTRACT

Extensive *in vivo* and *in vivo* experiments to find a specific binding site, or receptor, for abscisic acid (ABA) were largely unsuccessful. Some specific binding was detected in crude membrane fractions of barley aleurones, but the low levels of binding coupled with the difficulty of obtaining sufficient starting material inhibited further progress.

Spirodela polyrrhiza (a duckweed) was induced to form resting buds, or turions, by the addition of 10^{-6} M ABA to the culture medium. 10^{-6} M ABA caused a rapid inhibition of vegetative growth (visible after 10-15 hours) which was associated with a correspondingly rapid reduction in the cell wall plasticity of the smaller, fast-growing fronds.

A comparison of the non-cellulosic cell wall monosaccharides of fronds and turions revealed that the fronds contained a high proportion (21.4% of wall polysaccharides) of the branched-chain sugar, apiose, whereas the turion cell walls contained only trace amounts (0.1%). 10^{-6} M ABA was found to inhibit the incorporation of apiose into the cell walls of small fronds of turion-inducible size. By labelling with ^{3}H-glucuronic acid of a high specific activity and measuring the levels of radioactive soluble sugar intermediates and nucleotides, it was discovered that the activity of the enzyme UDP-apiose / UDP-xylose synthase was inhibited in ABA-induced turions.

CHAPTER 1

GENERAL INTRODUCTION

General Introduction

The plant hormone abscisic acid (ABA) is associated with a wide range of physiological responses in plants (reviewed by Addicott, 1983). These include abscission, seed dormancy, fruit ripening, stomatal closure and the control of elongation of both aerial and root growth. The justification for research into the biochemical reactions associated with these control mechanisms, with respect to the betterment of humankind, is probably predominantly connected with the role of ABA as an anti-water stress agent (Quarrie and Jones, 1977), which could be developed for beneficial use in countries where the crop-growing season is precariously short amidst long dry seasons.

The wide diversity of well-documented responses to ABA imposes a requirement on plant tissues to respond towards ABA in a specific manner and at certain developmental stages only. This predisposition of "sensitivity", a factor stressed by Hanson and Trewavas (1982), imposes an abstruse aspect to hormone-induced responses which is difficult to clarify by experimentation. However, considerable effort is now being directed towards determining tissue responses to hormones at the biochemical / genetic levels, rather than simply by describing gross morphological changes. Consequently, it is hoped that future knowledge concerning hormone action at the molecular level will further our understanding of how hormones can induce such a variety of physiologically expressed changes in plant growth and development.

The initial plant-hormone interaction is generally considered to require the presence of a specific receptor, and much attention has recently been directed to this aspect (Venis, 1985). Chapter 2 of this thesis concerns the search for an ABA-receptor.

How the hormone-receptor complex proceeds to affect the control of cellular processes is still largely uncertain, although it is increasingly considered that ABA specifically affects the synthesis of certain mRNA species, rather than inhibiting transcription generally (Jacobsen, 1977; Mozer, 1980). Smart and Trewavas (1984) suggest that turion formation in *Spirodela polyrrhiza* is signalled by the sequential inhibition of firstly DNA synthesis, secondly protein synthesis and thirdly RNA synthesis. The inhibition of protein synthesis was found to be selective, and there was also the concurrent appearance of new proteins.

Spirodela polyrrhiza provides an excellent material for ABA studies due to its physiological response of producing turions when exposed to ABA at specific external concentrations (Perry and Byrne, 1969; Smart and Trewavas, 1983a). ABA has the capability to transform the fate of developing primordia from frond to turion production. The developmental stage is critical in this transformation - only small fronds still undergoing cell division are predisposed to this sensitivity. In the transition from the meristematic to the cell expansion states the ABA-sensitivity, with respect to turion formation, is lost.

The inhibition of growth of *Lemna* (due to inhibition of cell division), has frequently been used as a bioassay for ABA, and Tilberg (1975) using *Lemna gibba* achieved a dose response curve which was linear for concentrations of 10^{-7} M to 10^{-11} M ABA. The initial speed of the growth inhibition, and the associated effect on tissue extensibility of the turion inducing concentration (10^{-6} M) of ABA on a clone of *Spirodela polyrrhiza* is described in Chapter 3. The resulting alteration in cell wall plastic extensibility is then further investigated in relation to the biochemical composition of cell walls of fronds and (ABA-induced) turions in Chapter 4. Analysis revealed that the apiosyl content of cell walls of developing fronds of turion-inducible size was selectively repressed on exposure to 10^{-6} M ABA. Further investigation revealed that the activity of the enzyme UDP-apiose / UDP-xylose synthase was suppressed in the ABA-induced transition from frond to turion production. This is thought to be the first reported example of a major chemical change in cell wall composition resulting from the addition of ABA.

There are more detailed Introductions, relevant to the sections following them, at the beginning of each chapter.

CHAPTER 2.

THE SEARCH FOR A RECEPTOR FOR ABSCISIC ACID.

2.1 INTRODUCTION

2.1.1 The requirement for hormone receptors.

It is generally accepted that, in order to exert their effects, hormones interact with cellular components called receptors. These bind to the hormone, and as a consequence, initiate the chain of biochemical events leading to the physiological response. The precision of the recognition process is such that only proteins are considered capable of recognising subtle structural differences between small molecules.

Some physiologists, prefer the term "plant growth regulators" to "plant hormones", but in this thesis the two terms will be used interchangeably. Trewavas (e.g. Trewavas, 1981) has argued that changes in tissue sensitivity towards plant growth regulators are of greater significance in the control of plant growth and development than are changes resulting purely from increases in hormone concentration. As a result, this implies that the presence of specific hormone receptors is of critical importance in determining hormonal effects.

2.1.2 Criteria for receptor status.

Certain generally accepted criteria for qualification as a receptor protein have been established (Stoddart and Venis, 1980; Lubke *et al.*, 1976). These are :

1) Binding should be of high affinity, of saturable capacity

and be reversible, in order to regulate the physiological effect.

2) The saturation concentration range of binding should correspond to the concentration range known to be active physiologically.

3) Binding should lead to a hormone-specific response. As plant growth and development is relatively slow, this factor is very difficult to demonstrate in plant systems, particularly when using *in vitro* binding techniques where the nature of the initial hormone-stimulated biochemical reaction is not known.

4) The relative biological activities of hormone analogues should be reflected by their binding affinities.

2.1.3 Hormone-receptor binding parameters.

The following mass action equation describes hormone-receptor interaction :

$$K_D = \frac{[L] \times [R]}{[RL]}$$

where K_D is the equilibrium dissociation constant, [L] the concentration of free ligand (hormone), [R] the unoccupied receptor concentration and [RL] the ligand-receptor complex concentration. The K_D is often calculated from binding assays using radioactive ligand, by rearrangement of the equation to allow a Scatchard analysis (Scatchard, 1949) (as shown in Figure 18) :

$$\frac{B}{F} = \frac{n}{K_D} - \frac{B}{K_D}$$

where B is the concentration of bound ligand, F the concentration of unbound (free) ligand and n is the total binding site concentration. The binding constants K_D and n are obtained from the slopes and intercepts of the Scatchard plot where B/F (ordinate) is plotted against B (abscissa). n is represented by the intercept on the x axis and the slope by $-(1/K_D)$.

2.1.4 Factors affecting binding parameters.

Important factors which have to be taken into consideration when investigating binding parameters are :

a) The ligand must have a radioactive specific activity sufficiently high to detect the expected number of binding sites.

b) The receptor concentration should be sufficiently high to be detectable.

c) The ligand concentrations used should be within a non-saturating, range.

d) The assay temperature should be chosen so that the degradation of both ligand and receptors is minimised, while still permitting convenient binding equilibrium times. Proteases may be present both in intact cells and in purified receptor preparations, and their activity increases with temperature. However, the time course of some binding equilibria is so slow at 0°C that a

compromise temperature has to be provided to achieve limited degradation together with a sufficiently rapid equilibration.

e) The method chosen for measuring both free and bound ligand should be appropriate for the ligand off-rate. Ligands with a K_D of 10^{-9} M or less (e.g. insulin) have dissociation rate constants with a half-life in excess of 10 min, whereas ligands with a K_D of 10^{-8} M with the same rate-on constant would have a half-life of only a few seconds for the ligand-receptor complex.

2.1.5 Specific versus non-specific binding proteins.

It is necessary to distinguish between receptors which are directly, or indirectly, responsible for producing developmental changes (= specific-binding proteins, or receptors) and binding proteins that are of low affinity or involved, e.g., in metabolism of the hormone (= non-specific binding proteins). Hence the terms "binding protein" and "receptor" are not necessarily synonymous, although, until the system under consideration has been well characterised, the assumption has to be that the two are the same. As already mentioned, a subsequent plant hormone-specific response is difficult to demonstrate conclusively.

2.1.6 Current knowledge of plant hormone receptors.

Our knowledge of receptors in the animal field is far in advance of that in plant research, and several animal hormone

receptors have been extensively characterised and purified (Jacobs and Cuatrecasas, 1981) including that of insulin (Rosen *et al.*, 1983). The insulin receptor consists of four sub-units, and the active site binding the insulin is a tyrosine-kinase. This suggests that the effect of insulin-receptor binding is initially a phosphorylation / dephosphorylation of the receptor complex. However, Trewavas (1981) has emphasized the danger of drawing too close a parallel between animal and plant hormones, bearing in mind that plants, unlike animals, use one hormone to regulate several diverse physiological activities, to extents not necessarily governed by the concentration of the hormone.

Although the plant hormone field lags behind its animal counterpart, the considerable interest and effort now being directed to the subject is reflected in the appearance of several recent reviews e.g. Kende and Gardner, 1976; Stoddart and Venis, 1980; Dodds and Hall, 1980; Trewavas, 1981; Venis, 1985.

A. Auxins.

Most of the progress with plant hormone receptors has been achieved with auxins (Rubery, 1981). Binding studies with auxins initially involved a wide variety of plant materials including dwarf beans (Wardrop and Polya, 1977), tobacco (Oostrom *et al.*, 1980; Maan *et al.*, 1983; Van der Linde *et al.*, 1984), soybean cotyledons (Ihl, 1976), immature coconut endosperm (Roy and Biswas, 1977) and zucchini hypocotyls (Jacobs and Hertel, 1978). The majority of auxin-binding work

has, however, been focused on maize coleoptiles. These constitute an excellent material to work with as they contain a high concentration of receptors. Since Hertel published his first report (Hertel *et al.*, 1972) of saturable binding of ^{14}C-NAA and ^{3}H-IAA to membrane preparations of maize coleoptiles, there has been controversy concerning the number and intracellular localisation of the receptors involved.

Dohrmann *et al.* (1978) have distinguished three types of auxin-binding sites - site I (associated with the endoplasmic reticulum), site II (tonoplast membrane) and site III (plasma membrane fraction) -and found that the three sites had different affinities for different auxin ligands.

Ray (1977) and Ray *et al.* (1977a) concluded that there was only one major NAA-binding site, with a K_D of 5 x 10^{-7} M, located on the endoplasmic reticulum, although it was conceded that a minority of sites might be located on Golgi and/or plasma membranes.

Batt and Venis (1976) and Batt *et al.* (1976) concluded that there were two binding sites for both NAA and IAA. Site I was thought to be on the Golgi membranes or endoplasmic reticulum, with site II associated with plasma membrane fractions. The K_D's calculated for NAA and IAA, sites I and II were 1.5 x 10^{-7} M and 1.6 x 10^{-6} M for NAA and 1.7 x 10^{-6} M and 5.8 x 10^{-6} M for IAA, respectively.

Murphy (1980) used a variety of computer curve-fitting analyses of this binding data to show a single class of binding sites, and claimed that all other binding was non-

specific. However, many workers e.g. Rubery *et al.* (1981) still believe that, even allowing for non-specific binding, there is more than one distinct membrane auxin-binding site in maize coleoptiles. It has been suggested (Venis, 1977a; Dohrmann *et al.*, 1978) that some of the binding sites detected may, in fact, be precursors of sites in another location, or may even act as preliminary selection binding sites.

Auxin-binding extracts from maize membranes can be prepared by extraction with Triton X-100 (Batt *et al.*, 1976; Ray *et al.*, 1977a; Cross and Briggs, 1978), or by using a modified acetone powder technique (Venis, 1977b). Further purifications of the binding sites have been achieved by a variety of chromatographic, ion-exchange, gel-filtration and affinity columns (Venis, 1985).

Recently, Lobler and Klambt (1985a,b) have used affinity chromatography to prepare monospecific antibodies to a purified auxin-binding protein (ABP) from *Zea mays*. These antibodies inhibit auxin responses in isolated *Zea* coleoptile segments and hence this ABP is claimed to be an auxin receptor. It is thought to consist of a 40,000 molecular weight dimer located on the plasmalemma of the outer epidermal cells.

B. Cytokinins.

Cytokinin-binding proteins have been detected in a number of plant tissues including wheat-germ (Polya and Davies, 1978; Moore, 1979; Erion and Fox, 1981; Fox and Gregerson, 1982), tobacco (Yoshida and Takegami, 1977; Sussman and Kende, 1978)

and barley embryos (Reddy *et al.*, 1983). However, many of these proteins have a very low affinity for cytokinin which casts doubt on their physiological role as receptors. The implications of cytokinin-binding in isolated ribosomes, and the resulting increase in protein synthesis have been reviewed by Stoddart and Venis (1980).

C. Ethylene.

Ethylene-binding sites have been isolated from a range of plants and tissues including cotyledons of legumes (Bengochea *et al.* 1980a,b; Thomas *et al.*, 1984 and 1985). Unfortunately, as these cotyledons are not known to be responsive to ethylene, a direct link between receptor sites and physiological expression cannot be made. The very slow dissociation rates of the ethylene-receptor complex, of up to 24 hours at 25°C for *Phaseolus vulgaris* (Bengochea *et al.*, 1980a), also does not suggest receptor status, although the methods used to determine the binding of ethylene, being a gas, have been unsuitable for measuring fast dissociation rates.

D. Gibberellins.

Jelsema *et al.* (1977) detected gibberellin (GA_1)-binding sites in extracts of wheat endosperm and Keith *et al.* (1981, 1982) have described GA_1-binding to soluble extracts (100,000g supernatants) of cucumber hypocotyls. Keith and Srivastava (1980) have also described *in vivo* binding of GA_1 to slices of dwarf pea epicotyls, following on from the preliminary work of Stoddart *et al.* (1974), and also to barley aleurone layers

(Keith *et al.*, 1980). It was these '*in vivo*' papers which inspired the *in vivo* ABA-binding approach tried initially in this thesis.

E. Abscisic acid.

Until 1984, only one report of ABA-binding had been published. Hocking *et al.* (1978) claimed to have found saturable binding of ^{3}H-ABA to membrane preparations from *Vicia faba* leaves. This claim has, however, not been substantiated, and attempts to repeat the work, even using ABA with a higher specific activity, have failed (Venis, 1985). It was at this stage, in 1982, that the ABA-binding work in this thesis was commenced. The characterisation of photoaffinity labelled ABA-receptors in *Vicia faba* guard cell protoplasts (Hornberg and Weiler, 1984) had not been published. Their paper is reviewed in the Discussion section of this chapter.

2.1.7 ABA-receptor detection - the approach adopted.

In the light of existing knowledge, the *in vivo* approach of Keith and Srivastava (1980) and Keith *et al.* (1980) used to study GA_1-receptor binding seemed a novel and attractive proposition with which to approach the problem of detecting ABA-receptor binding. The concept of using intact tissue sections, or, preferably, even entire plants e.g. duckweeds, to study ABA-binding, was considered to represent a physiologically realistic situation within which any ABA-binding might be expected to reflect biologically meaningful plant-ABA interactions. It was hoped that this *in vivo*

approach would avoid the artifacts encountered in *in vitro* studies arising from the grinding of plant tissues and resulting damage and disruption of the plant system. *Spirodela polyrrhiza*, in particular, seemed the ideal plant material to use as it represented an entire plant system which was genetically uniform, sterile, and easily cultured. It's "sensitivity window" (Smart, 1981) towards ABA with regard to turion induction embodies a specific event triggered by ABA, and hence it appeared to be an especially advantageous tissue for *in vivo* studies.

The ABA-binding work described here consequently began with *in vivo* binding studies, although it subsequently developed into *in vitro* studies. This chapter is therefore divided into A. *in vivo* and B. *in vitro* sub-sections in both the Methods 2.2 and the Results 2.3 sections to distinguish between these two approaches.

2.2 MATERIALS AND METHODS.

2.2A *IN VIVO* ^{3}H-ABA EQUILIBRIUM DIALYSIS MATERIALS AND METHODS.

2.2A.1 Chemicals.

Unless otherwise stated, all chemicals used were supplied by Sigma Ltd.

DL-cis,trans-[G-^{3}H]abscisic acid, specific activity of 33 Ci/mmol, was obtained from Amersham International plc.

2.2A.2 Plant Materials.

a) *Spirodela polyrrhiza* fronds.

The plants were grown in 2 litre rectangular Pyrex pie dishes containing 1 litre half-strength Hutners medium (H/2, see below) in a Fisons Fi-totron 600 Growth Cabinet. The cultures were illuminated for 20 hours per 24 hour growth cycle at 210 μeinsteins.s^{-1}.m^{-2} from 12 x 40 W white fluorescent tubes and 4 x 40 W tungsten bulbs. The temperature was kept constant at 25°C +/- 1°C. Plants were subcultured to fresh medium, using a sterile spatula, before they reached confluent growth. Both pie dishes and H/2 medium were autoclaved at 15 p.s.i. for 15 min prior to use.

Spirodela polyrrhiza culture medium :

Hutners medium – Composition

Compound	$mg.l^{-1}$	$\times 10^{-3}$ M
K_2SO_4	400	2.3
KOH	200	3.6
EDTA	500	1.7
NH_4NO_3	200	2.5
$Ca(NO_3)_2.4H_2O$	354	1.5
$MgSO_4.7H_2O$	500	2.0
$FeSO_4.7H_2O$	24.9	0.09
$MnCl_2.4H_2O$	17.9	0.09
$ZnSO_4.7H_2O$	65.9	0.23
H_3BO_3	14.2	0.23
$CuSO_4.5H_2O$	3.95	0.016
$Na_2MoO_4.2H_2O$	25.2	0.10
$Co(NO_3)_2.6H_2O$	0.2	0.0007

The following stock solutions were prepared :

1. A To 200 ml of water :

$Ca(NO_3)_2.4H_2O$	17.7 g
EDTA	25.0 g
K_2HPO_4	20.0 g
NH_4NO_3	10.0 g
KOH	12–15 g until solution was clear.

B To 150 ml of water :

$ZnSO_4.7H_2O$	3.295 g
H_3BO_3	0.710 g
$Na_2MoO_4.2H_2O$	1.260 g
$CuSO_4.5H_2O$	0.197 g

6 M HCl was added drop by drop until solution was clear.

Then the following were added :

$Co(NO_3)_2.6H_2O$	0.010 g
$MnCl_2.4H_2O$	0.897 g

C To 50 ml of water

$FeSO_4.H_2O$	1.245 g

Solutions A, B and C were mixed with constant stirring and made up to 1 litre with water (= Stock 1).

2. $MgSO_4.7H_2O$ 25.0 g made up to 1 litre with water.

To prepare 1 litre of H/2 medium, 10 ml of stocks 1 and 2 were mixed and made up to 1 litre with distilled water, adjusting the pH to 6.4 with 5 M KOH.

b) *Spirodela polyrrhiza* turions.

Turions were produced either by:

i) addition of 10^{-6} M ABA to the H/2 medium

or ii) allowing the plants to grow, in lower light conditions, for several days after reaching confluent growth. Lower light conditions = 25 to 50% of normal light conditions within growth cabinet.

c) *Lemna perpusilla* fronds.

Plants were grown, under aseptic conditions, in 250 ml conical flasks containing 100 ml H/2 medium and stoppered with foam bungs. Culture conditions were otherwise the same as for *S.polyrrhiza* fronds.

d) Pea epicotyls.

7 day old dark-grown (25°C) seedlings of *Pisum sativum* var. Feltham First were dissected with a razor blade to obtain 1 mm thick sections of epicotyls from the third internode.

e) Wheat coleoptiles.

5 day old dark-grown (25°C) seedlings of *Triticum aestivum* var. Highbury Spring were similarly dissected to obtain 1 mm thick sections of coleoptiles, after first removing the leaf rolls.

f) Maize coleoptiles.

Same procedure as for e).

g) Barley aleurone layers.

The preparation procedure used was essentially that of Chrispeels and Varner (1967). Seeds of *Hordeum vulgare* var. Proctor Spring were dehusked in 50% H_2SO_4 for 2 hours, then rinsed several times with distilled H_2O. The seeds were cut transversely into halves, and the halves containing the embryo discarded. The embryo-less halves were surface-sterilised in 1% sodium hypochlorite for 20 min and rinsed thoroughly with sterile distilled H_2O. The sterile half-seeds were then scattered on to sterile, water-saturated Whatman No.1 filter papers placed in 10 cm diameter plastic petri

dishes and incubated for 3 days at 25°C in the dark. The aleurone layers could then be peeled off the endosperm using a pair of small, sterile tweezers.

2.2A.3 Assay buffers.

All of the following assay buffers were sterilised by autoclaving at 15 p.s.i. for 15 min prior to use.

	pH		Composition
a)	6.4		H/2 medium (see 2.2A.2 a) above)
b)	4.8		0.002 M sodium acetate 0.001 M calcium chloride 50 $\mu g.ml^{-1}$ chloramphenicol
c)	5.0 6.0 7.0	obtained by mixing	0.1 M Tris-HCl 0.003 M magnesium chloride + 0.1 M MES 0.003 M magnesium chloride
d)	6.5 7.0 7.5		0.01 M Tris-HCl 0.001 M EDTA 0.25 M sucrose
e)	6.0		0.05 M MES 50 $\mu g.ml^{-1}$ chloramphenicol

2.2A.4 Incubation conditions.

The tissues were placed in 10 ml of the appropriate assay buffer in 50 ml beakers, and ice-cooled for 1 hour prior to the addition of ^{3}H-ABA to the required final concentration. In the subsequent incubation the beakers were covered with Parafilm and stood in ice within polystyrene ice-buckets. These were covered with polystyrene lids and placed in a cold-room (4°C) for the duration of the experiment. The actual temperature of the incubation medium was 0.5°C. Non-radioactive ABA, where added, was to a final concentration of [^{3}H-ABA concentration x 1000-fold].

2.2A.5 Sampling.

a) Incubation medium.

Three 100 µl aliquots of incubation medium were sampled and counted daily by liquid scintillation to check that the level of radioactivity in the medium remained constant, and was not being depleted significantly by uptake into the tissues.

b) Plant tissues.

At daily intervals, tissue samples were removed from the incubation medium, blotted dry, and weighed. The radioactivity was extracted by 3 x 20 min successive extractions in 1 ml boiling ethanol : acetic acid : water (80 : 5 : 15).

2.2A.6 Scintillation counting.

Both incubation medium and plant tissue samples were

transferred to plastic disposable scintillation vials. 10 ml of scintillation fluid (1 litre toluene + 0.5 litre Triton + 7 g Butyl-PBD) was added to each vial and the contents thoroughly mixed. The extracts containing chlorophyll were left in a dark cupboard for at least 2 hours before scintillation counting to allow any chemi-luminescence to subside.

Vials were counted in an Intertechnique SL-3000 Liquid Scintillation Counter against an internal ^{3}H-standard. Owing to the difference in quenching capacity between the incubation medium and plant extract samples, and hence the resulting counting efficiency of the counter, a quench calibration curve for ^{3}H was constucted (Figure 1). This was obtained by adding varying volumes of chloroform to a known, fixed specific activity of ^{3}H-ABA, and using the results to convert the c.p.m. (counts per minute) print-outs from the counter to d.p.m. (disintegrations per minute).

2.2A.7 Fluorography checks on metabolism of ABA.

At the end of each experiment, residual tissue samples were extracted (as in 2.2A.5. b) above), and the extract reduced to near-dryness and streaked on to glass TLC Silica Gel (Merck GF254) plates. These were developed in glass chromatography tanks using an ascending solvent system of toluene : ethyl acetate : acetic acid (25: 15 : 2) (Milborrow and Noddle, 1970) for 1 hour. The plates were dried, and then to enhance the efficiency of the autoradiographic detection of the ^{3}H-compounds, they were

FIGURE 1

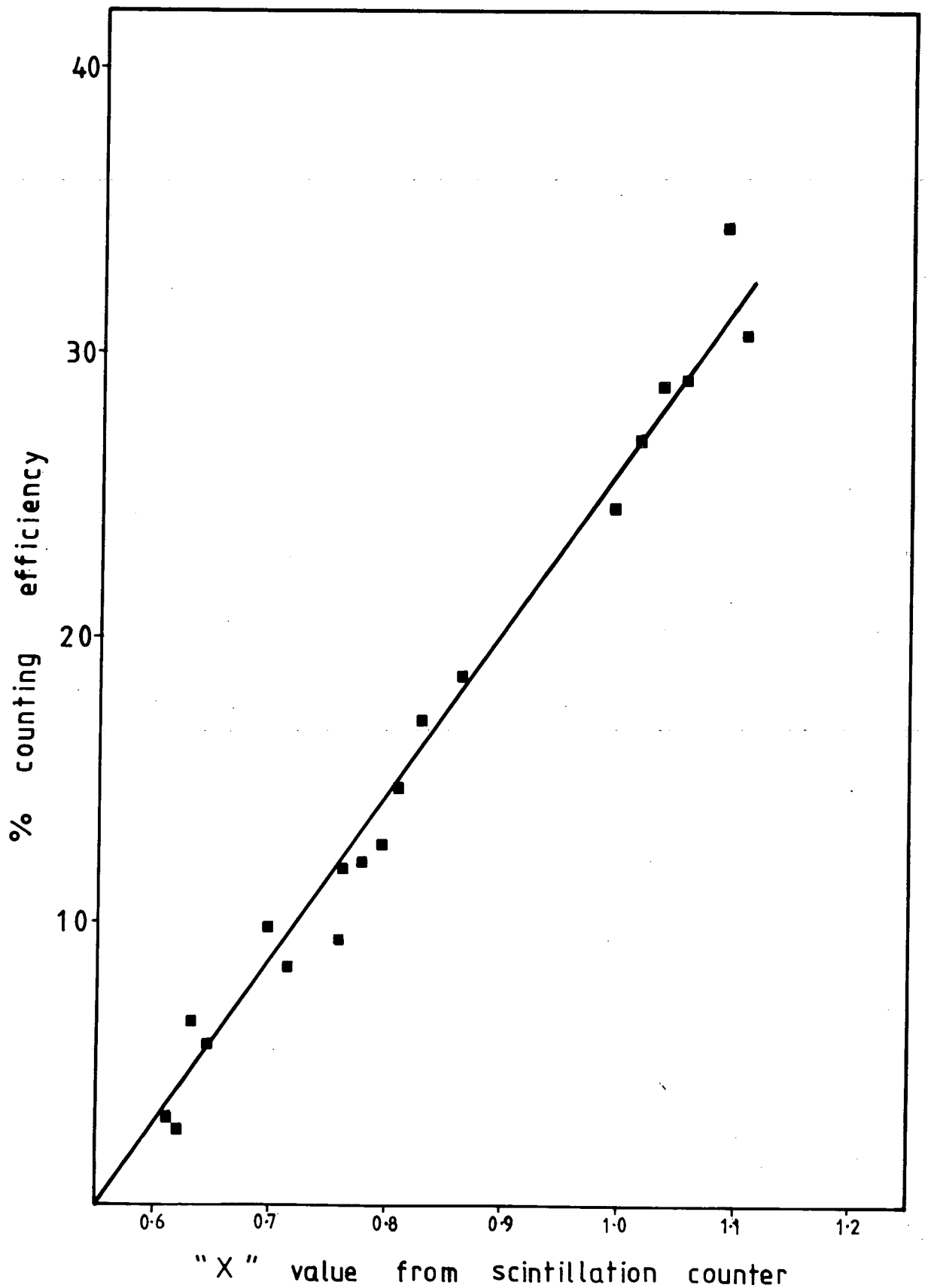
Quench Calibration Curve.

15 µl ^{3}H-ABA (specific activity 33 Ci/mmol., radioactive concentration 200 µCi/ml) was diluted to 1.5 ml with methanol. 0.1 ml aliquots were transferred to scintillation vials and chloroform added at volumes varying from 0 to 0.2 ml. 10 ml scintillation fluid (1 litre toluene + 0.5 litre Triton X-100 + 7 g Butyl-PBD) was added to each vial and the vials scintillation counted in an Intertechnique SL-3000 Liquid Scintillation Counter against an internal ^{3}H-standard.

The X-values (channel ratios) printed out by the counter are plotted against the % efficiency of the counting. The latter was obtained by dividing the c.p.m. on the print-out by the known d.p.m. added (= 440,000) and multiplying the result by 100%.

The data represent the cumulative results from 3 such experiments.

FIG 1


40
30
20
10
% counting efficiency
0·6
0·7
0·8
0·9
1·0
1·1
1·2
"X" value from scintillation counter

sprayed with 7% w/v PPO in acetone (Randerath, 1970) and re-dried. Pre-sensitised X-ray film (Laskey and Mills, 1975) was clamped on to the plates and they were left in the dark at -70°C to develop.

2.2B IN VITRO ^{3}H-ABA BINDING MATERIALS AND METHODS.

2.2B.1 Plant material.

		Variety
a)	Wheat coleoptiles (4 day old, dark grown)	Highbury Spring
b)	Wheatgerm	-
c)	Maize coleoptiles (5 day old, dark grown) minus leaf rolls	Beaupré
d)	Maize coleoptiles (ditto) leaf rolls only	Beaupré
e)	Maize mesocótyls (ditto)	Beaupré
f)	Pea epicotyls (8 day old, dark grown)	Feltham First, Alaska
g)	Pea apical hooks (ditto)	Feltham First, Alaska
h)	Zucchini hypocotyls (7 day old, dark grown)	Green Bush
i)	*Spirodela polyrrhiza* fronds. see 2.2A.2. a) for growth conditions	-
j)	*Spirodela polyrrhiza* turions	-
k)	Barley embryos	Proctor Spring
l)	Barley half-seeds	Proctor Spring
m)	Barley aleurone layers	Proctor Spring

2.2B.2 Grinding buffers.

a) 0.01 M Tris-HCl
0.25 M sucrose pH 7.5
0.001 M EDTA

b) 0.05 M Tris-acetate

0.25 M sucrose pH 8.0

0.001 M EDTA

0.0001 M $MgCl_2$

c) 0.1 M Tris-HCl

0.5 M sucrose pH 7.0

$MgCl_2$ at concentrations : 8.0

0.0003 M, 0.0005 M, 0.001 M,

0.003 M, or 0.005 M

d) 0.01 M Na-phosphate

0.001 M EDTA pH 6.6

0.25 M sucrose

e) 0.01 M MES pH 6.0

50 $\mu g.ml^{-1}$ chloramphenicol

f) 0.06 M MES

0.075 M Tris-HCl pH 7.7

0.003 M EDTA

0.3 M sorbitol

g) 0.05 M MES

0.05 M Tris-HCl pH 8.0

0.5 M sucrose

0.003 M $MgCl_2$

h) 0.1 M MES

0.01 M Tris-HCl pH 8.0

0.5 M sucrose

0.003 M $MgCl_2$

i) 0.01 M HEPES pH 7.0

50 $\mu g.ml^{-1}$ chloramphenicol

2.2B.3 Wash buffer.

0.01 M Na_3-citrate

0.25 M sucrose pH 6.0

0.0005 M $MgCl_2$

2.2B.4 Assay buffers.

a) 0.01 M Tris-HCl pH 7.5

0.001 M EDTA

b) 0.01 M Tris-HCl

$MgCl_2$ at concentrations : pH 7.0

0.001 M, 0.003 M, or 0.005 M

c) 0.01 M Tris-HCl pH 7.0

0.0003 M $MgCl_2$ 8.0

d) 0.1 M Tris-HCl pH 7.0

0.0003 M $MgCl_2$

e) 0.05 M Tris-acetate

0.25 M sucrose pH 7.0

0.0001 M $MgCl_2$

f) 0.05 M Tris-HCl 6.0

0.05 M MES pH 6.5

0.003 M $MgCl_2$ 7.0

g) 0.01 M MES pH 6.0

50 $\mu g.ml^{-1}$ chloramphenicol

h) 0.01 M HEPES pH 7.0

50 $\mu g.ml^{-1}$ chloramphenicol

i) 0.01 M Na_3-citrate 5.5

0.25 M sucrose pH 6.0

0.005 M $MgCl_2$ 7.0

j) 0.002 M Na-acetate

0.01 M $CaCl_2$ pH 4.8

50 $\mu g.ml^{-1}$ chloramphenicol

k) 0.01 M Na-phospate

0.001 M EDTA pH 6.6

0.25 M sucrose

l) 0.1 M MES pH 5.0

0.003 M $MgCl_2$ 5.5

6.0

2.2B.5 Preparation of tissue extract

From the time of harvesting the plant material, all operations were performed at 0 - 4°C. The harvested material was weighed, and then homogenised, using a mortar and pestle, in the chosen grinding buffer (see 2.2B.2). The homogenate was then spun in 50 ml centrifuge tubes in a Sorval RC-5B Superspeed Centrifuge. The resulting pellets and / or supernatants were used in the binding assays as the "tissue extracts". A wash step (see 2.2B.3) was incorporated with some of the pelleted fractions, prior to resuspension of the pellets in assay buffer (see 2.2B.4).

g force		length of spin (min)	tissue extract
0 - 500	x	10	i) 500 x g pellet ii) 500 x g supernatant (= crude extract)
0 / 500 - 4000	x	20	i) 4000 x g pellet ii) 4000 x g supernatant

g force		length of spin (min)	tissue extract
4000 - 39,000	x	30	i) 39000 x g pellet ii) 39000 x g supernatant
39,000 - 80,000	x	30	i) 80000 x g pellet ii) 80000 x g supernatant

2.2B.6 Protein estimation

Before assaying, part of the tissue extract was set aside for protein estimation, using the method of Lowry *et al.* (1951). The protein standard used was bovine serum albumin; Figure 2 shows the standard curve.

2.2B.7 Ammonium sulphate precipitation of proteins

In some cases, see Results 2.3B section, the proteins in the extracts were concentrated by ammonium sulphate treatment. $(NH_4)_2SO_4$ (specially low in heavy metals) was slowly added to 100% saturation at 4°C with constant stirring. The resulting precipitated proteins were pelleted by centrifuging at 20000 x g for 30 min. Before assaying for ABA-binding, the pellet was resuspended in assay buffer. The $(NH_4)_2SO_4$ was often removed by either :

(i) overnight dialysis in Visking tubing against 2 litres of assay buffer,

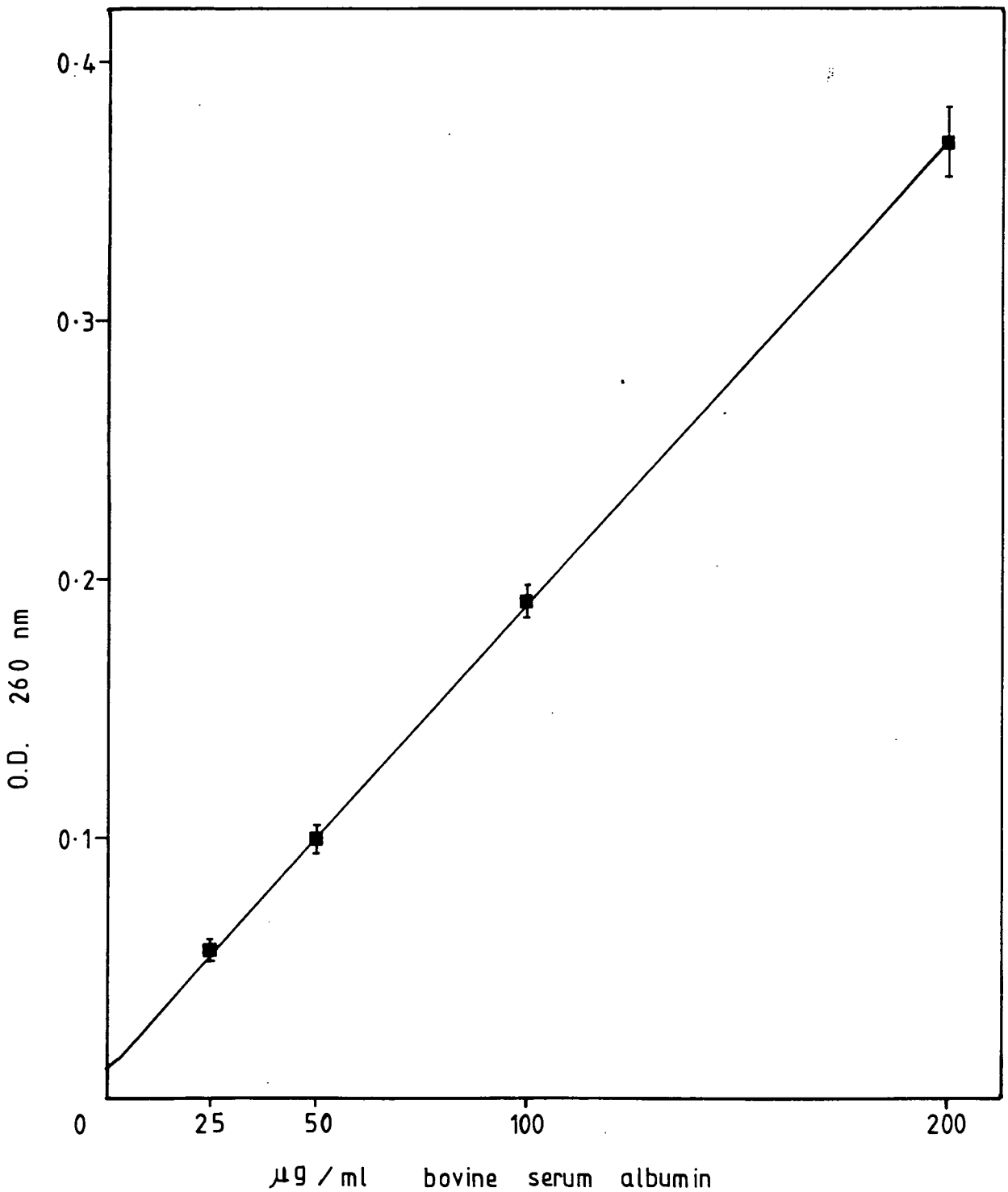
FIGURE 2

Standard Protein Curve - Lowry method.

The protein content of the various tissue extracts was estimated by the method of Lowry *et al.*, 1951. This involves the production of a blue colour resulting from the reduction of the Folin Reagent by the amino acids tyrosine, tryptophan and cysteine. The intensity of the blue colour, measured spectrophotometrically at 620 nm is representative of the total amount of protein present.

Bovine serum albumin was used as the standard protein. The results shown are the averages from 36 standard curves. Bars denote standard errors.

FIG 2



or (ii) passing through a Sephadex desalting column.

2.2B.8 Labelling with ^{3}H-ABA

Tissue extracts were divided into halves by volume :

a) ^{3}H-ABA was added to a concentration ranging from 10^{-9} M to 10^{-7} M (= "HOT" assay).

b) ^{3}H-ABA + [1000 x concentration of ^{3}H-ABA] non-radioactive ABA added (= "HOT + COLD" assay).

Both "HOT" and "HOT + COLD" assays were performed in triplicate at each ^{3}H-ABA concentration tested.

2.2B.9 Binding assays

A. DEAE-Cellulose filters - retention of ABA-protein bound complexes by ionic charge. (Santi *et al.*, 1973; Keith *et al.*, 1982).

A 2.5 mm diameter Whatman DE-81 filter was placed in a single metal filtration assembly attached to a water pump, and 20 ml ice-cooled assay buffer pulled through under vacuum. The "HOT" or "HOT + COLD" assay mixture was pulled through, followed by a further 20 ml wash with assay buffer. 20 ml was found to be the minimum volume required to remove unbound ^{3}H-ABA from the filters (see Figure 3). The wet filter was then removed to a scintillation vial containing 1 ml methanol. 6 ml scintillation fluid was added and the vial scintillation counted, as in 2.2A.6.

FIGURE 3

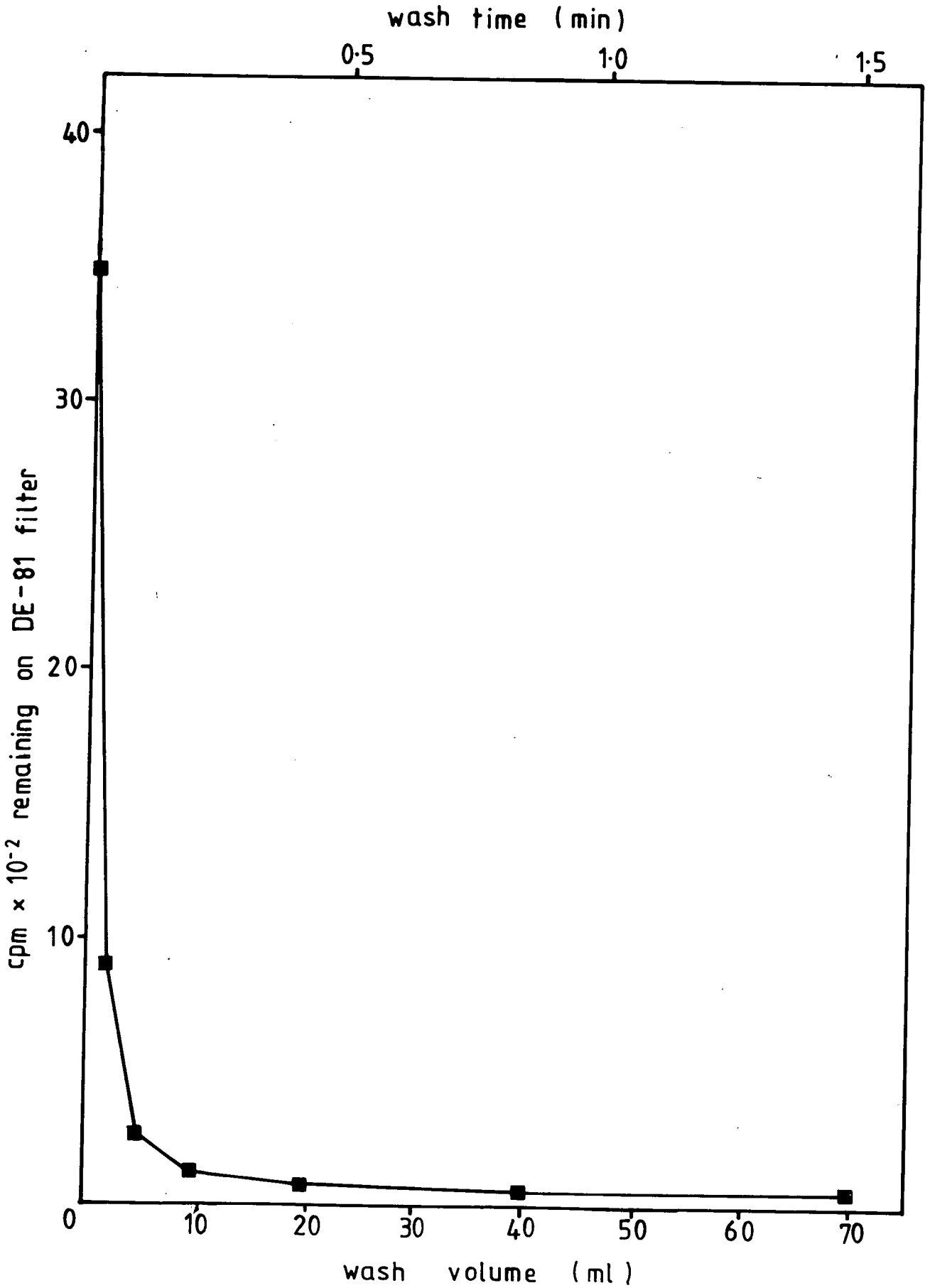
Wash volume required to remove ^{3}H-ABA from DE-81 filters

Whatman 2.5 cm diameter DE-81 filters were assembled in a metal filtration unit and fitted to a Buchner flask attached to a water vacuum pump.

8.3×10^{-8} M ^{3}H-ABA was diluted to 1.26 ml with assay buffer a) (10 mM Tris-HCl and 1 mM EDTA) pH 7.5, and applied to the DE-81 filters in 50 µl aliquots. The filters were then washed with known volumes of the assay buffer and the wash time required noted. The filters were removed to scintillation vials and 1 ml methanol added followed by 6 ml scintillation fluid, and the filters scintillation counted to check for retention of the ^{3}H-ABA by the filters.

The results are the averages of 3 replicates at each wash volume.

FIG 3


wash time (min)
0·5
1·0
1·5
40
30
20
10
0
cpm × 10⁻² remaining on DE-81 filter
10
20
30
40
50
60
70
wash volume (ml)

B. Amicon filtration with YMT membranes - retention of ABA-protein bound complexes by molecular size.

The YMT membranes were fitted into the Amicon micropartition system MPS-1 units and 1 ml of the "HOT" and "HOT + COLD" extracts added. The units were put into 50 ml MSE centrifuge tubes and spun for 10 min at 500 x g in a swing-out rotor in a pre-cooled (4°C) MSE Mistral 4L Centrifuge. The filters were then removed and scintillation counted, as in 2.2A.6.

C. Photoaffinity labelling. (Gronemeyer and Pongs, 1980).

This method was tried once only, as follows. All operations (excluding exposure to UV irradiation) were carried out at 0 - 4°C. 6.4 g fresh weight of barley aleurone layers were prepared as in 2.2A.2 g). They were ground up in 20 ml grinding buffer c) above (0.003 M $MgCl_2$) pH 7.0. The homogenate was filtered through 3 layers of muslin, spun in 50 ml centrifuge tubes in a Sorval RC-5B Refrigerated Superspeed Centrifuge at 500 x g for 10 min, and the resulting supernatant used in the assays. 0.2 ml was reserved for protein estimation, and 18.5 ml of the remainder was removed and ^{3}H-ABA added to a final concentration of 5×10^{-9} M (= "HOT" extract). 9.25 ml of this "HOT" extract was removed and non-radioactive ABA added to a final concentration of 5×10^{-6} M ABA (= "HOT + COLD" extract). Both "HOT" and "HOT + COLD" extracts were divided into 8 x 1 ml in Pyrex glass tubes and exposed, in duplicate tubes, to irradiation from a broad

spectrum u.v. lamp* for 1, 2, 3, and 5 min respectively. The extracts were transferred to 50 ml centrifuge tubes and spun at 39000 x g for 30 min in a Sorval RC-5B Centrifuge. The tubes were drained, 2 ml methanol added to each and left for 1 hour at 0°C to extract. The methanol was transferred to scintillation vials, 12 ml scintillation fluid added, and the vials scintillation counted, as in 2.2A.6.

* 8W blacklight blue lamp from Sylvania Ltd (British agent Langrex Supplies Ltd., London SW16 6ED).

D. Centrifugal method.

This method was used for the pelletable extracts only i.e. the 500 x g, 4000 x g, 39,000 x g and 80,000 x g pelleted "tissue extracts". Following resuspension in a binding buffer and addition of the ^{3}H-ABA +/- non-radioactive ABA, the extracts were re-pelleted using the same g force in 1.5 ml centrifuge tubes in 8 x 50 ml Sorval adapters to re-pellet the original fraction. The pellets were resuspended in 3 x 0.1 ml aliquots of H_2O, and scintillation counted as in Methods 2.2A.6.

E. Equilibrium dialysis.

The following equilibrium dialysis assay methods (all carried out at 4°C) were employed :

a) Dianorm cells. (from Langnau Ltd, Zurich)

The apparatus contained 5 pairs of half-cells separated by Visking tubing. Each half-cell had a volume capacity of 1 ml. The tissue extracts, in assay buffer, were put in one side

of a pair of half-cells, and the other side filled with assay buffer containing either :

i) ^{3}H-ABA only (= "HOT" assay).

or ii) ^{3}H-ABA + non-radioactive ABA at a concentration of x 1000 that of the ^{3}H-ABA concentration (= "HOT + COLD" assay).

The apparatus was revolved at 12 r.p.m., using a motor drive, and left to dialyse overnight at 4°C. The contents of all the half-cells were then removed and scintillation counted, as before.

b) Hoefer equilibrium dialysis apparatus. (from Hoefer Ltd, U.S.A.)

The half-cells were separated by EM104 membranes (exclusion size of 6-8000 daltons). Each half-cell had a volume capacity of 0.25 ml. The assay procedure was the same as that used in a) above.

c) Visking tubing.

Extracts were secured inside Visking tubing (size 8/32) which was suspended in 2 litre beakers containing 1.5 litres of assay buffer and ^{3}H-ABA +/- non-radioactive ABA. The beakers were stirred on a magnetic stirrer, and sampled at regular intervals until the entire system had reached equilibrium.

F. Sephadex G-25 column.

Bovine serum albumin (BSA) was passed through a 50 cm column of Sephadex G25 to find the exclusion volume for proteins. BSA was detected by its absorbance at 280 nm. The

following were then passed through the column using assay buffer :

(i) ^{3}H-ABA in assay buffer, then

(ii) ^{3}H-ABA plus tissue extract in assay buffer.

0.5 ml fractions were collected and scintillation counted to to determine whether any of the ^{3}H-ABA in (ii) was protein -bound and hence eluted in the column void volume.

2.3 ^{3}H-ABA BINDING RESULTS

2.3.A *IN VIVO* EQUILIBRIUM DIALYSIS RESULTS

The *in vivo* equilibrium dialysis involved the incubation of whole plants, or intact tissues, at 0.5°C in radioactive (^{3}H-) ABA. The principle involved in detecting ABA-binding using this method relies on the plasma membrane acting as a semi-permeable membrane, equivalent to the dialysis tubing as used in dialysis techniques. At a steady state of equilibrium, the concentration of "free" ^{3}H-ABA inside the plant tissues should be the same as that in the external medium, due to passive diffusion. Any excess ^{3}H-ABA found within the tissues is therefore due either to active uptake, or to binding (specific or non-specific) of the ABA by the tissues. Any specific ABA-binding should be displaced by an excess of non-radioactive ABA. Keeping the incubation temperatures at 0.5°C was intended to ensure that the ABA could not be actively metabolised by the tissues.

Figure 4 shows the results of *in vivo* equilbrium dialysis of pea epicotyls pH 7.0, wheat coleoptiles pH 6.5, and maize coleoptiles pH 7.5, all in assay buffer b). None of these tissues, although sliced into 1 mm sections in the hope of facilitating rapid diffusion, reached 100% equilibrium with the external medium, although the internal levels of ^{3}H-ABA did seem to level off after about 4 days.

It was wondered whether it was necessary to induce the

FIGURE 4

In vivo equilibrium dialysis of pea epicotyls, wheat coleoptiles and maize coleoptiles.

1 mm thick sections of dark-grown :

A. Pea epicotyls

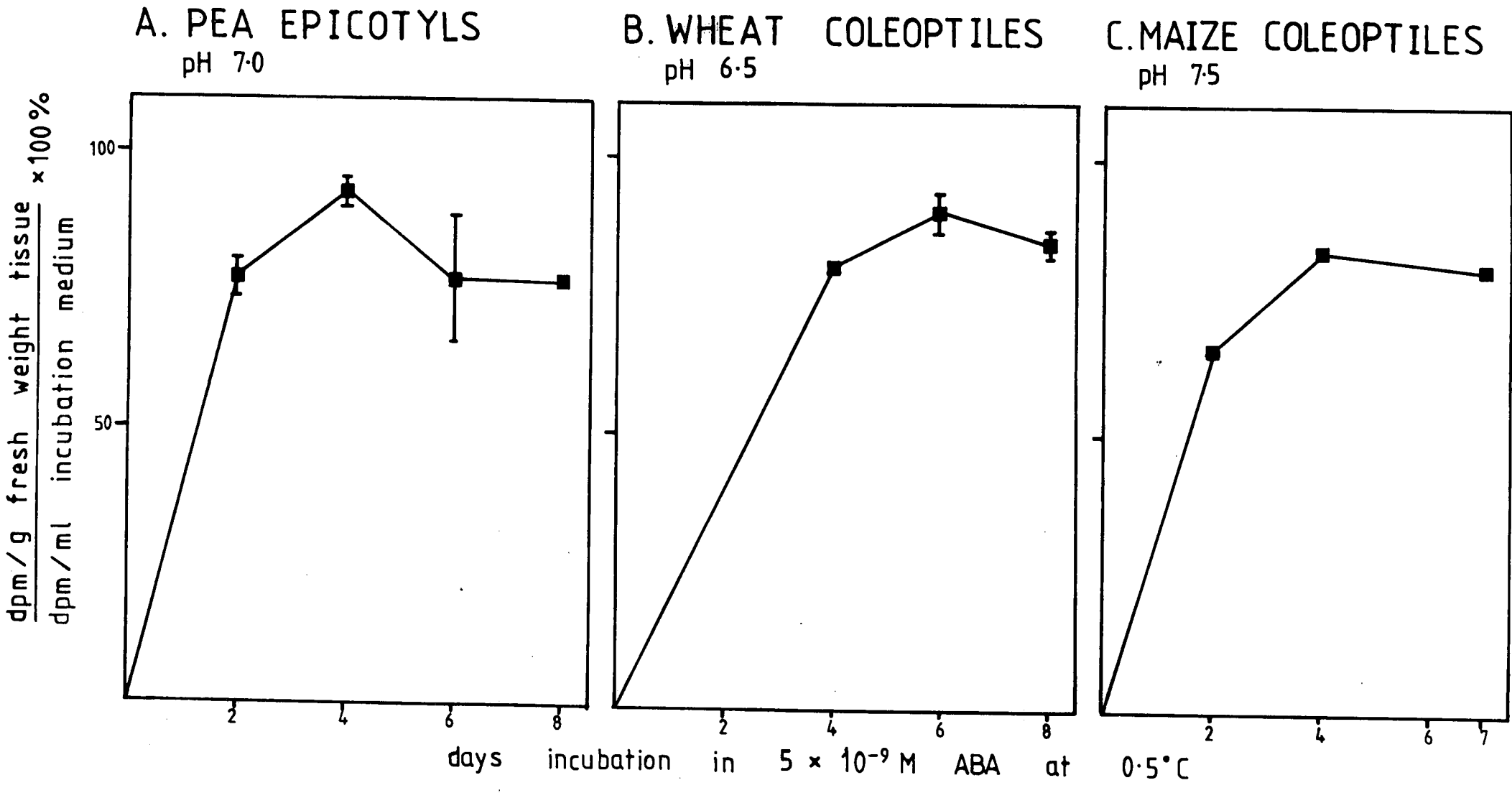
B. Wheat coleoptiles

C. Maize coleoptiles

were incubated at 0.5°C in assay buffer d) (see *In vivo* Methods 2.2A) containing 5×10^{-9} M ^{3}H-ABA at pH 7.0, 6.5 and 7.5 respectively. Samples of tissues and incubation medium were taken daily, and scintillation counted for ^{3}H-ABA content.

100% represents the relative level of radioactivity in the incubation medium. This remained constant throughout the duration of the experiment. Bars, where present, show the difference between duplicate samples within the same experiment.

FIG 4


A. PEA EPICOTYLS
pH 7·0
B. WHEAT COLEOPTILES
pH 6·5
C. MAIZE COLEOPTILES
pH 7·5
dpm/g fresh weight tissue / dpm/ml incubation medium ×100%
100
50
2
4
6
8
2
4
6
8
2
4
6
7
days incubation in 5 × 10⁻⁹ M ABA at 0·5°C

presence of ABA-receptors under conditions of active metabolism of the plant tissues. Maybe the receptors are not normally present in an active form capable of binding ABA, but need to be "switched on" by some active process; and this would be impossible under the low-temperature conditions used in the incubation assays. To investigate this, in many of the *in vivo* experiments, plants or plant tissues were pre-incubated, or pre-imbibed, in non-radioactive ABA at a temperature of 25°C to see if ABA-binding proteins could be induced in this way.

The results obtained from pre-incubating *Lemna perpusilla* in H/2 medium pH 6.4 containing 10^{-6} M non-radioactive ABA for 2 days, prior to equilibrium dialysis in ^{3}H-ABA at 0.5°C are shown in Figure 5, along with a control group of plants which received no ABA pre-incubation. It should be noted that all the *in vivo* experiments were carried out in covered ice-buckets to maintain the temperature at 0.5°C, and hence the plants / plant tissues were kept in the dark during this period. Figure 5 suggests that pre-incubation in ABA may have caused some eventual increase in the final levels of ^{3}H-ABA taken up by the plants in A, as compared with those not pre-incubated, in B. However, the addition of 10^{-6} M non-radioactive ABA after 8 days did not prevent the levels of ^{3}H-ABA in A from continuing to rise. Whether the non-radioactive ABA displaced any ^{3}H-ABA in B is not clear due to the variable results obtained.

Figure 6 shows the uptake of 10^{-9} M ^{3}H-ABA by *Spirodela*

FIGURE 5

In vivo equilibrium dialysis of *Lemna perpusilla* with ^{3}H-ABA - the effect of pre-incubation in ABA.

Lemna perpusilla fronds were split into two groups :

A. Pre-incubated in 10^{-6} M ABA for 2 days.

B. Not pre-incubated.

Plants from both A and B were removed to H/2 medium containing 10^{-6} M ^{3}H-ABA and incubated at 0.5°C for 6 days, sampling at daily intervals for internal ^{3}H-ABA levels. After 6 days, 10^{-5}M non-radioactive ABA was added to the medium. Further samples were taken 4 days later.

These results are the compilation of 4 separate experiments. Bars denote standard errors.

FIG 5

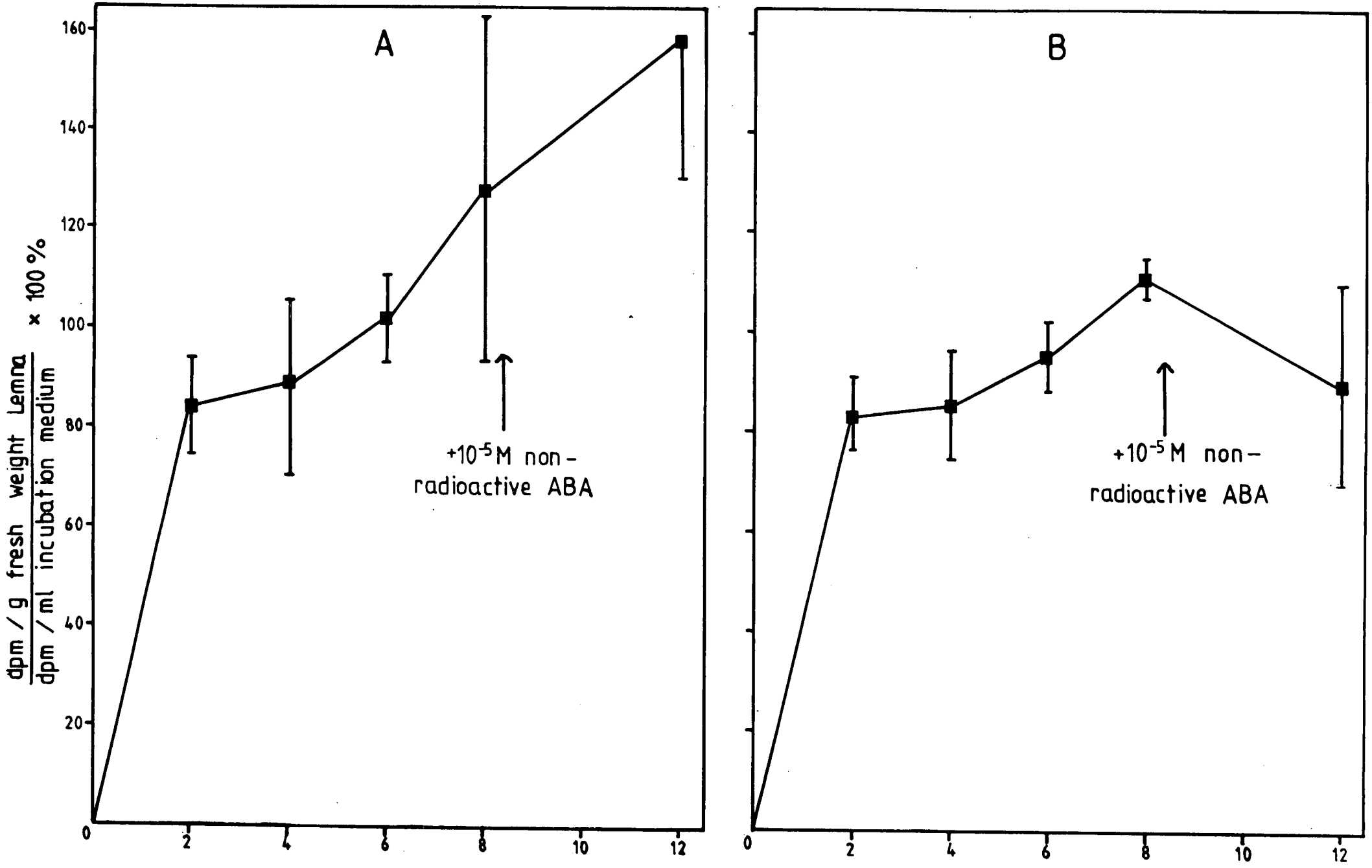

A
B
160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
dpm / g fresh weight Lemna / dpm / ml incubation medium × 100 %
+10⁻⁵ M non-radioactive ABA
+10⁻⁵ M non-radioactive ABA

FIGURE 6

In vivo equilibrium dialysis of ^{3}H-ABA with *S. polyrrhiza* fronds + / - pre-incubation in ABA.

Spirodela polyrrhiza fronds were divided into two groups :

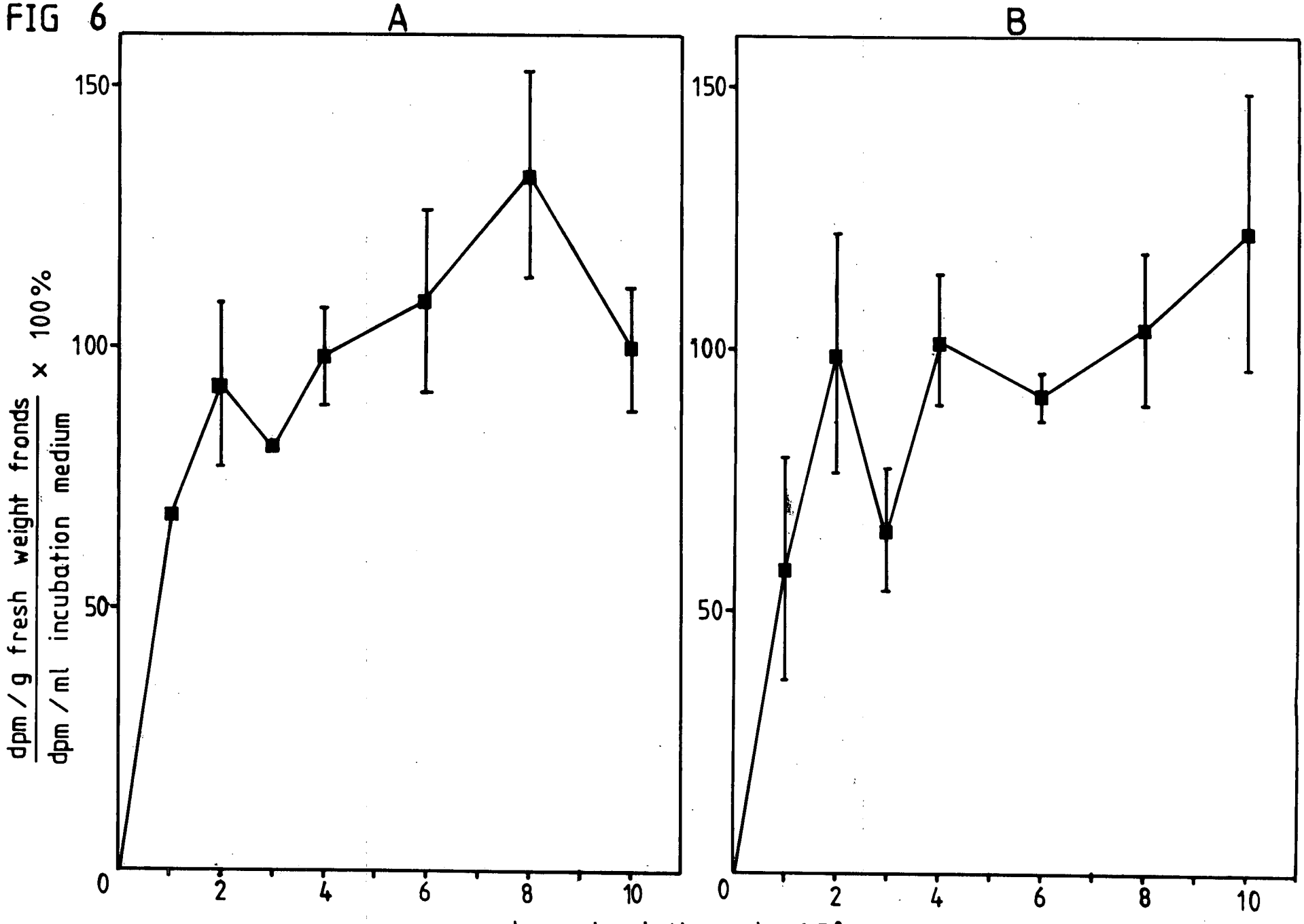
A. Pre-incubated in 10^{-8} M ABA for 2 days.

B. Not pre-incubated.

A and B fronds were removed to fresh H/2 medium and 10^{-9} M ^{3}H-ABA added to the medium. They were incubated at 0.5°C for 10 days, sampling at daily intervals to measure internal ^{3}H-ABA levels by scintillation counting.

The results are the compilation of 6 different experiments. Bars denote standard errors.

FIG 6


A
B
150
100
50
0
2
4
6
8
10
dpm / g fresh weight fronds / dpm / ml incubation medium x 100%

polyrrhiza fronds, both with and without pre-incubation in 10^{-8} M ABA. There does not appear to be any significant difference in uptake between the two groups.

The importance of keeping the incubation temperature at 0.5°C, and also of keeping constant checks on the levels of available ^{3}H-ABA in the incubation medium is demonstrated in Figure 7. Here, using an incubation temperature of 4°C the level of radioactivity accumulated by the fronds increased until a point was reached, after 5 days, where the ^{3}H-ABA amount in the medium was no longer in great excess of that within the plant tissues, and hence not effectively constant with respect to the fronds for the duration of the experiment. To overcome this problem, either higher levels of ^{3}H-ABA, or larger volumes of incubation medium, were used. Figure 7 also demonstrates that a steady state of equilibrium was not obtained at 4°C. This was presumably due to active metabolism of the ^{3}H-ABA by the tissues.

It was found that the single most important factor responsible for uptake into plant tissues at 0.5°C was the external pH (Astle and Rubery, 1980). The reasons for this are reviewed in the Discussion section. Figure 8 shows the effect on uptake of ^{3}H-ABA by *S. polyrrhiza* fronds of varying the pH of the incubation medium (H/2) between 5, 6, and 7. Over a period of 3 days, only at pH 5 did the levels of ^{3}H-ABA inside the fronds exceed that in the incubation medium. At pH 7, uptake was very slow. Another interesting result here was

FIGURE 7

Uptake of ^{3}H-ABA by *S. polyrrhiza* fronds at 4°C.

Spirodela polyrrhiza fronds were incubated at 4°C in 10 ml H/2 medium pH 6.4 containing 10^{-9} M ^{3}H-ABA. Samples of fronds and incubation medium were taken at daily intervals, and scintillation counted.

■——————■ *S. polyrrhiza* fronds.

□— — — —□ H/2 medium + ^{3}H-ABA.

FIG 7

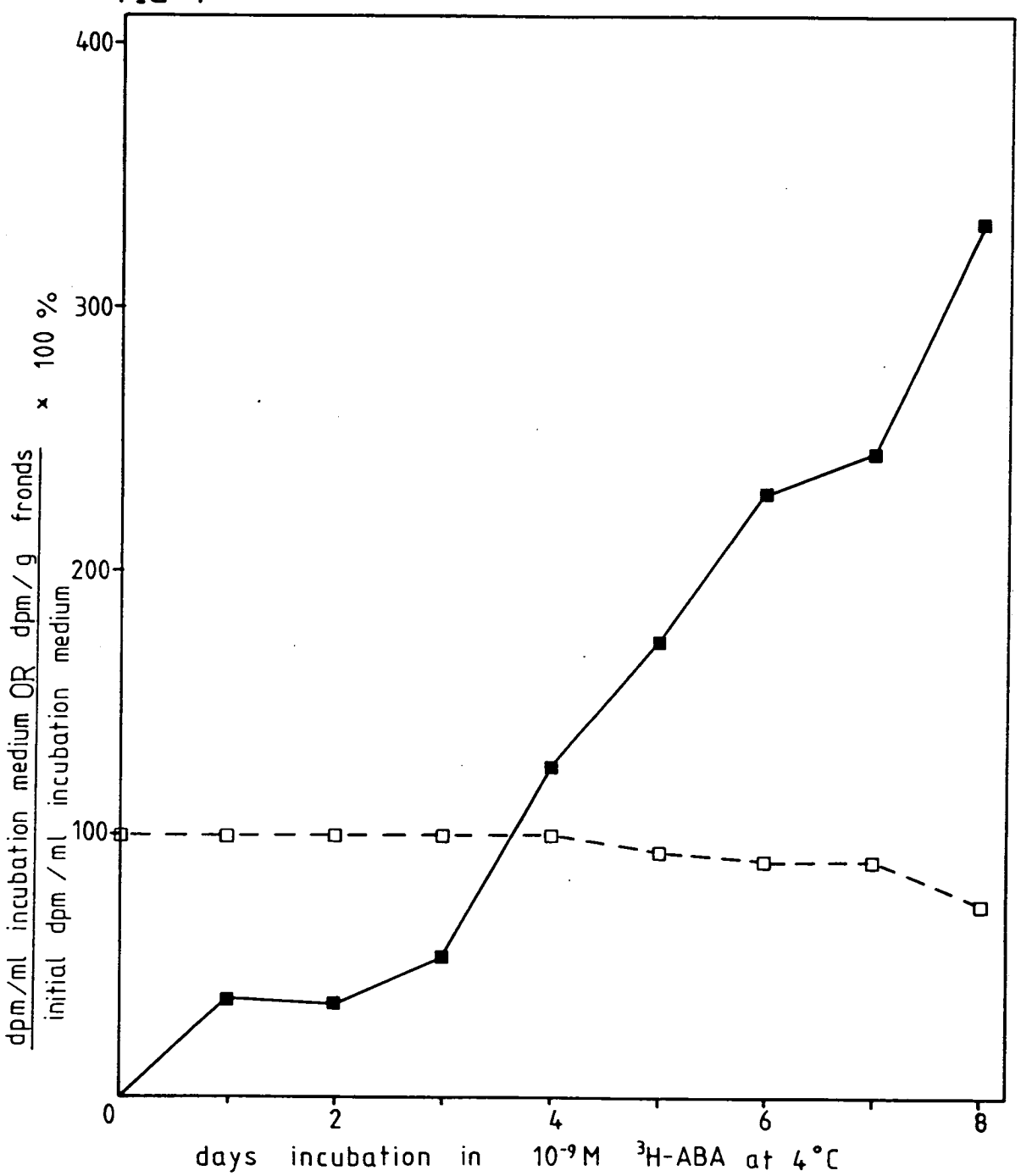

400
300
200
100
0
2
4
6
8
dpm/ml incubation medium OR dpm/g fronds
initial dpm/ml incubation medium
x 100 %
days incubation in 10⁻⁹M ³H-ABA at 4°C

FIGURE 8

In vivo equilibrium dialysis of *S. polyrrhiza* fronds with ^{3}H-ABA - at pH's 5.0, 6.0 and 7.0.

Spirodela polyrrhiza fronds were incubated at 0.5°C in H/2 medium at pH 5.0, 6.0 and 7.0. At each pH, parallel incubations were carried out containing :

A. 10^{-9} M ^{3}H-ABA ("HOT")

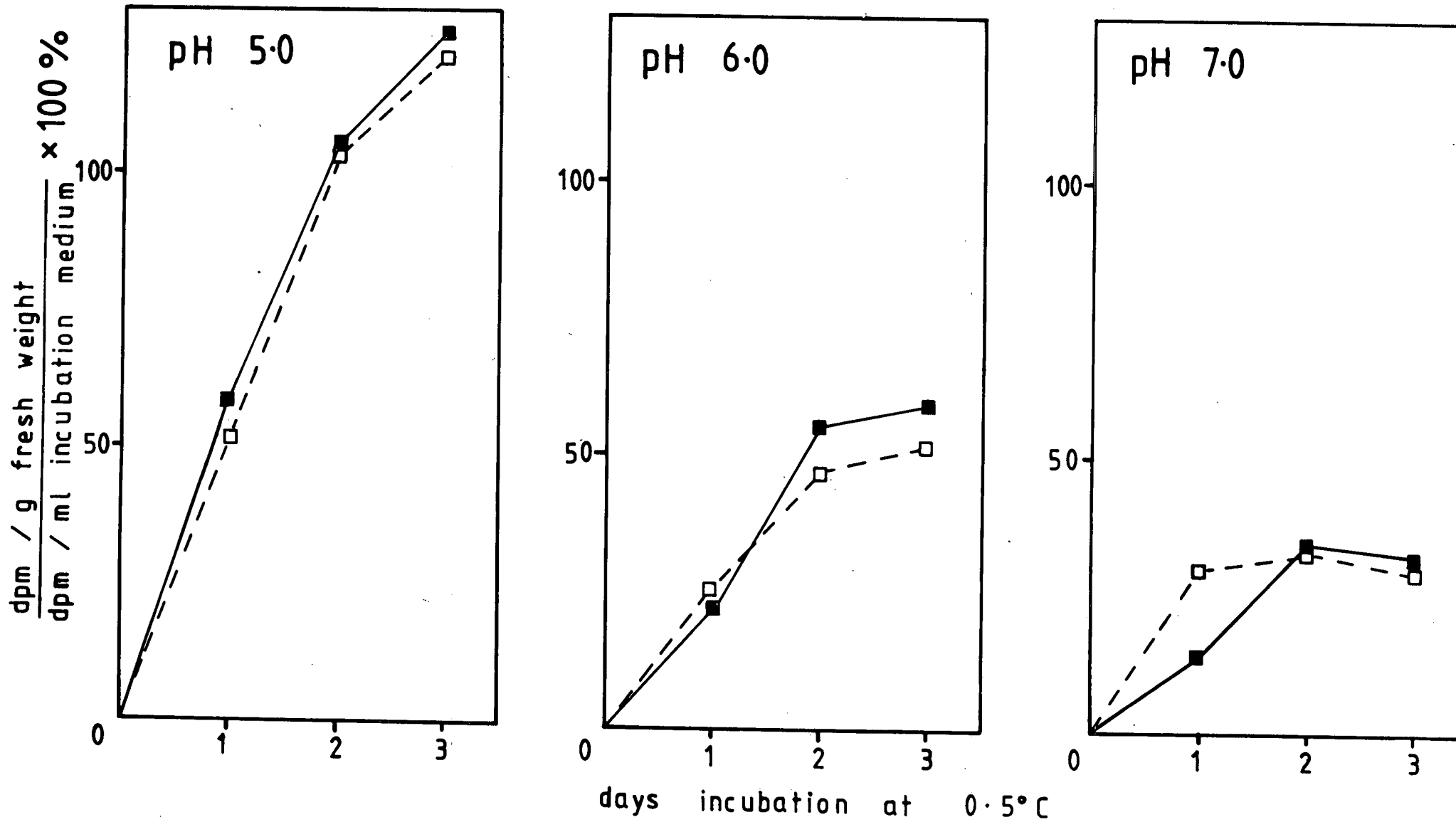
B. 10^{-9} M ^{3}H-ABA + 10^{-6} M non-radioactive ABA ("HOT + COLD")

Samples of fronds and incubation media were taken at daily intervals, and scintillation counted.

■——————■ 10^{-9} M ^{3}H-ABA only.

□— — — —□ 10^{-9} M ^{3}H-ABA + 10^{-6} M non-radioactive ABA.

FIG 8


pH 5·0
pH 6·0
pH 7·0
dpm / g fresh weight / dpm / ml incubation medium × 100 %
100
50
0
1
2
3
days incubation at 0·5°C

that, at all pH's, the levels of internal ^{3}H-ABA were not reduced by parallel incubations that also included 10^{-6} M non-radioactive ABA. This suggests that the uptake capacity of the fronds for ABA was not saturated at 10^{-6} M ABA.

Figure 9 shows a similar pattern involving pH-dependent uptake of ^{3}H-ABA by barley aleurone layers. Whilst the final levels of internal ^{3}H-ABA isolated from the aleurones were far in excess of those reached by fronds :

$$\frac{\text{dpm / g fresh weight}}{\text{dpm / ml incubation medium}} \times 100\ \%$$

	pH 5.0	pH 6.0	pH 7.0
FRONDS	130	60	50
ALEURONES	400	150	10

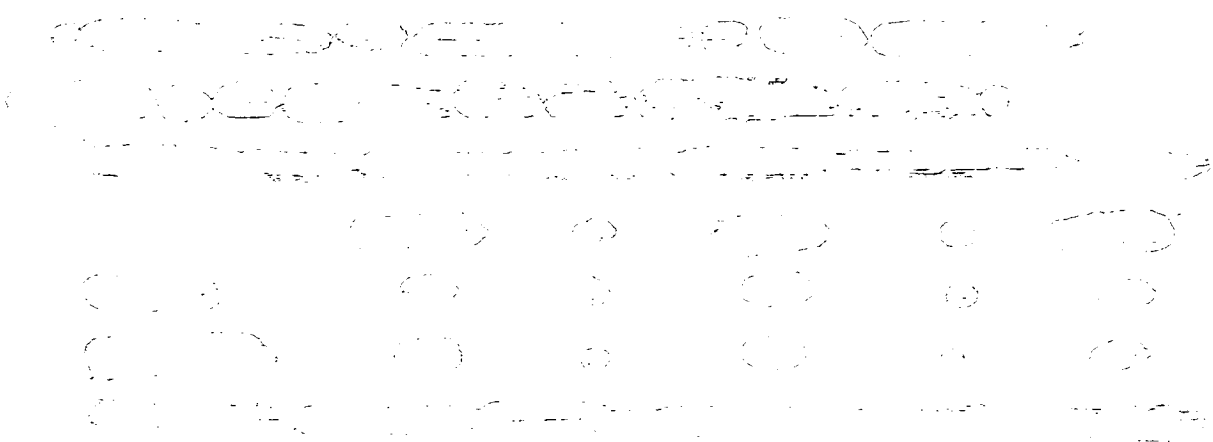


Figure 10 shows the uptake, at pH 6.4, of 2.5×10^{-9} M ^{3}H-ABA by turions induced :

A. by the addition of 10^{-6} M ABA to fronds,

FIGURE 9

In vivo equilibrium dialysis of barley aleurone layers with ^{3}H-ABA - at pH's 5.0, 6.0 and 7.0.

Barley aleurone layers were incubated at 0.5°C in assay buffer c) (100 mM Tris-HCl, 100 mM MES, 3 mM $MgCl_2$) containing 10^{-9} M ^{3}H-ABA at pH 5.0, 6.0 and 7.0. Samples of aleurones and incubation medium were taken at daily intervals, and scintillation counted for ^{3}H-ABA content.

FIG 9

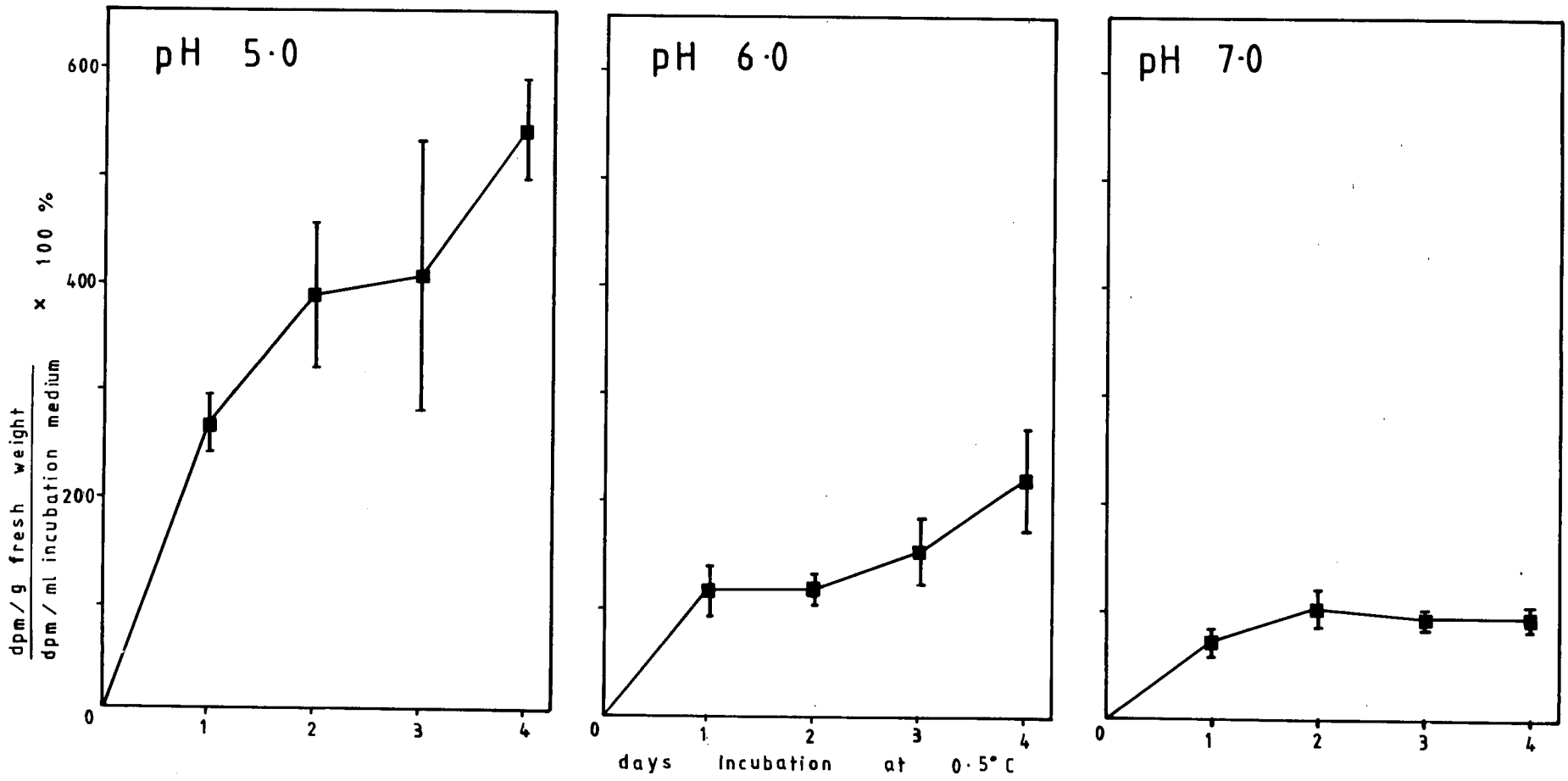

pH 5·0
pH 6·0
pH 7·0
600
400
200
0
dpm / g fresh weight / dpm / ml incubation medium × 100 %
1
2
3
4
days Incubation at 0·5° C

FIGURE 10

In vivo equilibrium dialysis of turions with ^{3}H-ABA. Effect of pre-exposure to ABA, and displacement of ^{3}H-ABA by non-radioactive ABA.

Spirodela polyrrhiza turions were induced from fronds either by :

A. Addition of 10^{-4} M ABA to the H/2 medium,

or B. Overcrowding fronds in low light conditions.

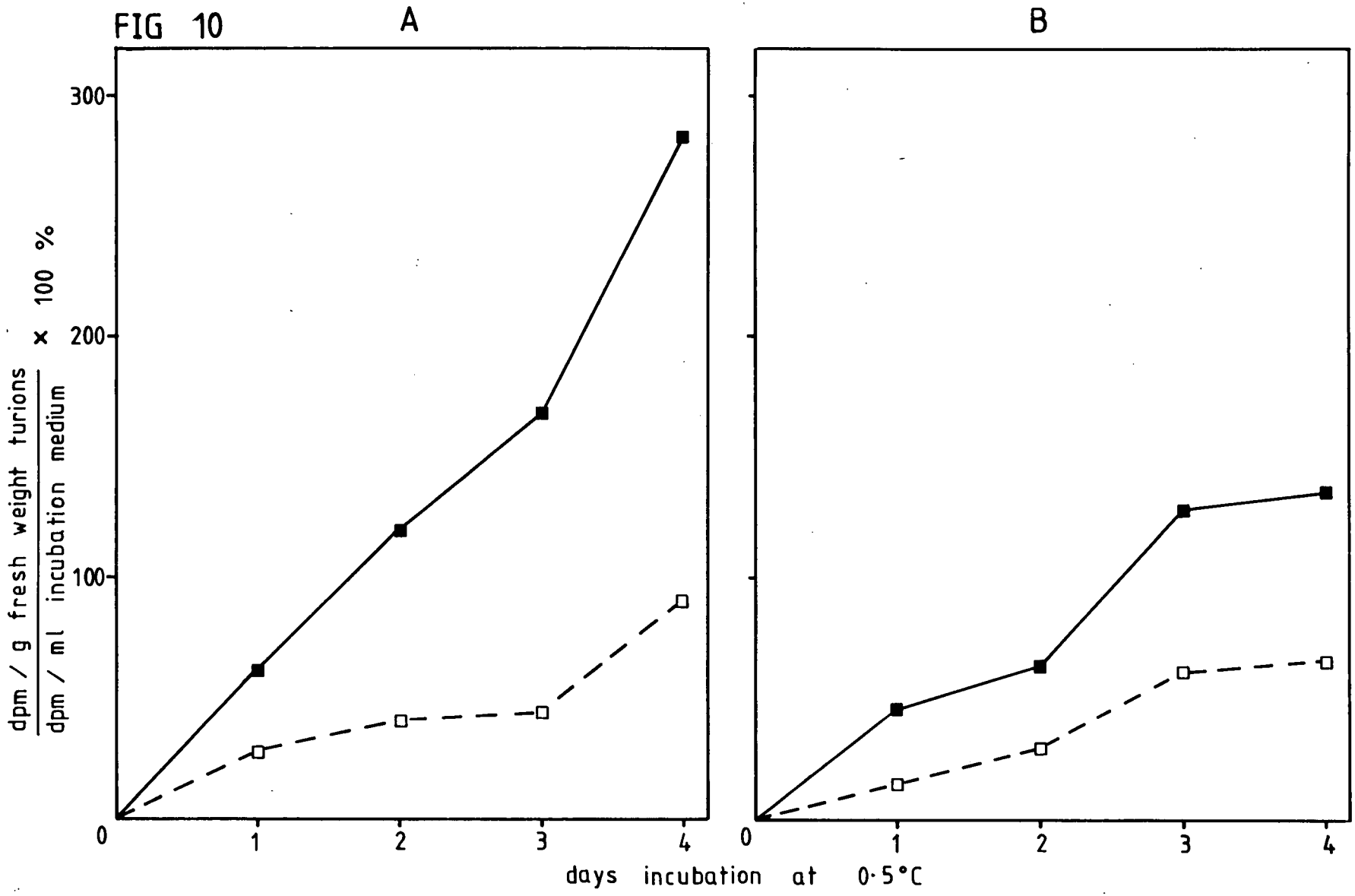
The turions from both groups, A and B, were incubated at 0.5°C in 10 ml H/2 medium pH 6.4 containing either :

i) 2.5×10^{-9} M ^{3}H-ABA (■——————■)

or ii) 2.5×10^{-9} M ^{3}H-ABA plus 10^{-4} M non-radioactive ABA (□— — — —□).

Turions and incubation media were sampled daily for ^{3}H-ABA content, as measured by scintillation counting.

FIG 10


A
B
300
200
100
0
1
2
3
4
dpm / g fresh weight turions / dpm / ml incubation medium × 100 %
days incubation at 0·5 °C

and B. by overcrowding fronds in low light conditions i.e. no pre-exposure to external ABA.

It appeared that turions previously exposed to ABA do have a greater capacity for uptake of ^{3}H-ABA. Also, in both A and B, parallel incubations also containing 10^{-4} M non-radioactive ABA depressed the uptake of ^{3}H-ABA to below the level of the incubation medium (= 100%). Hence, the capacity for uptake of ABA by turions shows saturation at 10^{-4} M, unlike that of fronds (Figure 8). The possibility that 10^{-5} M would displace more was not investigated.

Figure 11 shows a similar experiment with turions. In graphs III A and III B the amount of ^{3}H-ABA displaced by 10^{-4} M non-radioactive ABA is plotted. Whilst the results in III A appear erratic, overall, in both III A and III B, there does appear to be a component of ^{3}H-ABA which can be displaced.

The effect of incubating barley aleurones with 5×10^{-9} M ^{3}H-ABA plus increasing concentrations of non-radioactive ABA is shown in Figure 12. From 10^{-8} M to 10^{-4} M non-radioactive ABA, there appears to be an effect of "co-operative binding" - the greater the concentration of non-radioactive ABA, the greater the ^{3}H-ABA binding; although it is suspected that this effect may be an artifact. However, at 10^{-5} M non-radioactive ABA there was a difference between A : ABA pre-incubated, and B : not pre-incubated, aleurones. Whilst the uptake of ^{3}H-ABA increased in A i.e. the uptake capacity was not saturated at 10^{-4} M, the uptake decreased in B. However, this decrease in B resulted in a new level of internal ^{3}H-ABA below that achieved

FIGURE 11

In vivo equilibrium dialysis of turions + / - pre-exposure to ABA, and the resulting displacement of ^{3}H-ABA by non-radioactive ABA.

Spirodela polyrrhiza turions were induced from fronds by either :

A. Addition of 10^{-6}M ABA to the H/2 medium,

or B. Overcrowding fronds in low light conditions.

The turions from both groups A and B were incubated at 0.5°C in 10 ml H/2 medium pH 6.4 containing either :

I 2.5×10^{-9} M ^{3}H-ABA only,

or II 2.5×10^{-9} M ^{3}H-ABA + 10^{-6} M non-radioactive ABA.

The graphs in III (A and B) represent the differences between I and II (I-II) i.e. the ^{3}H-ABA displaced by the non-radioactive 10^{-6} M ABA.

FIG 11

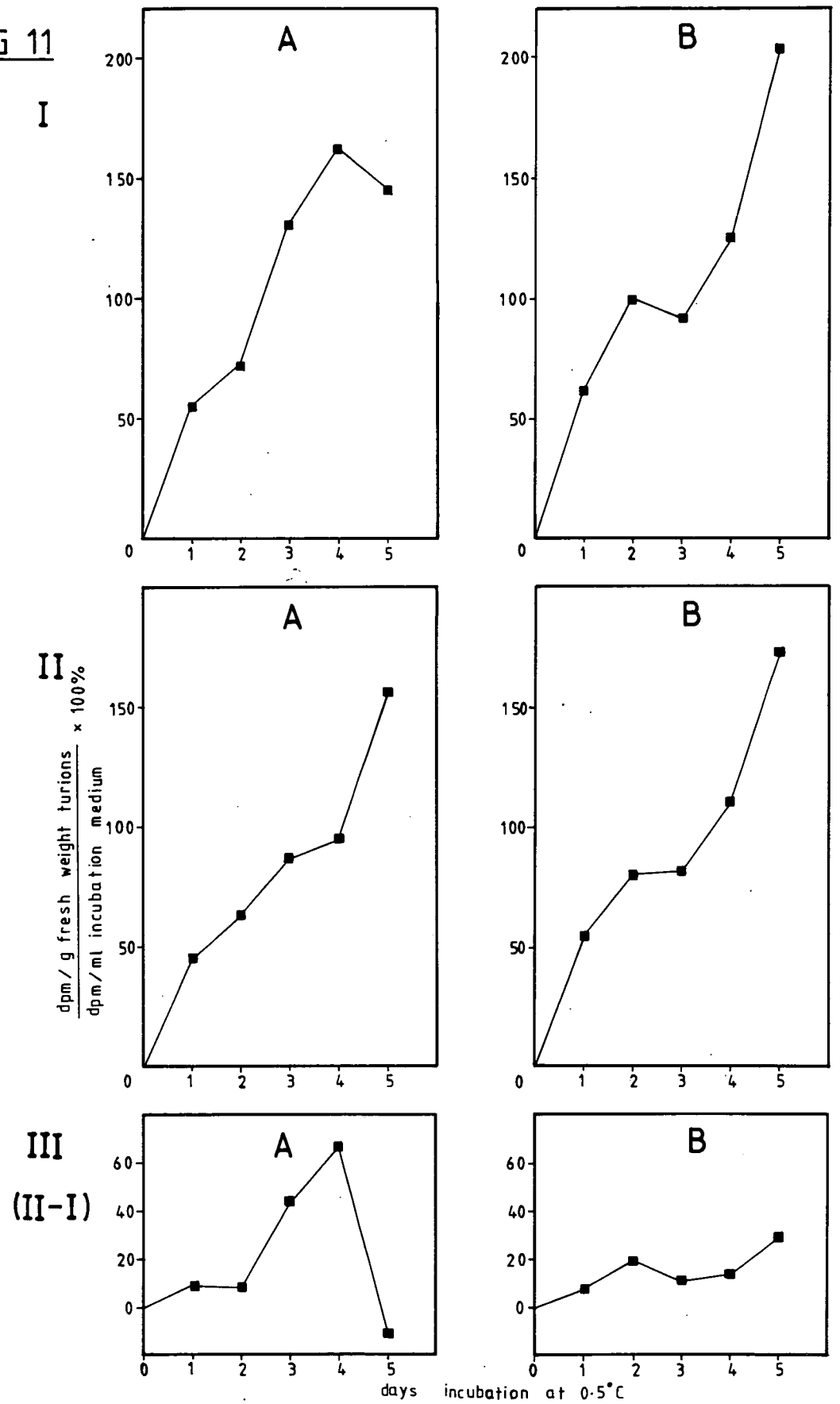

I
A
B
200
150
100
50
0
1
2
3
4
5
II
$\frac{\text{dpm/g fresh weight turions}}{\text{dpm/ml incubation medium}} \times 100\%$
III
(II-I)
60
40
20
days incubation at 0·5°C

FIGURE 12

In vivo equilibrium dialysis of ^{3}H-ABA with barley aleurones + / - pre-exposure to ABA, and the displacement of ^{3}H-ABA by non-radioactive ABA.

Barley aleurones were split into two groups :

A. Pre-imbibed in 10^{-6} M ABA at 25°C for 24 hours.

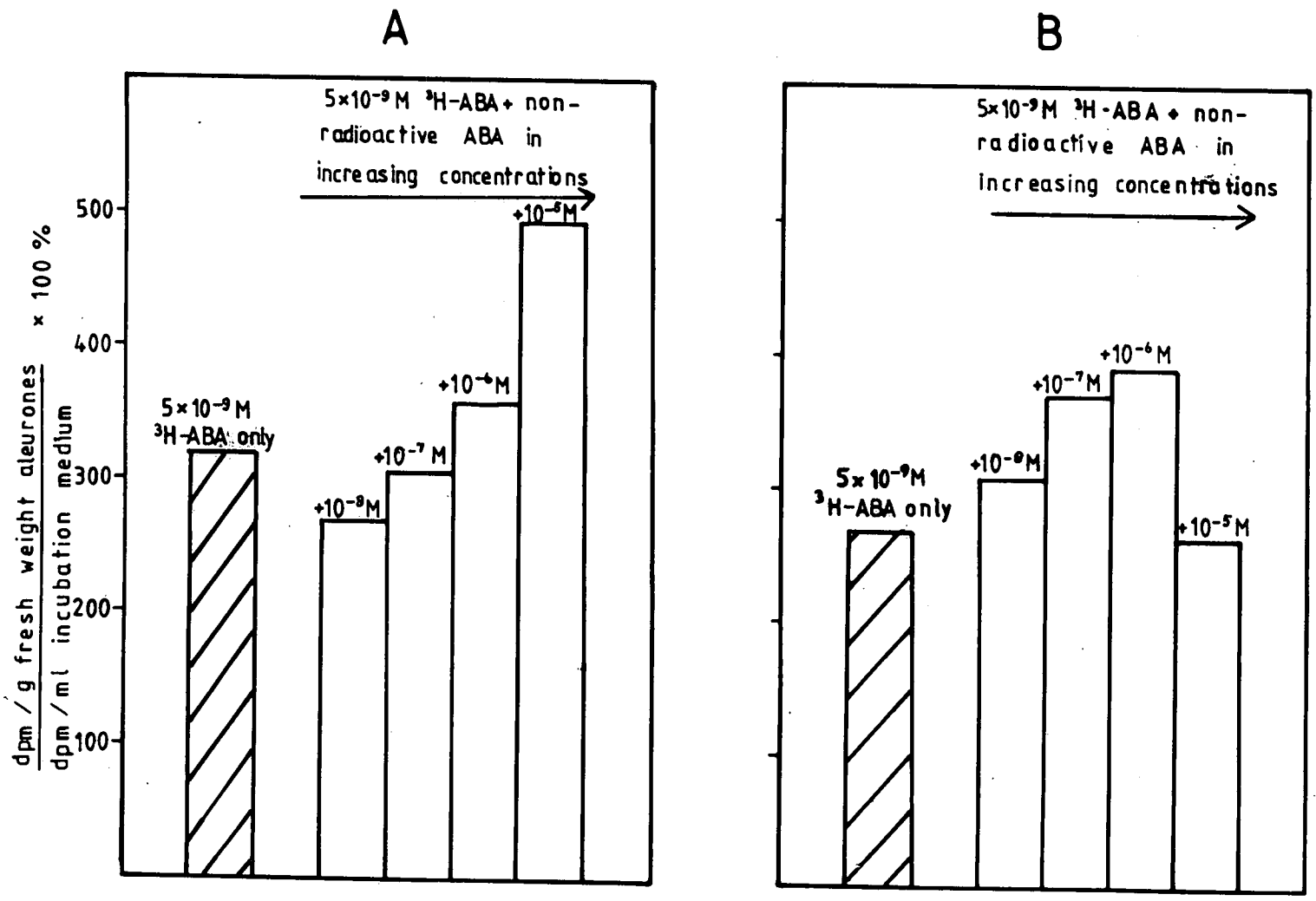
B. Not pre-imbibed.

Both A and B were then incubated in buffer b) pH 4.8 (2 mM sodium acetate, 10 mM calcium chloride and 50 ug.ml^{-1} chloramphenicol) containing 5 x 10^{-9} M ^{3}H-ABA plus non-radioactive ABA at each of the following concentrations :

0 (control), 10^{-6} M, 10^{-7} M, 10^{-6} M, 10^{-5} M.

All combinations were incubated at 0.5°C for 2 days. The aleurones and incubation medium were then sampled and scintillation counted as in *In vivo* Methods 2.2A.6.

FIG 12


A
B
5×10⁻⁹ M ³H-ABA + non-radioactive ABA in increasing concentrations
5×10⁻⁹ M ³H-ABA only
+10⁻⁸M
+10⁻⁷ M
+10⁻⁶M
+10⁻⁵M
dpm / g fresh weight aleurones / dpm / ml incubation medium × 100 %
100
200
300
400
500

with 10^{-8} M non-radioactive ABA which seems inexplicable, and so this result is probably artifactual.

The problems of obtaining reproducible results are further demonstrated in Figure 13. The two experiments shown, both with aleurones + / - pre-imbibition in 10^{-8} M ABA, were carried out using the same procedure, although on different dates and with different sampling frequencies. The results obtained were not very similar. Although it could be concluded that, in the pre-imbibed aleurones at least, there was some displacement of ^{3}H-ABA by the non-radioactive ABA, any interpretation of minor effects would have to be treated with caution.

In view of a report (Addicott and Lyon, 1969) of a synergistic promotion of growth using ABA plus GA_3 on cucumber hypocotyls, the effect of pre-imbibing aleurones in GA_3 was investigated. Figure 14 shows the effect of pre-imbibition of aleurones in 10^{-7} M GA_3 on the subsequent uptake of ^{3}H-ABA at pH 6.0 and pH 7.0. Pre-imbibition in GA_3 at pH 6.0 led to a higher uptake of ^{3}H-ABA than no pre-imbibition. This experiment was not repeated.

Figure 15 shows the effect of increasing the incubation temperature on the uptake of ^{3}H-ABA by barley aleurone layers. As in Figure 7 (*S. polyrrhiza* fronds at 4°C), the result of raising the temperature above 0.5°C was to increase this uptake sharply. It can be seen from Figure 12 that after only 2 days in 5 x 10^{-9} M ^{3}H-ABA, the level of radioactivity detected within the aleurones was nearly x 10 that in the

FIGURE 13

In vivo equilibrium dialysis using barley aleurones to demonstrate the difficulty if obtaining reproducible *in vivo* results.

Both experiments were carried out in the manner described below.

Barley aleurones were split into 2 groups :

A. Pre-imbibed in 10^{-6} M ABA at 25°C for 24 hours. ■————

B. Not pre-imbibed. □— — —□

Both A and B aleurones were then incubated in buffer b) pH 4.8 (2 mM sodium acetate, 10 mM calcium chloride and 50 ug.ml^{-1} chloramphenicol) containing 5 x 10^{-9} M ^{3}H-ABA for 4 days, sampling both aleurones and incubation media at regular intervals for radioactive content. After 4 days, 5 x 10^{-6} M non-radioactive ABA was added, and the incubation and sampling continued as before.

FIG 13

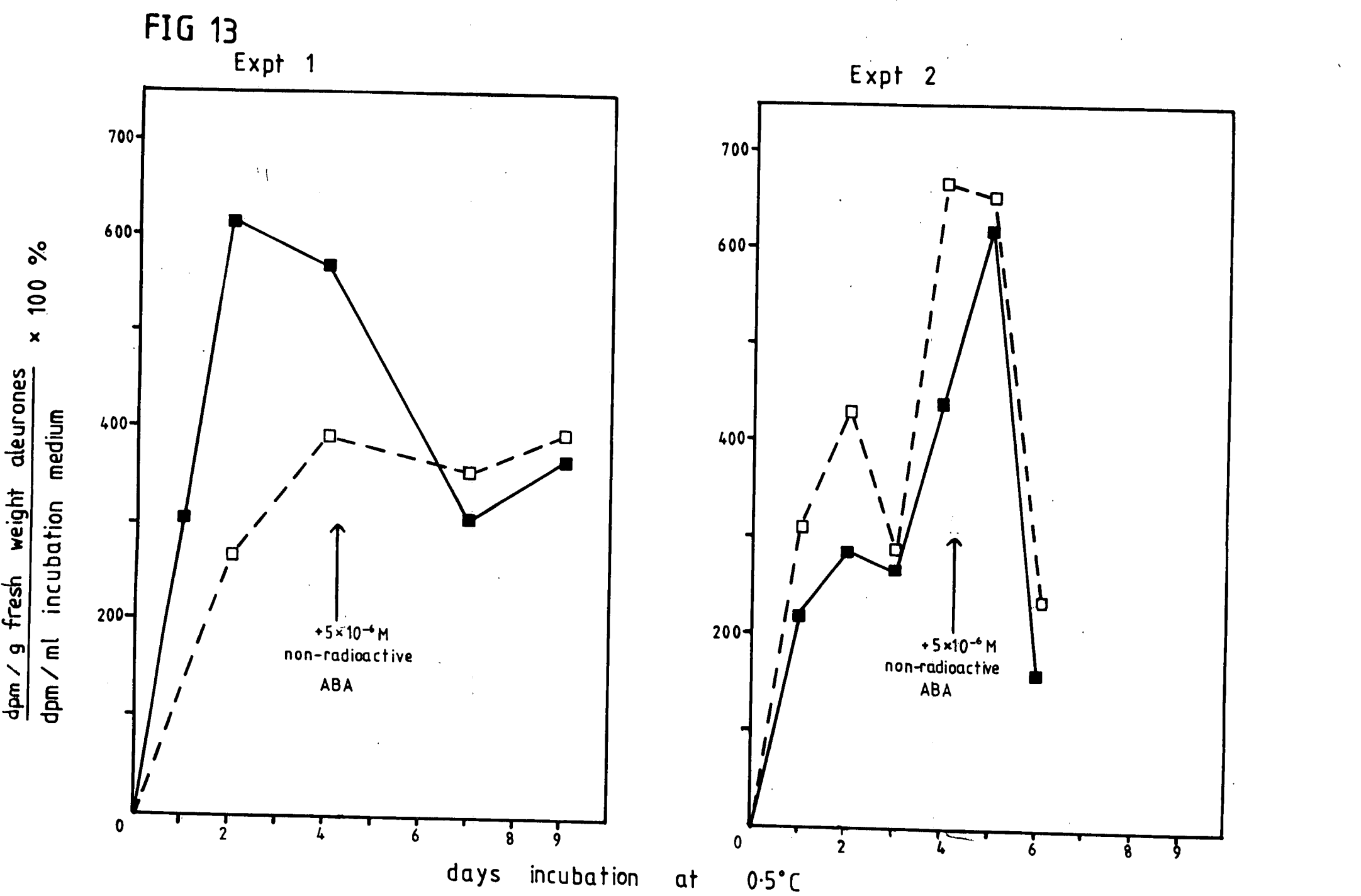

Expt 1
Expt 2
dpm / g fresh weight aleurones / dpm / ml incubation medium × 100 %
700
600
400
200
0
2
4
6
8
9
+5×10⁻⁶M non-radioactive ABA
days incubation at 0·5°C

FIGURE 14

In vivo equilibrium dialysis of ^{3}H-ABA with barley aleurones (+ / − pre-imbibition in GA_3) − pH 6.0 and 7.0.

Barley aleurones were divided into two groups :

I. Pre-imbibed at 25°C for 24 hours in 10^{-7} M GA_3. (■————)

II. Not pre-imbibed. (□– — —□)

Aleurones from Both A and B were then incubated with 10^{-8}M ^{3}H-ABA at 0.5°C in either :

A Buffer e) (50 mM MES, 50 µg.ml.$^{-1}$ chloramphenicol) pH 6.0,

or B Buffer d) (10 mM Tris-HCl, 1 mM EDTA and 0.25 M sucrose) pH 7.0.

Samples of aleurones and incubation medium were taken daily, and scintillation counted.

FIG 14

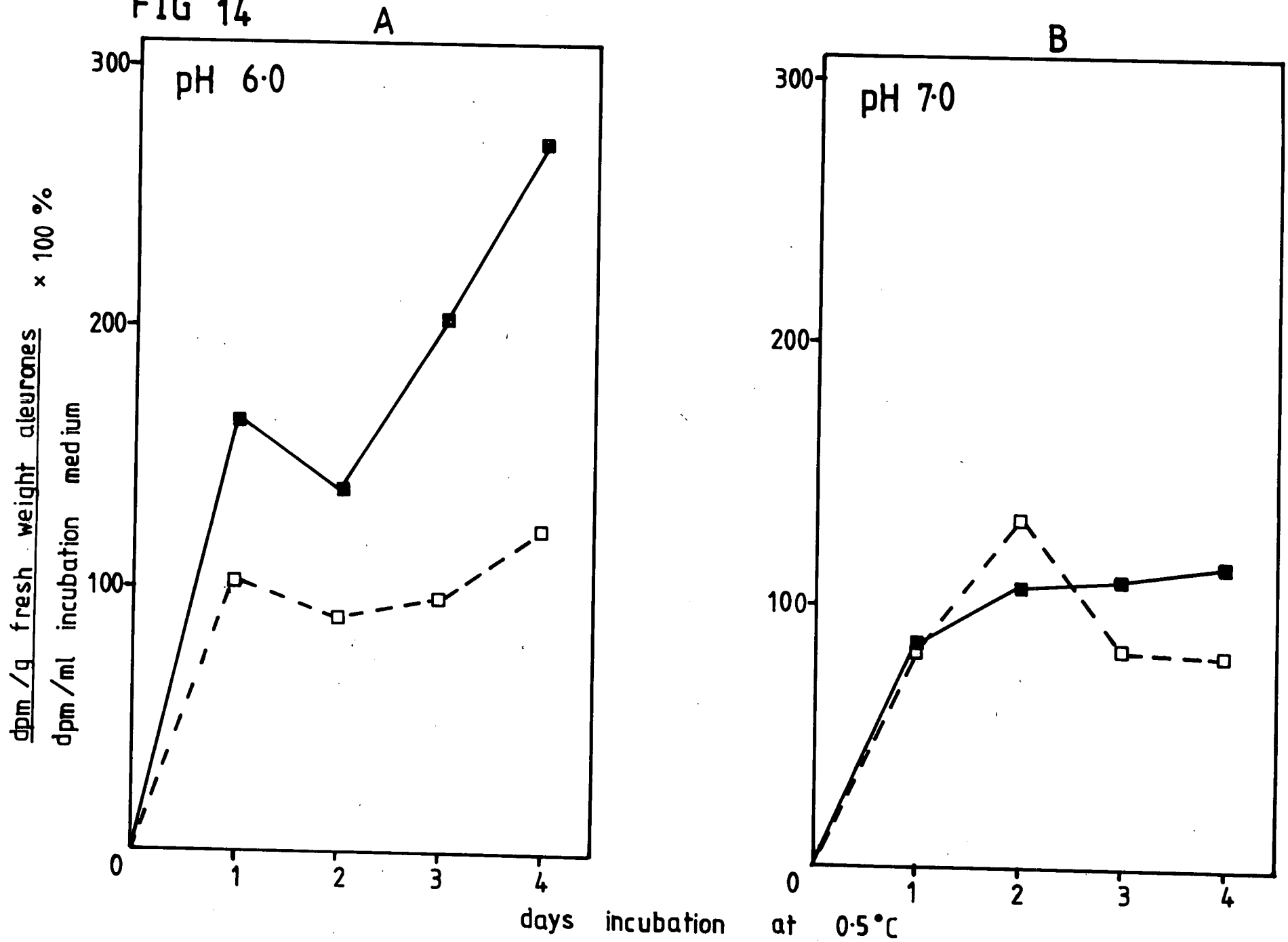
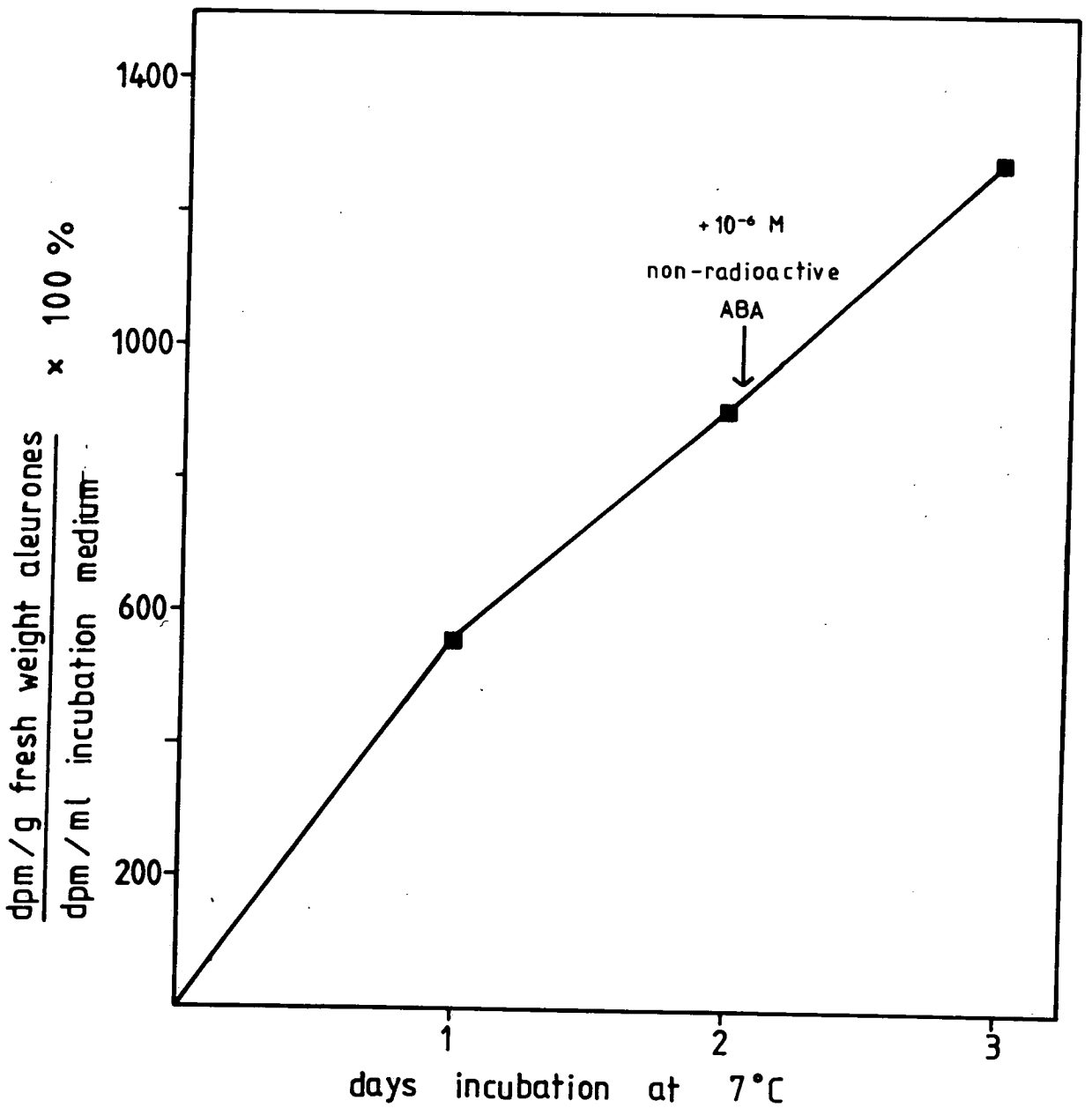

A
pH 6·0
B
pH 7·0
300
200
100
0
1
2
3
4
dpm /g fresh weight aleurones × 100 %
dpm /ml incubation medium
days incubation at 0·5 °C

FIGURE 15

Effect of temperature on the uptake of ^{3}H-ABA by barley aleurones.

Barley aleurones were incubated in assay buffer d) (10 mM Tris-HCl, 1 mM EDTA and 0.25 M sucrose) pH 7.0 containing 5 x 10^{-9} M ^{3}H-ABA at 7°C. Samples of incubation medium and aleurones were taken daily for 2 days, and the samples scintillation counted. After 2 days, 10^{-6} M non-radioactive ABA was added, and samples again taken and scintillation counted a further 24 hours later.

FIG 15


1400
1000
600
200
dpm/g fresh weight aleurones / dpm/ml incubation medium × 100 %
+10⁻⁶ M
non-radioactive
ABA
1
2
3
days incubation at 7°C

corresponding volume of incubation medium. The addition of 10^{-4} M non-radioactive ABA did not appear to depress the rate of further uptake of ^{3}H-ABA. This temperature-dependent uptake of ^{3}H-ABA is similar to the findings of Keith et al (1980a), who reported that at temperatures above 1.5°C there was considerable metabolism of ^{3}H-GA_1 by barley aleurones. However, the relatively high levels of uptake of ^{3}H-ABA by aleurones, even at 0.5°C, that I have found, suggest that even at this low temperature, aleurones may be capable of metabolising ABA.

Figure 16 shows the fluorographs taken of extracts of *S. polyrrhiza*, *L. perpusilla* and barley aleurone layers, following *in vivo* equilibrium dialysis experiments at 0.5°C. Two bands of radioactive material not corresponding to the standard of ^{3}H-ABA are apparent in the barley aleurone extracts. No further analysis of this material was made. However, the majority of the radioactive material present was still in the form of ^{3}H-ABA, and therefore metabolism probably cannot account entirely for the very high levels of accumulation observed in aleurones. No break-down products were evident in the *S. polyrrhiza* and *L. perpusilla* extracts, although low concentrations of any such material would not be detectable by this method.

FIGURE 16

Fluorography checks on metabolism of ABA.

After 4 days of *in vivo* equilibrium dialysis, tissue samples were extracted into methanol, reduced to near dryness and submitted to T.L.C. using the solvent system toluene : ethyl acetate : acetic acid (25 : 15 : 2). The plates were sprayed with 7% PPO in acetone to enhance the detection of ^{3}H-ABA compounds, and pre-flashed X-ray film clamped firmly to them. The films were left at -70°C in the dark for 5 months to develop.

A ABA standard

B *Lemna perpusilla* (not pre-incubated in ABA prior to expt.)

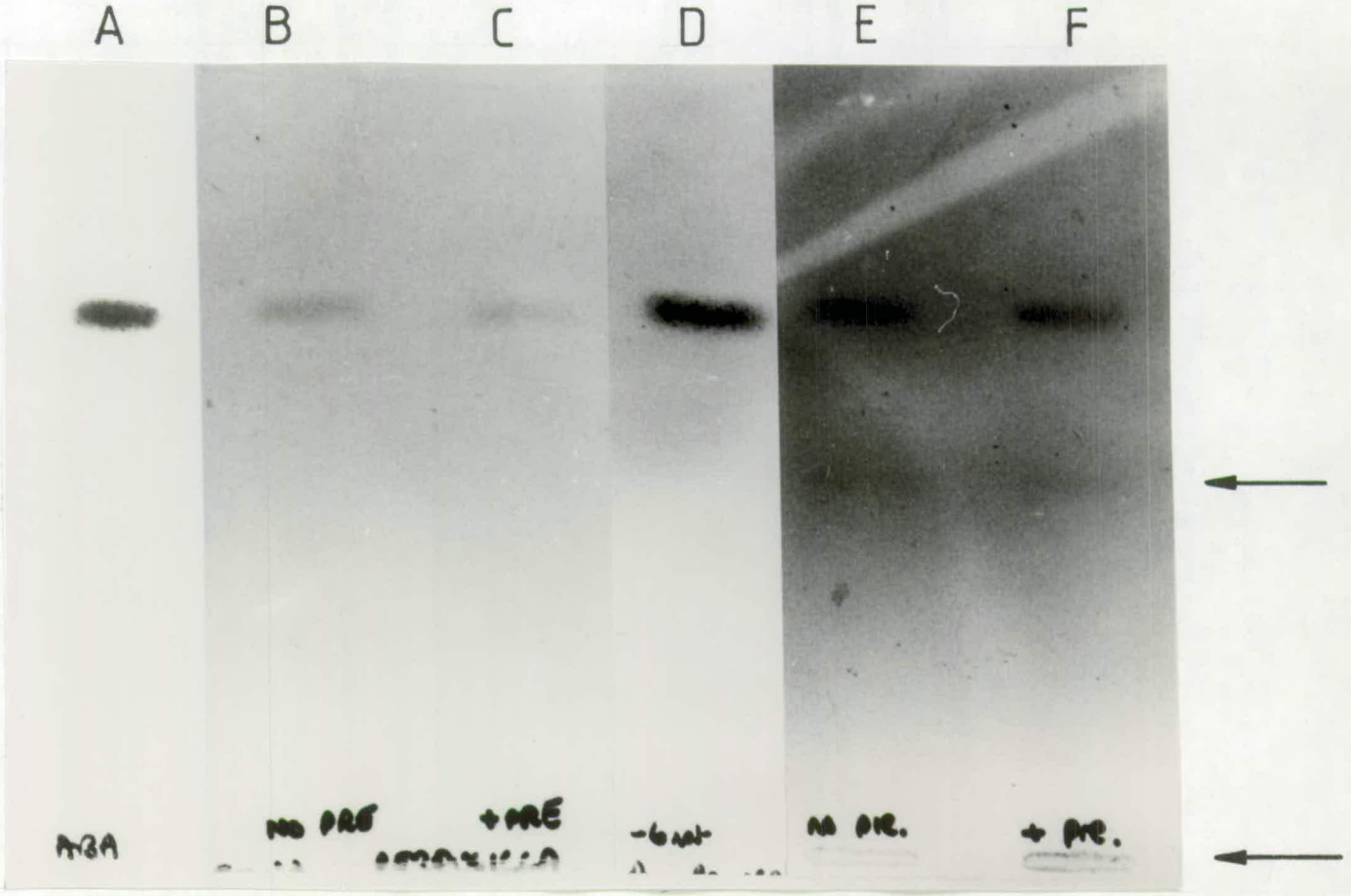
C *Lemna perpusilla* (pre-incubated in 10^{-6}M ABA)

D *Spirodela polyrrhiza* (not pre-incubated in ABA)

E Barley aleurone layers (not pre-incubated in ABA)

F Barley aleurone layers (pre-incubated in 10^{-6}M ABA)

FIG 16



2.3B *IN VITRO* ^{3}H-ABA BINDING RESULTS

2.3B.1 ^{3}H-ABA binding experiments which gave negative results.

The following ABA-binding experiments gave negative results i.e. no ^{3}H-ABA binding in the "HOT" assays in excess of that in the "HOT + COLD" assays. For the sake of brevity, these are in list form.

See appropriate sections of Materials and Methods 2.2B for details.

tissue extract	grinding buffer no.	type of binding assay	assay buffer no.
a) Wheat coleoptiles.			
39000 x g pellet	a) pH 7.5	B. Amicon filtration YMT membranes	a) pH 7.5
39000 x g supernatant	1)&2) a) pH 7.5	A. DE-81 filters	grinding buffer a) pH 7.5
b) Wheatgerm.			
500 x g pellet	1) c) pH 7.0 (0.001 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.001 M $MgCl_2$)

tissue extract		grinding buffer no.	type of binding assay	assay buffer no.
b) Wheatgerm. (cont.)				
500 x g pellet	2)	c) pH 7.0 (0.005 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.001 M $MgCl_2$)
4000 x g pellet		c) pH 7.0 (0.005 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.005 M $MgCl_2$)
39000 x g pellet		c) pH 7.0 (0.005 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.005 M $MgCl_2$)
c) Maize coleoptiles, minus leaf rolls.				
39000 x g pellet		b) pH 8.0	B. Amicon filtration YMT membranes	i) pH 6.0
80000 x g pellet	1)	b) pH 8.0	D. Centrifugal	i) pH 5.5
	2)	b) pH 8.0	D. Centrifugal	i) pH 7.0
	3) 4)	As 1) and 2) above, but incorporating a wash step using the wash buffer pH 6.0 (2.2B.4) before resuspending in assay buffer.		

tissue extract	grinding buffer no.	type of binding assay	assay buffer no.
	c) Maize coleoptiles, minus leaf rolls. (cont.)		
80000 x g supernatant	1) b) pH 8.0	E. Equilibrium Dialysis - Dianorm cells	grinding buffer b) pH'd to 5.5
	2)		or 7.0
	d) Maize coleoptiles, leaf rolls only.		
80000 x g pellet	1)&2) b) pH 8.0	D. Centrifugal	i) pH 5.5
	3)&4) b) pH 8.0	D. Centrifugal	i) pH 7.0
80000 x g supernatant	1)&2) b) pH 8.0	E. Equilibrium Dialysis - Dianorm cells	grinding buffer b) pH'd to 5.5
	3)&4) b) pH 8.0	E. Equilibrium Dialysis - Dianorm cells	grinding buffer b) pH'd to 7.0
	e) Maize mesocotyls.		
80000 x g pellet	1) b) pH 8.0	D. Centrifugal	i) pH 5.5
	2) b) pH 8.0	D. Centrifugal	i) pH 7.0

Both 1) and 2) incorporated a wash step in the wash buffer pH 6.0 (2.2B.4) before resuspension in assay buffer.

tissue extract		grinding buffer no.	type of binding assay	assay buffer no.
f) Pea epicotyls (3rd internode).				
		(i) Feltham First		
500 x g pellet	1)&2)	f) pH 7.7	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
4000 x g pellet	1)2)&3)	f) pH 7.7	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
	4)	g) pH 8.0	D. Centrifugal	f) pH 6.0
	5)	g) pH 8.0	D. Centrifugal	f) pH 6.5
	6)	g) pH 8.0	D. Centrifugal	f) pH 7.0
39000 x g pellet	1)	g) pH 8.0	D. Centrifugal	f) pH 6.0
	2)	g) pH 8.0	D. Centrifugal	f) pH 6.5
	3)	g) pH 8.0	D. Centrifugal	f) pH 7.0
	4)5)&6)	f) pH 7.7	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
80000 x g pellet		b) pH 8.0 f) pH 7.7	D. Centrifugal E. Equilibrium Dialysis - Dianorm cells	i) pH 5.5 b) pH 7.0

tissue extract	grinding buffer no.	type of binding assay	assay buffer no.
	(ii) Alaska		
80000 x g pellet 1)	b) pH 8.0	D. Centrifugal	i) pH 5.5
80000 x g pellet 2)	b) pH 8.0	D. Centrifugal	i) pH 7.0
80000 x g supernatant 1)	b) pH 8.0	E. Equilibrium Dialysis - Dianorm cells	grinding buffer adjusted to pH 5.5
2)			or pH 7.0

g) Pea apical hooks.

Both Alaska and Feltham First varieties were tested in all the combinations below :

80000 x g pellet 1)&2)	b) pH 8.0	D. Centrifugal	i) pH 5.5
80000 x g pellet 3)	b) pH 8.0	D. Centrifugal	i) pH 7.0

1)2) & 3) incorporated a wash step in the wash buffer pH 6.0 (2.2B.4) before resuspension in assay buffer.

80000 x g supernatant 1)	b) pH 8.0	E. Equilibrium Dialysis - Dianorm cells	grinding buffer adjusted to pH 5.5
2)			or pH 7.0

tissue extract		grinding buffer no.	type of binding assay	assay buffer no.
		h) Zucchini hypocotyls.		
80000 x g pellet	1)	b) pH 8.0	D. Centrifugal	i) pH 5.5
	2)	b) pH 8.0	D. Centrifugal	i) pH 7.0
80000 x g supernatant	1)	b) pH 8.0	E. Equilibrium Dialysis - Dianorm cells	grinding buffer adjusted to pH 5.5
	2)			or pH 7.0
	3)	b) pH 8.0	B.Amicon filtration	grinding buffer adjusted to pH 5.5
	4)			or pH 7.0
		i) *Spirodela polyrrhiza* fronds.		
500 x g pellet	1)	c) pH 7.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
	2)	c) pH 8.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
500 x g supernatant	1)&2)	c) pH 7.0 (0.003 M $MgCl_2$)	E. Equilibrium Dialysis - Visking tubing	b) pH 7.0 (0.003 M $MgCl_2$)

tissue extract		grinding buffer no.	type of binding buffer	assay buffer no.
i) *Spirodela polyrrhiza* fronds. (cont.)				
4000 x g pellet	1)	c) pH 8.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
	2)	g) pH 8.0	D. Centrifugal	f) pH 6.0
	3)	g) pH 8.0	D. Centrifugal	f) pH 6.5
	4)	g) pH 8.0	D. Centrifugal	f) pH 7.0
39000 x g pellet	1)	d) pH 6.6	D. Centrifugal	f) pH 6.5
	2)	c) pH 7.0 (0.003 M $MgCl_2$)	E. Equilibrium Dialysis - Visking tubing	b) pH 7.0 (0.003 M $MgCl_2$)
		2) incorporated an $(NH_4)_2SO_4$ pptn. (2.2B.8) before resusupension in assay buffer		
	3)&4)	c) pH 7.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
	5)	c) pH 8.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
	6)	g) pH 8.0	D. Centrifugal	f) pH 5.0
	7)	g) pH 8.0	D. Centrifugal	f) pH 5.5
	8)9)&10)	g) pH 8.0	D. Centrifugal	f) pH 6.0
	11)&12)	g) pH 8.0	D. Centrifugal	f) pH 6.5
	13)&14)	g) pH 8.0	D. Centrifugal	f) pH 7.0

tissue extract		grinding buffer no.	type of binding assay	assay buffer no.
i) *S. polyrrhiza* fronds (cont.)				
39000 x g supernatant	1)	d) pH 6.6	B. Amicon filtration	grinding buffer d) pH 6.6
	2)3)&4)	c) pH 7.0 (0.0003 M $MgCl_2$)	E. Equilibrium Dialysis - Visking tubing	b) pH 7.0 (0.003 M $MgCl_2$)
2)3)&4) incorporated an $(NH_4)_2SO_4$ pptn. (2.2B.8) before resuspension in assay buffer				
j) *Spirodela polyrrhiza* turions.				
500 x g pellet	1)	b) pH 8.0	E. Equilibrium Dialysis - Visking tubing	grinding buffer b)
	2)	b) pH 8.0	E. Equilibrium Dialysis - Visking tubing	i) pH5.5
2) incorporated an $(NH_4)_2SO_4$ pptn. (2.2B.8) before resuspension in assay buffer				
4000 x g supernatant		b) pH 8.0	E. Equilibrium Dialysis - Hoefer apparatus	grinding buffer b)

tissue extract		grinding buffer no.	type of binding assay	assay buffer no.
		j) *Spirodela polyrrhiza* turions (cont.)		
39000 x g pellet	1)	c) pH 7.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
	2)	b) pH 8.0	F. Sephadex column	i) pH 6.0
	3)	b) pH 8.0	D. Centrifugal	i) pH 5.5
		3) incorporated a wash step in the wash buffer pH 6.0 before resuspension in assay buffer		
		k) Barley embryos.		
39000 x g pellet	1)&2)	c) pH 7.0 (0.003 M $MgCl_2$)	D. Centrifugal	b) pH 7.0 (0.003 M $MgCl_2$)
		1) Barley 1/2-seeds.		
4000 x g supernatant	1)	e) pH 6.0	E. Equilibrium Dialysis - Visking tubing	g) pH 6.0
	2)	i) pH 7.0	E. Equilibrium Dialysis - Visking tubing	h) pH 7.0
		Both 1)&2) incorporated an $(NH_4)_2SO_4$ pptn. (2.2B.8) before resuspension in assay buffer		

Addition of enzyme inhibitors

The addition of protease inhibitors leupeptin (1 μM) (Alpi and Beevers, 1981) and para-methyl sulphonyl fluoride (250 μM), and of the reducing agent dithiothreitol (100 μM), to the grinding buffer in randomly selected experiments, made no apparent difference to the failure to detect binding.

2.3B.2 *In vitro* ^{3}H-ABA binding to extracts of barley aleurone layers.

The following experiments gave promising results, using extracts of barley aleurone layers :

In vitro equilibrium dialysis of 500 g crude extracts of barley aleurones in dialysis tubing at pH 6.0, 7.0 and 8.0 gave the results in Table 1. There appeared to be some binding of ^{3}H-ABA by the extract at pH 7.0 and 8.0 which was apparently displaced by an excess of non-radioactive ABA, although the method was too crude to yield accurate results. Problems of swelling of the dialysis bag and non-displaceable uptake of ABA did occur when too concentrated an extract was placed inside the tubing. The latter effect could be removed by the addition of 10 mM NaCl to both extract and incubation medium, showing that this was probably a Donnan effect.

The Hoefer Equilbrium Dialysis Apparatus gave similar results. Although the results were variable, a 0 - 3 % difference in radioactivity ("HOT" - "HOT + COLD") could be detected using 500 x g crude extracts. However, this low level of binding could not be improved upon.

Two experiments on 4000 x g supernatant fractions of aleurones were carried out in assay buffers i) pH 5.5 and e) pH 7.0 using a Sephadex G-25 column (see 2.2B.9 F). No radioactivity was detected in the eluate aliquots corresponding to the BSA standard i.e. none of the ^{3}H-ABA was protein-bound.

TABLE 1

In vitro equilibrium dialysis of crude extracts of barley aleurones at pH's 6.0, 7.0 and 8.0.

3 g barley aleurones were ground up in buffer g) (0.05 M MES, 0.05 M Tris-HCl, 0.5 M sucrose, 0.003 M $MgCl_2$) pH 8.0. The homogenate was spun at 500 x g for 10 min, and $(NH_4)_2SO_4$ added to the resulting supernatant to 100% saturation at 4°C. After spinning at 20,000 x g for 30 min the resulting pellet was resuspended in one of the following assay buffers :

f) (0.05 M Tris-HCl, 0.05 M MES, 0.003 M $MgCl_2$) pH 6.0 or pH 7.0

or c) (0.01 M Tris-HCl, 0.0003 M $MgCl_2$) pH 8.0

The assay mixture was then dialysed overnight at 4°C against 2 litres of the same buffer to remove the $(NH_4)_2SO_4$. The extract was then re-dialysed against the same buffer containing 10^{-9} M ^{3}H-ABA with constant stirring at 4°C, and left for 24 hours for the system to reach equilibrium. The radioactivity in both the incubation medium and in the extract in the dialysis tubing was scintillation counted.

The results are expressed as {(dpm/ml inside dialysis tubing) / (dpm/ml in incubation medium)} x 100%. Figures over 100% represent ABA-binding by the extract. Figures in brackets represent new equilibrium values after addition of 10^{-6} M non-radioactive ABA to displace specifically bound ABA.

* 167% here represents 1 x 10^{-12} mole of ^{3}H-ABA bound per mg of protein in the extract.

TABLE 1

pH 6.0 (MES)	pH 7.0 (Hepes)	pH 7.0 (Tris)	pH 8.0 (Tris)
82	97	122	116 (97)
82	85	104	112 (96)
91		111	
96		104	
106 (98)		108	
		155	
		136 (97)	
		*167 (94)	

Table 2 shows results obtained from crude membrane extracts (500 x g - 39000 x g pellet) using the centrifugal method 2.2B.9 D of measuring ABA-binding. It must be stated that this method frequently gave negative results, but that on balance there did appear to be some specific ABA-binding.

To validate my experimental technique, a classical auxin-binding experiment with maize coleoptiles (Batt and Venis, 1976) was tried whilst visiting Shell Research, Sittingbourne. Dr. Mike Venis and myself carried out parallel centrifugal binding experiment using a crude membrane (4000-80000 x g) fraction from maize coleoptiles (minus leaf rolls). The experiment showed specific binding of the ^{14}C-NAA, and our two independent sets of data were within 4% of each other.

It was found that incubating the ^{3}H-ABA with the barley aleurone extracts for 2 hours at 25°C resulted in a higher frequency of positive data. Figure 17 shows the relationship between the concentration of ^{3}H-ABA added and the resulting specifically bound ABA, showing a trend towards saturation at around 10^{-7} M.

Kinetic analysis.

Whilst it was considered that the data only marginally warranted kinetic analysis, Figure 18 shows two interpretations of a Scatchard analysis (Scatchard, 1949). In A, a single straight line was fitted to the data, although the linear correlation coefficient (0.4783) was just under the 95% significance level of 0.497. This line gave a resulting K_D of 6.9×10^{-8} M and n (number of binding sites) of 3.6×10^{-12}

TABLE 2

In vitro binding of ^{3}H-ABA to crude membrane fractions of barley aleurones - Centrifugal method.

3 g barley aleurones were ground up in grinding buffer c) (0.1 M Tris-HCl, 0.5 M sucrose and 0.003 M $MgCl_2$) pH 8.0. After differential centrifugation 500 - 39000 x g, the 39000 x g pellet was resuspended in assay buffer b) (0.01 M Tris-HCl and 0.003 M $MgCl_2$) pH 7.0 and ^{3}H-ABA added to a final concentration of between 10^{-7} M to 10^{-9} M (= "FREE" ^{3}H-ABA). After 2 hours incubation at 25°C, the extract was centrifuged at 39000 x g for 30 min and the radioactivity in the pellet extracted into methanol. Parallel incubations containing non-radioactive ABA at concentrations of [x 1000 that of the ^{3}H-ABA concentration], determined the amount of specifically-bound ^{3}H-ABA by displacement (= "BOUND" ^{3}H-ABA). Each figure in the table represents the mean of 4 replicates within an experiment. A maximum of 2 "FREE" ^{3}H-ABA concentrations were investigated for binding within any one experiment. This limitation was imposed by the problems of obtaining sufficient quantities of starting material.

1 g fresh weight corresponds to 0.7 mg protein.

TABLE 2

FREE ^{3}H-ABA (M)	BOUND ^{3}H-ABA (mol/g f.wt.)	(BOUND / FREE)
10^{-7}	9.8×10^{-13}	0.10×10^{-4}
	12.7×10^{-13}	0.13×10^{-4}
5×10^{-8}	8.7×10^{-13}	0.17×10^{-4}
2.5×10^{-8}	1.8×10^{-13}	0.07×10^{-4}
10^{-8}	9.3×10^{-13}	0.93×10^{-4}
	2.1×10^{-13}	0.21×10^{-4}
7.5×10^{-9}	5.9×10^{-13}	0.79×10^{-4}
	0.9×10^{-13}	0.18×10^{-4}
5×10^{-9}	1.0×10^{-13}	0.20×10^{-4}
	1.5×10^{-13}	0.30×10^{-4}
	2.0×10^{-13}	0.40×10^{-4}
	1.8×10^{-13}	0.72×10^{-4}
2.5×10^{-9}	0.9×10^{-13}	0.36×10^{-4}
	1.2×10^{-13}	0.48×10^{-4}
2×10^{-9}	1.4×10^{-13}	0.70×10^{-4}
10^{-9}	1.6×10^{-13}	1.60×10^{-4}

FIGURE 17

In vitro ^{3}H-ABA binding (centrifugal method) to a crude membrane fraction of barley aleurones.

A barley aleurone crude membrane fraction (500-39000 x g) was resuspended in 0.1 M Tris-HCl (pH 7.0) and 0.003 M $MgCl_2$. ^{3}H-ABA was added to a final concentration ranging between 10^{-7} M - 10^{-9} M (= FREE ABA). Following incubation at 25°C for 2 hours the fractions were re-pelleted and the radioactivity in the pellet extracted into methanol and scintillation counted. Parallel incubations containing non-radioactive ABA at concentrations of [x 1000 concentration of FREE ABA] determined the specifically bound ^{3}H-ABA (= BOUND ABA) by displacing it.

Table 2 summarises the results obtained from several individual experiments, which are shown here as a semi-log plot.

FIG 17

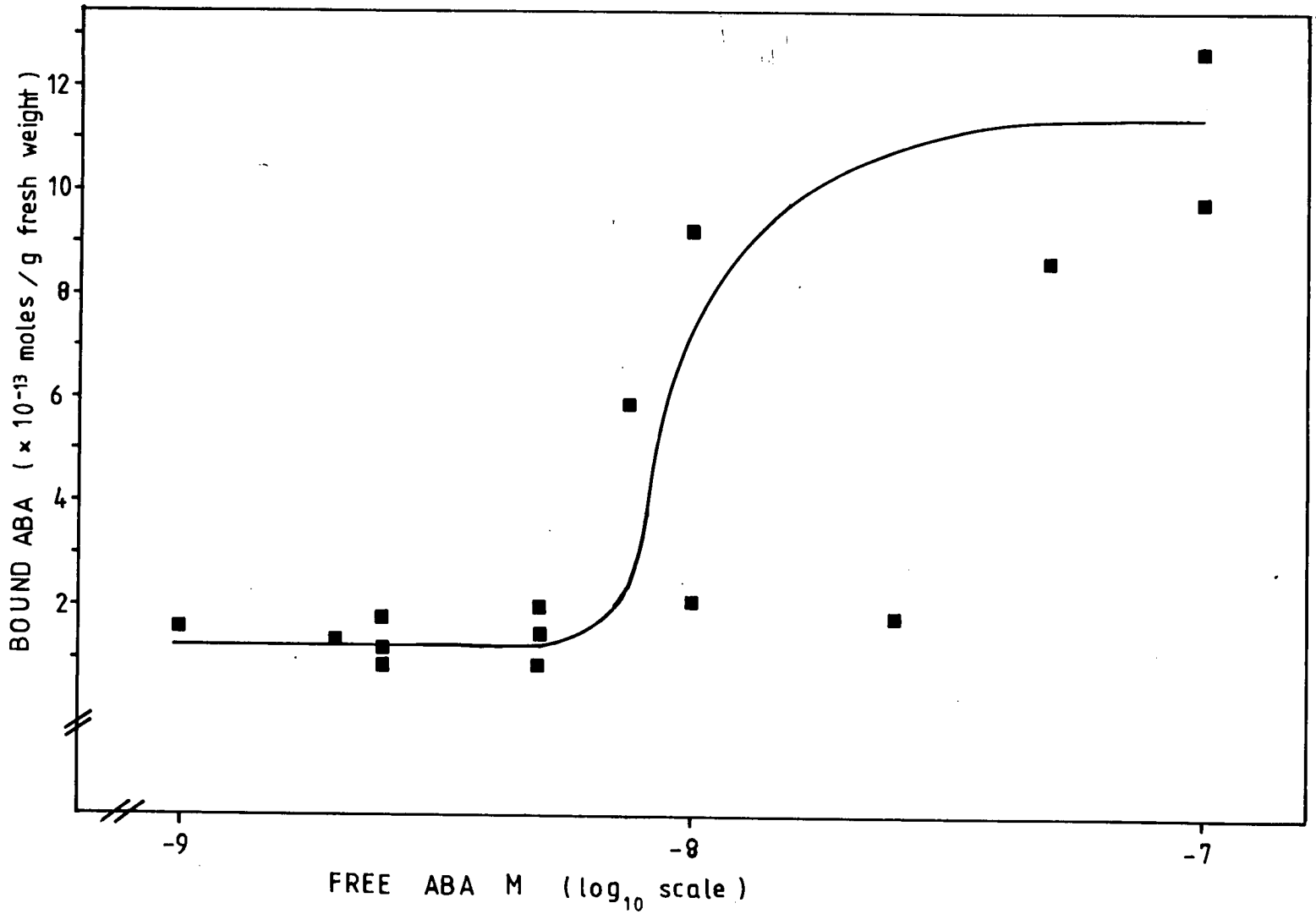

BOUND ABA (× 10⁻¹³ moles / g fresh weight)
12
10
8
6
4
2
-9
-8
-7
FREE ABA M (log10 scale)

FIGURE 18

Scatchard analysis of ^{3}H-ABA binding to crude membrane fractions of barley aleurone layers.

A barley aleurone (500-39000 x g) crude membrane fraction was resuspended in 0.01 M Tris-HCl (pH 7.0) and 0.003 M $MgCl_2$. ^{3}H-ABA was added to a final concentration ranging between 10^{-7} M and 10^{-9} M (= FREE ABA). After 2 hours incubation at 25°C the membrane fraction was re-pelleted and the radioactivity in the pellet extracted into methanol. Parallel incubations containing non-radioactive ABA at concentrations of [x 1000 concentration of FREE ABA] determined the amount of specifically bound ^{3}H-ABA (= BOUND ABA) by displacing it.

The results are summarised in Table 2, and represent a compilation of results from several experiments.

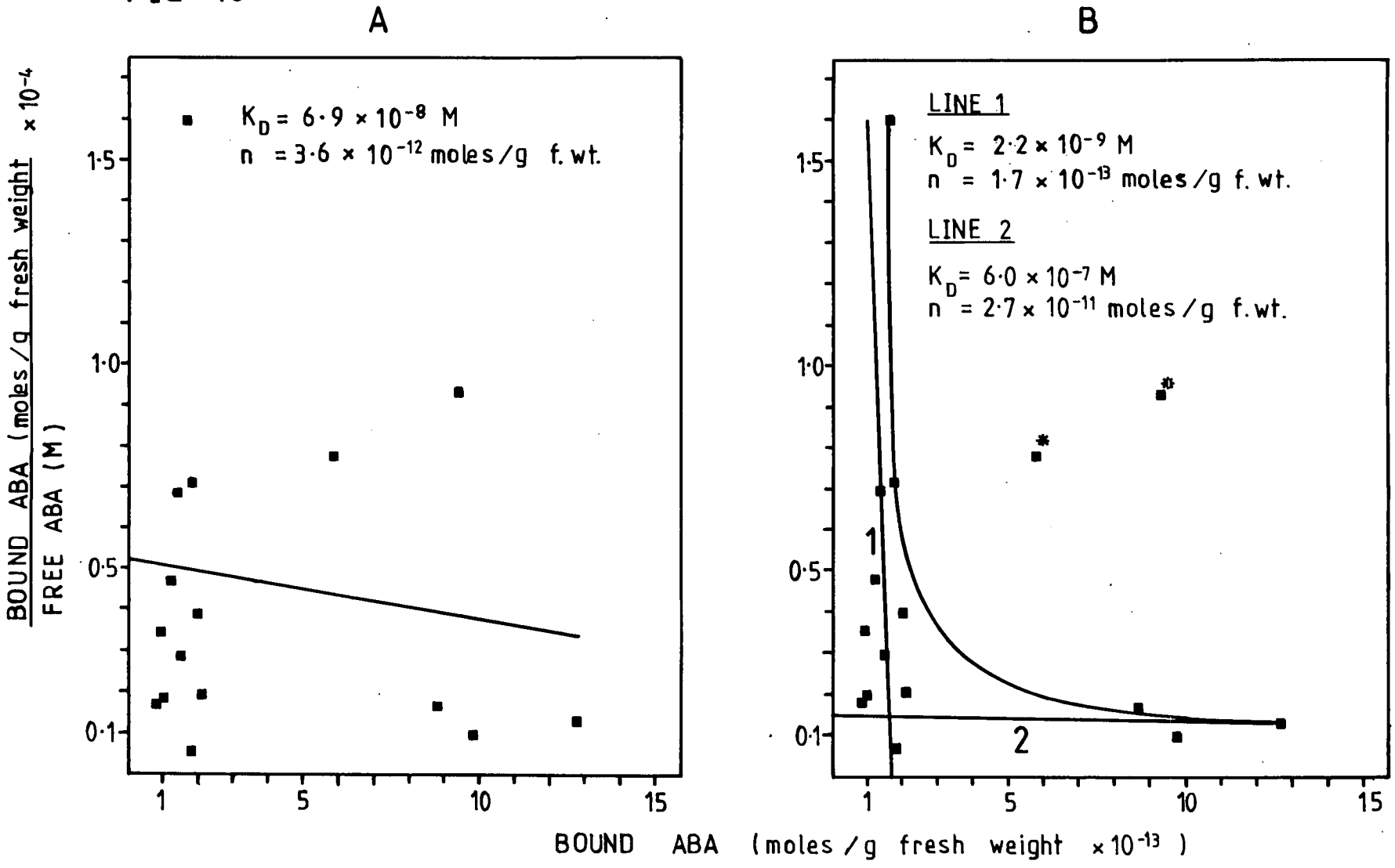
K_D (dissociation constant) = - (1 / slope)

n (concentration of binding sites) = intercept on x axis

A shows one line representing a single binding site.

B is an interpretation of two separate binding sites, represented by the two lines.

FIG 18


A
B
$K_D = 6{\cdot}9 \times 10^{-8}$ M
$n = 3{\cdot}6 \times 10^{-12}$ moles/g f. wt.
LINE 1
$K_D = 2{\cdot}2 \times 10^{-9}$ M
$n = 1{\cdot}7 \times 10^{-13}$ moles/g f. wt.
LINE 2
$K_D = 6{\cdot}0 \times 10^{-7}$ M
$n = 2{\cdot}7 \times 10^{-11}$ moles/g f. wt.
1
2
1·5
1·0
0·5
0·1
1
5
10
15
BOUND ABA / FREE ABA (M) × 10^{-4}
BOUND ABA (moles/g fresh weight × 10^{-13})

moles/g fresh weight.

An alternative interpretation assuming two ABA-binding sites is shown in B. In the absence of available, suitable computer packages, the curve was fitted by eye, and similarly separated into 2 linear components, lines 1 and 2. The two data points marked with an asterisk did not seem in accord with the rest of the data in this analysis, and were consequently not taken into consideration. Line 1 produced a K_D of 2.2×10^{-9} M and n of 1.7×10^{-13} moles/g fresh weight, and line 2 a K_D of 6.0×10^{-7} M and n of 2.7×10^{-11} moles/g fresh weight. Whilst the validity of these calculations is questionable, it did appear that there may be a component of ABA-binding with a very high affinity and low concentration of binding sites (line 1), and another component with a lower affinity and higher binding site concentration (line 2).

Which, if either, of the interpretations in A and B is representative of *in vivo* ABA-receptor binding, is not known.

The low concentration of binding sites, coupled with the difficulty in obtaining sufficient quantities of aleurone layers to compensate for this low concentration, resulted in the inability to make further progress in this direction. In the absence of a starting material supplying a sufficiently high concentration of ABA-binding sites, the methods employed were not sensitive enough to detect the very low levels of binding encountered.

2.4 DISCUSSION

The results of the *in vivo* equilibrium dialyses showed that this approach to the detection of ABA-receptors was not successful in the tissues investigated.

The fluorographs showed considerable levels of radioactive break-down products from the ^{3}H-ABA in barley aleurones, suggesting active metabolism at 0.5°C. The other plants / tissues may also have been metabolising at this low temperature but below the detection limits of the fluorography procedure.

It was difficult to demonstrate the reversibility of any apparent ABA-binding, or even of any saturable uptake. Increasing the temperature increased both the rate of uptake and the final levels of internal ^{3}H-ABA. The time scale of the experiments, spanning several days, cast doubt on whether the experimental circumstances (no light, 0.5°C) would evoke responses relevant to *in vivo* situations. *S. polyrrhiza* fronds remaining at the end of *in vivo* experiments and consequently returned to the growth cabinet and normal growth conditions (minus ABA), did survive. However, there was a considerable time lag before growth resumed, and whilst the daughter progeny grew and multiplied as usual, the original parent fronds died soon after producing them.

Although the results were very variable, pre-incubation, or pre-imbibition, in ABA, may have increased the subsequent uptake of ^{3}H-ABA. This might have been due to the induction of

carrier or metabolic binding proteins, but as this additional uptake was not displaced by non-radioactive ABA, it was not due to specific receptors.

The main conclusion that can be drawn from these experiments is that uptake of ABA *in vivo* is pH-dependent. This finding is in agreement with that of Astle and Rubery (1980) who found that uptake of ABA into segments of runner bean roots was dependent on the extracellular pH being lower than that of the cytoplasm, which acted as an anion trap. As well as a non-saturable, diffusible component of ABA uptake, they also detected a saturable component (also dependent on lower external pH for activity), which they described as an ABA-carrier protein across the plasma membrane. As ABA is freely lipid soluble and membranes are hence intrinsically permeable to ABA, the requirement for ABA membrane carrier proteins would seem unnecessary. Astle and Rubery suggest that their carrier proteins might be involved with the channeling of ABA through certain tissues, particularly in relatively undifferentiated plant regions lacking a fully developed stele, such as in root apices. However, Lea and Collins (1979) incorporated ABA into black lipid membranes, and from their measurements of ionic conductivity, hypothesized that the alignment of three ABA molecules across the membrane formed an ion transport channel. The presence of such channels would affect water permeability and ionic sensitivity, and introduces a concept of ABA effect (e.g. on flux of K^+ in stomata), without the need for ABA receptors.

Working with suspension-cultured Runner bean cells, Astle and Rubery (1985) have further described a saturable component involved with ABA uptake. This was only active at below pH 6.0. They found that both diffusive (non-saturable) and saturable components were present, and that both were modified in their activity by changing pH i.e. pH was a common driving force. The presence of proton ionophores (2,4-dinitrophenol or carbonylcyanide p-trifluoromethoxy phenylhydrazone) blocked the carrier-mediated ABA uptake, and suggested that the carrier transport involved ABA^- / H^+ symport. The role of ABA in the modification of membrane systems, and its inhibition of an electrogenic pump coupled to a K^+ exchange mechanism, has been reviewed by Van Steveninck and Van Steveninck (1983).

It is considered that the *in vivo* ABA uptake described in this thesis may consist of a similar combination of diffusive (non-saturable) and carrier-mediated (saturable) components, particularly in barley aleurones which were metabolically active under the experimental conditions. The addition of proton ionophores to the incubation medium might have given a clearer indication of any ABA^- / H^+ co-transport mechanisms. However, amidst this ABA uptake by diffusion, carrier or metabolic binding proteins, the reliably reproducible detection of any low levels of specific ABA receptors was not possible using this method.

The results obtained by Srivastava's group (Keith *et al.*, 1980b) using *in vivo* equilibrium dialysis with barley aleurones to show saturable uptake of GA_1 at 0.5°C, could not

be repeated using ABA. Keith *et al* expressed their uptake results on a volume : volume basis, as dpm ml^{-1} aleurone tissue / dpm ml^{-1} incubation medium, using a conversion factor of 1 g aleurones being equivalent to 0.67 ml aleurones (i.e. 149 g aleurones = 100 ml aleurones). Taking this conversion factor into account, the GA_1 uptake into the tissues was very low compared to my results with ABA - even at 1.5°C, uptake of GA_1 was only just 100% on a weight to volume basis, where 100% represents the amount of radioactivity in the medium.

To show displacement of specific ^3H-GA_1 binding, Keith *et al.* used non-radioactive GA_1 at only a [x 50 fold] carrier excess. Their paper includes a table of results showing displacement of increasing concentrations of specific $^3GA_1$ binding at concentrations of $^3GA_1$ between 5×10^{-9} M and 5×10^{-7} M. If saturation of GA_1 receptors was not achieved until 5×10^{-7} M it seems difficult to explain the detection of specific binding at concentrations of 10^{-8} M or less, using a [x 50] fold carrier excess.

Following the failure to detect specific ABA-binding with the *in vivo* approach, the ensuing *in vitro* methods employed were not much more productive. Although specific binding could be detected using barley aleurone crude membrane preparations, the levels of binding were so low that a quantitative analysis was difficult. It became clear that, using the binding methods described, it was necessary to use a tissue with a high concentration of ABA receptors together with optimal binding conditions in order to detect specific ABA-binding. As the barley aleurone is only three cells thick it seems unlikely

that there would be a differential distribution of receptors within the tissue structure.

Since the completion of the work for this chapter, Hornberg and Weiler (1984) have published a report of photoaffinity labelling of ABA to guard cell protoplasts of *Vicia faba*, showing both saturable, and (+)ABA-enantiomer specific, binding. They used monoclonal antibodies to distinguish between, and separate, the biologically active (+)ABA, and the biologically inactive (-)ABA, enantiomers. They then covalently bound ^{3}H-ABA to the protoplasts by photoactivation of the α,β-unsaturated ketone group at position C-4[1] in the ABA molecules using UV light (330 nm). This principle has frequently been used in animal research e.g. to photolabel ecdysterone receptors on *Drosophila* chromosomal puffs (Gronemeyer and Pongs, 1980). Hornberg and Weiler's photoaffinity ^{3}H-ABA binding showed saturation with increasing concentration, and addition of unlabelled (+)ABA prior to photolysis competed with the ^{3}H-(+)ABA for photo-induced covalent attachment whereas (-)ABA did not compete.

SDS-gel analysis revealed three protein bands labelled wih ^{3}H-ABA suggesting three different binding sites. The amount of ^{3}H-ABA labelling to each of these bands changed with varying pH, and it was deduced from this that one of the sites bound ABA^{-} whilst the other two probably bound ABAH. It was suggested that this would explain how ABA can cause stomatal closure over a wide pH range.

The resulting K_D of the guard cell protoplasts for (+)ABA

was 3-4 x 10^{-9} M, and the number (n) of these sites was very high - 1.95 x 10^{6} sites per guard cell, compared with the concentration I found in barley aleurone extracts (see Figure 18A) of 3.6 x 10^{-12} moles per g fresh weight. Treatment of the protoplasts with trypsin removed the binding capability, and so the binding sites were postulated to be on the outside of the plasma membrane. This again raises the question as to why plant cells need membrane-bound ABA receptors when ABA is freely permeable in lipid environments, unless, as suggested by Hanke (1984), these binding proteins might be ABA-carrier proteins which were responsible for making ABA accessible to internal receptors. As Hanke also points out, progress in the field of ABA-receptors has been focused on carrier systems, because transport is more readily measurable than the slower, developmental consequences of ABA-binding. The importance of carrier sites with respect to ABA-induced physiological changes in growth and development is not, in general, clear.

Hornberg and Weiler claim their protoplast receptors are of direct physiological importance. To substantiate this claim, they used a range of ABA analogues to demonstrate that the relative degrees of competition for ^{3}H-ABA receptors in the protoplasts, were directly related to the relative extent of stomatal closure in *Vicia faba* resulting from exposure to the analogues. Hornberg and Weiler's binding sites hence meet with all the standard requirements of "receptor" status (see Introduction 2.1.2). They appear to have made the first great innovative advance in the ABA-receptor field, and as such,

their work has been greeted with much acclaim (Hanke, 1984; Venis, 1985).

However, since in a range of tissues sensitive to abscisic acid, I was unable to detect binding of this magnitude using a variety of conventionally accepted procedures, and in one case, photoaffinity labelling, it may be that the stomatal ABA-receptor is unusual. Also, the low yield of stomatal protoplasts which can normally be isolated from leaf tissue may prevent further purification of this tentative receptor. Affinity labelling is not without its problems and the use of radioactive azido auxins by Jones *et al.* (1984) resulted in the labelling of tens if not hundreds of proteins.

To conclude, my results from the *in vivo* experiments showed pH-dependent uptake of ABA, but the method was too crude to obtain reliable data, and there was no clear indication of specific ABA-receptor binding. Also, there was the problem of ABA metabolism by the tissues. The *in vitro* binding experiments detected some high-affinity ABA-binding sites in crude membrane fractions of barley aleurone layers. However, the low concentration of binding sites, coupled with the difficulty of obtaining even a few grams of starting material, made further progress impossible.

CHAPTER 3

RAPID EFFECTS OF ABA ON THE GROWTH AND CELL WALL PLASTICITY OF *SPIRODELA POLYRRHIZA*

3.1 INTRODUCTION

3.1.1 Growth of *Spirodela polyrrhiza*

The growth of the *Lemnaceae* as measured by frond number is generally considered to be exponential (Ashby and Wangermann, 1949). This is an over-simplification, as each frond has a finite life-time during which it produces a certain number of daughter fronds, each of which is smaller in area than the one preceding it, before dying (Ashby and Wangermann, 1951). The length of life, frond size, and rate of production of daughter fronds, are characteristic of the clone and of its environment. As a means of measuring growth, total frond areas have frequently been monitored in *Lemna* and *Spirodela* cultures (Hillman, 1961). This has been done by projecting images of the floating fronds on to a ground glass screen, and tracings made for subsequent measurement, or by focussing their shadows directly over a photocell and relating area to the decrease in light transmitted (Ashby *et al.*, 1928; Ashby and Oxley, 1935). Gorham (1941) further refined the technique by incorporating planimetric or photoelectric estimations of area. However, the recent development of computerised digitiser pens which trace outlines of objects and are programmed to calculate the areas within the traces, has made the task of measuring frond areas much simpler. It is this latter technique that I have employed.

3.1.2 Effect of ABA on growth, and the importance of endogenous ABA

Perry and Byrne (1969) proposed that old *Spirodela* cultures might contain a factor which could induce turion formation. This factor was attributed to be ABA by Saks *et al.* (1980) and, under laboratory conditions, they correlated the time of release of ABA from the fronds into the external medium, with the beginning of turion formation. However, it seems doubtful whether, under the natural environmental conditions of a lake or pond, the localisation of ABA at turion-inducing concentrations could be established adjacent to the plants, even though *Spirodela* is known to frequent still waters (Jacobs, 1947).

Hence, whilst the link between the addition of exogenous ABA at certain concentrations and the subsequent turion formation in various clones of *Spirodela* has been firmly established under controlled growth conditions (Perry, 1968; Smart, 1981; Stewart, 1969; Perry and Byrne, 1969), the link between endogenous ABA levels and turion production in nature, has not been proven.

Bartels and Watson (1978) discovered that the weedkillers fluridone and norflurazon inhibited carotenoid biosynthesis in wheat seedlings. Carotenoids protect chloroplasts from photodestruction which would result in the disruption of the chloroplasts and loss of chlorophyll. Fong *et al.* (1982, 1983) used fluridone to induce vivipary in maize seeds. They produced a working hypothesis that the fluridone acted by

inhibiting the conversion of phytoene to phytofluene in the biosynthesis of carotenoids.

Phytoene -> Phytofluene -> Xanthophylls -> ABA -> DORMANCY

(Fong *et al.*, 1982)

fluridone inhibition

Moore and Smith (1984) found that *Zea mays* seedlings treated with fluridone contained no endogenous ABA. Using high pressure liquid chromatography they characterised the ABA content of leaves, roots and root tips of control plants, but failed to detect any ABA in the fluridone-treated tissues.

Fluridone and norflurazon, being inhibitors of the carotenoid (and hence also ABA) synthetic pathways, hence appeared to provide a suitable probe with which to ensure artificially the absence of endogenous ABA within *S. polyrrhiza* fronds. It was hoped that by this means, it could be established whether endogenous ABA was required to initiate turion induction.

3.1.3 Effect of ABA on cell wall plasticity

With regard to the ABA-imposed inhibition of growth of *S. polyrrhiza*, attention was then directed towards the effect of ABA on the cell wall extensibility of ABA-treated plants. Van Volkenburgh and Davies (1983) found that *Phaseolus vulgaris* leaf tissue exposed to ABA was less extensible than control tissue, and concluded that ABA diminished the capacity of cell walls to undergo acid-induced wall loosening. However, they found that ABA did not interfere with the light

stimulated acidification mechanism and suggested that it might inhibit other metabolic processes necessary for maintaining the capacity of the walls to be loosened by acid. MacRobbie (1981) also concluded that ABA had no effect on proton efflux, but rather increased the rate of K^+ efflux from guard cells.

Cell expansion *in vivo* is driven by turgor pressure which is non-directional, but any resulting cellular extension is dependent on the cell shape and on the structure and alignment of the components of the retaining cell walls.

The extensibility of cell walls is comprised of 2 separate components :

1. Elastic extensibility.

This is a characteristic of all cell walls, and is reversible. It can be linked to the properties of an elastic band which yields when stretched but returns to its original length once the stress is removed. It is a property used by plant cells to accommodate changes in turgor pressure and cell volume (Taiz and Metraux, 1979). Mature cells, which have stopped growing, have cell walls whose extensibility is comprised solely of elastic extensibility.

2. Plastic extensibility.

This describes the ability of cell walls to expand irreversibly in response to an applied stress. Once extended, the plastic extensibility utilised in the process is exhausted, and on removal of the stress, the walls do not return to their original size.

Plastic extensibility is considered to reflect a measure of

the potential of the tissue for irreversible expansion i.e. growth, and it decreases with age. It is usually associated with, and attributed to, acid-induced wall loosening (Rayle and Cleland, 1977; Van Volkenburgh *et al.*, 1985), although the capacity of the wall to be loosened at acid pH (CAWL) is itself under metabolic and hormonal control (Cleland, 1981; Taiz, 1984). These wall loosening processes require constant metabolic input. The relationship between cell wall composition and wall extensibility is further discussed in Chapter 4.

Uni-axial mechanical testing of cell wall extensibility has been of 3 types :

1) Instron analysis (application of a constant rate of strain and measurement of associated wall stress).
2) Stress relaxation (application of predetermined strain which remains constant whilst the decay in tension is measured).
3) Creep (consists of the plastic and delayed elastic extensibilities following application of constant load).

It has been argued (Taiz, 1984) that, of these 3, creep most closely resembles *in vivo* extension. However, as the stress relaxation and Instron techniques are more rapid and easier to carry out, these two methods are generally used. The method employed here is of the Instron-type.

3.2 MATERIALS AND METHODS

3.2.1 Growth of fronds

Growth was determined by daily counting the number of fronds which visibly protruded beyond the margin of the mother frond.

3.2.2 Induction of turions

Turions could be induced by either of the following methods

a) by the addition of 10^{-4} M ABA to fronds.

or b) by allowing fronds to reach confluent growth in low light conditions.

Photographs of fronds, and of turions formed by method a) above are shown in Figure 19.

3.2.3 Inhibition of turion formation by Fluridone and Norflurazon

The herbicides, which inhibit carotenoid synthesis (Bartels and Watson, 1978), Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) and Norflurazon [4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone] were gifts from Lilly Research Laboratories, Greenfield, Indiana, U.S.A. and Sandoz Ltd., Basle, Switzerland, respectively. They were added, at 10^{-5} M strength, to cultures of *S. polyrrhiza* which had just reached confluent growth, had been removed to a low light intensity, and were about to start producing turions. The number of

FIGURE 19

Spirodela polyrrhiza Fronds and Turions

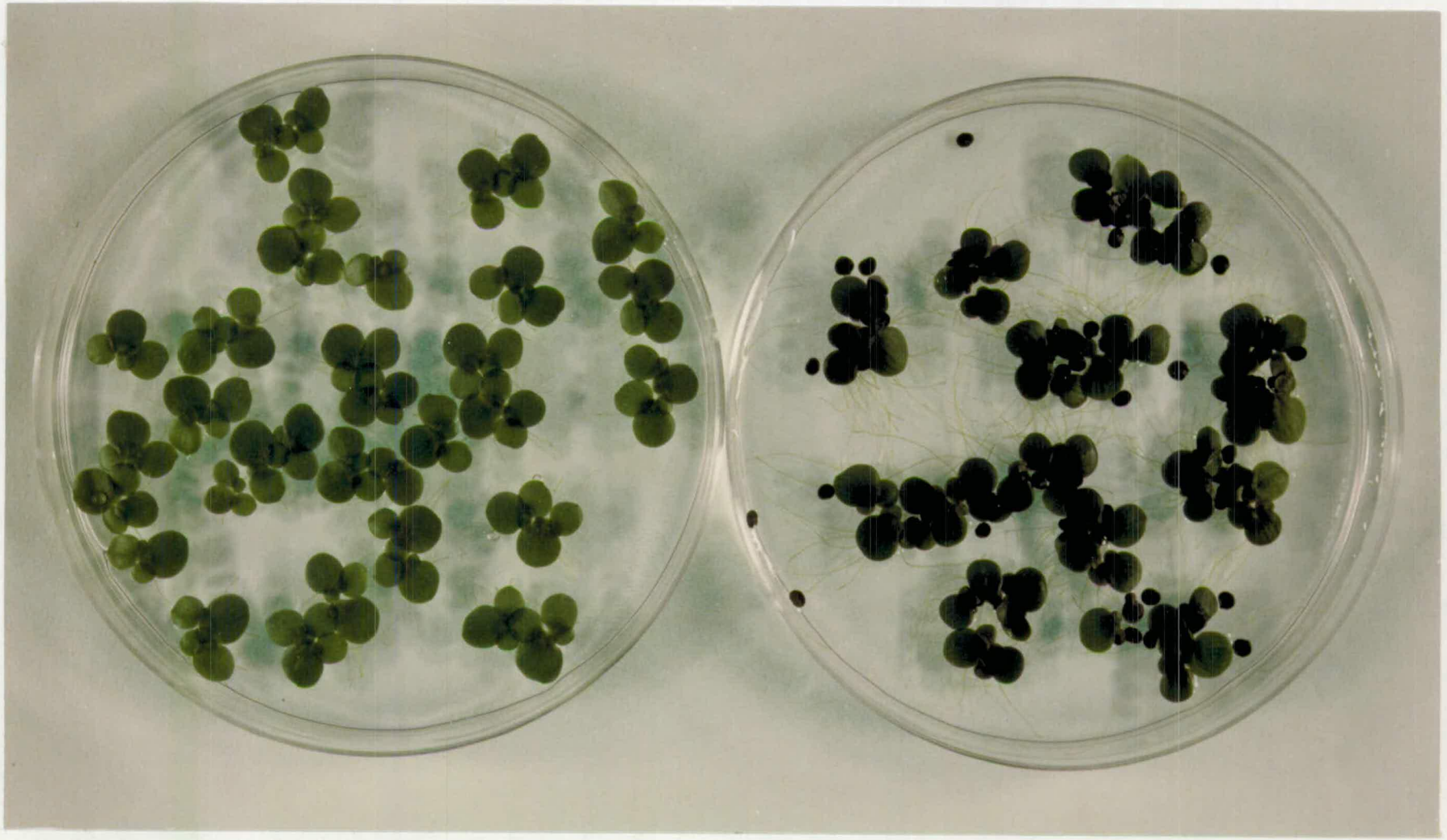
A. Fronds.

B. Fronds with turions formed as a result of addition of 10^{-6} M ABA to the H/2 medium.

FIG 19

A

B



turions subsequently released free into the medium were counted, in parallel with control cultures lacking herbicide.

3.2.4 Short-term inhibition of growth of fronds by ABA as measured by time-lapse photography

Four clumps of *S. polyrrhiza* were inoculated into each of four x 50 mm petri dishes containing 10 ml H/2. The dishes were placed in the growth cabinet for 3 hours prior to the addition of ABA (= time 0) at the following concentrations :

1) 0 (control)

2) 10^{-6} M

3) 10^{-7} M

4) 10^{-8} M

From time 0 to 15 hours later the growth of the fronds was monitored by taking photographs at 30 min intervals. A Minolta X-700 Camera with multifunction back and autowinder G (lens 50 mm f/3.5 MD MACRO) was clamped vertically above the petri dishes within the growth cabinet. The camera was loaded with PAN X film and set on automatic mode to take one exposure every 30 min. A ruler placed alongside the petri dishes acted as a scale marker. The film was developed using Kodak developer and fixer, and the negatives cut up and mounted in individual slide brackets. The slides were then projected on to a screen and the outlines of the enlarged clumps of fronds traced on to paper. Figure 20 shows prints developed from the negatives at times 0 and 14.5 hours.

The paper traces were then re-traced using a computer image

FIGURE 20

Time-lapse photography to show rapid inhibition of growth of *S. polyrrhiza* by ABA

The experimental details are described in section 3.2.4.

Prints of the negatives from times 0 and 14.5 hours are shown opposite.

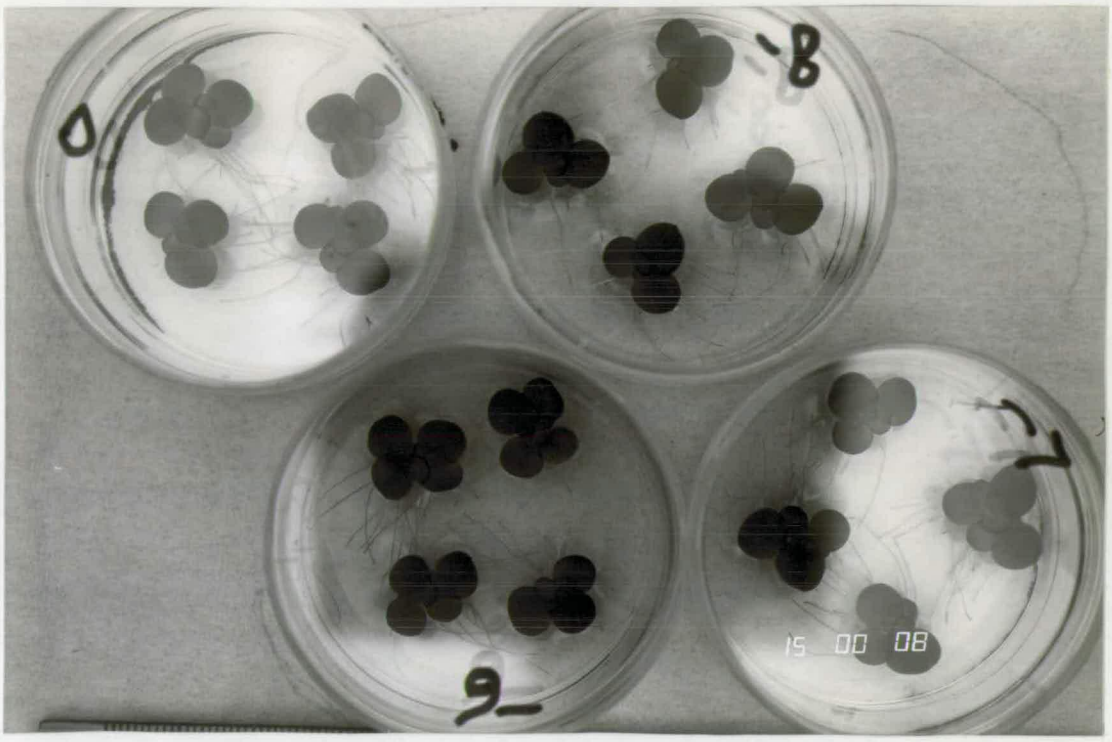
A. Time 0.

B. After 14.5 hours incubation.

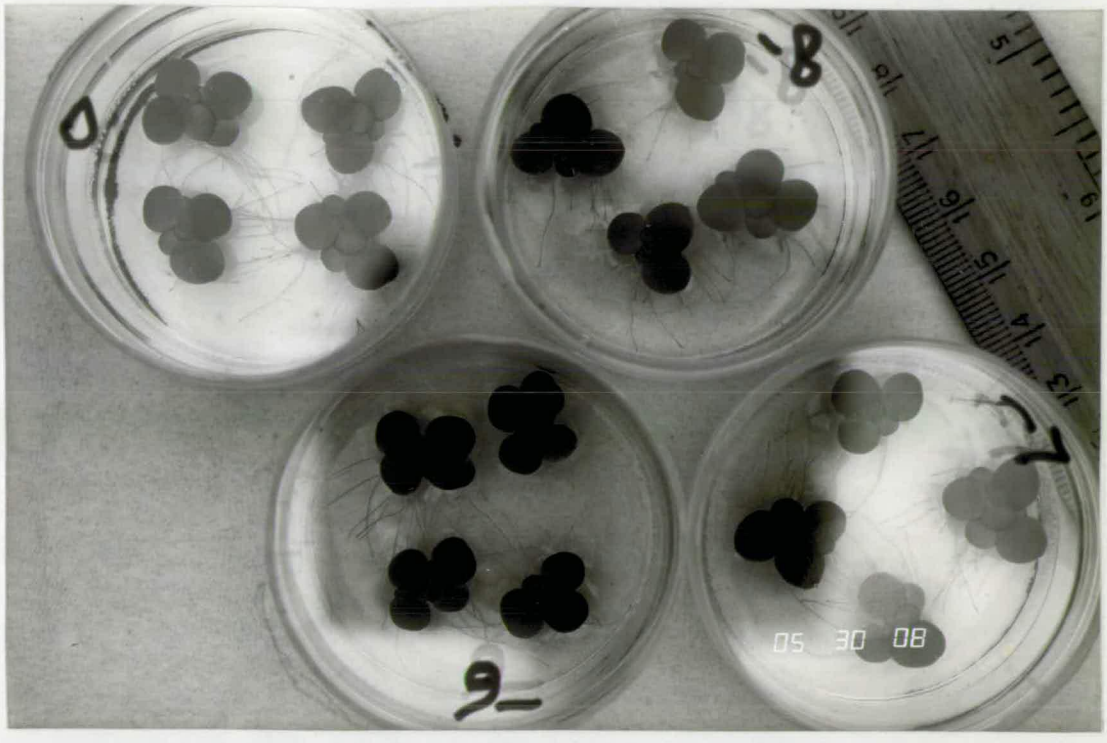
The dishes marked 0 contain the control plants (no ABA). Those marked -6, -7, and -8 correspond to the dishes containing plants with 10^{-6} M, 10^{-7} M and 10^{-8} M ABA, respectively.

FIG 20

A



B



analyser with a built in digitiser, and the computer calculated the areas within the outline traces, and converted these, by reference to the scale marker, to correspond to the total photosynthetic area of the fronds, as viewed from above. It was this latter parameter which was used as a measure of frond growth.

3.2.5 Effect of ABA on plastic extensibility of cell walls

A Preparation of tissue

Fronds and turions were killed, to eliminate turgor, by immersion in boiling methanol. After 3 min they were transferred to fresh methanol in which they were stored. The plants were re-hydrated in H_2O just prior to extensibility measurements.

B Apparatus and procedure

The design of the Edinburgh Tensiometer (Milligan, 1986) (Figure 21) was based on the Instron-type apparatus used by Van Volkenburgh *et al.*, 1983.

The plants were inserted, nodes uppermost, between two vertical clamps and stretched to a maximum load of 10 g by the action of a motor. The load on the tissue was measured by a strain gouge attached to the upper (immobile) clamp and recorded against time on a chart recorder. The motor speed was constant and hence the trace of load against time represents a "load-extension curve". Each frond/turion was stretched twice. The first extension revealed the total (elastic plus plastic) extensibility, and the second the elastic only, as any plastic

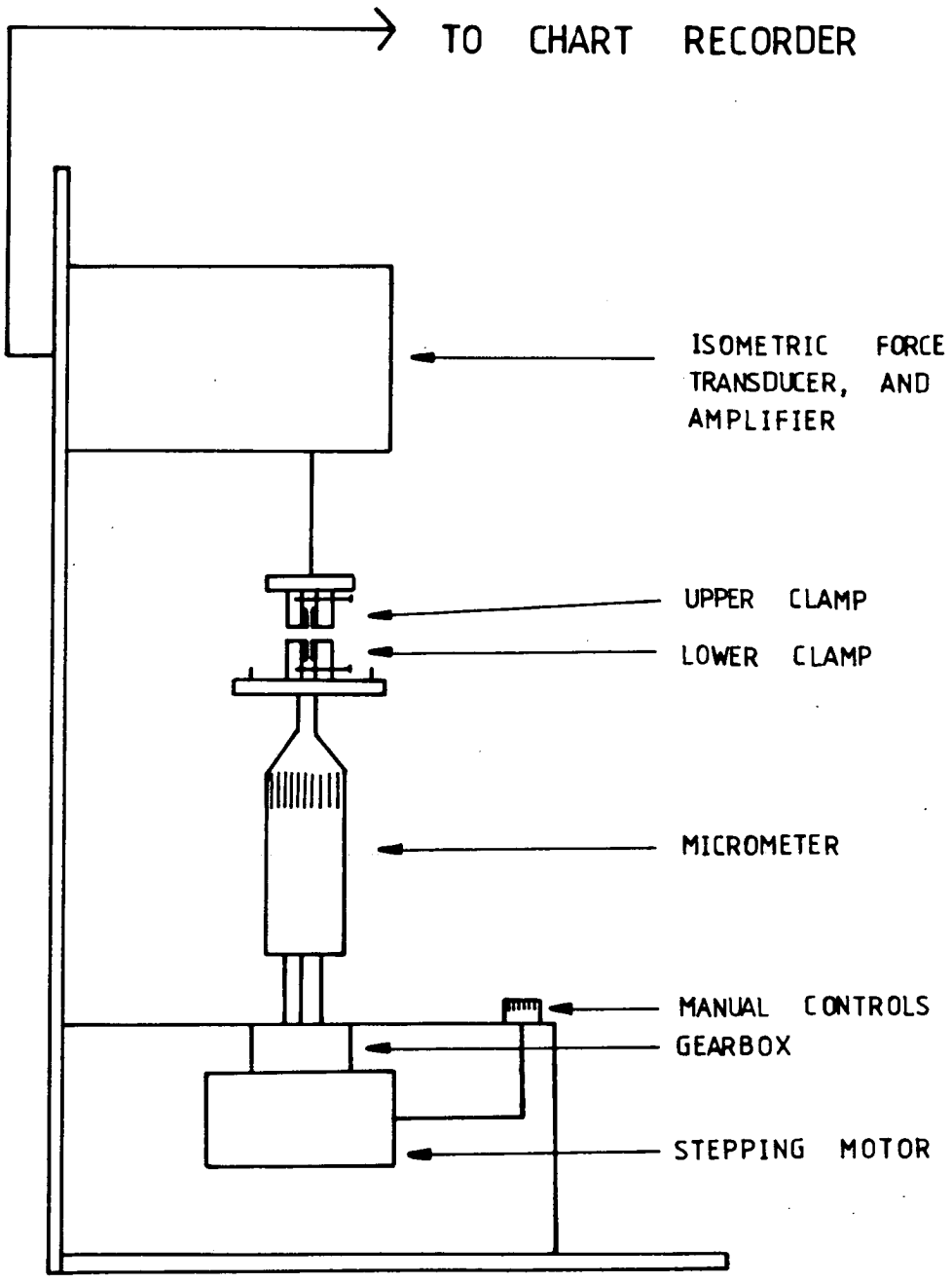
FIGURE 21

The Edinburgh Tensiometer

The apparatus consisted of a pair of clamps which held the frond/turion, a screw micrometer gauge which described the motor-driven distance between the clamps, and a force transducer which translated the load applied. The clamps had neoprene rubber ends which held the small plants firmly without tearing or damaging the tissues. The lower clamp was attached to a motor, via a gearbox and micrometer gauge, which had an accuracy of 0.01 mm. When the plants were extended, the output from the force transducer was amplified and plotted against time on a chart recorder. As the motor speed was at a known, constant rate, extension of the tissue was directly proportional to time i.e. the load/time trace obtained is equivalent to a load/ extension relationship.

The instrument design and operation is described in greater detail by Milligan (1986).

FIG 21


TO CHART RECORDER
ISOMETRIC FORCE TRANSDUCER, AND AMPLIFIER
UPPER CLAMP
LOWER CLAMP
MICROMETER
MANUAL CONTROLS
GEARBOX
STEPPING MOTOR

extensibility was irreversibly exhausted after the first extension. The plastic extensibility was therefore obtained by subtracting the second extensibility from the first. Figure 22 shows in diagrammatic form the traces obtained from the chart recorder, and details how the extensibility measurements were calculated from these. The results are expressed as : % plastic extensibility / 10 g load / mm^2 cross-sectional area. To calculate the cross-sectional area the dorsal-ventral thickness of the different sized fronds and of the turions was measured using a Moore and Wright micrometer screw gauge. The resulting thickness of the tissues has been plotted in Figure 23.

FIGURE 22

Calculation of plastic extensibilities of fronds and turions using the Edinburgh Tensiometer

Turions and fronds of different ages/sizes were killed by immersion in boiling methanol, then re-hydrated in H_2O and stretched in an Instron-type tensiometer (Figure 21). A full-scale deflection on the chart recorder using 500 millivolts corresponded to a load of 10 g. From the traces :

n represents the starting distance between the clamps as marked on the micrometer.

l_i represents the initial length of the taut, but unextended tissue. It is found by extrapolating the linear part of the "load-extension curve" to the point where it crosses the horizontal axis. The distance n plus the "slack" distance represented by x gives l_i.

l_i = initial length of tissue at zero load.

l_f represents the final length of the tissue and is found by drawing a vertical line from the point at which a load of 10 g was reached to the horizontal axis. The sum of the distances n plus y gives l_f.

l_f = final length of tissue at 10 g load.

The percentage extensibility of the tissue for 10 g load is hence :

$$\% \text{ extensibility} = \{(l_f - l_i) / l_i\} \times 100\%$$

and

$$\% \text{ plastic extensibility} = [(\% \text{ extensibility 1st extension}) - (\% \text{ extensibility 2nd extension})]$$

FIG 22

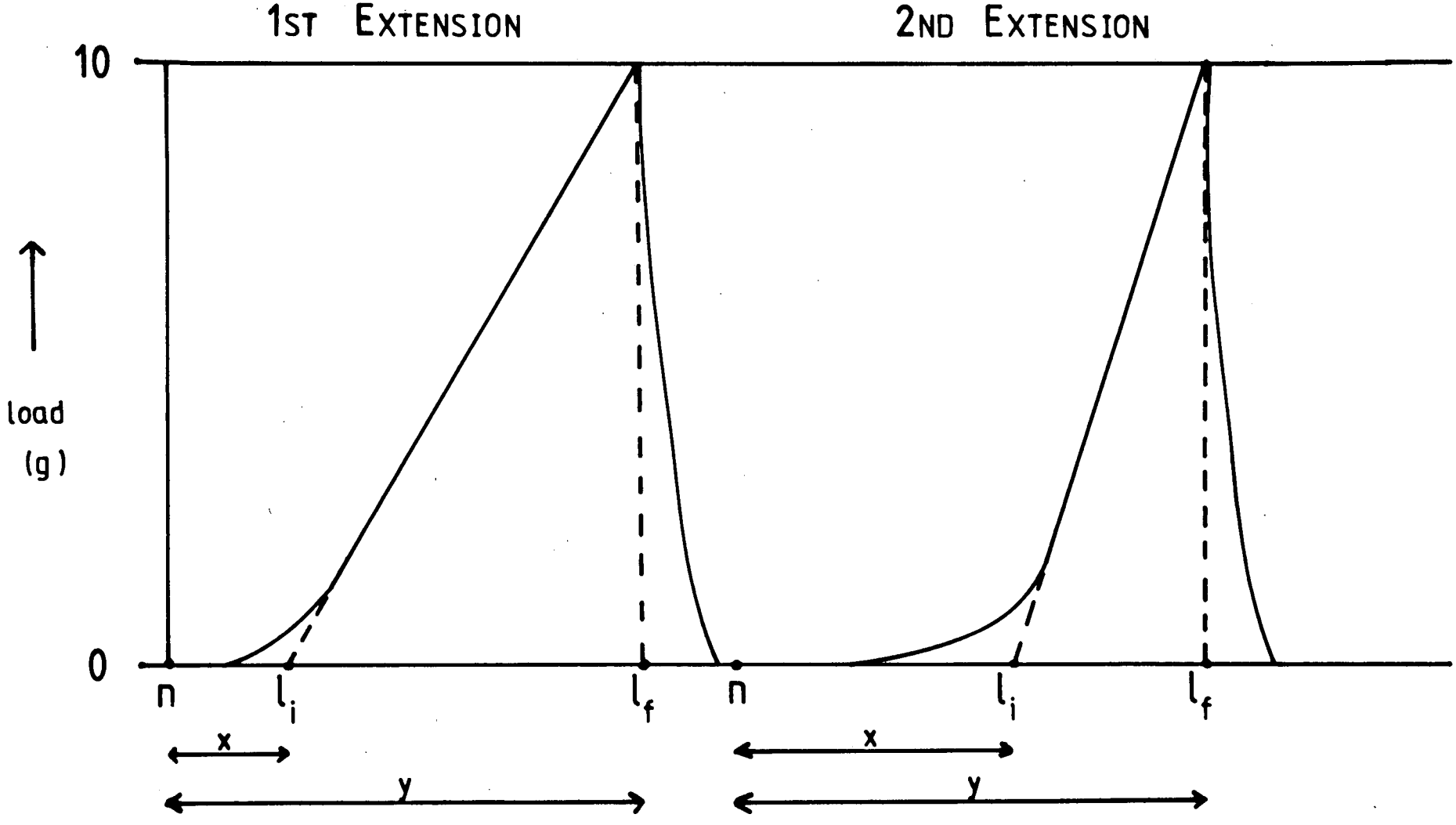

1ST EXTENSION
2ND EXTENSION
10
0
load
(g)
n
l_i
l_f
n
l_i
l_f
x
y
x
y
time / distance between clamps
(calibrated from chart speed)

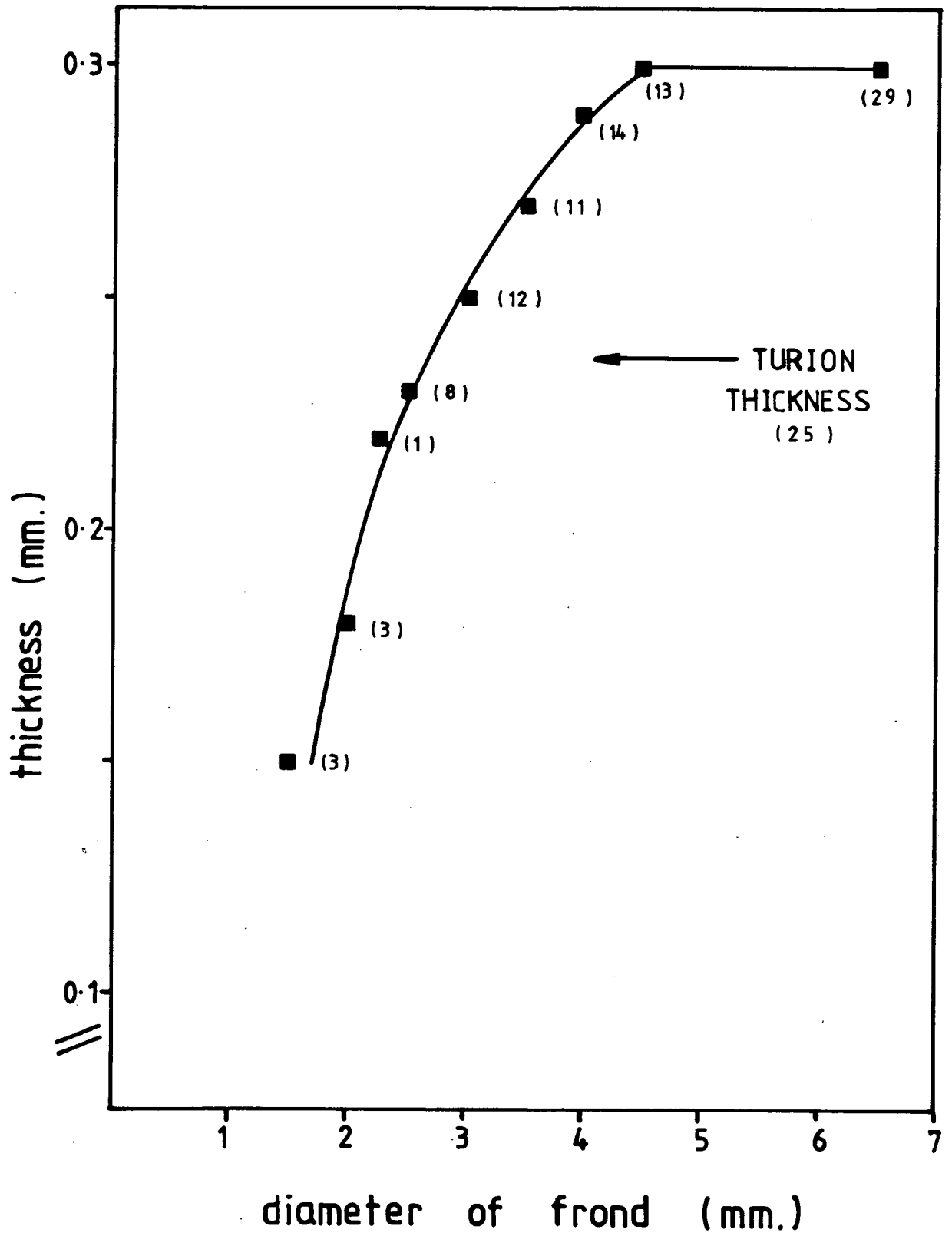
FIGURE 23

Thickness of turions and of fronds of different sizes/ages.

Fronds and turions were killed by immersion in boiling methanol. They were re-hydrated in water and their dorsal-ventral thickness measured using a sensitive micrometer screw guage.

The data points plotted represent average values. The figures in brackets correspond to the number of plants contributing to each point. The diameter of fronds was measured as the distance between the distal and proximal ends of the plants.

FIG 23


0·3
0·2
0·1
thickness (mm.)
(13)
(29)
(14)
(11)
(12)
(8)
(1)
(3)
(3)
TURION
THICKNESS
(25)
1
2
3
4
5
6
7
diameter of frond (mm.)

3.3 RESULTS

3.3.1 Growth of fronds

The growth of fronds as measured by frond number is essentially exponential, each parent frond producing two daughter fronds. Figure 24 shows the growth curve obtained for the *S. polyrrhiza* clone described in this thesis. The rate of growth is described by the equation : $F_t = F_o e^{kt}$

where F_t = frond number at time t

F_o = original frond number

k = growth constant

t = time (days)

A logarithmic interpretation gives the equation :

$$\log F_t = \log F_o + kt$$

Figure 25 describes a plot of log frond number against time. This straight line has an intercept of log F_o and a slope of k. The growth constant k is 0.103. It should be noted here that the *S. polyrrhiza* strain used in this thesis was inherited as the same strain used by Cheryl Smart as *S. polyrrhiza* (N) in her Ph.D. Thesis (Smart, 1981). It has, however, changed in various aspects of its growth behaviour during the 18 months between Smart's departure and my arrival and subsequent research. For example, the growth constant k has decreased from 0.175 to 0.103. The sensitivity towards ABA has also decreased - I found 10^{-6} M ABA to be the concentration required to induce turion production, whilst the previous turion-inducing concentration of 10^{-7} M found by

FIGURE 24

The growth rate of *Spirodela polyrrhiza*

The number of fronds visibly protruding from underneath the parent frond, as viewed from above, was counted on a daily basis. Bars denote standard errors and sample number of clumps of fronds = 10.

FIG 24

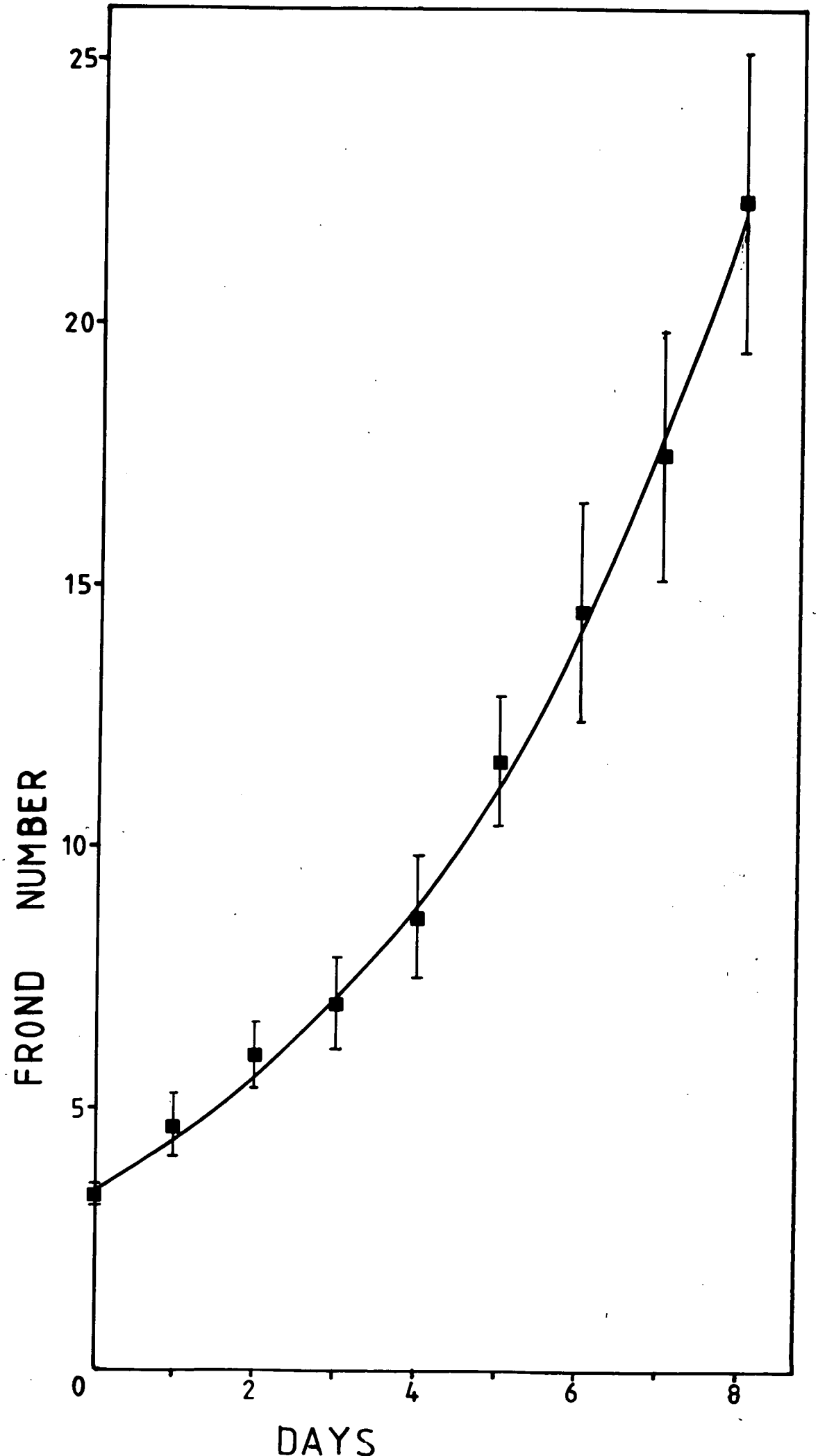


FIGURE 25

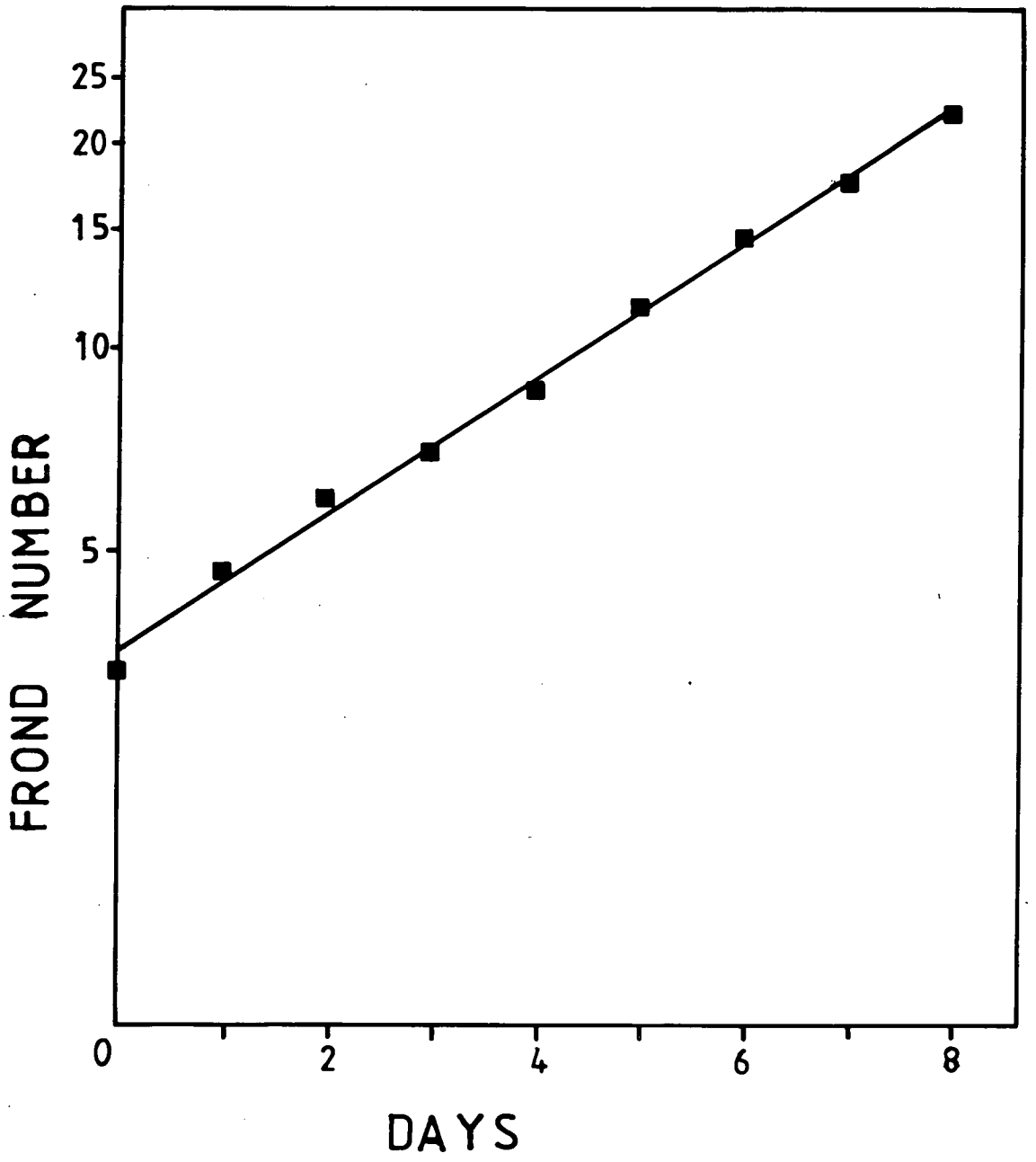
The growth rate of *Spirodela polyrrhiza* - semi-log plot.

The number of fronds visibly protruding from underneath the mother frond, as viewed from above, was counted on a daily basis. Log. frond number is plotted against time (days) to show the exponential nature of the growth pattern.

Growth constant (k) = 0.103 (slope).

Correlation coefficient r(9) = 0.953.

FIG 25



Smart, was found only to slow the growth rate. The sensitivity of different strains of *S. polyrrhiza* is known to be very variable. Stewart (1969) reported a strain which formed turions at 5×10^{-11} M ABA (with no apparent associated inhibition of growth), whilst Perry and Byrne (1969) described a strain, 2-66, from South America, which did not produce turions, or even exhibit growth inhibition, in response to any ABA concentration tested.

It would appear that during its 18 months of relative neglect in Edinburgh, a slower growing, less ABA-sensitive strain has been selected out, and hence the detailed characterisation of the strain as documented in Smart's Thesis unfortunately cannot be assumed to relate 100% to the strain examined herein.

3.3.2 Inhibition of turion formation by Fluridone and Norflurazon

The results obtained from treating *S. polyrrhiza* fronds about to form turions with the weedkillers fluridone and norflurazon, which inhibit carotenoid (and ABA) biosynthesis, are shown below.

The effect of 10^{-5} M Fluridone and 10^{-5} M Norflurazon on turion induction.

	number of dissociated turions				
days -->	7	10	17	22	25
Control	110	134	180	202	202
Fluridone	24	24	24	24	24
Norflurazon	34	34	34	34	34

These results show that turion production was inhibited by both herbicides. However, despite being kept in shaded conditions, the treated plants showed severe chlorosis of the tissues and so it was impossible to discern whether the lack of turion production was due to a specific inhibition of ABA, or to a more general inhibition of total metabolism. Plants treated with the herbicides and kept under normal light and growth cabinet conditions for 7 days were completely bleached of pigment, and the entire colony beyond recovery. Unfortunately, therefore, the action of fluridone and norflurazon was not sufficiently specific to promote them as manipulative tools with which to investigate the effect that a depletion of endogenous ABA levels would have on subsequent turion formation.

3.3.3 Rapid ABA inhibition of frond growth - Time-lapse photography

The data relating to total photosynthetic areas of the fronds obtained by computer analysis (Methods 3.2.4) was fed into the Edinburgh Computing Centre EMAS computer and analysed using a Genstat 4.04B statistical package. The individual growth curves were drawn, and then all data reduced, by subtraction, by a value such that the initial area was 0 at time 0, in order to standardise the starting areas for all clumps of fronds. This was achieved by computerised extrapolation of the linear parts of the curves for 0, 10^{-8} and 10^{-7} M ABA, and by assuming immediate cessation of growth

with 10^{-6} M ABA. The data from the four replicates within each treatment were then averaged and the resulting curves shown in Figure 26. There was no significant difference between the curves for 0 (control), 10^{-7} M and 10^{-8} M ABA (F test value $F_{(87,2)} = 5.59$, which is within the 95% confidence limits for a two-tailed F test of 39.48.) Hence, one line has been drawn to represent these three treatments, each consisting of four replicates. There was, however, a significant difference, using data from times 0 to 9 hours only, between the treatments 0, 10^{-8} M and 10^{-7} M; and the 10^{-6} M ABA treatment ($F_{(116,3)} = 56.51$, which is above the 95% confidence limit of 13.95). It appears, therefore, that with the concentrations tested, ABA has an almost immediate effect on growth at a concentration of 10^{-6} M only (the turion-inducing concentration). At 10^{-6} M, for the first 2 hours there appeared to be a "shock" reaction and no growth at all, followed by a partial recovery and a reduced rate of growth for about 9 hours, and then cessation of visible growth for the remaining 4 hours.

Smart (1981) found that the addition of ABA at a turion-inducing concentration did not result in the production of turions if the fronds were removed to ABA-free medium following a 48 hour incubation. As turion induction is a reversible process up to this point, it appears that the initial inhibition of growth measured here at 10^{-6} M ABA is not an essential pre-requisite of turion formation. It should be noted that only those fronds protruding beyond the limits

FIGURE 26

Time-lapse photography to demonstrate the rapid inhibition of growth of *S. polyrrhiza* fronds by 10^{-6} M ABA.

Four clumps of fronds were incubated in each of four petri dishes containing H/2 medium plus :

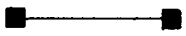
A. 0 (control)

B. 10^{-8} M ABA

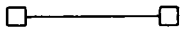
C. 10^{-7} M ABA

D. 10^{-6} M ABA

A Minolta X-700 camera with multi-function back and auto-winder was clamped above the plants in the growth cabinet, and one exposure taken automatically every 30 min. The frond outlines on the resulting negatives were traced by a computerised digitiser pen which calculated the areas within the outlines to give the total photosynthetic areas of the fronds.

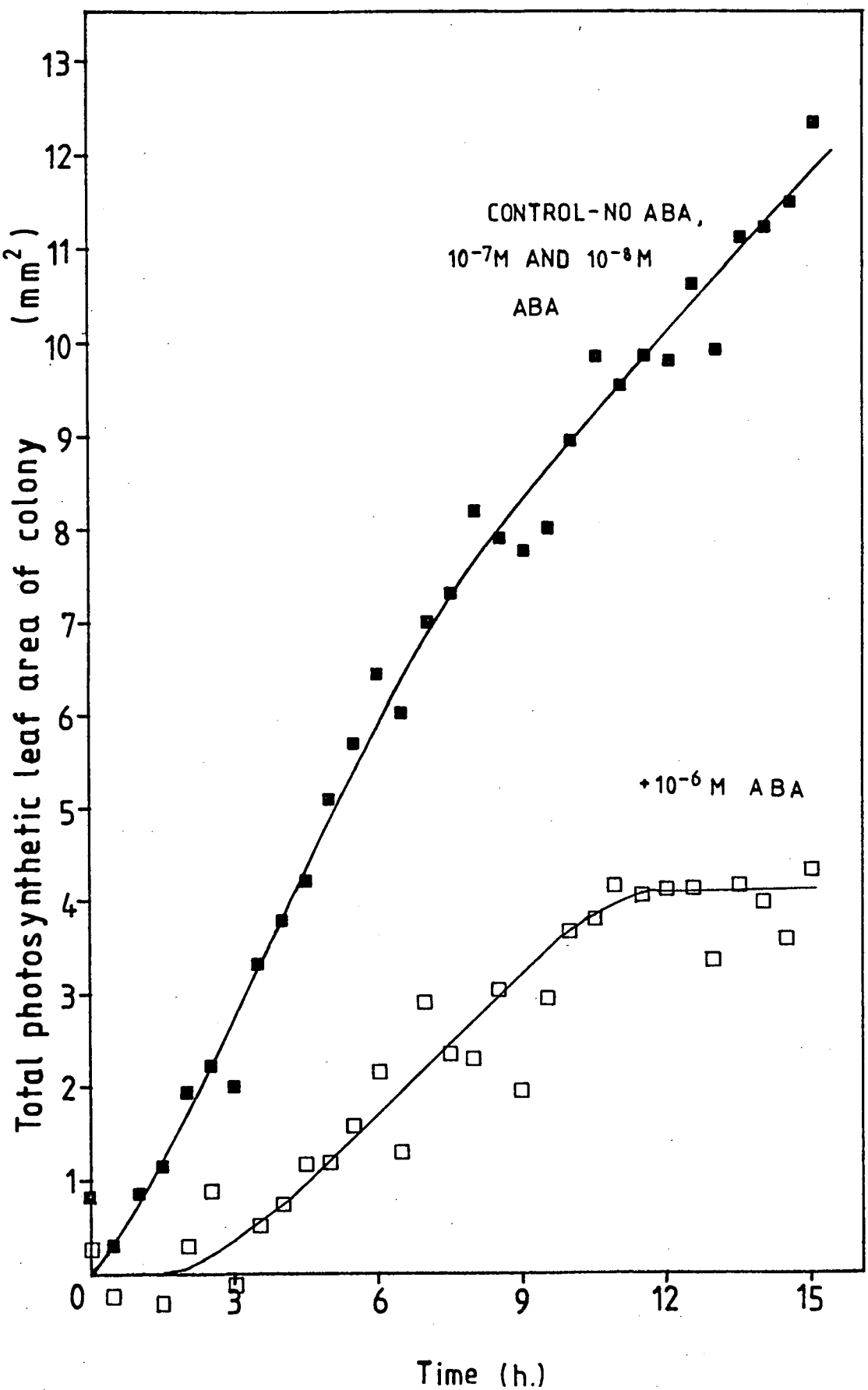


The four replicates from each of the A, B, and C treatments. There was no significant difference between these treatments (see Results 3.3.3 for F test values).



The average of the four replicates for treatment D (10^{-6} M ABA).

FIG 26


Total photosynthetic leaf area of colony (mm²)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
3
6
9
12
15
Time (h.)
CONTROL-NO ABA, 10⁻⁷M AND 10⁻⁸M ABA
+10⁻⁶ M ABA

of the mother frond could be detected using this method i.e. fronds which were too large to be converted into turions. The small fronds which did have the potential to develop into turions by ABA induction would be at that stage still be contained within the meristematic pockets and hidden underneath the parent fronds. However, due to the positioning of the meristematic pockets at the sides of the proximal end of the frond, as soon as the young frond outgrew the confines of the parental pockets, they should have protruded visibly, as viewed from above. At this stage a frond would be about 0.7 mm in size (Smart, 1981). Hence, young fronds have participated in the outcome of these results. The orientation of un-abscised fronds within a clump may, however, have delayed this protrusion, and hence the contribution, of some young fronds.

3.3.4 Effect of ABA on plastic extensibility of cell walls

The results of the plastic extensibility measurements of different sized fronds when incubated in 10^{-4} M ABA are shown in Figures 27A and B and Figure 28. They show that the cell wall plasticity of the fronds decreases as they enlarge and mature, which is in accordance with the expected associated growth potential of the different aged fronds. The smallest fronds measured (2.0 - 2.5 mm diameter) had 46.2% plastic extensibility / 10 g load / mm^2 cross-sectional area, as compared with the values for adult fronds and turions at time 0, which were 1.8% and 10.3%, respectively. The extensibility

FIGURE 27A.

The effect of 10^{-6} M ABA on the plastic extensibility of *S. polyrrhiza* fronds of different sizes.

10^{-6} M ABA was added to a group of fronds. Samples of fronds were taken at intervals up until 15 hours after ABA addition. The fronds were removed and killed immediately in boiling methanol. The different sized fronds were then separated out according to their diameter size. They were re-hydrated, and their plastic extensibility measured using the Instron technique on the Edinburgh Tensiometer (see Methods 3.2.5, and Figures 21 and 22 for details of this technique).

The plastic extensibility of different sized fronds has been plotted against time.

Frond sizes :

A. Up to 2.5 mm diameter

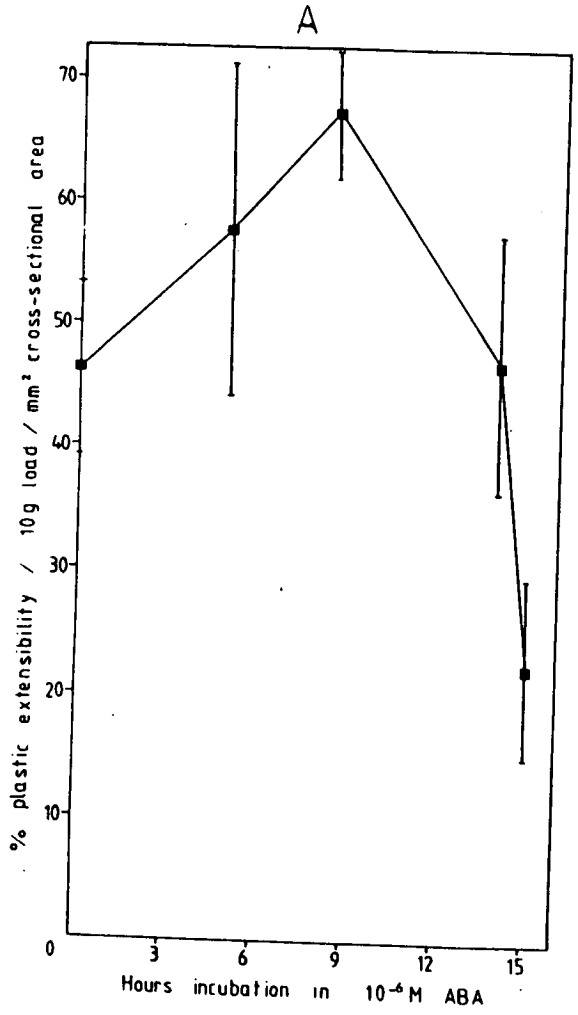
B. 2.6 - 3.5 mm diameter

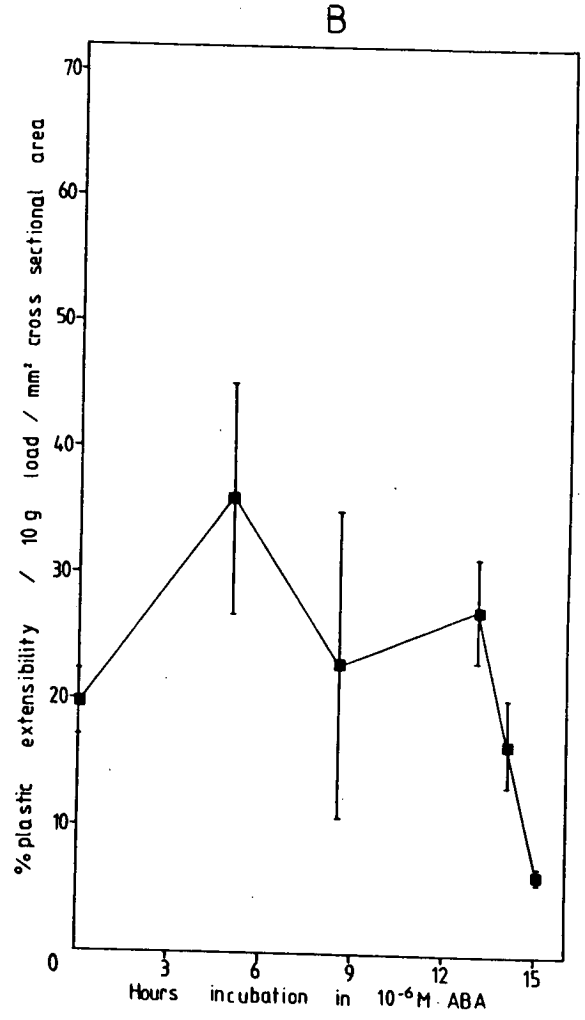
C. 3.6 - 4.5 mm diameter

Bars denote standard errors

Continued on Figure 27B.

FIG 27 A


A
% plastic extensibility / 10 g load / mm² cross-sectional area
0
10
20
30
40
50
60
70
3
6
9
12
15
Hours incubation in 10⁻⁶ M ABA


B
% plastic extensibility / 10 g load / mm² cross sectional area
0
10
20
30
40
50
60
70
3
6
9
12
15
Hours incubation in 10⁻⁶ M ABA

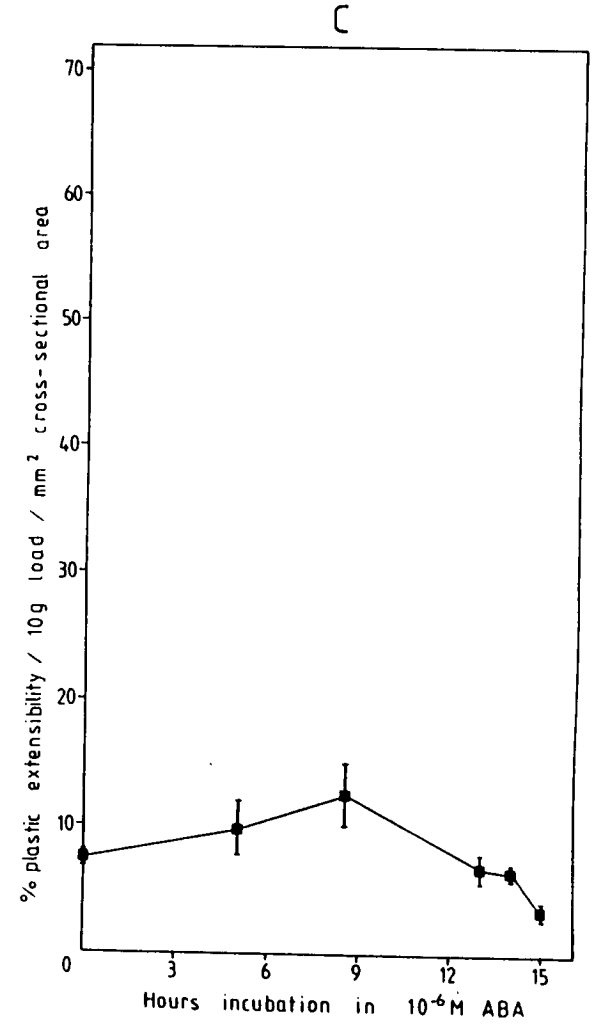

C
% plastic extensibility / 10 g load / mm² cross-sectional area
0
10
20
30
40
50
60
70
3
6
9
12
15
Hours incubation in 10⁻⁶ M ABA

FIGURE 27B.

The effect of 10^{-6} M ABA on the plastic extensibility of *S. polyrrhiza* fronds of different sizes.

Continued from Figure 27A.

Frond sizes :

D. 4.6 - 5.5 mm diameter

E. 5.6 - 6.5 mm diameter

F. 6.6 - 7.0 mm diameter (adult fronds). The plastic extensibilities of turions formed as a result of the exposure of fronds to 10^{-6} M ABA, but with no subsequent ABA treatment, is also indicated in F.

Bars denote standard errors.

FIG 27 B

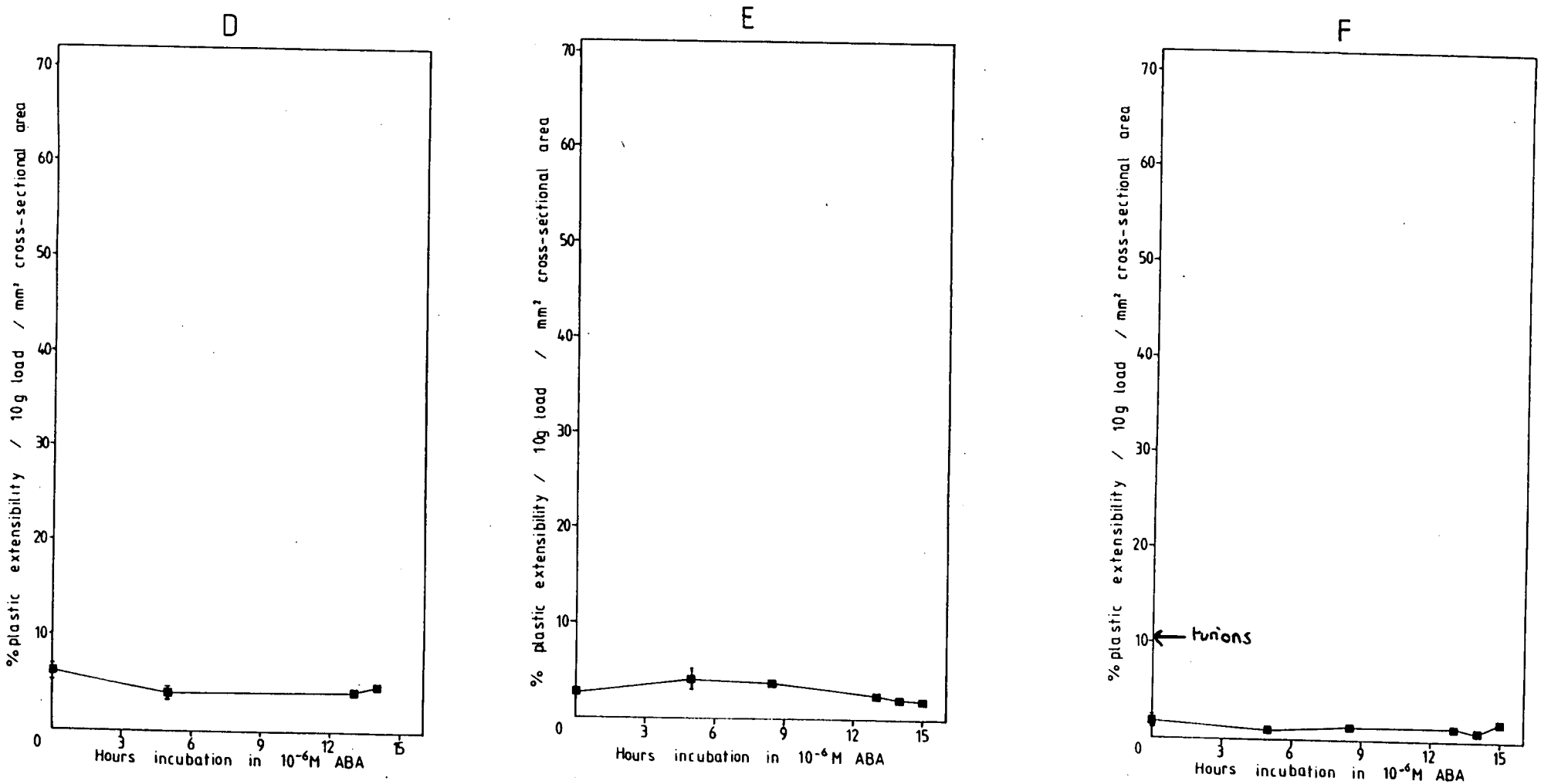

D
E
F
% plastic extensibility / 10g load / mm² cross-sectional area
Hours incubation in 10⁻⁶M ABA
0
10
20
30
40
50
60
70
3
6
9
12
15
← tunions

FIGURE 28

The rate of inhibition of plastic extensibilities of *S. polyrrhiza* fronds of varying age following exposure to 10^{-6} M ABA.

10^{-6} M ABA was added to a group of fronds in H/2 medium. Samples of fronds were taken at intervals up until 15 hours after addition of ABA. The fronds were removed and killed immediately by immersion in boiling methanol. The different sized fronds were then dissected out and grouped according to their diameter size. After re-hydration, their plastic extensibilities were measured on the Edinburgh Tensiometer using the Instron technique (see Methods 3.2.5, and Figures 21 and 22 for details of this technique).

The plastic extensibility has been plotted against frond size (mm diameter), for incubation times in 10^{-6} M ABA of :

A. 0

B. 5 hours

C. 8/9 hours

D. 13 hours

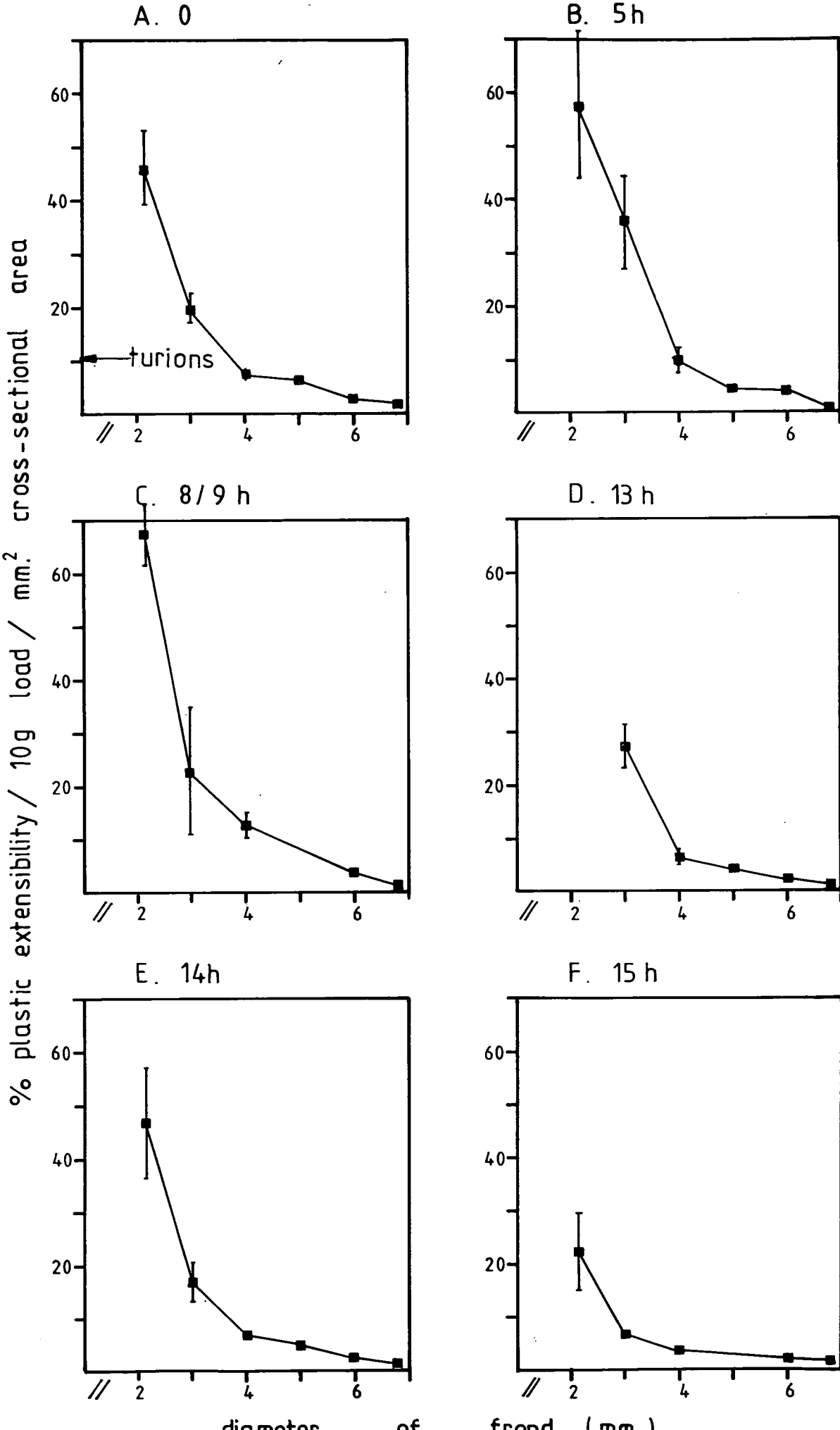
E. 14 hours

F. 15 hours.

The plastic extensibility of turions, formed as a result of the addition of 10^{-6} M ABA to the medium, and examined approx. one week after abscission from the parent fronds, is indicated in A.

Bars denote standard errors

FIG 28


A. 0
B. 5h
C. 8/9 h
D. 13 h
E. 14h
F. 15 h
turions
% plastic extensibility / 10g load / mm.² cross-sectional area
60
40
20
2
4
6

of turions cannot be compared with those of the fronds due to the difference in tissue structure (discussed later in 3.4).

It should be noted that the Edinburgh Tensiometer was designed for use with strips of leaves approximately 10 mm long and that the small size of the turions (1.5 mm diameter) in particular, meant that placing the material evenly and securely between the clamps was a painstakingly difficult task. It also meant that the narrow scale range of measurements taken were stretching (sic) the accuracy of the apparatus to its limit. For this reason, with regard to the small fronds in particular, the standard errors shown in Figures 27A and B are high.

Fronds over 4.5 mm in diameter (distance between proximal and distal ends of fronds) had a very low initial plastic extensibility (Figure 27B), presumably because they had almost reached their complete mature size, and these fronds will not be further discussed here.

For the smaller fronds (Figure 27A), it appeared that, following the addition of 10^{-6} M ABA, there was a general increase in plastic extensibility for the first 9 hours, then a decrease until after 12 hours the values were approximately the same as at time 0. During the remaining 3 hours of the experiment (12 - 15 hours after ABA addition) the extensibilities dropped to below the starting values.

However, even after 15 hours in 10^{-6} M ABA, fronds smaller than 4 mm in diameter still retained a plastic extensibility higher than that of the mature fronds (see Figure 28). Whilst

there was a reduction in plastic extensibility after 15 hours, it is difficult to relate the changes in extensibility during the course of the experiment with the associated reduction in growth as measured by time-lapse photography, shown in Figure 26.

Figure 29 summarises in diagrammatic form the two different experiments, which tested the immediate effects (0 - 15 hours) of 10^{-6} M ABA on a) growth and b) plastic extensibility. It appears that the inhibition of growth is not solely related to the plastic extensibility of the tissue, although by the end (after 15 hours in 10^{-6} M ABA) of these relatively short-term experiments, there did appear to be a correlation between growth inhibition and loss of cell wall plasticity.

The plastic extensibilities of the smaller fronds (up to 4.5 mm in diameter) <u>increased</u> from the time of addition of ABA to about 9 hours later (Figure 27A), decreased again from 9 hours to 12 hours, at which point the values were approximately the same as at time 0, and then continued to decrease during the remaining 3 hours. This last 3 hour period during which the plastic extensibility decreased to values below those at time 0, corresponds with the time-lapse photography results where from time 12 - 15 hours the fronds completely stopped growing.

However, the reduction in growth rate between 2 and 9 hours is not associated with a corresponding reduction in plastic extensibility. This lack of correlation, as shown in Figure

FIGURE 29

A Summary of the Short-term Effects on of 10^{-6} M ABA on

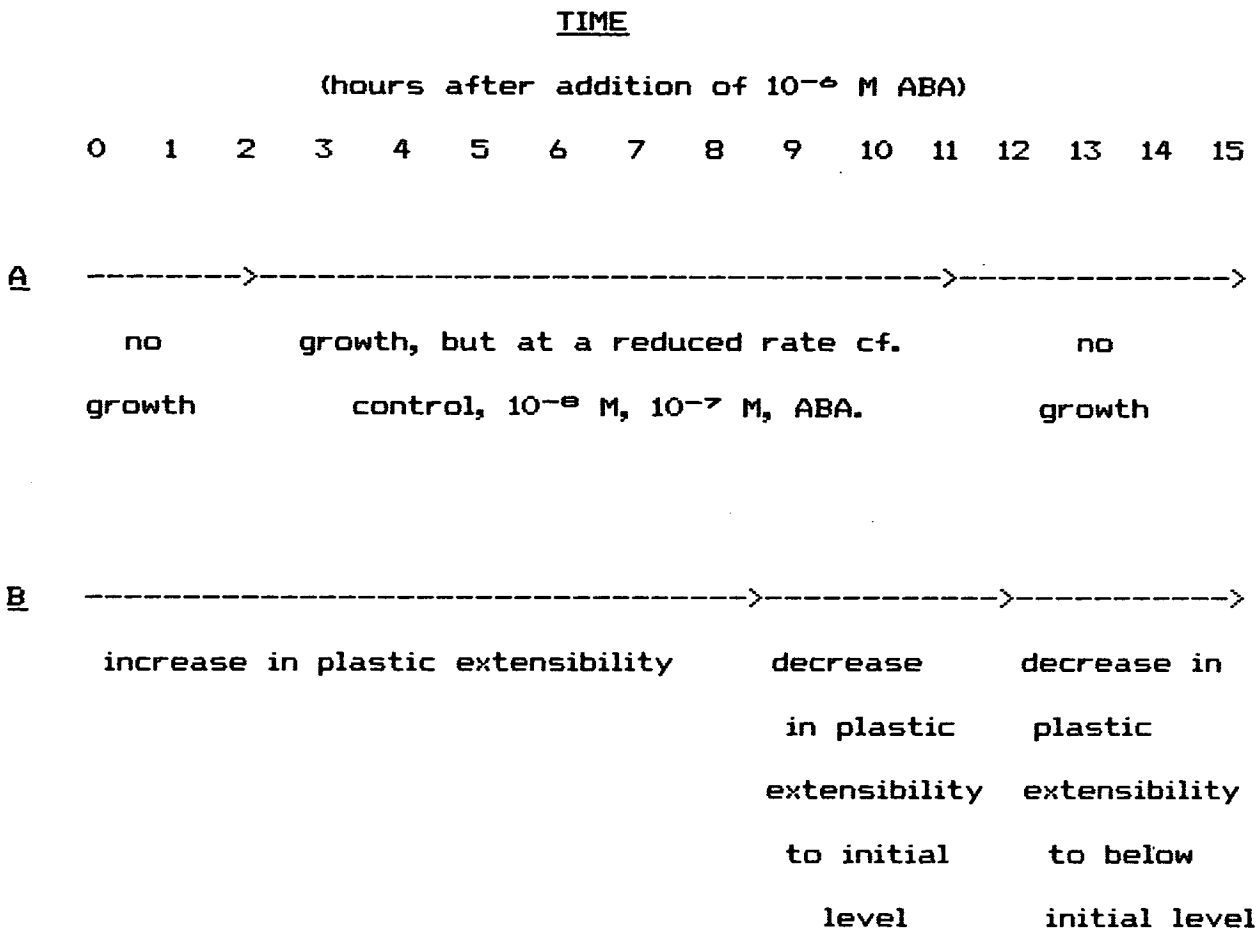
A. Growth, and

B. Plastic Extensibility

of *S. polyrrhiza* fronds.

ABA was added to fronds of *S. polyrrhiza* in H/2 medium to a final concentration of 10^{-6} M. The resulting inhibition of growth (total photosynthetic area), as shown in A, was monitored by time-lapse photography (3.2.4). The plastic extensibilities of the smaller (up to 4.5 mm in diameter) methanol-killed fronds, as shown in B, was measured by an Instron-type apparatus (3.2.5).

FIGURE 29


TIME
(hours after addition of 10^{-6} M ABA)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A
no growth
growth, but at a reduced rate cf. control, 10^{-8} M, 10^{-7} M, ABA.
no growth
B
increase in plastic extensibility
decrease in plastic extensibility to initial level
decrease in plastic extensibility to below initial level

29, is not surprising considering the complexity of factors controlling plant growth. For this reason, these measurements of plastic extensibility must be considered in context as being just one contributory factor in the general regulation of plant growth control. Evidently, growth inhibition during the first 9 hours after ABA addition is not regulated by cell wall extensibility. The reason for the increase in extensibility during this period is not known, but it might be that there has to be some re-arrangement within the wall structure prior to cessation of growth and that during this re-structuring period, the plasticity is increased rather than decreased. Other growth regulating factors must be in control at this time to ensure that the potential of the cell wall to extend is not realised. Turgor pressure is probably an important factor here. If ABA addition resulted in a decrease in turgor pressure (Pierce and Raschke, 1980) the walls would not have been stretched to the level of control plants (no ABA), and the ABA-treated plants would therefore have more "unused" plastic extensibility. This would account for the initial rise in plastic extensibility, as shown by my measurements.

3.4 DISCUSSION

ABA added at a turion-inducing concentration (10^{-6} M) had the effect of very rapidly inhibiting the growth of *S. polyrrhiza*, whilst lower concentrations, which had been observed to slow the growth rate over the time-span of several days, produced no observable effect for the first 15 hours. The developing primordia, destined to become turions, were not visible at this stage as they would be ensconced within the parental pocket. As they were completely dependent upon the parent frond for growth requirements, any visible effect on the parent frond population might be expected to have a direct effect upon these developing primordia, unless the parents could somehow selectively determine what their offspring are exposed to. The destiny of a primordium (frond or turion) is decided by the time it has reached approximately 1 mm in diameter (Smart, 1981).

Following the addition of 10^{-7} M ABA, Smart found that fronds of 0.7 mm or less in diameter would form turions. Those of 0.7 - 1.3 mm diameter formed semi-turions. At about 0.7 mm diameter, at the cellular level, cell expansion takes over from cell division, and air spaces begin to form. The sensitivity of the cells towards ABA apparently decreases with the onset of these developmental changes, and as a consequence the primordium proceeds to develop into a frond. The key to this sensitivity change remains a mystery.

The biochemical changes occurring within fronds and

developing primordia have been well documented by Smart (1981) and Smart and Trewavas (1983a and 1984). They found that DNA synthesis in the developing primordium, destined to become a turion, was inhibited within 3 hours of addition of ABA at turion-inducing concentrations. There was a repression of protein synthesis after 24 hours, but RNA synthesis was not inhibited until 3 days after ABA addition. It was only at this stage, 3 days later, that the developing primordium was committed to becoming a turion. Up until 3 days, the process was reversible, and on removal of the ABA, fronds rather than turions would result. As my investigations concerned only the first 15 hours effect, the resulting inhibition of growth found only at the concentration sufficient to induce turions, cannot be directly linked with turion formation, which at that stage would have been a reversible phenomenon. Clearly, there are several sequential but independent biochemical changes involved in ABA-induced growth inhibition, and turion production. It would have been interesting to investigate the reaction to a higher concentration of ABA e.g. 10^{-5} M, to see if the speed and pattern of growth inhibition were the same as for 10^{-6} M. 10^{-5} M ABA stops the growth of *S. polyrrhiza* without the formation of turions.

The results of the plastic extensibility measurements reflected an equally rapid reaction to exogenous ABA at 10^{-6} M. These results were expressed as % plastic extensibility / 10 g load / mm^2 cross-sectional area of tissue. This basis of standardisation does not, however, take into account the

difference in tissue structure between young fronds and adult fronds, and turions. Ultra-microscopical examination of thin sections (Smart, 1981; Smart and Trewavas, 1983b) revealed marked structural differences. For example, adult fronds contain a large proportion of aerenchyma i.e. parenchyma separated by large intercellular spaces, whilst the smaller fronds have a denser structure with fewer air spaces. Turions, which have no buoyancy requirement, have starch-packed, mesophyll cells which lack both differentiation in structure and intercellular air spaces. Turion cell walls are also twice as thick as those of fronds and are constituently different (see Chapter 4). The comparison of tissue extensibility based on tissue volume, as used here to standardise measurements, must have its limitations. Cellulose content might possibly have been a more relevant index of cell wall content, but as any attempt at standardisation for the various sized fronds and the turions represented a compromise between similar and dissimilar characteristics, it was decided to use the relatively easily obtainable index of cross-sectional area as a standard in expressing the results.

It should be remembered that the Instron method used killed tissue. When living fronds were placed in the apparatus, they tore as soon as any strain was put on them. Cleland (1967) found that boiling and re-hydrating tissues profoundly altered the cell walls native gel structure, and he proposed that the Instron technique was merely measuring a strain-hardening function of the wall. The apparatus itself is designed to

measure the response to a uni-axially applied stress, whereas the stress (turgor) initiating cell expansion *in vivo* is non-axial. Dead tissue stretched in this way cannot therefore be assumed to reflect the *in vivo* extension, in which wall-loosening and the synthesis and / or release of wall loosening factors (Taiz, 1984) requires active metabolic processes. Therefore, this method should only be considered to reflect some potential extensibility of the cell walls at the time of death.

CHAPTER 4

Cell Wall Monosaccharide Composition of Fronds and Turions of *Spirodela polyrrhiza*, and the Effect of ABA on the Incorporation of Apiose into Cell Walls of Young (Turion-Inducible) Fronds.

4.1 INTRODUCTION

4.1.1 The approach adopted for the analysis of *S. polyrrhiza* cell walls.

The cell walls of plants are responsible for the maintenance of cell shape, and their structural and mechanical properties are of critical importance in the determination of cell growth potential. The effect of 10^{-6} M ABA on cell wall plastic extensibility in *Spirodela polyrrhiza* was discussed in Chapter 3. Wall extension and plasticity have traditionally been associated with auxin-promoted proton pumps (Rayle and Cleland, 1977), but Fry (1979) has suggested that gibberellic acid (GA_3) at 10^{-6} M to 10^{-11} M will promote the expansion of cultured spinach cells by the inhibition of peroxidase secretion and its associated rigidification of the cell wall. This chapter similarly aims to focus attention towards the ABA-stimulated inhibition of growth and turion formation in *S. polyrrhiza*, as reflected in aspects of the chemical cell wall composition.

In *S. polyrrhiza*, Smart (1981) showed that the cell walls of turions were twice as thick as those of fronds on a volumetric basis, frond cell walls having a volume density of 0.05 cm^3/cm^3 cell compared with that of turion cell walls of 0.1 cm^3/cm^3 cell. Smart's data, coupled with my results in Chapter 3 showing altered wall plastic extensibilities in fronds treated with turion-inducing concentrations of ABA, initiated a programme of analyses of cell wall composition of

fronds and turions. The intention was to analyse and compare the monosaccharide cell wall composition of fronds and ABA-induced turions to determine whether the composition was altered in the transition from vegetative to dormant physiological states. It was hoped to investigate further any differences in chemical constitution by determining the differences in levels of associated sugar intermediates and nucleotides in fronds and in turions which have been induced by ABA.

4.1.2 Cell wall structure.

Plant cell walls consist of components which are generally categorised into pectic substances, cellulose, hemicelluloses, glycoproteins and "other polymers" (e.g. lignin). The hemicelluloses are found non-covalently associated with cellulose, whilst pectic polysaccharides are those polymers with galacturonosyl-containing backbones. However, this rigid classification now seems less favourably accepted, and walls are becoming increasingly considered as a network of covalently and non-covalently linked polysaccharides and proteins.

The primary cell walls of monocots generally contain only about 3% pectin, which is about one-tenth the amount found in dicots. For example, the total amount of galacturonic acid has been estimated to be 3% of the cell walls of oat coleoptiles (Ray and Rottenberg, 1964), and only 1.3% of those of maize root meristem (Dever *et al.*, 1968). For this reason,

there is a commonly held view, expressed here by Darvill *et al.* (1980), that "the pectic polysaccharides of monocots do not appear to play as important a role in primary cell wall structure of monocots as they do in dicots." They do, however, make the mistake of classifying *Lemna minor* as a dicot! Whilst it may be true that most monocot cell walls appear to contain less than 10% of the pectic polysaccharide content of dicot primary cell walls, duckweeds appear to be the exception to the rule. The reason for this is that duckweeds contain large quantities of wall apiogalacturonans.

4.1.3 Isolated pectic wall fragments.

Recent attention has been directed towards fragments isolated from pectin fractions of walls which have regulatory effects on plant tissues and are termed "biologically-active" fragments. One such example is a cell wall fragment released by partial hydrolysis of the walls of suspension-cultured sycamore cells (Albersheim *et al.*, 1983). Cultures of *Lemna minor* G3 were induced to flower by growing under continuous light conditions, but when the sycamore wall fragments were added to concentrations of 0.05 - 0.1 mg/ml, they had the effect of both inhibiting flowering and approximately doubling vegetative growth i.e. they redirected the fate of developing primordia. This suggests that the Lemnaceae may respond to biologically-active cell wall fragments.

Other fragments isolated from dicot pectin wall fractions have included rhamnogalacturonan I (RG I) and

rhamnogalacturonan II (RG II). RG II is a complex polysaccharide which consists of twelve different monosaccharides including apiose, 2-O-methylfucose and 2-O-methylxylose.

4.1.4 Duckweed cell walls

Interest in the cell walls of duckweeds has essentially been focused entirely on one striking characteristic - the possession of large amounts of the branched chain aldopentose, D-apiose (Duff, 1965).

4.1.5 Discovery and isolation of apiose.

The monosaccharide apiose was first isolated from its soluble derivative, apiin, from parsley *Petroselinum hortense* (Vongerichten, 1901). Parsley was then known as *Apium petroselinum* and hence the name apiose. Vongerichten (1902) largely established its structure, and its acyclic character was later verified by Schmidt (1930). However, only the cyclic D-*erythro*-furanose form of apiose (Figure 30) has ever been found in nature. Until the 1950's, apiose was the only branched chain sugar to have been isolated from plants, although others e.g. D-hamamelose (van Scherpenberg *et al.*, 1965), laminitol (Schweiger, 1967), mytilitol (Wickerberg, 1957) and aceric acid (Spellman *et al.*, 1983) are now known to exist.

4.1.6 The form in which apiose is found in plants.

Apiose occurs in plants either in soluble form or as a cell wall polysaccharide component :

1) Soluble apiose derivatives.

Apiose occurs in glycosides which are linked to flavone, isoflavone, phenol or anthraquinone derivatives (Watson and Orenstein, 1975). The most common of these is apiin, 7-O-(2-O-β-D-apiofuranosyl-β-D-glucopyranosyl)-apigenin, which has been isolated from a wide range of plant species throughout the plant kingdom, though usually only in trace amounts (Duff, 1965; Watson and Orenstein, 1975). *Petroselinum hortense* has regularly been found to contain soluble apiose in the form of both apiin and petroselinin (an apioside of 3′,4′,5,7-tetrahydroxyflavone) (Norstrom *et al.*, 1953).

2) Apiose as a cell wall component.

Apiose has been isolated from the pectic fractions of cell walls. It is found in dicots in low concentrations in wall polysaccharides such as RG II, as previously mentioned. Apiose also occurs as a major cell wall constituent in some fresh- and salt-water hydrophytic monocots, and seems to be mainly restricted to the orders Helobiae and Spathiflora (Grisebach, 1980). The total proportions of apiose isolated from walls has been reported to vary both between species and within species, and particularly in the latter case between the same species in varying habitats (Duff and Knight, 1963). In these hydrophytic plants, apiose occurs primarily in apiogalacturonans. Apiogalacturonans consist of α,1-4 linked

poly-galacturonic acid chains with an apiose or apiobiose linked to C-2 or C-3 (Figure 30). Apiobiose has the structure D-api$_f$-β(1-3)-D-api$_f$ (Hart and Kindel, 1970b). Most of the apiose is thought to be accounted for by this dimer although it may occur as singly attached molecules. This uncertainty exists as the furanosyl molecule of apiose is easily hydrolysed, and may be removed during the extraction procedure, hence making the elucidation of the original, intact structure difficult.

Apiogalacturonans have been isolated from *Lemna minor* with apiosyl and galacturonosyl residues as the only components (Beck, 1967; Duff, 1965; Hart and Kindel, 1970a), although xylose has frequently been isolated as a constituent of apiogalacturonans (Mascaro and Kindel, 1977; Hart and Kindel, 1970a). How the xylose is contained within the molecular structure, or even whether it is an obligatory component or merely a contaminant, is not known.

Zosterine is another apiose containing pectic polysaccharide(s) which has been found in several members of the Zosteraceae (sea-wracks) (Ovodova *et al.*, 1968). Pectinase digestion of zosterine released an apiogalacturonan consisting of a (1-4) linked D-galacturonan backbone with apiose on O-2 or O-3, or both (Ovodova *et al.*, 1968).

4.1.7 Role of apiose

The role of apiose is still uncertain, although the following suggestions have been made :

FIGURE 30

A. D-Apiose

The structure of apiose isolated from nature - the D-erythro-furanose form. The L-threo-furanose structure has not been found.

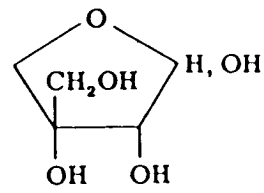
B. An apiogalacturonan

The structure of an apiogalacturonan isolated from *Lemna minor* (Hart and Kindel, 1970a,b).

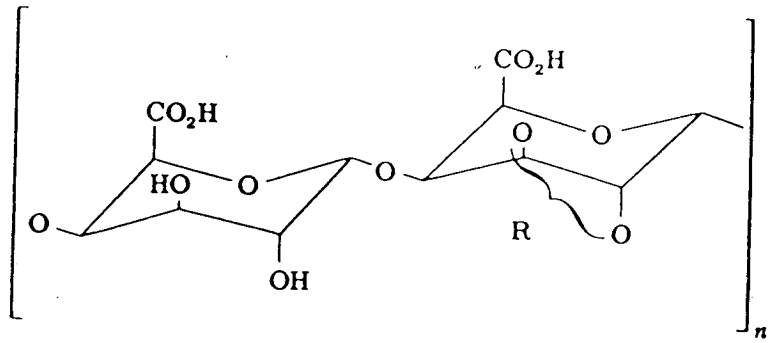
The backbone consists of an α,1-4 linked (practically unesterified) polygalacturonic acid chain. Apiobiose (R) - a dimer of apiose - is linked to C-2 or C-3 of the galacturonic acid backbone.

FIG 30

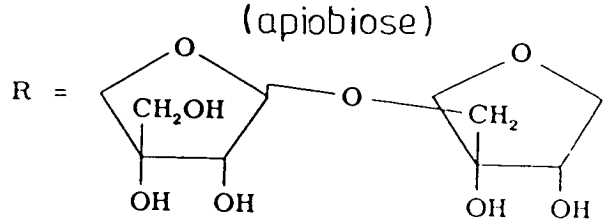
A. Apiose (D-erythro-furanose form)



B. Apiogalacturonan (from L. minor)



(apiobiose)



1) Watson and Orenstein (1975) suggested that it might play a role in winter-hardiness as it was found in winter-hardy plants, namely duckweed, celery and parsley. However, as celery and parsley are herbaceous plants which die back each winter, and as temperate duckweeds overwinter either by producing turions (e.g. *S. polyrrhiza*) or by relying on the survival of a small percentage of vegetative fronds (e.g. *L. minor*), this suggestion seems unlikely.

2) Hart and Kindel (1970a), and Bacon and Cheshire (1971) proposed that apiose conferred resistance to attack by the pectinases of pathogenic fungi. Isolated apiogalacturonan fragments were found to have a greater resistance to digestion by a crude pectinase preparation than did the isolated homogalacturonan backbone stripped of apiose. The ecological significance of this - whether hydrophytic plants are especially susceptable to fungal attack due to their aquatic environment - is not known.

3) Duff and Knight (1963) suggested that apiose in cell walls, rather than acting as a storage sugar, fulfilled a structural role connected with the fast expansion required of these rapidly growing monocots.

4) Hanna *et al.*, (1973) found that *Lemna minor* was capable of metabolising free D-[U-^{14}C]apiose to $^{14}CO_2$ (the other plants investigated - carrot, spinach and lettuce, could not do this). After 24 hours in Norris medium (Norris *et al.*, 1955) containing the D-[U-^{14}C]apiose, 50% had been converted into $^{14}CO_2$. There was, however, negligible incorporation of the

free D-[U-^{14}C]apiose into cell wall polysaccharides (less than 0.01% incorporation after 48 hours). The results suggest that whilst apiose cannot be re-incorporated into the wall structure once hydrolysed from it, there is nevertheless a very efficient and rapidly mobilised system for its subsequent utilisation as an energy source. It may therefore be regarded as an energy reserve.

4.1.8 Biosynthesis of apiose.

Radioisotope tracer studies, particularly using cell-free extracts, have shown that D-glucuronic acid is involved in apiose biosynthesis in *Petroselinum crispum* (Baron *et al.*, 1973), *Lemna gibba* (Roberts *et al.*, 1967) and *Lemna minor* (Picken and Mendicino, 1967; Gustine and Kindel, 1968; Watson, 1971). Both myo-inositol and D-glucose can act as substrates.

The alternative biosynthetic pathways from D-glucose to UDP-glucuronic acid and its associated UDP-sugar products are shown in Figure 31. The step from UDP-glucuronic acid to UDP-apiose is catalysed by a bifunctional enzyme, UDP-apiose/UDP-xylose synthase, which also catalyses the production of UDP-xylose. Another enzyme, UDP-glucuronic acid decarboxylase, produces UDP-xylose only. Grisebach (1980) has proposed a schematic mechanism (Figure 32) to explain the complex action of the UDP-apiose/UDP-xylose synthase.

The two enzymes, UDP-apiose/UDP-xylose synthase and UDP-glucuronic acid decarboxylase, have been isolated from *Lemna minor* (Gustine *et al.*, 1975; Wellmann *et al.*, 1971). They

FIGURE 31

The biosynthetic pathways from glucose / inositol to UDP-apiose.

The diagram shows the alternative known pathways from glucose to UDP-glucuronic acid. The relative importance of these in apiose biosynthesis in duckweeds is not known.

1. UDP-glucuronic acid decarboxylase.
2. UDP-apiose / UDP-xylose synthase.

FIG 31

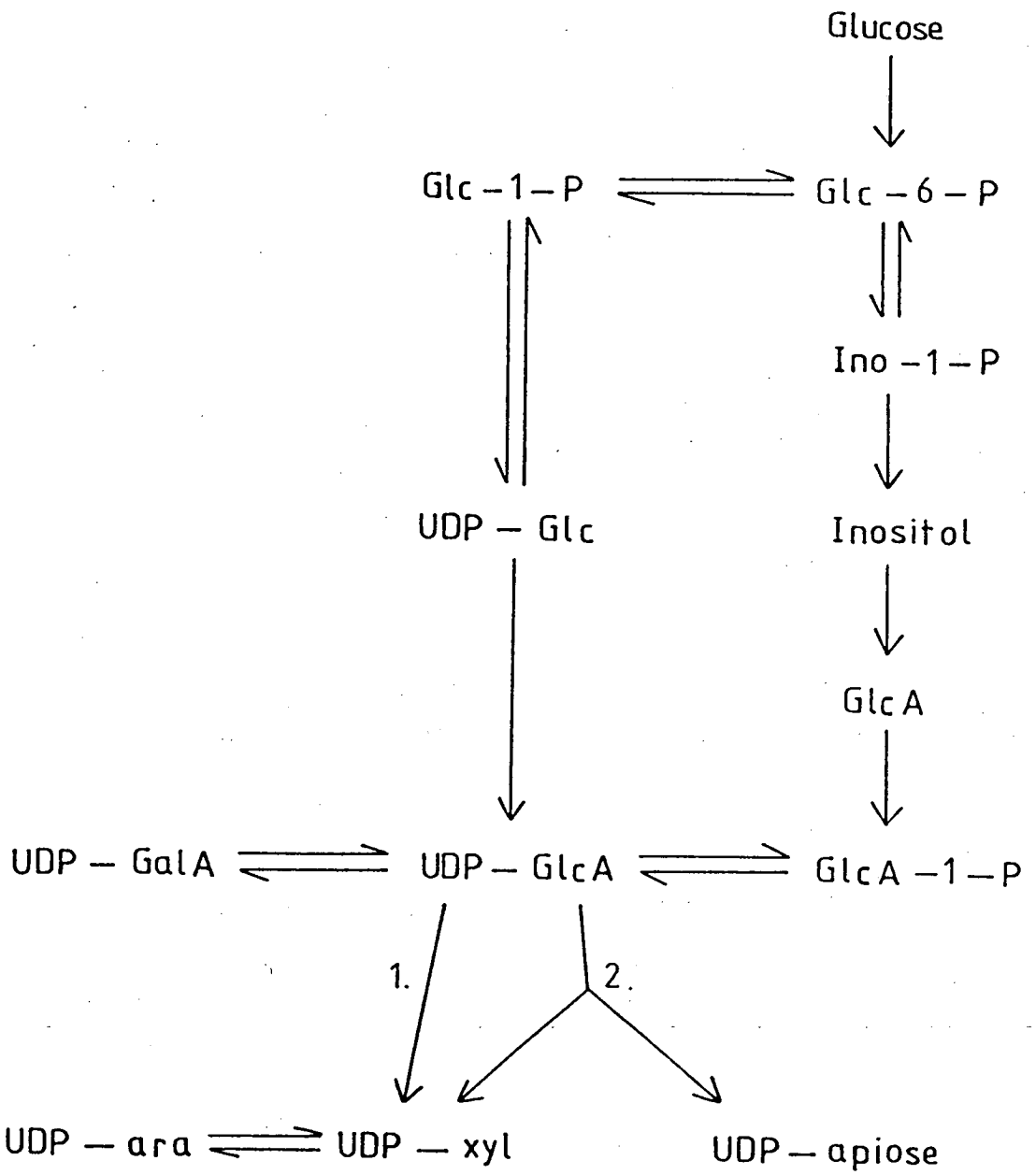

Glucose
Glc – 1 – P
Glc – 6 – P
Ino – 1 – P
UDP – Glc
Inositol
GlcA
UDP – GalA
UDP – GlcA
GlcA – 1 – P
1.
2.
UDP – ara
UDP – xyl
UDP – apiose

FIGURE 32

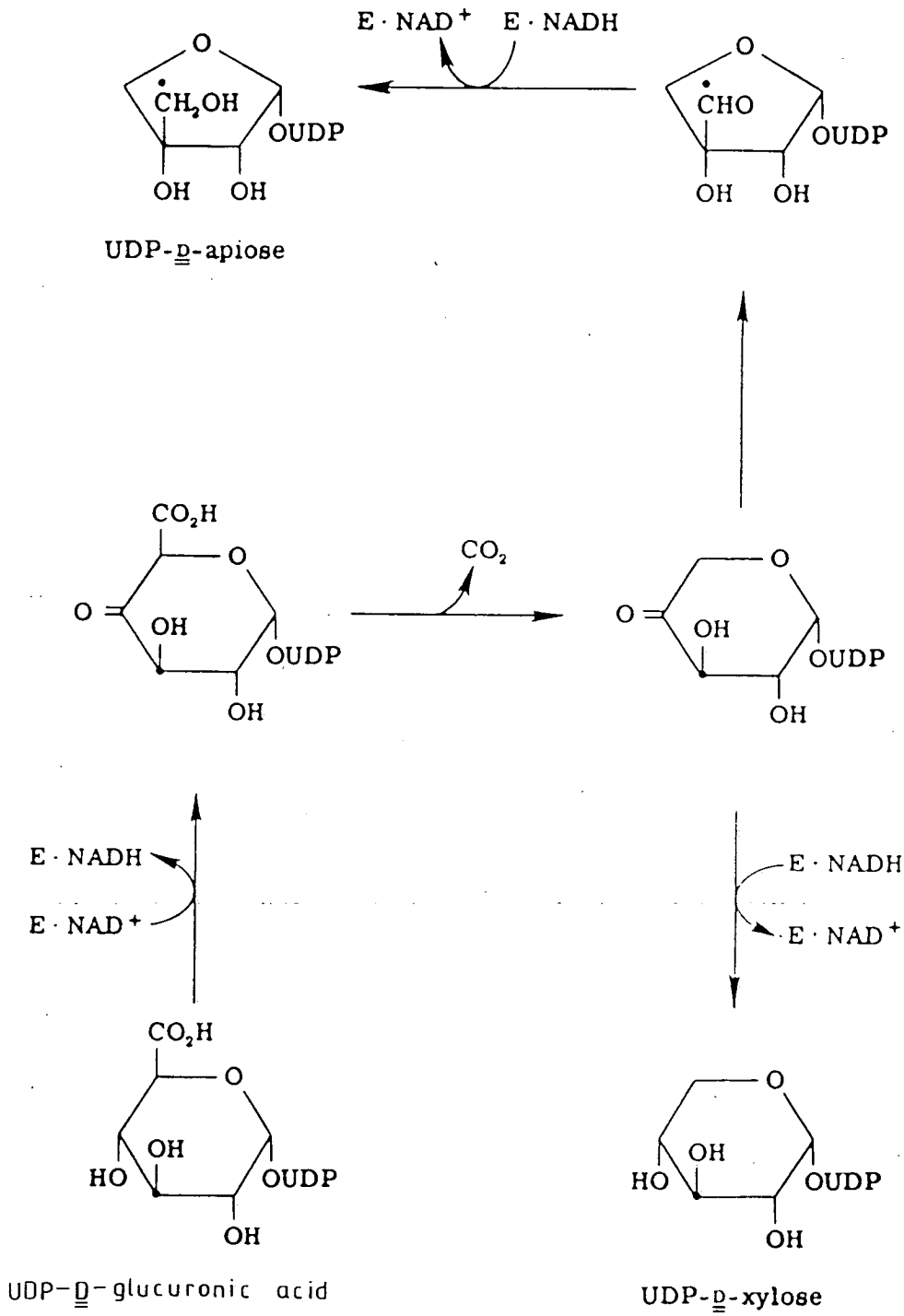
A proposed mechanism for the UDP-apiose / UDP-xylose synthase reaction.

(Taken from H. Grisebach, The Biochemistry of Plants 3, ed. J. Preiss, 1980. Academic Press.)

UDP-glucuronic acid is oxidised to the 4-keto intermediate with the formation of enzyme-bound NADH. The 4-keto intermediate undergoes a decarboxylation reaction, and subsequently is either reduced by the NADH to UDP-D-xylose, or undergoes a ring contraction to an aldehyde intermediate which is then reduced by the enzyme-bound NADH to UDP-D-apiose.

The dotted carbon atoms show the origin of the branched methyl group of apiose from C-3 of glucuronic acid.

FIG 32


E · NAD+
E · NADH
CH2OH
CHO
OUDP
OH
OH
UDP-D-apiose
CO2H
CO2
O
OH
OUDP
OH
E · NADH
E · NAD+
HO
UDP-D-glucuronic acid
UDP-D-xylose

have different pH optima, NAD^+ requirements, responses to illumination, and respond differently to ammonium ions. The UDP-apiose/UDP-xylose synthase enzyme from *L.minor* was found to have a pH optimum of 7.0-8.5 (Wellmann and Grisebach, 1971), and (unlike the UDP-glucuronic acid decarboxylase) a requirement for NAD^+ at catalytic concentrations (Sandermann and Grisebach, 1970; Kindel *et al.*, 1971). UDP-apiose/UDP-xylose synthase isolated from cell suspension cultures of parsley was found to be composed of two proteins with molecular weights of 65,000 and 86,000 (Matern and Grisebach, 1977). Only the 86,000 protein was required for activity, but the associated presence of the 65,000 protein was needed to maintain enzyme stability. However, Gardiner *et al.* (1980), have suggested that the 65,000 protein is a separate entity from the synthase enzyme despite the fact that they co-purify together in the ratio of 1 : 1. They do not rule out the possibility that it has a stabilising influence on the synthase enzyme, but do suggest that UDP-xylose is possibly an artificial product of the *in vitro* assay.

The ratio of UDP-apiose : UDP-xylose *in vitro* products could be artificially manipulated in a UDP-apiose/UDP-xylose synthase extract prepared from a parsley cell suspension culture (Matern and Grisebach, 1977; Grisebach, 1980). Ammonium ions were found to stimulate xylose production and inhibit apiose synthesis in the pH range 8.2-7.5 and stimulate both activities at pH 7, whilst the apiose / xylose ratio was higher in phosphate buffer (2.3-2.7) than in Tris-HCl (1.2-

1.4). This artificial manipulation of the ratios of reaction products suggests the existence of similar *in vivo* regulation mechanisms, which may be brought into operation to cope with changing natural environmental conditions. However, such an extrapolation of results from *in vitro* systems to the *in vivo* situation must be regarded with caution.

Transferase systems operate to transfer the D-apiose from UDP-apiose to either soluble acceptors (e.g. as in apiin) or into cell wall polysaccharides (e.g. as in apiogalacturonans). Whilst the transferase systems involved in the formation of apiin in cell cultures of parsley have been investigated (Hahlbrock *et al.*, 1971; Ortmann *et al.*, 1970), less is known concerning the transfer of apiose to wall polysaccharides. Pan and Kindel (1977) isolated a particulate enzyme preparation from *Lemna minor* which catalysed the transfer of D-[U-^{14}C]apiose from UDP-[^{14}C]apiose to endogenous acceptors. The result was the formation of [^{14}C]-labelled apiogalacturonans, but the number of intermediate acceptors is not known.

4.2 MATERIALS AND METHODS

MATERIALS

4.2.1 Chemicals.

The following radiochemicals were obtained from Amersham International p.l.c. :

1. D-[U-^{14}C]Glucose (250 mCi/mmol).

2. D-[1(n)-^{3}H]Glucuronic acid, sodium salt, was prepared by the catalytic exchange of 25 mg of the pure sugar in solution with tritium gas (method TR.7). It was purified by chromatography in system 6 (4.2.7) to yield 105 mCi (72% recovery) at approximately 5 Ci/mmol, and was always re-purified immediately prior to its experimental use by electrophoresis in system 2 (4.2.7) (90 - 95% recovery).

All other chemicals were from Sigma Ltd.

METHODS

4.2.2 Radioisotope labelling of fronds and turions.

Fronds of *Spirodela polyrrhiza* were cultured, and turions induced, as in Methods 2.2A.2.

^{14}C-Glucose

20 µCi was added to 25 ml H/2 incubation medium for 24 hours.

^{3}H-Glucuronic acid (^{3}H-glcA)

Where no vacuum-infiltration was required, 1 mCi was added to 25 ml H/2 medium for 24 hours.

For vacuum-infiltration, the plants were placed in a

crystallising dish containing 25 ml H/2 medium plus 1 mCi ^{3}H-glcA, and then forced below the surface of the medium by placing a wire mesh grid over them to weight them down. The dish was placed in a vacuum chamber attached to a vacuum pump, and the plants were vacuum-infiltrated for 2 min. By this time air bubbles had been expressed from the fronds which had a "wetted" appearance and they sank to the bottom of the dish. The vacuum was then released, the grid removed, and the timing of the experiment started (= time 0). The plants were maintained in the growth cabinet at 25°C in normal light conditions for the duration of the experiment.

4.2.3 Sampling of plant material.

Fronds or turions were removed from the medium, and the following procedures carried out, all in ice-cooled containers and using ice-cooled solutions.

Extraction procedures.

The plants were rinsed briefly with H_2O, blotted with tissue paper and weighed. They were ground up in a mortar and pestle with a total of 1 ml 90% aqueous phenol : 10 mM potassium phosphate buffer pH 6.5, shaken into an emulsion in the ratio 1 : 1. The homogenates were spun in Eppendorf tubes for 2 min at 6000 x g and the water-soluble fractions (top layer) analysed for radioactive content of free ^{3}H-glcA and low molecular weight sugar intermediates. The (lower) phenol layers were shaken in a water bath (85-95°C) for 1 hour before re-pelleting cell wall material. The pellets were washed

several times with ethanol until the smell of phenol had disappeared (usually five x 1 ml ethanol were required).

4.2.4 De-starching of turion cell wall components.

Whilst the frond cell wall pellets contained a negligible proportion of starch, the turion pellets were found to contain heavy starch granules which had spun down with the cell wall fraction. These accounted for approximately 75% of the dry weight of the crude cell wall extract. It was necessary to remove this starch before hydrolysis of the wall fraction to free monosaccharides. Failure to do so resulted in an over-estimate of ^{14}C-glucose in the walls and caused an overloading of total sugars on to the chromatography paper. This, in turn, resulted in "streaking" of the sample on the paper and subsequent failure to resolve the monosaccharide components into discrete peaks.

1. Amylase digestion of starch

Incubation with a combination of α- and β- amylases (ex *Bacillus* sp.) proved unsatisfactory as a means of totally removing the starch. The digestion mixture used was :

10 mg cell walls

1 ml 100 mM potassium phosphate buffer, pH 7.0

40 μg α-amylase

40 μg β-amylase.

The mixture was incubated in a covered test tube at 25°C for 48 hours with constant magnetic stirring. 2 drops of toluene were added to prevent bacterial contamination.

2. DMSO solubilisation of starch

The following procedure using dimethylsulphoxide (DMSO) (Leach and Schoch, 1962; Ring and Selvendran, 1978) was subsequently found to be effective in solubilising the starch from turion cell wall pellets. After washing the turion pellet with H_2O, 3 ml 90% aqueous DMSO (per 1 g fresh weight of turions) was added and the mixture magnetically stirred for 1 hour at 25°C. The solubilised starch was removed after re-pelleting insoluble cell wall material, and the DMSO treatment repeated until there was no remaining detectable starch in solution after incubation. The presence of starch was confirmed by the production of a blue colour with I_2/KI solution. Three extractions with DMSO were found to be sufficient to remove all detectable starch.

4.2.5 Hydrolysis of cell wall components.

The washed pellets were resuspended in 2 M trifluoroacetic acid (TFA) (1 ml TFA per 1 g fresh weight material) and placed in an autoclave set for 1 hour at 20 p.s.i. After re-centrifuging to pellet insoluble material (including cellulose and some uronic-acid containing polymers), the solubilised monosaccharide fraction was reduced to near-dryness *in vacuo* with a 'Speed-Vac' (Savant Instruments). It was then applied to chromatography paper by streaking on in 2 cm strips, and dried using a hair dryer. The paper was chromatographed along with relevant sugar standards using the chosen system (4.2.7). Table 3 lists the mobilities

TABLE 3

Mobilities of standards on paper electrophoresis and descending paper chromatography

Aqueous stock solutions of the standards were prepared, samples spotted on to Whatman Chromatography paper (either No. 1 or 3 MM), and run on the systems below :

A Electrophoresis systems

Mobilities are given relative to picric acid (pH 2.1, 3.5) and bromophenol blue (pH 6.5). The effective paper length was approximately 40 cm. The buffers, which were white spirit (pH 2.1, 3.5) and toluene (pH 6.5), were water-cooled to 20 - 30 °C. No corrections have been made for electroendo-osmosis.

1. pH 2.1 at 3 Kv for 30 min.
2. pH 3.5 at 3 Kv for 30 min.
3. pH 6.5 at 1.5 Kv for 30 min.

B Descending Chromatography systems

Mobilities are given relative to picric acid. The effective path length was approximately 40 cm.

1. EtOAc : pyridine : H_2O (8 : 2 : 1) for 20 hours.
2. BuOH : AcOH : H_2O (12 : 3 : 5) for 9 hours; paper dried then run in the same direction in EtOAc : pyridine : H_2O (8 : 2 : 1) for 7 hours.
3. EtOAc : AcOH : H_2O (9 : 2 : 2) + 0.25% phenylboronic acid for 9.5 hours. The bracketed figure denotes a faint spot.

TABLE 3

	A ELECTROPHORESIS SYSTEMS, 1-3			B CHROMATOGRAPHY SYSTEMS, 1-3		
	1	2	3	1	2	3
apiose	0.08	0.05	0.40	0.64	0.72	1.21 (0.78)
glcA	0.18	0.69	1.00	0	0.19	0.23
galA	0.17	0.56	0.84	0	–	0.20
xylose	0.10	0.04	0.40	0.32	0.48	0.40
ara	0.12	0.04	0.40	0.27	0.42	0.33
glc	0.12	0.04	0.37	0.18	0.29	0.23
gal	–	–	0.40	0.13	–	0.29
UDP-xyl	0.99	1.02	1.10			
UDP-glcA	0.94	1.08	1.60			
UDP-galA	0.90	0.93	1.50			
xly-1-P	0.65	0.92	1.00			
glcA-1-P	0.67	1.20	1.70			
gal-1-P	0.76	0.78	1.00			
glc-6-P	0.64	0.84	1.00			
ara-5-P	–	1.37	–			
UMP	–	0.85	–			
P (Na)	–	1.36	–			
P-P (Na)	–	1.75	–			

of the sugar standards in some of the systems used.

4.2.6 Analysis of soluble nucleotide sugars and sugar intermediates.

UDP-apiose is unstable and this necessitated special precautions to ensure its detection and assay. The aqueous fraction from 4.2.3 was reduced *in vacuo* with a 'Speed-Vac' (no heat applied) to near-dryness and then streaked on to chromatography paper in a cold room (4°C). The samples were dried on to the paper using a cold air-stream from a hair dryer, and then immediately electrophoresed at pH 6.5 in system 3 (4.2.7). As UDP-apiose is unstable, it is not available commercially for use as a standard. However, it is known to migrate electrophoretically in a manner similarly to UDP-xylose (Kindel and Watson, 1973), and so UDP-xylose was used as a standard for establishing the electrophoretic migration of UDP-apiose. Once separated by electrophoresis at pH 6.5 from uncharged sugars, the soluble nucleotide sugars and sugar-phosphates were eluted from the appropriate sections of paper into 2 M TFA by the method of Eshdat and Mirelman (1972) as described in section 4.2.7. The eluates were then placed in a boiling water bath for 20 min to hydrolyse them to their respective monosaccharides. These were subsequently resolved using the descending chromatographic systems (4.2.7) used for monosaccharide identification.

For the analysis of soluble sugar intermediates, other than UDP-apiose, electrophoresis at pH 2.1 or 3.5 gave a better

initial separation of soluble components than did pH 6.5.

Table 3 lists the electrophoretic mobilities of the standards used.

4.2.7 Chromatographic techniques.

Whatman No. 1 or 3 MM paper (unwashed) was used. The effective paper length for both chromatography and electrophoresis systems was approximately 40 cm. Thin Layer Chromatography systems were tried, but these had two drawbacks compared with the paper systems :

A. Their total loading capacity was low.

B. They did not separate the individual components within such crude mixtures well.

Consequently, paper systems were used :

1. Descending paper chromatography.

Systems used :

1) EtOAc/pyridine/H_2O (8:2:1); 20 hours.

2) EtOAc/pyridine/H_2O (8:2:1); 60 hours.

3) EtOAc/AcOH/H_2O (9:2:2) + 0.25% phenylboronic acid; 9.5 hours.

4) Bu-1-OH/EtOAc/H_2O (40:11:19); 60 hours.

5) BuOH/AcOH/H_2O (12:3:5); 9 hours, followed by EtOAc/pyridine/H_2O (8:2:1); 7 hours in same direction.

6) EtOAc/HOAc/H_2O (3:3:1); 12 hours.

For a complete cell wall monosaccharide analysis, systems 1 or 5 were used for the initial separation of hydrolysates (5 yielded a poorer qualitative resolution than 1, but permitted

the separation of a more concentrated loading of material). Subsequent to this : System 4 separated xylose from any putative fucose; system 2 resolved glucose, galactose and mannose; and system 3 was used for the identification of apiose (the borate specifically attaches to cis-diol groups which the apiose molecule (Figure 30) contains). System 6 was used in the initial purification of ^{3}H-glcA (see 4.2.1).

2. Paper electrophoresis.

Systems used :

1) pH 2.1; formic acid/HOAc/H_2O (1:4:45); 3 KV 30 min.

2) pH 3.5; HOAc/pyridine/H_2O (10:1:189); 3 KV 30 min.

3) pH 6.5; HOAc/pyridine/H_2O (3:100:897); 1.5 KV 30 min.

Picric acid was used as a reference marker at pH 2.1 and 3.5, and bromophenol blue at pH 6.5 (as picric acid was soluble in the toluene coolant buffer used at this pH).

Systems 1 and 2 were used for the separation of all soluble sugars, nucleotide sugars and sugar intermediates with the exception of UDP-apiose, which decomposed to free apiose + UDP at pH 2.1 and 3.5 (Watson, 1971). System 3 (pH 6.5) had to be used for the initial separation of UDP-apiose from any free apiose, before proceeding to the chromatographic separation systems.

High-voltage electrophoresis (equipment from Locarte, Edinburgh) was performed in tanks filled with white spirit (pH 2.1 and 3.5) or toluene (pH 6.5), and continuously water-cooled to between 20 and 30°C. The cathode was sited at the top of the tanks. Papers which had been run in

solvent systems / buffers containing a high proportion of pyridine were dried, and then dipped in butan-1-ol to remove traces of residual pyridine and re-dried before proceding to the next stage.

Transfer of material from one system to another was achieved by the method of Eshdat and Mirelman, (1972). This involved cutting out the relevant sections of paper and placing them in a rolled up form inside the barrels of empty 5 ml plastic syringes. The syringes were balanced on top of Pyrex centrifuge tubes and H_2O or 2 M TFA was then added until the paper was just saturated. After leaving for 10 min, the tubes were spun at low speed on a bench centrifuge, hence spinning through the eluted material. It was found that three such elutions performed successively were sufficient to remove all detectable radioactive material from the paper. The eluate was then reduced using a 'Speed Vac' and re-applied to chromatography paper to run on a fresh system.

The electrophoretic and chromatographic mobilities of the monosaccharides and their associated intermediates and nucleotides are shown in Table 3.

4.2.8 Detection of monosaccharides

Monosaccharides were detected by dipping the paper with Wilson's dip - an aniline-phthalate dip (see below), followed by heating at 100°C for 10 min. Free sugars appeared as spots on the paper, ranging in colour from pink (xylose / arabinose) to orange (glucuronic acid). Apiose reacted with

the spray to give a fluorescent yellow colour on exposure to u.v. light (Sandermann, 1969).

Wilson's dip (Partridge, 1949) - detection of monosaccharides

The following were mixed to produce a stock solution :

16 g phthalic acid

490 ml diethyl ether

490 ml butan-1-ol

20 ml H_2O

On the day of use, aniline was added to a final concentration of 0.92%.

4.2.9 Detection of sugar-nucleotides and sugar-phosphates

Sugar-1-phosphates and UDP-sugars were identified by dipping with a perchloric acid-molybdate spray (see below). On exposure of the dried paper to ultra-violet light, the sugar intermediates appeared as blue spots.

Perchloric acid-molybdate dip (Bandurski and Axelrod, 1951) - detection of phosphates (in the form of nucleotide sugars and sugar phospates)

The following produced a stock solution :

12 ml H_2O

3 ml concentrated hydrochloric acid

2 g ammonium molybdate in 5 ml H_2O

6 ml 60% perchloric acid

After mixing the above, 180 ml acetone was added.

4.2.10 Detection of radioactivity.

The chromatography / electrophoresis papers were pencil marked off into 1 cm horizontal strips from the origin to the solvent front. Vertical sections corresponding to the loaded 2 cm strips plus 1 cm on either side i.e. 4 cm strips, were then also marked off, and the resulting 4 cm x 1 cm oblong strips identified individually with pencil marks, cut out and removed to disposable scintillation vials. The paper strips were saturated in 0.5% PPO (2,5-diphenyloxazole) in toluene and scintillation counted in an Intertechnique SL-3000 Liquid Scintillation Counter. The resulting peaks of radioactivity were identified by comparison with with the associated positions of the non-radioactive standards on the paper.

4.2.11 Micro-dissection of fronds

Samples were removed from a population of fronds, and killed immediately by immersion in boiling methanol for 5 min. The small fronds (< 1 mm in diameter) were then dissected out from inside the parental meristematic pockets using a scalpel, whilst viewing through a binocular microscope.

4.2.12 Cellulose estimation.

The cellulose content of the (destarched) cell wall fractions was measured using the method of Updegraff (1969). Assuming 1 g dry weight of cell walls, the procedure was as follows :

1. 6 ml acetic nitric reagent (prepared by mixing 150 ml 80%

acetic acid and 15 ml concentrated nitric acid) was added to the sample in a Pyrex tube by the successive addition of three x 2 ml, with mixing on a Vortex mixer after each addition.

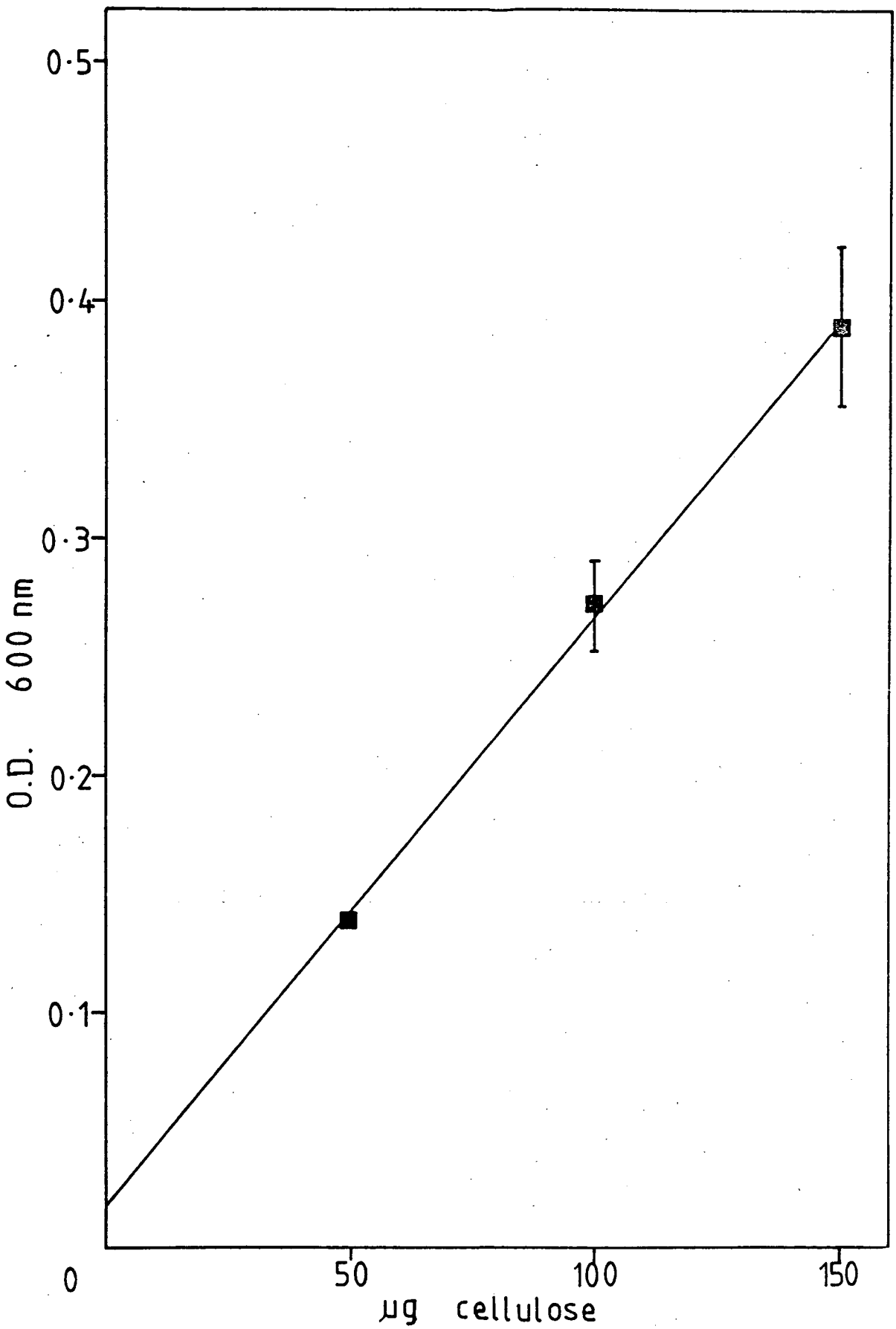
2. The stoppered tube was placed in a boiling water bath for 30 min.
3. After centrifuging for 5 min at high speed on a bench centrifuge, the supernatant was decanted and discarded.
4. The pellet was washed by re-suspension in 20 ml distilled water, adding four x 5 ml in successive steps as in 1, then spun and the supernatant decanted and discarded.
5. 20 ml of 72% sulphuric acid was added in a stepwise fashion as in 1.
6. The tube was left for 1 hour.
7. 1 ml was diluted to 100 ml with H_2O. Any precipitate was removed by centrifugation.
8. 1 ml was diluted with 4 ml H_2O in a screw-cap Pyrex tube and cooled in an ice-bucket.
9. 10 ml cold anthrone reagent (0.2 g anthrone in 100 ml concentrated sulphuric acid - freshly prepared and refrigerated before use) was layered on top using a pipette.
10. The tube was Vortexed and then placed in a boiling water bath for 16 min.
11. After cooling in an ice-bucket, the tube was allowed to return to room temperature.
12. The tube was read on a bench spectrophotometer at 600 nm against a reagent blank. A standard curve (Figure 33) was

FIGURE 33

Standard cellulose curve.

Following the method of Updegraff (1969) as described in Methods 4.2.12 the standard curve corresponding to 50, 100 and 150 µg cellulose is shown. The results are the average values from four such estimations. Bars denote standard errors, and the line was fitted by linear regression (intercept at 0.017; slope = 0.002484).

FIG 33



obtained by dissolving 50 mg cellulose in 10 ml 67% sulphuric acid with gentle heat. This was diluted to 100 ml with H_2O and 0.5, 1.0 and 1.5 ml, corresponding to 50, 100 and 150 µg cellulose, was used in step 8 of the procedure. The volume was made up to 5 ml with H_2O and the steps 9 - 12 were followed.

4.3 RESULTS

4.3.1 Non-cellulosic monosaccharide composition of fronds and turions as measured by D-[U-^{14}C]-glucose labelling.

Populations of fronds and turions were radio-labelled by incubation in H/2 medium containing D-[U-^{14}C]-glucose for 24 hours, and their cell walls analysed (Methods 4.2). The results are shown in Table 4, and this data has also been plotted as histograms in Figures 34 (fronds) and 35 (turions). Although the method of acid hydrolysis employed (autoclaving for 1 hour in 2 M TFA) may have resulted in incomplete hydrolysis of some of the uronic acid-containing polymers and hence an under-estimation of their content, the results, nevertheless, do illustrate several interesting differences in the relative ratios of labelling of the wall components.

Notable is the high glucose content, on average 63.3% of total non-cellulosic monosaccharides in turions, compared with only 13.0% in fronds. This high percentage in turions should not be attributable to starch due to the preceding destarching techniques employed. However, the figures obtained using amylase starch digestion treatment (72.9% and 68.3%), are higher than that obtained following DMSO treatment (48.6%), which was subsequently found to be more effective in removing starch from cell wall preparations. It is therefore possible that the results in columns 4 and 5 may have over-estimated the non-cellulosic glucose content. Despite this factor, glucose is undoubtably still the major constituent of turion

TABLE 4.

Non-cellulosic monosaccharide composition of fronds and turions.

Fronds and turions were incubated in 20 ml H/2 medium with D-[U-^{14}C]-glucose (40 μCi) and ABA (to 10^{-6} M) as follows :

Column	Description of starting material
1	Fronds in D-[U-^{14}C]-glucose + ABA for 8 days; then removed to D-[U-^{14}C]-glucose only for a further 7 days.
2 & 3	Fronds incubated in D-[U-^{14}C]-glucose for 15 days.
4	Turions resulting from the exposure of fronds in (1) to 10^{-6} M ABA were incubated in D-[U-^{14}C]-glucose for 15 days.
5 & 6	Turions formed by overcrowding fronds in low light conditions (no exogenous ABA) were incubated in D-[U-^{14}C]-glucose for 15 days.

The fronds and turions were fresh-weighed and homogenised in an emulsion of 1 ml 90% aqueous phenol : 10 mM $(K)PO_4$, pH 6.5. In 1, 2, 4 and 5, the lower (phenol) layer was shaken in a water-bath (85-95°C) for 1 hour. This stage was omitted in 3 and 6. The cell wall material in the phenol layer was pelleted by centrifugation at 2000 x g for 5 min, then washed five times with ethanol. The turion cell wall extract also contained large starch granules, which were solubilised and removed in 4 and 5 by amylase treatment, and in 6 by treatment with 90% DMSO (Methods 4.2.4). The non-cellulosic monosaccharides were hydrolysed by autoclaving for 1 hour in 2 M trifluoroacetic acid. A series of paper chromatographic and electrophoretic systems (4.2.7 and Table 3) resolved the resulting monosaccharides qualitatively. The quantitative analysis was obtained by the scintillation counting of 1 cm sections along the length of the chromatography paper, and relating these to the position of standard monosaccharides (4.2.8).

TABLE 4.

% COMPOSITION OF LABELLED NON-CELLULOSIC MONOSACCHARIDES.

	FRONDS		%	TURIONS		
	1	2	3	4	5	6
APIOSE	19.9	18.3	25.9	0.2	0	0.2
ARABINOSE	11.1	9.6	13.2	8.9	6.8	0.4
FUCOSE	1.9	1.1	1.0	0.6	0.1	1.2
GALACTOSE	8.8	8.8	8.5	4.9	4.5	4.9
GALACTURONIC ACID	25.0	26.5	25.2	6.5	14.8	23.6
GLUCOSE	11.9	16.8	10.3	72.9	68.3	48.6
GLUCURONIC ACID	2.7	4.0	9.8	1.2	2.5	12.2
MANNOSE	0	0	0	0	0	0
RHAMNOSE	6.5	4.0	3.3	0.6	0	0.6
XYLOSE	12.2	10.9	2.8	4.2	3.0	8.3
	100	100	100	100	100	100

	AVERAGE VALUES (%)	
	FRONDS	TURIONS
APIOSE	21.4	0.1
ARABINOSE	11.3	5.4
FUCOSE	1.3	0.6
GALACTOSE	8.4	4.8
GALACTURONIC ACID	25.6	15.0
GLUCOSE	13.0	63.2
GLUCURONIC ACID	5.8	5.3
RHAMNOSE	4.6	0.4
XYLOSE	8.6	5.2
	100	100

FIGURE 34.

Non-cellulosic cell wall composition of *S. polyrrhiza* fronds radio-labelled with D-[U-^{14}C]-glucose.

The histogram shows the average values for the fronds' non-cellulosic cell wall monosaccharide labelling, as listed in Table 4.

FIG 34

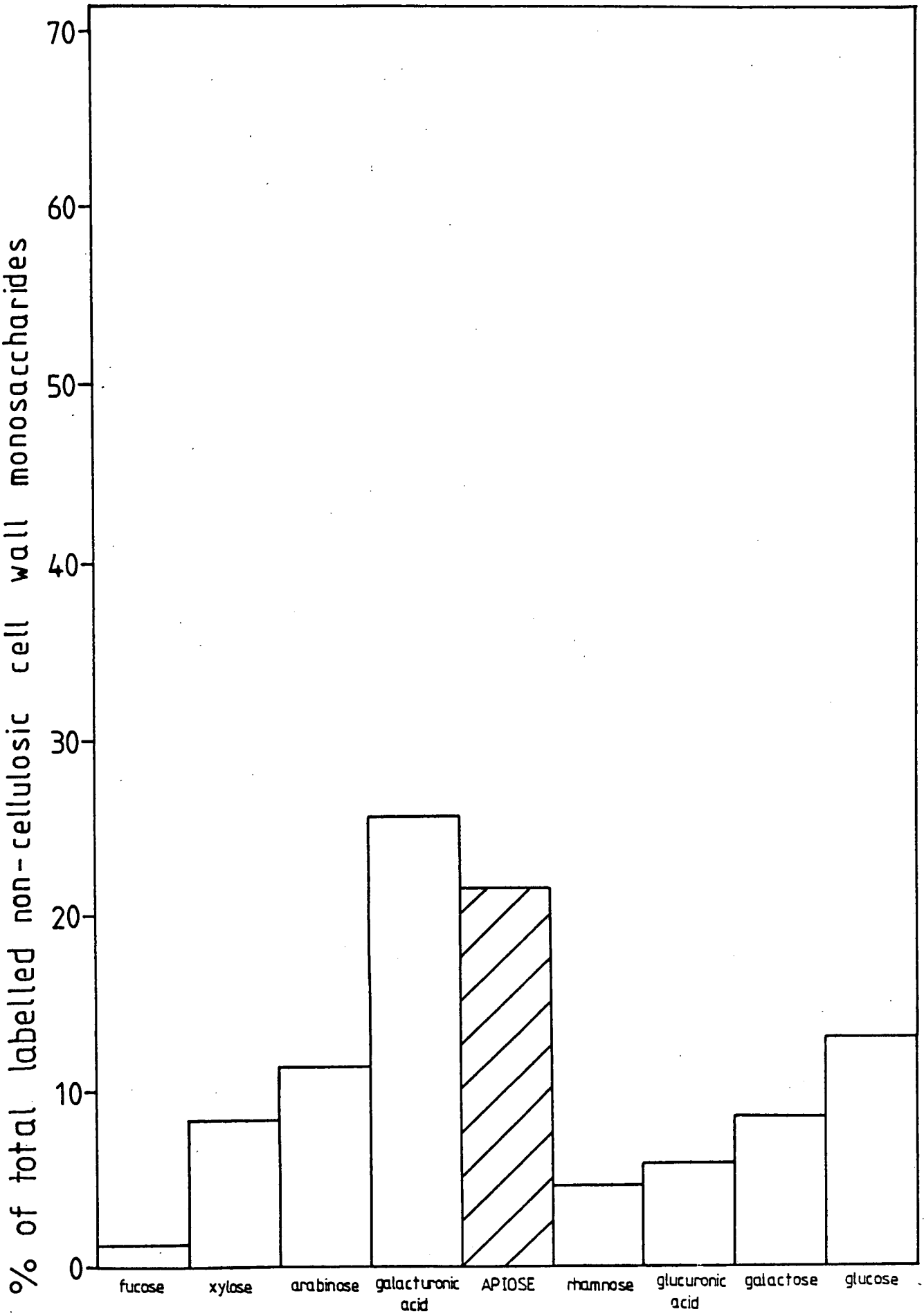
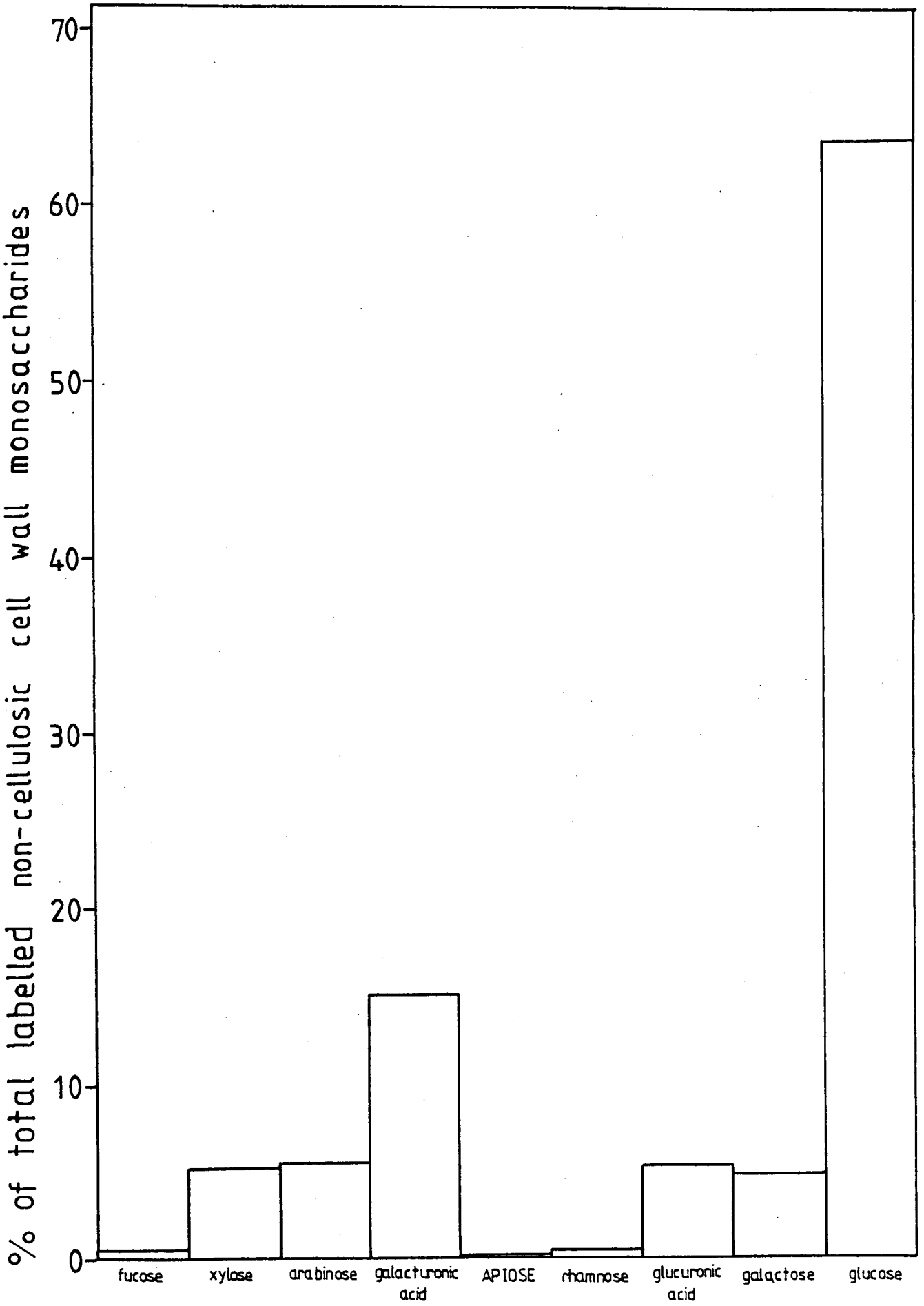

% of total labelled non-cellulosic cell wall monosaccharides
70
60
50
40
30
20
10
0
fucose
xylose
arabinose
galacturonic acid
APIOSE
rhamnose
glucuronic acid
galactose
glucose

FIGURE 35.

Non-cellulosic cell wall composition of *S. polyrrhiza* turions radio-labelled with D-[U-^{14}C]-glucose.

The histogram shows the average values for the turions' non-cellulosic cell wall monosaccharide labelling, as listed in Table 4.

FIG 35


% of total labelled non-cellulosic cell wall monosaccharides
70
60
50
40
30
20
10
0
fucose
xylose
arabinose
galacturonic acid
APIOSE
rhamnose
glucuronic acid
galactose
glucose

cell walls non-cellulosic polymers. The nature of the glycosyl linkages was not determined. However, as turions may spend many months at the bottom of muddy ponds, where they are unable to photosynthesize, awaiting the return of favourable germinating conditions, it seems likely that this large amount of glucan constitutes an energy reserve. Mixed β-(1-3,1-4) linked glucans, which have frequently been isolated from monocot walls, are thought to constitute energy reserves rather than being structural polymers (Nevins *et al.*, 1977). The ratio of 3-linked to 4-linked glucosyl residues can be determined by both quantitative methylation and periodate oxidation analyses (Buchala and Meier, 1973). Such methods could have elucidated the structure of the glucan reserves in turions.

There was no detectable mannose in fronds or turions, and only low levels of fucose (fronds 1.3%; turions 0.6%). The arabinose : xylose ratio was 1 : 0.76 in fronds and 1 : 0.96 in turions, i.e. approximately 1 : 1 in both cases which is in accord with the findings of Burke *et al.* (1974), using 6 suspension-cultured monocots.

However, the most striking difference between fronds and turions is that involving apiose. The fronds were found to contain, on average, 21.4% apiose, whilst in turions it was virtually absent (0.1%). In separate experiments, the phenol extraction was found not to remove significant apiose from the cell wall pellets into solution as it was buffered at pH 6.5. The high levels of galacturonic acid (separated from glcA by

electrophoresis at pH 3.5, 3 Kv for 30 min) particularly in fronds (25.6%) (although the turions were found to have 15.0%), is compatible with the view that the high levels of apiose present is in the form of apiogalacturonan. The levels of glucuronic acid were much lower (frond 5.8%; turion 5.3%).

It should be noted here that Table 4 shows results using turions formed both as a result of adding 10^{-6} M ABA to fronds in H/2 medium, and by overcrowding fronds in low light conditions (i.e. no exogenous ABA). Similarly, included in the table are fronds which had been exposed to exogenous ABA for 8 days, and others which had no such exposure. The results show that exposure to 10^{-6} M ABA alone is not sufficient to alter cell wall labelling. Whilst ABA can be regarded as a tool with which turion production can be reliably artificially induced, once the destiny of fronds and turions has been irreversibly decided (by whatever means), then ABA has no influence on the resulting wall composition. However, as ABA has the ability to decide irrevocably the fate of developing primordia, it was considered that the cell wall composition of fronds and turions, with particular reference to apiose incorporation, was worthy of further consideration. Two possibilities exist to explain the difference in apiose content between fronds and turions. Either :

1) In the transition from frond to turion production, the incorporation of apiose into cell wall polysaccharides is drastically repressed in the developing meristem,

or

2) The potential for apiose incorporation into the wall is not realised until the developing primordium is irreversibly destined to becoming a frond i.e. is greater than 1 mm in diameter.

4.3.2 Cellulose content of cell walls of fronds and turions.

Using the Updegraff method of cellulose estimation (Updegraff, 1969) described in Methods 4.2.12, frond cell walls were found to contain 25.5% cellulose and turions 37.0% cellulose.

4.3.3 A new approach to the study of apiose biosynthesis in duckweeds - the *in vivo* approach.

In depth investigations into apiose biosynthesis in duckweeds have previously involved only cell-free systems, as mentioned in the Introduction to this Chapter (4.1). *In vivo* studies were previously difficult, due to the lack of availability of a suitable radioactive precursor in adequate quantities to detect the low levels of UDP-apiose. However, Amersham International have a service whereby high levels of tritium can be introduced into small quantities of monosaccharides. This has enabled me to detect low levels of sugar intermediates labelled *in vivo* in *Spirodela* using ^{3}H-glucuronic acid (^{3}H-glcA) as a precursor.

4.3.4 ^{3}H-glucuronic acid labelling of cell wall monosaccharides and associated intermediates / nucleotides.

The pathway to apiose biosynthesis has already been outlined in 4.1.8. In Figure 36, all the compounds radioactively labelled as a result of exposure to ^{3}H-glcA are shown. These are :

a) Cell wall monosaccharides (underlined in Figure 36). Glucuronic acid, galacturonic acid, arabinose, xylose and apiose.

b) Soluble sugar nucleotides / sugar intermediates. Glucuronic acid (added radio-label), glucuronic acid-1-phosphate, UDP-glucuronic acid, UDP-galacturonic acid, galacturonic acid-1-phosphate, UDP-arabinose, arabinose-1-phosphate, UDP-xylose, xylose-1-phosphate and UDP-apiose.

4.3.5 ^{3}H-glucuronic acid versus ^{14}C-glucose labelling - advantages and disadvantages

^{14}C-glucose had the primary advantage of being easily assimilated by the plants. However, it serves as a precursor for many biosynthetic pathways, and its use to study just one of these pathways (glucuronic acid -> apiose) is limited by other non-participating radioactive products. The high cost, coupled with the relatively low specific activity (250 mCi/mmol), of the ^{14}C-glucose did not permit its use to follow changes in concentrations of the soluble sugar nucleotides and sugar phosphate intermediates.

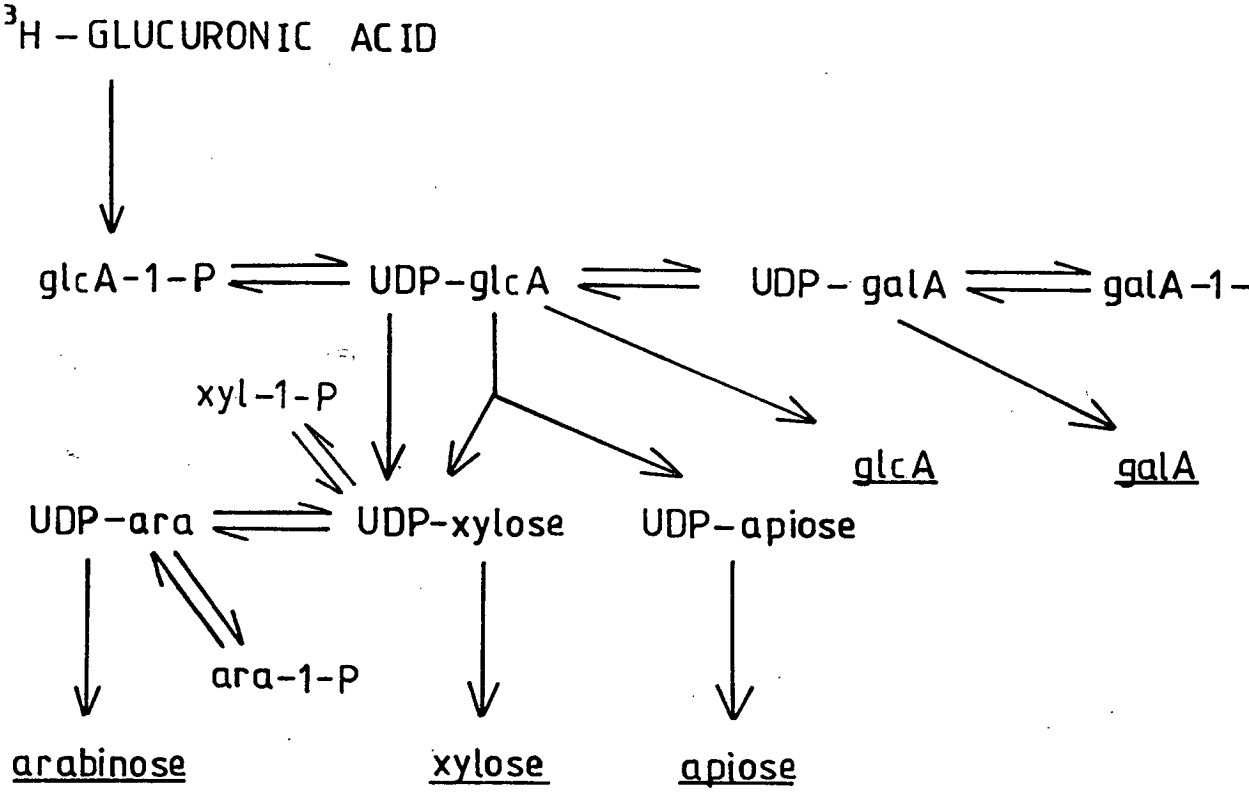
The ^{3}H-glcA had a higher specific activity (5 Ci/mmol) and

FIGURE 36

D-^{3}H-glucuronic acid labelling of *S. polyrrhiza* cell wall monosaccharides, and associated UDP-sugars and sugar-phosphates.

The compounds shown opposite are ^{3}H-labelled as a result of D-^{3}H-glcA labelling of fronds or turions. The compounds underlined are the resulting cell wall ^{3}H-monosaccharides, whilst the (not underlined) UDP-sugars and sugar-phosphates are found in the soluble fraction of the cytoplasm.

FIG 36


^{3}H – GLUCURONIC ACID
glcA-1-P
UDP-glcA
UDP – galA
galA-1-
xyl-1-P
glcA
galA
UDP-ara
UDP-xylose
UDP-apiose
ara-1-P
arabinose
xylose
apiose

as a total of 105 mCi was available, there was sufficient label present to follow the fate of radioactive intermediaries between glcA and apiose. However, it was found necessary to vacuum-infiltrate the plants with the ^{3}H-glcA (Methods 4.2.2). Otherwise, they were very slow to internalise the radio-label, and even though adult fronds would eventually incorporate the ^{3}H into wall constituents, the smaller, parent-dependent fronds did not accumulate radioactivity without vacuum-infiltration. This is assumed to be due to the high negative charge on the glcA molecule, which at pH 6.4 would make transport ac ross cell membranes a slow process. This problem of transport could have been overcome by using inositol as the initial radio-label, but unfortunately inositol containing a sufficiently high amount of radioactivity was not available commercially.

The importance of the inositol pathway (Figure 31) to my *Spirodela* culture *in vivo* is not known. It might be that glucose is a preferred substrate (via glucose-1-phosphate) rather than glcA, and that the plants were not accustomed to metabolising glcA. Hence, the required enzyme systems or transport mechanisms associated with glcA assimilation and biosynthesis were either not present, or were activated slowly. A pre-incubation in non-radioactive glcA might have resolved any such problem of enzyme activation requirement.

FIGURE 37

The rate of incorporation of radioactivity from D-^{3}H-glcA into *S. polyrrhiza* fronds cell wall glucuronic/galacturonic acid, apiose, arabinose and xylose moieties.

S. polyrrhiza fronds were placed in 25 ml H/2 medium containing 1 mCi D-^{3}H-glcA and vacuum-infiltrated. After 10, 20, 30 and 60 min incubation at 25°C, fronds were removed and their cell walls extracted, hydrolysed and analysed for the distribution of radioactivity in wall monosaccharides. See Methods 4.2 for details.

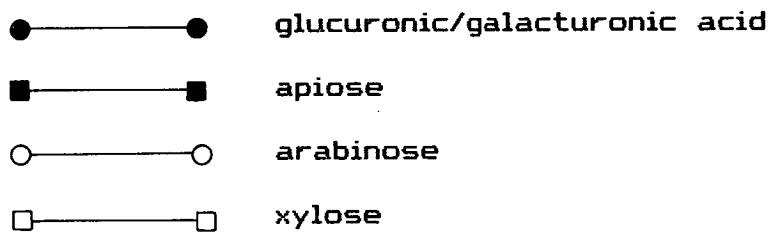


FIG 37

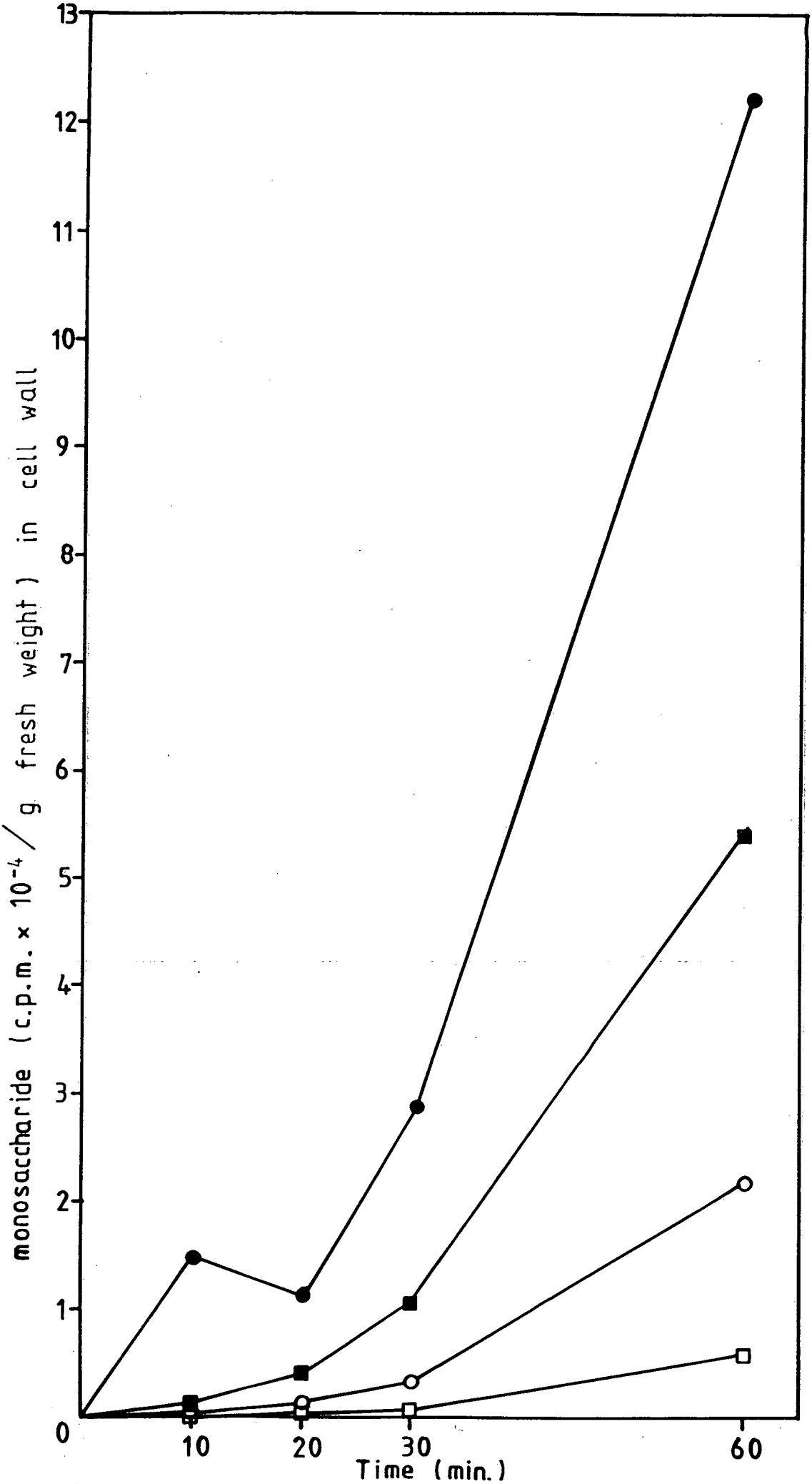

monosaccharide (c.p.m. × 10⁻⁴ / g fresh weight) in cell wall
0
1
2
3
4
5
6
7
8
9
10
11
12
13
10
20
30
60
Time (min.)

FIGURE 38

The relative rates of incorporation of radioactivity from D-^{3}H-glcA into the cell wall monosaccharides of *S.polyrrhiza* fronds.

The results from Figure 37 are expressed as percentages of the total radioactive cell wall monosaccharides at the individual sampling times (10, 20, 30 and 60 min). They show that from between 20 - 60 min the relative rates of incorporation of radioactivity into glucuronic/galacturonic acid, apiose, arabinose and xylose were at a steady level.

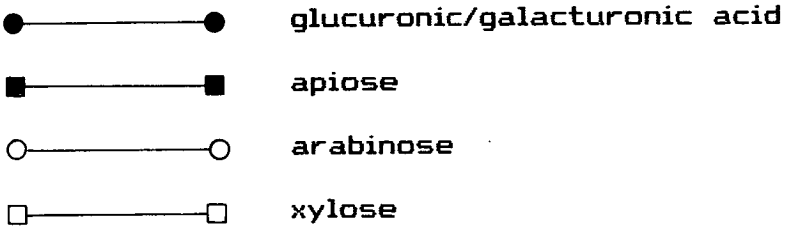


FIG 38

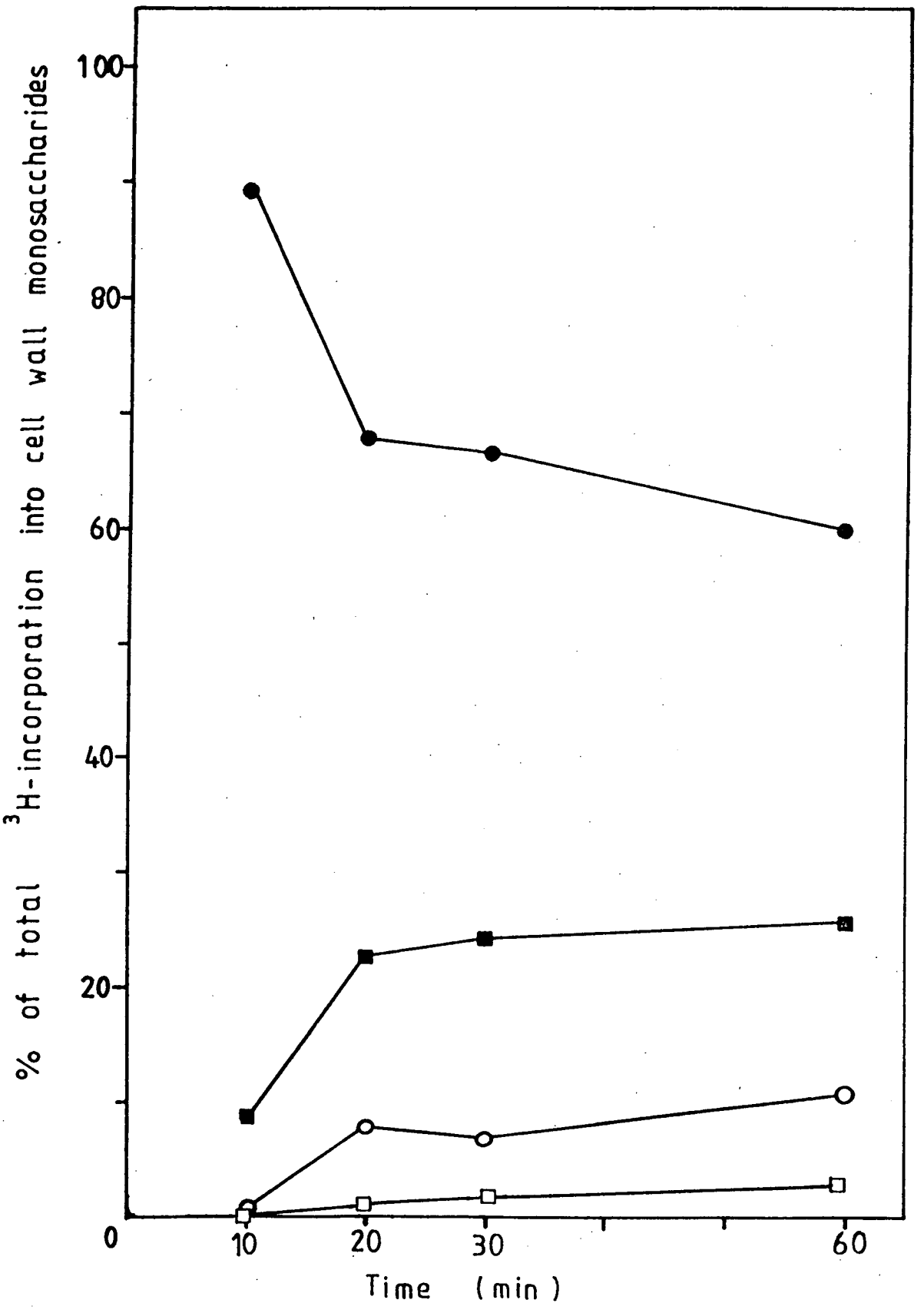

% of total ^{3}H-incorporation into cell wall monosaccharides
100
80
60
40
20
0
10
20
30
60
Time (min)

FIGURE 39

Fronds versus turions of *S. polyrrhiza* - the rate of incorporation of radioactivity from D-^{3}H-glcA into arabinose and xylose cell wall components.

S. polyrrhiza fronds and turions were vacuum-infiltrated in 25 ml H/2 medium containing 1 mCi D-^{3}H-glcA. They were incubated for 60 min at 25°C, during which time samples were taken at regular intervals for cell wall analysis. The cell walls were extracted, hydrolysed and analysed for arabinose, xylose (and apiose - Figure 40) content. The cell walls of turions were de-starched using DMSO prior to hydrolysis. See section 4.2 for details of methods.

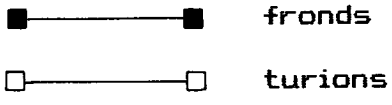
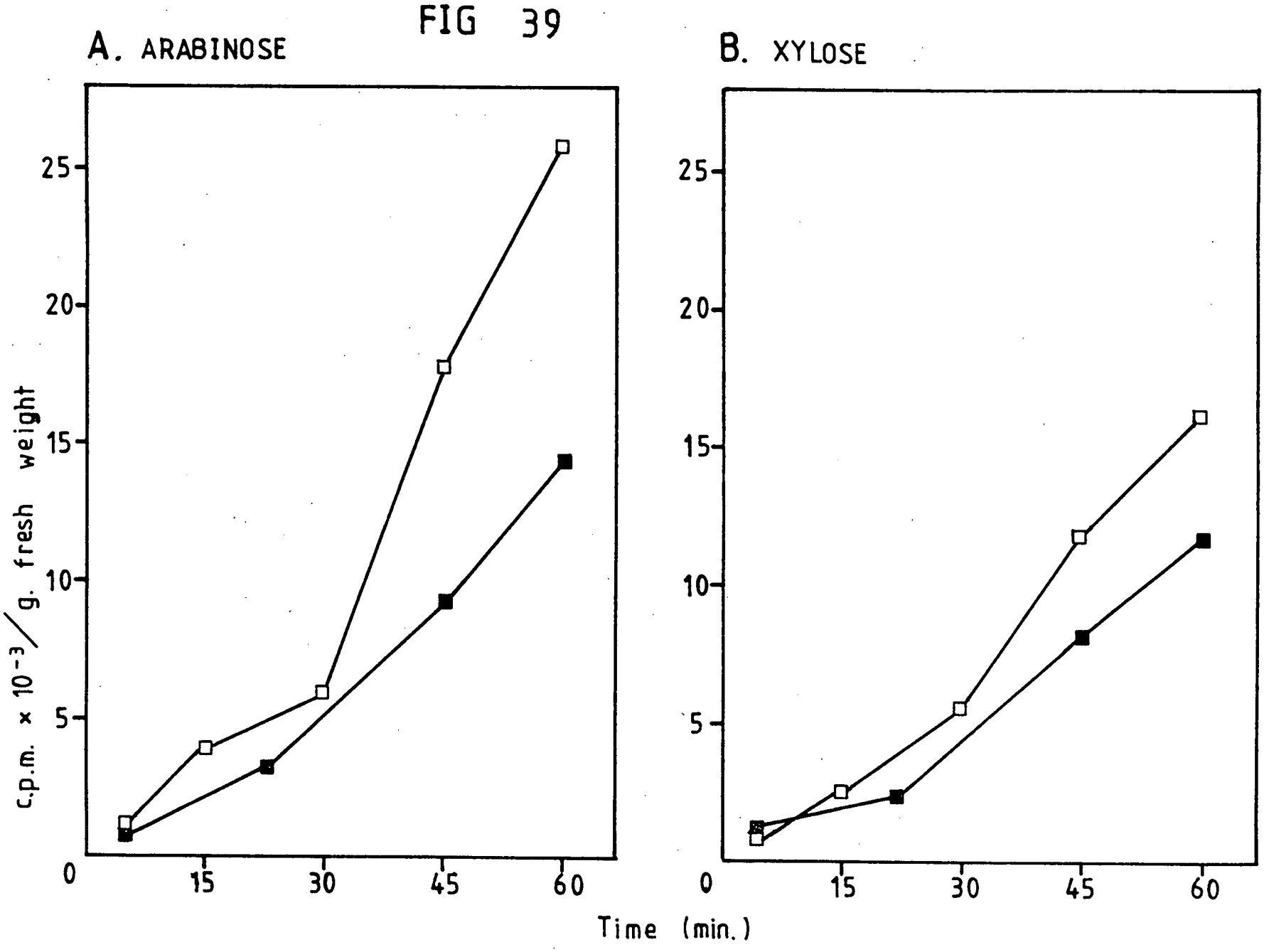


FIG 39


A. ARABINOSE
B. XYLOSE
c.p.m. × 10⁻³ / g. fresh weight
0
5
10
15
20
25
15
30
45
60
Time (min.)

4.3.6 Rate of incorporation of ^{3}H-glcA into cell wall components.

1) Fronds only

A time course showing the incorporation of radioactive monosaccharides into cell walls of fronds vacuum-infiltrated and radio-labelled with ^{3}H-glcA for 60 min is shown in Figure 37. The ^{3}H was incorporated preferentially into the wall components in the sequence glcA/galA > apiose > arabinose / xylose. Figure 38 shows the % of total incorporation into the wall polymer residues listed in Figure 37, and demonstrates how, after about 20 min, the relative rates of incorporation of the monosaccharides into the walls have reached a constant level.

2) Fronds versus turions

Fronds and turions were vacuum-infiltrated and incubated for 60 min (as above) to compare the rates of incorporation of arabinose, xylose and apiose into their cell walls. Figure 39 shows the rates of arabinose and xylose incorporation. It shows that the rate of incorporation by turions is faster (per g fresh weight) than that of fronds. However, the rate of incorporation of label is not synonymous with the rate of synthesis of the monosaccharides. If the precursor pool size was a lot smaller in turions than in fronds, then the initial levels of labelling in turion walls would appear artificially high compared to those in fronds. However, the turions do represent dormant buds and would, perhaps, be expected to behave in a more sluggish fashion. Evidently they were

metabolising actively, although no distinction was made between young (newly formed) and old turions. It should also be remembered that the cell walls of turions are twice as thick as those of fronds on a cell volume : volume comparative basis (Smart, 1981) and hence any interpretation of such analytical results depends on the criteria chosen for comparison. In this instance g fresh weight fronds/turions was used to standardise the results.

The rate of apiose incorporation into frond and turion walls obtained in the same experiment (Figure 40) is clearly very different from that of xylose and arabinose (Figure 39). Whilst the turions have incorporated some ^{3}H into apiose, the fronds have incorporated considerably more, i.e. the turions selectively incorporated radioactivity from ^{3}H-glcA into xylosyl and arabinosyl wall components rather than into apiosyl ones, relative to the same situation in fronds.

4.3.7 Rate of incorporation of radio-label from ^{3}H-glcA into wall components of fronds of different sizes.

The ^{14}C-glucose feeding experiments (4.3.1) were evaluating the cell walls of populations of fronds of differing age and size. No distinction was made between different frond sizes and growth stages to determine whether deposition of wall apiose was an age related factor, for example, whether it was only associated with full maturity. As mature fronds constituted the bulk weight of material used in these experiments, their composition dominated the final analysis.

FIGURE 40

Fronds versus turions of *S. polyrrhiza* - rate of incorporation of radioactivity from D-^{3}H-glcA into cell wall apiose.

Continued from Figure 39.

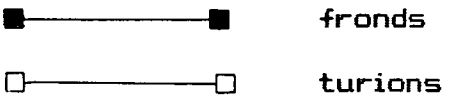


FIG 40

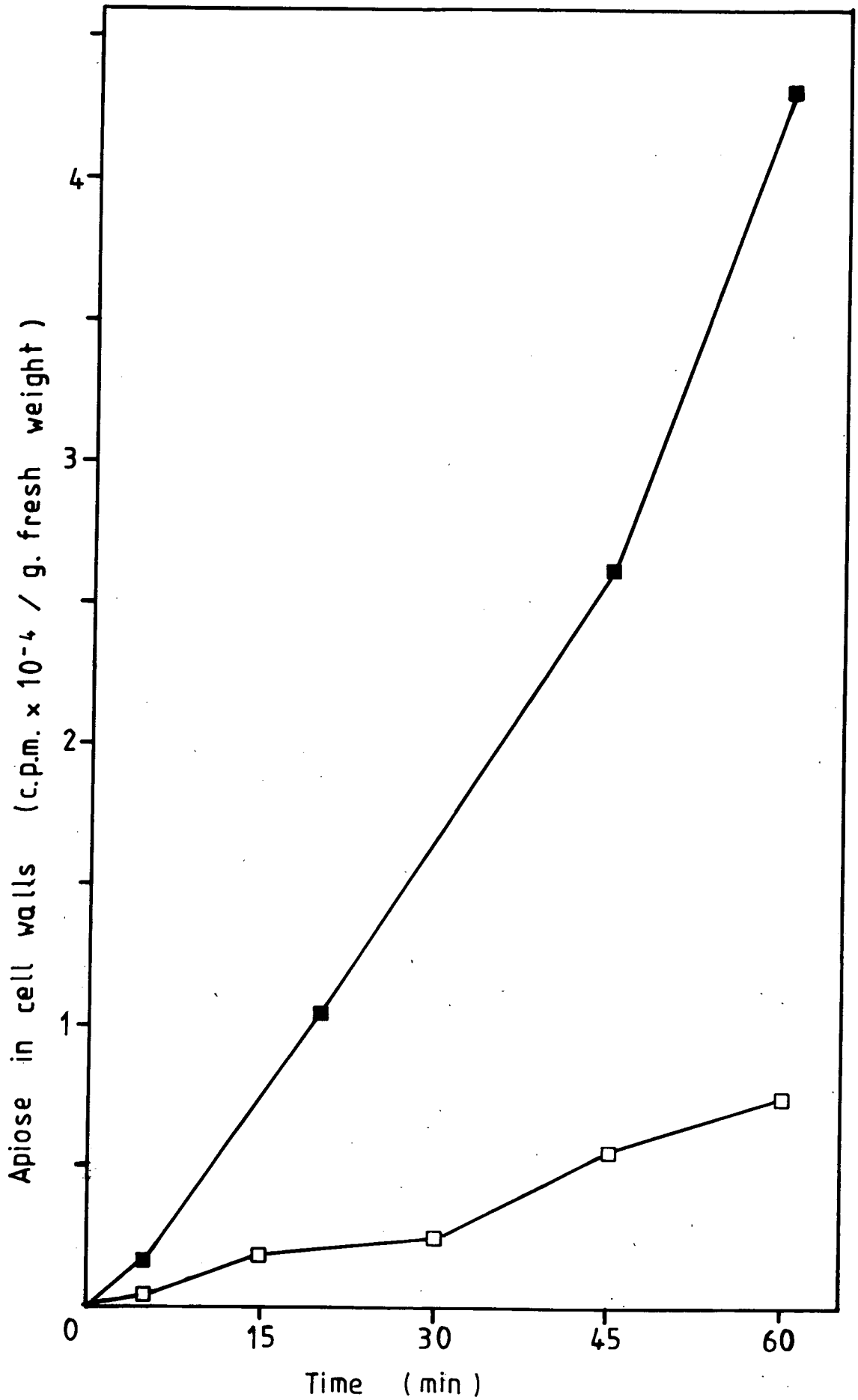

Apiose in cell walls (c.p.m. x 10⁻⁴ / g. fresh weight)
0
1
2
3
4
15
30
45
60
Time (min)

Figure 41 shows the incorporation of ^{3}H into xylose and arabinose in the walls of fronds of varying sizes, following addition of ^{3}H-glcA to the H/2 medium and subsequent 24 hours incubation (no vacuum-infiltration). Figure 42 likewise shows the incorporation into apiose in different sized fronds. From both Figures it appears that whilst the radio-label was readily assimilated by larger fronds, it was not subsequently being passed on to their smaller, dependent daughter fronds. The problems of using ^{3}H-glcA as a substrate have already been discussed in section 4.3.5, and this is an example of the failure to transport radioactive material between (not vacuum-infiltrated) parent fronds, and their dependent vegetative offspring following incubation in ^{3}H-glcA.

This "nursing" effect posed a problem, as in order to investigate the effects of ABA on the cell walls of developing primordia during the turion-inducible phase (up to 1 mm in diameter) it was necessary to study the incorporation of radio-label into these very small fronds, and it seemed as though, for this purpose, ^{3}H-glcA was not a suitable substrate.

Table 5 summarises the relative ratios of arabinose, xylose and apiose found in the different sized fronds, as shown in Figures 41 and 42. As the frond diameter increased from 2.2 mm to 7.0 mm the relative ratio of arabinose decreased from (approximately) 0.4 to 0.2, whilst that of apiose increased from 0.2 to 0.4. The xylose levels remained constant (average 0.36). The ratio of arabinose : xylose : apiose was similar in

FIGURE 41

The incorporation of radioactivity from D-^{3}H-glcA into arabinose and xylose residues of cell wall components of different sized fronds of *S. polyrrhiza*.

A population of *S. polyrrhiza* fronds was incubated in 25 ml H/2 medium containing 1 mCi D-^{3}H-glcA (no vacuum-infiltration) at 25°C for 24 hours. They were then removed and dissected out into the individual fronds. These were grouped according to diameter size and the cell walls extracted, hydrolysed and analysed for ^{3}H-xylose, arabinose (and apiose - Figure 42) content. See Methods 4.2 for details.

FIG. 41

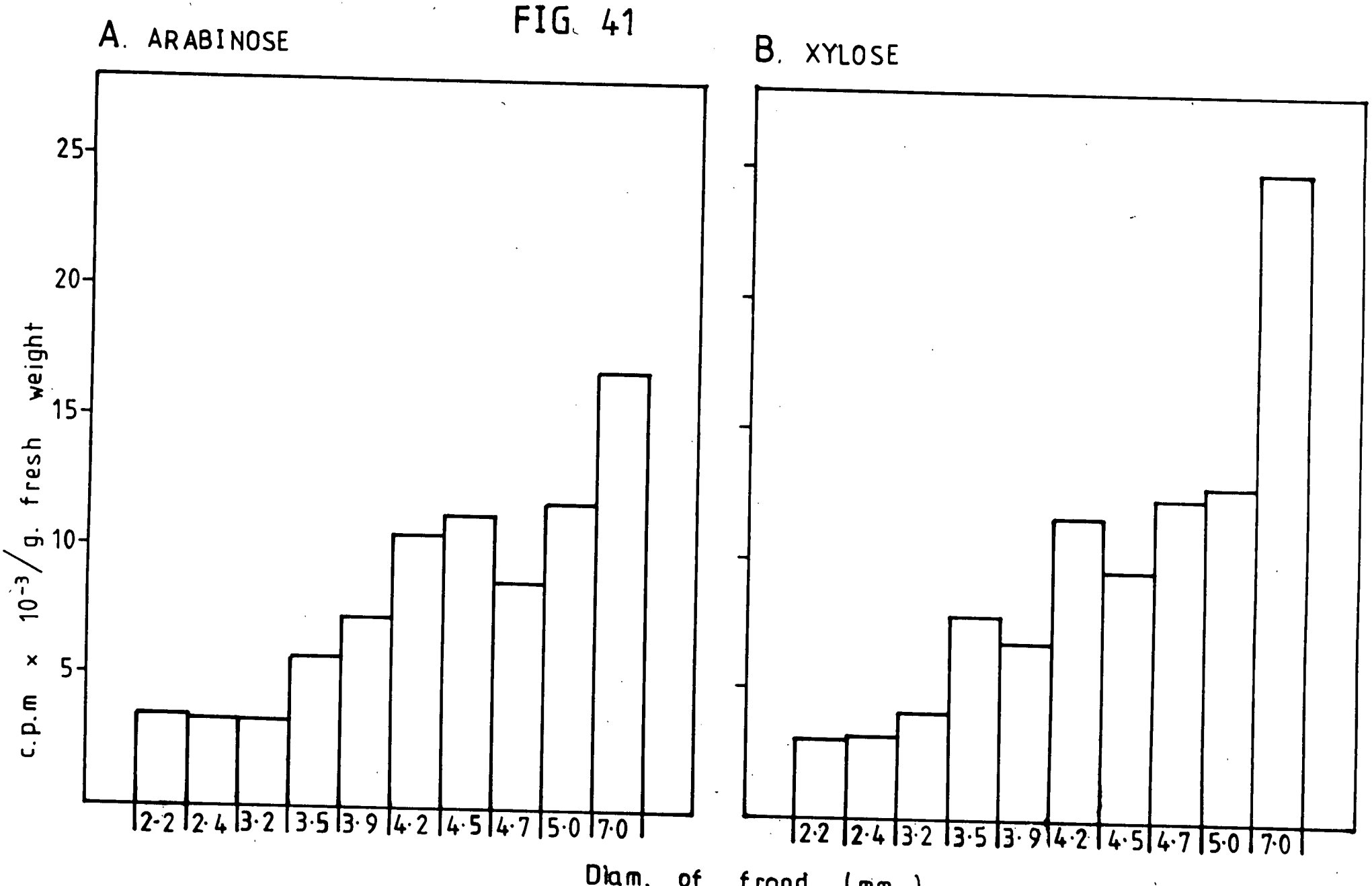

A. ARABINOSE
B. XYLOSE
c.p.m x 10^{-3}/g. fresh weight
25
20
15
10
5
2·2
2·4
3·2
3·5
3·9
4·2
4·5
4·7
5·0
7·0
Diam. of frond (mm.)

FIGURE 42

The incorporation of radioactivity from D-^{3}H-glcA into cell wall apiose residues in different sized fronds of *S. polyrrhiza*.

Continued from Figure 41.

FIG 42

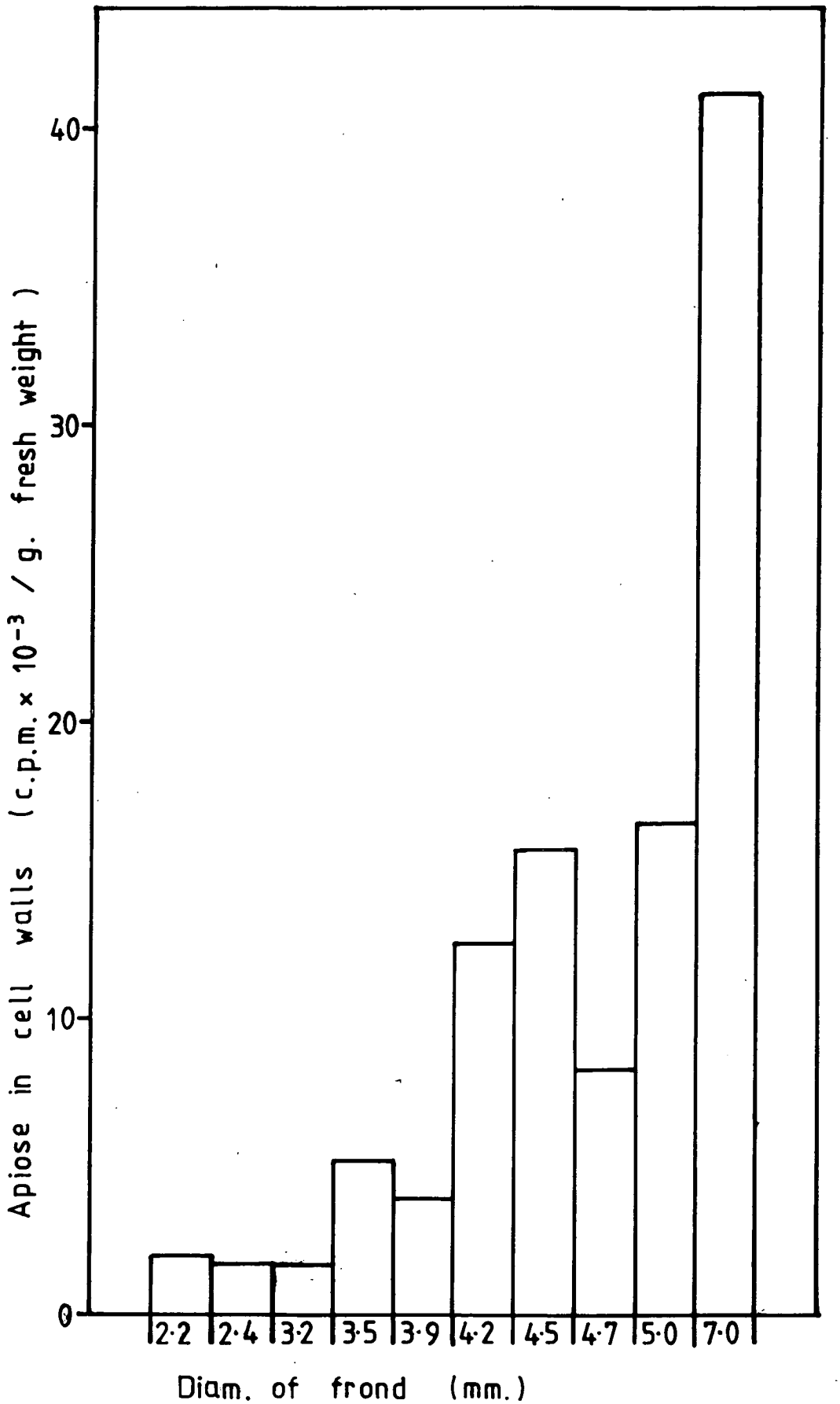

40
30
20
10
0
Apiose in cell walls (c.p.m. x 10^{-3} / g. fresh weight)
2·2
2·4
3·2
3·5
3·9
4·2
4·5
4·7
5·0
7·0
Diam. of frond (mm.)

TABLE 5

The ratios of radiolabelling of arabinose, xylose and apiose moieties in :

1. Different sized fronds following exposure to ^{3}H-glcA.

2. A population of fronds exposed to ^{14}C-glc.

1. Different sized fronds of *S. polyrrhiza* were labelled in ^{3}H-glcA for 24 hours, and analysed for labelled arabinose, xylose and apiose in the cell walls. Details of methods are outlined in Figure 41, and the results are shown in histogram form in Figures 41 and 42. Also included is data for roots of mature fronds.

2. A population of *S. polyrrhiza* fronds were labelled in ^{14}C-glc for 15 days and analysed for labelled arabinose, xylose and apiose in the cell walls. The data is taken from Table 4 (average values).

TABLE 5

	1. ^{3}H-glcA (24 hours) diameter of frond (mm)											2. ^{14}C-glc (15 days)
	2·2	2·4	3·2	3·5	3·9	4·2	4·5	4·7	5·0	7·0	roots	mixed sizes of fronds
arabinose	0·41	0·42	0·37	0·31	0·40	0·30	0·31	0·30	0·29	0·19	0·24	0·27
xylose	0·36	0·39	0·45	0·42	0·34	0·34	0·26	0·42	0·31	0·35	0·35	0·21
apiose	0·23	0·19	0·18	0·27	0·22	0·36	0·43	0·28	0·40	0·46	0·41	0·52
TOTAL	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0

roots and mature fronds. It therefore appears that as well as there being less total label assimilated by the smaller fronds (Figures 41, 42) there is also a difference in the proportion of labelled arabinose, xylose and apiose components of small and mature frond cell walls. As a comparison, the same relative ratios found from ^{14}C-glc labelling of a mixed population of fronds (taken from Table 4) shows a higher proportion of labelled apiose (0.52) - at the expense of xylose labelling (0.21). This demonstrates the problem of comparing results between labelling experiments using different radioisotopes and precursors, and which may also involve different metabolic pathways.

4.3.8 A comparison between the uptake of radio-label using A) ^{14}C-glucose and B) ^{3}H-glcA, by fronds of varying size.

Figure 43 shows incorporation into apiose residues by fronds of different sizes following incubation with :

A. ^{14}C-glucose for 24 hours.

B. ^{3}H-glucuronic acid for 24 hours (no vacuum-infiltration).

The data show that ^{14}C-glc was readily incorporated into the wall polymers of (rapidly growing) small fronds, whereas ^{3}H-glcA was not. For this reason, and those mentioned in 4.3.5 above, it was decided to investigate the effects of ABA on the incorporation of ^{14}C-glc into the apiose residues of cell walls of small fronds. Whilst the low levels of soluble sugar intermediates and nucleotides could not be detected due to the lower specific activity of the ^{14}C-glc

FIGURE 43

A comparison between the incorporation of radioactivity into apiosyl cell wall components of *S. polyrrhiza* fronds of varying sizes following incubation for 24 hours in :

A. D-^{14}C-glucose.

B. D-^{3}H-glucuronic acid (no vacuum-infiltration).

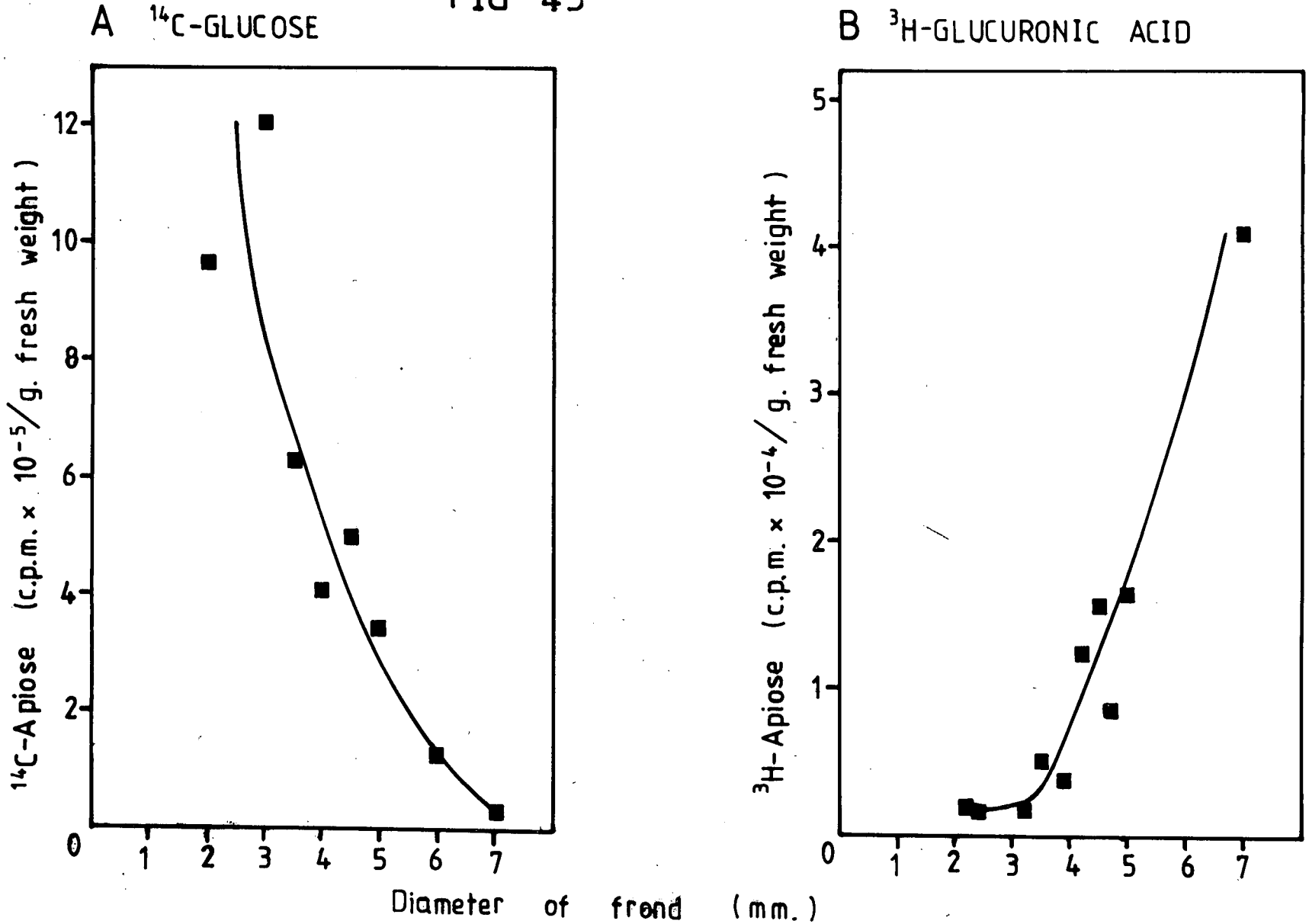
A population of *S. polyrrhiza* fronds was incubated for 24 hours in 25 ml H/2 medium containing either

A. 20 μCi D-^{14}C-glucose,

or B. 1 mCi D-^{3}H-glucuronic acid.

The fronds were removed, and killed by immersion in boiling methanol for 5 min, before dissecting out the individual fronds. These were grouped according to diameter size, and the cell walls extracted, hydrolysed and analysed (Methods 4.2) for radioactive apiose content.

FIG 43


A ^{14}C-GLUCOSE
^{14}C-Apiose (c.p.m. x 10^{-5}/g. fresh weight)
0
2
4
6
8
10
12
1
2
3
4
5
6
7
Diameter of frond (mm.)
B ^{3}H-GLUCURONIC ACID
^{3}H-Apiose (c.p.m. x 10^{-4}/g. fresh weight)
0
1
2
3
4
5
1
2
3
4
5
6
7

compared with that of ^{3}H-glcA, the apiose incorporated into wall components, being an accumulative fraction, could be measured.

4.3.9 The effect of ABA on the incorporation of ^{14}C-glc into apiose residues of cell walls of small (turion-inducible) fronds.

^{14}C-Glucose was added to a population of fronds in H/2 medium and incubated for 24 hours. 10^{-6} M ABA was then added, and at regular intervals for the subsequent 72 hours, samples of fronds were removed and killed immediately in boiling methanol. The frond population was then micro-dissected to obtain all of the small fronds sized between 0.25-1.0 mm in diameter, which were pooled (pool size of 5-15 mg at each sampling) and analysed for apiose content. Figure 44 shows the resulting levels of apiose found in these developing primordia.

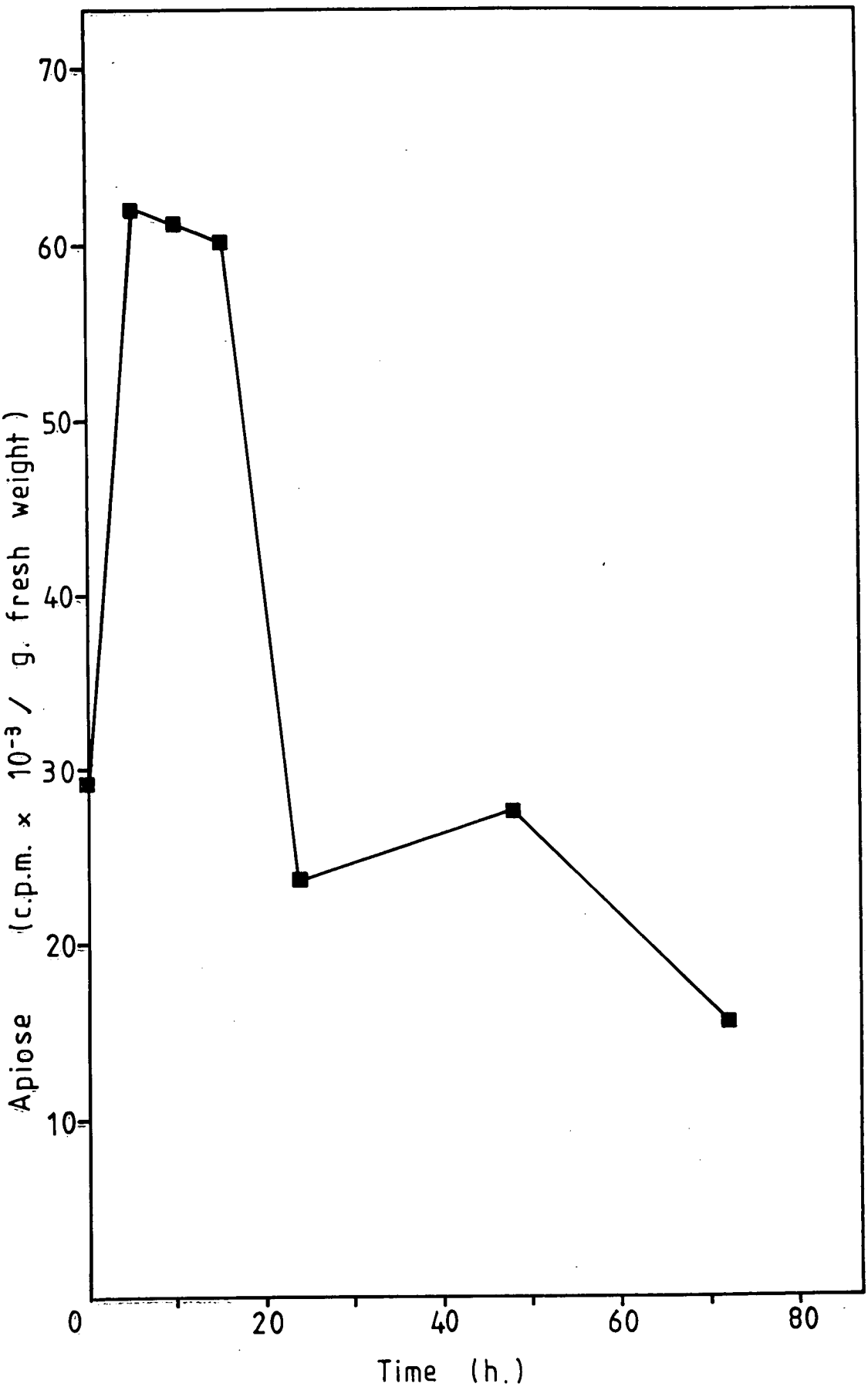
The results show an initial increase in apiose content above the initial level at time 0 (= time of ABA addition) to 15 hours, and then a decrease after 15 hours in ABA, until after 22 hours the level had decreased to that of the starting level. It stayed at, or just below, the initial level for the remainder of the experiment (another 50 hours). This interpretation places considerable importance on the accuracy of the data, in particular at time 0. The sample sizes were small, and inaccuracies in blotting and fresh weighing the material may have occurred. Unfortunately, a control

FIGURE 44

The effect of 10^{-6} M ABA on the D- ^{14}C-apiose content in cell walls of small fronds of turion-inducible size.

S. polyrrhiza fronds were incubated at 25°C in 25 ml H/2 medium containing 20 µCi D-^{14}C-glucose. ABA was then added to a final concentration of 10^{-6} M, and samples of fronds removed, and killed by immersion in boiling methanol for 5 min, at regular intervals during the next 72 hours. The small, turion-inducible fronds (< 1 mm but > 0.25 mm in diameter) were micro-dissected out from within the parental meristematic pockets, and analysed for D-^{14}C-apiose cell wall content. Details of methods are in 4.2.

FIG 44


70
60
50
40
30
20
10
0
20
40
60
80
Apiose (c.p.m. x 10^{-3} / g. fresh weight)
Time (h.)

experiment (without ABA) was not performed in parallel. Such an experiment may have confirmed if the early rise in apiose content in ABA-treated plants was due to addition of ABA, or purely due to continued accumulation of radio-labelled apiose with time. It may also have shown whether the fall in apiose content after 20 hours was due to depletion of the ^{14}C-glucose supply, rather than to an ABA-effected inhibition of apiose incorporation. However, the results do confirm that small fronds of turion-inducible size contain apiose in their cell walls.

4.3.10 The pathway from UDP-glcA -> apiose in the cell wall.

Figure 32 shows in detail the biosynthetic pathway between UDP-glucuronic acid and UDP-apiose. In order to determine at which enzymatic stage apiose biosynthesis is inhibited during the switch from frond to turion production in the developing primordia, it was necessary to detect and quantify the relevant intermediates to determine the position of any specific "block". Bearing in mind that xylose is incorporated into turion wall material there are two possible control points between UDP-glucuronic acid and apiose incorporation into wall polysaccharides -

1) the UDP-apiose/UDP-xylose synthase,

or 2) the UDP-apiose transferase.

In order to determine which stage the incorporation of wall apiose was being repressed during turion production, it was necessary to establish either the presence, or the absence,

of UDP-apiose in ABA-treated developing primordia (< 1 mm in diameter). The presence of UDP-apiose would suggest that the transferase system was the control point, whereas its absence would imply that the synthase enzyme was the regulating factor.

4.3.11 Problems associated with working with UDP-apiose

The isolation of UDP-apiose is difficult due to its extreme instability compared with other sugar nucleotides (Kindel *et al.*, 1970). Watson (1971) found that at below pH 7, UDP-apiose decomposes to give D-apiose + UDP, and at alkaline pH to give α-D-apio-furanosyl cyclic-1:2-phosphate + UMP. After 90 min incubation at 25°C, 50% of the original UDP-apiose had decomposed to apiose at pH 3; whilst at pH 9, 50% had decomposed to the apiosyl cyclic-1:2 phosphate.

UDP-apiose in the soluble extracts of fronds/turions was established by rapid electrophoretic separation, at neutral pH, from any uncharged soluble apiose (details in Methods 4.2.6). Once separated in this way, the UDP-apiose was hydrolysed in 2 M TFA to free apiose + UDP, and any apiose identified by the chromatographic techniques listed in Table 3.

4.3.12 Measurement of relative UDP-apiose levels in fronds and turions.

Whilst both fronds and turions constitute the same plant species, they are of very different anatomical composition.

This factor made it difficult to devise a means of satisfactorily comparing any alterations in the relative levels of UDP-apiose in fronds and turions.

It was decided that the ratio of :

$$\frac{\text{UDP-apiose}}{[\text{UDP-arabinose} + \text{arabinose-1-P} + \text{UDP-xylose} + \text{xylose-1-P}]}$$

found in turions (which do not incorporate apiose into wall components) and the same ratio found in fronds, would be the best method of determining any inhibition of UDP-apiose production. This, in turn, would be indicative of which enzyme (either the synthase or the transferase systems) was the controlling element in the process. A similar ratio in both fronds and turions i.e. UDP-apiose present in relatively undiminished concentrations would infer that the "block" to apiosyl cell wall incorporation in turions was at the transferase level, whilst the relative repression of UDP-apiose synthesis would point to the UDP-apiose/UDP-xylose synthase enzyme as being a regulating factor.

The levels of UDP-apiose, UDP-arabinose + arabinose-1-P, UDP-xylose and xylose-1-P in populations of fronds and turions (formed as a result of the exposure of fronds to 10^{-6} M ABA) was determined by vacuum-infiltration and subsequent 30 min incubation with ^{3}H-glcA. The resulting cpm / g fresh weight of label in the compounds listed above are shown in Table 6. The ratios of :

TABLE 6

The ratio of labelled UDP-apiose : [UDP-arabinose + arabinose-1-P + UDP-xylose + xylose-1-P] in fronds and turions following labelling with ^{3}H-glucuronic acid.

Fronds and turions were vacuum-infiltrated with ^{3}H-glcA (4.2.2) and incubated for 30 min at 25°C. The soluble fractions were extracted (4.2.3) and analysed for the radioactive content of arabinose, xylose and apiose moieties (4.2.6, 4.2.7). The results show the cpm/g fresh weight in arabinosyl, xylosyl and apiosyl soluble nucleotides / intermediates.

TABLE 6

(figures represent cpm per g fresh weight)

	A	B	C	C / A + B
	UDP-ara + ara-1-P	UDP-xyl + xyl-1-P	UDP-api	
FRONDS				
	15329	11991	8223	0.301
	13780	5820	4310	0.220
	15000	8320	9120	0.391
	9990	7500	6165	0.352
	11410	10070	7330	0.341
	16260	17340	9190	0.274
	12805	7770	8595	0.418
	10885	16448	18900	0.691
			AVERAGE	0.374
			(S.E. = 0.051)	
TURIONS				
	14896	10145	1471	0.059
	15467	10391	1068	0.041
	17112	29815	826	0.018
			AVERAGE	0.039
			(S.E. = 0.012)	

UDP-apiose : [UDP-xylose + UDP-arabinose + arabinose-1-P]

were

FRONDS 0.374 : 1 (n = 8; S.E. = 0.050)

TURIONS 0.039 : 1 (n = 3; S.E. = 0.012).

This gives a difference in ratios of fronds : turions corresponding to 0.374 : 0.038

or 9.8 : 1.

This represents a substantial and specific suppression of UDP-apiose biosynthesis in turions, and indicates that the UDP-apiose/UDP-xylose synthase enzyme is not as active in turions as in fronds. There is the possibility that UDP-apiose is synthesised but rapidly turned over in turions, but as apiose was not detected in the soluble fraction, e.g. as apiin, in turions (or fronds), this possibility seems unlikely. Whether the synthase enzyme is not being synthesised i.e. diminished in quantity, or whether its activity is being lessened by some modification, is not known. It is also possible that its path of action is being re-directed to produce UDP-xylose rather than UDP-apiose. Matern and Grisebach (1977), using an isolated UDP-apiose/UDP-xylose synthase system from cell suspension cultured parsley managed to alter artificially the ratio of xylose : apiose formed by a factor of two only (4.1.8). It consequently seems unlikely that, in the frond/turion comparison, such a re-direction of activity as caused by Grisebach's additives could account for the large suppression of UDP-apiose synthesis in turions described here. The *in vivo* synthesis of UDP-apiose in fronds

may be regulated by native compounds, such as proteins, which were not investigated by Grisebach.

Whether the transferase system is also a regulating factor is not known. For the UDP-apiose/UDP-xylose synthase enzyme to be the sole regulatory factor the difference in relative UDP-apiose content as calculated above would need to be sufficient to result in the difference in final wall apiose content between fronds and turions. This was 214 : 1, respectively, using ^{14}C-glc labelling (Table 4) - although only about 7 : 1, respectively, using ^{3}H-glcA labelling (Figure 40).

It would have been very interesting to carry out a time course to monitor the relative levels of UDP-apiose in small fronds treated with 10^{-6} M ABA, so that the rate at which the ABA affected UDP-apiose production could be determined. Unfortunately, as these small fronds were not assimilating ^{3}H-glcA or its products, and as ^{14}C-glucose was not available in sufficiently high radioactive concentrations, this experiment was not a feasible proposition.

4.4 DISCUSSION

Whilst considerable interest has been directed towards the apiosyl content of *L. minor* (which does not form turions), there are no previous reports concerning the analysis of turion cell wall monosaccharides. The results with *Spirodela polyrrhiza* have shown that the monosaccharide components of fronds and turions are qualitatively very different; in particular, that the large proportion of cell wall apiose found in the vegetative fronds of *S. polyrrhiza* (21.4% of total non-cellulosic monosaccharides) is virtually absent in the turions (0.1%).

The developing primordia of fronds which were of turion-inducible size (i.e. < 1 mm in diameter) were found to contain considerable amounts of wall apiose (Figure 44, time 0). This confirms that apiose biosynthesis is switched off in turion production, rather than switched on in fronds once they are beyond the turion-inducible phase. Also, after 20 hours exposure to 10^{-6} M ABA the level of D-apiose in wall polysaccharides of these small fronds had decreased, although during the initial 0 - 15 hour period there was an apparent increase in apiose content.

Whilst turions were found to contain detectable levels of UDP-apiose, the levels were considerably reduced in comparison with fronds, and it is concluded that the UDP-apiose/UDP-xylose synthase activity is repressed in turions. It is not known whether this was due to the enzyme being inactivated, re-directed to form UDP-xylose, or simply not synthesised. Gardiner

et al. (1980), working with cell suspension cultures of parsley, determined that irradiation with ultra-violet light for 2.5 hours caused a large increase in UDP-apiose/UDP-xylose synthase mRNA activity, and that this change in activity was caused by a corresponding change in the rate of synthesis, and so regulation by controlling the rate of apiose biosynthesis may be a strong possibility in *Spirodela*. As turions were found to contain considerable amounts of galacturonic acid, it appears that the block in wall apiose incorporation was not due to the unavailability of a suitable apiosyl acceptor molecule.

During the transition from frond to turion induction, apiose biosynthesis is almost totally switched off. This is a dramatic change in the chemical composition of the cell walls, and can be directly brought about by the addition of 10^{-6} M ABA to the medium. The rationale for this alteration in apiose content poses an interesting problem. Evidently, in the change from the vegetative frond - an actively metabolising, rapidly reproducing plant floating on top of ponds - to the dormant turion resting at the bottom of ponds, the requirement for apiose in the cell wall is lost. This does suggest, in agreement with Duff and Knight 1963) that apiose may be connected with rapid growth in an aqueous environment. Apiogalacturonan is a branched chain molecule in the pectin fraction and it might be that the loss and gain of apiose from within this structure is associated with a corresponding loss and gain of growth potential. However, as our knowledge of the apiogalacturonan-containing wall structure is still limited at the primary level, this can only be a

tentative hypothesis.

The reported inhibition of flowering, and associated promotion of vegetative growth, of *Lemna gibba* by a plant cell wall fragment from suspension-cultured sycamore cells (Albersheim *et al.*, 1983) has already been mentioned in the Introduction, 4.1. It may be that the cell walls of duckweeds play a very sensitive regulatory role in the growth and development of duckweeds. As such, they may prove to be a excellent plant material for future cell wall studies.

CHAPTER 5

FINAL DISCUSSION

FINAL DISCUSSION

5.1 Summary

The work described in this thesis is of varied content. The ABA-binding work proved extremely difficult to obtain fruitful data from, and the direction of research was moved to another area of the ABA-dormancy association. It was felt desirable to obtain some positive data concerning ABA effects at the molecular level, and it was also becoming increasingly questionable as to whether ABA receptors, in the form of specific molecular entities, even existed.

Veldstra (1944) was the first to suggest that the hydrophobic nature of plant hormones might result in them partitioning into biological membranes thereby altering the permeability of the latter. The discovery of Lea and Collins (1979), already mentioned in 2.1, that ABA created ion channels by spanning artificial bilipid membranes, reinforces this concept. Of associated interest is the recent isolation of ABA from rat and pig brain tissues (Le Page-Devry *et al.*, 1986) which raises the possibility that ABA may be a mammalian neurotransmitter. In the light of such results, there is a school of thought (e.g. Trewavas, 1979) that ABA acts primarily by affecting membrane permeability. As ABA is known to alter ion fluxes across membranes (Van Steveninck and Van Steveninck, 1983) and does cause physiological effects over a wide range of concentrations, this seems an attractive possibility which would explain the difficulties encountered

in ABA-receptor detection, as receptors would not be required for the initial ABA interaction.

However, this hypothesis does not explain the highly specific nature of the ABA-binding response (Hornberg and Weiler, 1984). Bittner *et al.* (1977) found that there was a large difference in bioassay responses towards 36 aromatic analogues of ABA which differed only slightly in minor structural variations. This underlines the requirement for a very precise molecular interaction, and implies a specific recognition site, or receptor. It is considered that the conflict of ideas concerning the nature of this initial ABA-tissue interaction - is a receptor really always necessary to produce a physiological response? - is not convincingly resolved at this current moment in time.

The endogenous levels of ABA in hydrophytic plants are generally low, even when subjected to drought conditions (Millborrow and Robinson, 1973), although there are conflicting reports concerning *Lemna minor*. In nonstressed *Lemna minor* Weiler (1979) found 37 ng/g fresh weight endogenous ABA (a typical concentration in hydrophytes) whereas Huber and Sankla (1979) found 5280 ng/g fresh weight. As hydrophytic plants are not normally exposed to water-stress, low endogenous levels might be expected. This poses the question as to why *Spirodela* should express such a specific sensitivity to certain concentrations of exogenously applied ABA. It has to be assumed that the physiological and morphogenetical changes associated with specific

concentrations of added ABA do in some way reflect the *in vivo* situation with endogenous levels. It may also be that ABA has different molecular modes of action according to the applied concentration, and it does seem likely that the inhibition of growth, and turion formation, proceed by completely different sets of biochemical reactions. The developmental stage of the cells, and the genes expressed at the time of exposure to ABA must be of importance with regard to the associated sensitivity of the tissue.

Turion-inducing concentrations (10^{-6} M) of ABA rapidly reduced both growth and cell wall plastic extensibility of fronds (Chapter 3), and also reduced the incorporation of apiose into cell walls of fronds of turion-inducible size (Chapter 4). Whilst the link between cell growth and wall apiose content is an attractive speculation, as mature fronds were found to contain large quantities of apiose but had cell walls which were virtually inextensible (although they had previously been extensible during immaturity), any connection is not a simple one. It is felt, however, that the similarity in shape of the graphs shown in Figures 27A and 44 deserves further comment. At first glance it appears that the effect of 10^{-6} M ABA on wall plastic extensibility of fronds (Figure 27A) is directly related to its effect on apiose content (Figure 44). However :

1) The extensibility measurements were, for practical limitations, restricted to fronds over 1 mm in diameter i.e. not turion-inducible, whereas the apiose measurements were of

fronds of turion-inducible size (0.25 - 1.0 mm in diameter).

2) In Figure 44, the initial rise in apiose content is reliant on one data point only (time 0).

3) In Figure 44, the external levels of ^{14}C-glucose were not monitored to check for exhaustion of radioactive "stock" material. The initial rise in radio-label could be due to accumulative uptake of ^{14}C into apiosyl components, and the subsequent fall due not to lowered apiose content, but rather to a dilution factor where the apiose incorporated was derived from a non-radioactive source in preference to from a dwindling ^{14}C-glucose source.

In view of the above points, it is considered that a more speculative claim linking the effect of ABA on wall extensibility and wall apiosyl content is not, at present, realistic.

There is currently little information regarding the relationship between the activity of wall-associated enzymes and wall extension. Fry (1979) has suggested that growth in spinach suspension-cultured cells is regulated by peroxidase catalysed wall rigidification in which feruloylated pectins become cross-linked in the cell wall via diferulate bridges. The feruloylation sites on the wall polymers are proposed (Fry, 1982) to be specifically associated with arabinogalactan components. Whether a similar mechanism operates in duckweeds is not known, but it does appear that apiose could not be the only controlling factor in wall extensibility.

The establishment of the control point involving apiose

biosynthesis in ABA-induced turions as being the inhibition of UDP-apiose/UDP-xylose synthase activity, is felt to be an important discovery. It represents the identification of a cell wall related enzymic change during the striking developmental transition from frond to turion production; a transition which can be only dictated to "sensitive" meristematic tissue by certain concentrations of ABA (here 10^{-6} M). This is an example of the molecular mode of action of ABA in the induction of the dormant state. It also confirms that control of wall synthesis can be by regulation of the NDP-sugar supply, in contrast with the idea that regulation is at the polysaccharide synthase level only (e.g. Bolwell and Northcote, 1984).

5.2 Criticism of work and suggestions for future progress

The main problem in understanding hormone action has been to relate the hormone-effected end physiological result back to the initial hormone recognition site and primary response. The work here has described how ABA can affect cell wall extensibility and initiate physiological changes which result in altered cell wall composition. Progress at the molecular level has been achieved in the identification of one enzyme, concerned with wall synthesis, whose activity is specifically repressed in an ABA-effected physiological transition. Attempts to detect specific receptor sites were not so successful. With the benefit of hindsight, in view of the success of Hornberg and Weiler (1984) with photoaffinity

labelling of ABA-receptors in guard cell protoplasts, it would have been pertinent to have devoted more time to ABA-binding studies using photoaffinity labelling. However, it should not now be long before their ABA-binding fractions have been isolated. This will enable the production of monoclonal antibodies to detect ABA receptors in tissue fractions where the concentration of receptors is low.

Another immunological technique which might prove useful involves the "auto-anti-idiotypic" response of immune systems, in which, in addition to the production of antibodies to antigens, antibodies ("anti-idiotypic antibodies") are also produced to the antigen-binding sites of the first antibodies. Such a system applied to ABA-antibody production should produce from amongst these anti-idiotypic types some which are ABA receptor antibodies. After separation of the "active" antibodies from the "inactive" anti-idiotypic antibodies, the anti-receptor activity of the latter could be established by raising monoclonal antibodies against ABA and checking for inhibition of ABA/ABA-monoclonal antibody binding. Any such anti-idiotypic ABA-receptor antibodies could then be used to detect receptor sites, if they do in fact exist, in plant tissues. This immunological technique has already been used with success in acetylcholine receptors (Cleveland *et al.*, 1983). With respect to *Spirodela polyrrhiza* such an ABA-receptor antibody would also be useful to determine whether the ABA-insensitive clone from Puerto Rico described by Perry and Byrne (1969) had receptors and hence point with more

assurance to the importance (or otherwise) of the presence of active ABA receptors in effecting the physiological response.

The establishment of receptor importance remains a critical link in the chain of biochemical reactions between hormone and physiological response. However, despite the possibility that endogenous ABA levels may not be important in determining the sensitivity of the tissue to the hormone, it is felt that it would be important to measure the endogenous levels of ABA in fronds and turions. That the levels of exogenously added ABA to fronds should be so critical in the determination of turion induction suggests considerable sensitivity to endogenous levels, in developing primordia, at least. Careful measurements, following a time course, might reveal a rise in endogenous ABA levels in developing primordia of turion-inducible size within a population of fronds initiating turion production as a result of exposure to a "natural" stressful situation e.g. overcrowding in low light. However, as the vacuole may act as a sink for ABA, it would be advisable to also check the intra-cellular distribution of the ABA. Autoradiography of sections of fronds and turions radio-labelled with ^{3}H-ABA would show the position of the radio-label within the plant tissue (and possibly the site of ABA-receptors?), although the sectioning and subsequent damage of the tissue could result in artifacts. Also the rapid metabolism of the ^{3}H-ABA would result in the presence of radio-labelled, and generally inactive, ^{3}H-metabolites.

The involvement of ABA in producing non-floating dormant

buds instead of floating fronds in *S. polyrrhiza* is in sharp contrast with the findings of Anderson (1978). Anderson discovered that 10^{-5} - 10^{-4} M ABA induced the formation of floating, rather than submersed, leaves in totally submersed germinating tubers of *Potamogeton nodosus* (American pond weed). The floating leaves were broader, had stomata on their upper surfaces, and were the result of a more complex differentiation of leaf tissue than the submersed leaves. This ABA effect was wholly or partially overcome by simultaneous exposure of ABA with gibberellic acid, kinetin or benzyladenine. As apiose has been detected in the cell walls of *Potamogeton pectinatus* (Van Beusekom, 1967) it seems likely that *P. nodosus* would also have apiose-containing wall polymers. It would be especially interesting to determine if there was any difference in apiose content between the floating leaves and the submersed leaves. It may be that apiose is not required in totally submersed leaf tissue, although I detected it in *S. polyrrhiza* roots (Table 5). If this was found to be the case, however, it would follow that ABA was, in *P. nodosus*, responsible for the <u>incorporation</u> of apiose into wall polysaccharides - the reverse of the case in *S. polyrrhiza* turion formation. Alternatively, ABA might inhibit the incorporation of apiose into the floating leaves. In any case, that ABA alone can cause these two seemingly opposite physiological effects in two aquatic monocots, suggests that ABA does not act directly on leaf differentiation.

The total structure of the apiose-containing polymers in fronds and turions of *S. polyrrhiza* was not determined, and it has been assumed that all the apiose was in the form of apiogalacturonans. It would have been interesting to discover if all the apiose in fronds was contained within identical units, and also whether the small amount of apiose in turion cell walls is a component of an apiogalacturonan(s) or a different polymer e.g. RG II. Different apiose-containing polymers may have different functions, for example, defence, wall extensibility, or food reserves.

That ABA can cause both rapid and longer-term effects is already known. Considerable interest has been focused on the rapid closure of stomata in response to ABA (reviewed by Davies and Mansfield, 1983), whilst the effect of ABA on ion transport is known to be much slower (Van Steveninck, 1974). The rapid stomatal response is due to alterations in membrane permeability, whilst the latter effect is due to control of the carrier proteins syntheses. The work in this thesis has investigated ABA effects concerned with the induction of dormancy in *S. polyrrhiza* which have also shown a difference in the speed of effect. The effect on growth inhibition and plastic extensibility was almost immediate, whilst the alteration in cell wall composition associated with ABA-induced turion production involves a slower morphological change. This difference in kinetics questions the feasibility of a universal ABA-receptor and of a single molecular mode of action of ABA.

Turion induction is known to be a reversible phenomenon following removal of ABA within three days, and also to be the result of regulation of gene transcription (Smart and Trewavas, 1984). Recombinant DNA methods have led to the preparation of cDNA libraries from poly (A) RNA obtained from auxin-treated soybean hypocotyls (Hagen *et al.*, 1984). Differential hybridisation techniques showed that the auxin treatment resulted in quantitative changes in mRNA levels (rather than changes in activation). Similar studies could be directed towards the ABA effect on gene transcription, and subsequent sequencing of cDNA clones and matching against libraries could lead to the identification of the affected gene products.

The complexity of plant growth and development is such that it is difficult to obtain a system which will respond to a single hormone in a specific and reliably reproducible manner. The effect of 10^{-6} M ABA on turion induction in a clone of *S. polyrrhiza*, as described herein, provides such a system. However, the complexity of the various biochemical changes involved in even this system provides ample scope for further study. The most profitable approaches to furthering our understanding of the molecular mode of action of ABA - its cellular recognition and isolation of any receptors, and the identification of the resulting sequence of biochemical changes - currently appear to consist of a combination of immunological and recombinant DNA techniques.

BIBLIOGRAPHY

Abdul-Baki, A.A. (1974). Plant Physiol. 53, 768-771.

Addicott, F.T. (1983). *In* Abscisic acid, ed. F.T. Addicott. Praeger Press, N.Y.

Addicott, F.T. and J.L. Lyon (1969). Annu. Rev. Plant Physiol. 20, 139-164.

Albersheim, P., A.G. Darvill, M. McNeil, B. Valent, J. Sharp *et al.* (1983). *In* Structure and Function of Plant Genomes, ed. O. Ciferri, L. Dure. Plenum, N.Y. pp. 293-312.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson (1983). *In* Molecular Biology of the Cell. Garland Pub. Inc. p. 724.

Alpi, A. and H. Beevers (1981). Plant Physiol. 67, 499-502.

Anderson, L.W.J. (1978). Science 201, 1135-1138.

Ashby, E. and T.A. Oxley (1935). Ann. Bot. 49, 309-336.

Ashby, E. and E. Wangermann (1949). Nature 164, 187.

Ashby, E. and E. Wangermann (1951). New Phytol. 5, 200-209.

Ashby, E., B.D. Bolas and F.Y. Henderson (1928). Ann. Bot. 42, 771-782.

Astle, M.C. and P.H. Rubery (1980). Planta 150, 312-320.

Astle, M.C. and P.H. Rubery (1985). J. Exp. Bot. 36, 469-485.

Bacon, J.S.D. and M.V. Cheshire (1971). Biochem. J. 124, 555-562.

Bandurski, R.S. and B. Axelrod (1951). J. Biol. Chem. 193, 405.

Bartels, P.G. and C.W. Watson (1978). Weed Sci. 26, 198-203.

Baron, D., U. Streitberger and H. Grisebach (1973). Biochim. Biophys. Acta 293, 526-533.

Batt, S. and M.A. Venis (1976). Planta 130, 15-21.

Batt, S., M.B. Wilkins and M.A. Venis (1976). Planta 130, 7-13.

Beck, E. (1967). Z. Pflanzenphysiol. 57, 444-461.

Bengochea, T., J.H. Dodds, D.E. Evans, P.H. Jerie and M.A. Hall (1980a). Planta 148, 397-406.

Bengochea, T., J.H. Dodds, D.E. Evans, P.H. Jerie and M.A. Hall (1980b). Planta 148, 407-411.

Bittner, S., M. Gorodetsky, I. Har-Paz, Y. Mizrahi and A.E. Richmond (1977). Phytochem. 16, 1143-1151.

Bolwell, G.P. and D.H. Northcote (1984). Planta 162, 139-146.

Burke, D., P. Kaufman, M. McNeil and P. Albersheim (1974). Plant Physiol. 54, 109-115.

Chrispeels, M.J. and J.E. Varner (1967). Plant Physiol. 42, 398-406.

Cleland, R.E. (1967). Planta 74, 197-209.

Cleland, R.E. (1981). In Encyclopaedia of Plant Physiol., New Ser. Plant Carbohydrates II eds. W. Tanner, F.A. Loewus, 13B 255-273. Berlin, Springer-Verlag.

Cleveland, W.L., N.H. Wassermann, R. Sarangarajan, A.S. Penn and B.F. Erlanger (1983). Nature 305, 56-57.

Cross, J.W. and W.R. Briggs (1978). Plant Physiol. 62, 152-157.

Darvill A.G., M. McNeil, P. Albersheim and D.P. Delmer (1980). *In* The Biochemistry of Plants I, ed. N.E. Tolbert. N.Y., Academic Press. pp 91-162.

Davies, W.J. and T.A. Mansfield (1983). *In* Abscisic Acid, ed., F.T. Addicott, Chap. 7. Praeger Publishers, N.Y. pp 237-268.

Dever, J.E., R.S. Bandurski and A. Kivilaan (1968). Plant Physiol. 43, 50-56.

Dohrmann, U.C., R. Hertel and H. Kowalik (1978). Planta 140, 97-106.

Dodds, J.H. and M.A. Hall (1980). Sci. Prog., Oxf. 66, 513-535.

Duff, R.B. (1965). Biochem. J. 94, 768-772.

Duff, R.B. and A.H. Knight (1963). Biochem. J. 88, 33-34.

Erion, J.L. and J.E. Fox (1981). Plant Physiol. 67, 156-163.

Eshdat, Y. and D. Mirelman (1972). J. Chromatogr. 65, 458-459.

Fong, F., D.E. Koehler and J.D. Smith (1982). Int. Symp. on Pre-Harvest Sprouting in Cereals, 3rd. Boulder, Colorado 188-196.

Fong, F., J.D. Smith and D.E. Koehler (1983). Plant Physiol. 73, 899-901.

Fox, J.E. and E. Gregerson (1982). *In* P.F. Wareing ed., Plant Growth Substances. Academic Press, London. pp. 207-214.

Fry, S.C. (1979). Planta 146, 343-351.

Fry, S.C. (1982). Biochem. J. 203, 493-504.

Fry, S.C. (1985). Oxf. Sur. Plant Mol. and Cell Biol. 2, 1-42.

Gardiner, S.E., J. Schroder, U. Matern, D. Hammer and K. Hahlbrock (1980). J. Biol. Chem. 255, 10752-10757.

Gorham, P.R. (1941). American J. Bot. 28, 98-101.

Grisebach, H. (1980). *In* The Biochemistry of Plants 3, ed. J. Preiss. Academic Press. pp. 171-197.

Gronemeyer H. and O. Pongs (1980). Proc. Natl. Acad. Sci. 77, 2108-2112.

Gustine, D.L. and P.K. Kindel (1968). Fed. Proc. 27, 595.

Gustine, D.L., D. Hso-Freng Yuan and P.K. Kindel (1975). Arch. Biochem. Biophys. 170, 82-91.

Hagen, G., A. Kleinschmidt and T. Guilfoyle (1984). Planta 162, 147-153.

Hahlbrock, K., J. Ebel, R. Ortmann, A. Sutter, E. Wellmann and H. Grisebach (1971). Biochim. Biophys. Acta 244, 7-15.

Hall, H.A., A.J. Cairns, D.E. Evans, A.R. Smith, P.G. Smith, J.E. Taylor and C.J.R. Thomas (1982). *In* P.F. Wareing ed., Plant Growth Substances. Academic Press, London. pp. 375-383.

Hanke, D. (1984). Nature 310, 272-273.

Hanna, R., M. Picken and J. Mendicino (1973). Biochem. Biophys. Acta 315, 259-271.

Hanson, J.B. and A.J. Trewavas (1982). New Phytol. 90, 1-18.

Hart, D.A. and P.K. Kindel (1970a). Biochem. J. 116, 569-579.

Hart, P.K. and P.K. Kindel (1970b). Biochemistry 9, 2190-2196.

Hertel, R., K-St. Thomson and V.E.A. Russo (1972). Planta 107, 325-340.

Hillman, W.S. (1961). Bot. Rev. 27, 221-287.

Hocking, T.J., J. Clapham and K.J. Cattell (1978). Planta 138, 303-304.

Hornberg, C. and E.W. Weiler (1984). Nature 310, 321-324.

Huber, W. and N. Sankhla (1979). Z. Pflanzenphysiol. 91, 147-156.

Ihl, M. (1976). Planta 131, 223-228.

Jacobs, D.L. (1947). Ecol. Monog. 17, 437-469.

Jacobs, S. and P. Cuatrecasas (1981). *In* Receptors and Recognition. Series B:Vol. 11 (Membrane Receptors). Chapman and Hall, London and New York. pp. 1-31.

Jacobs, M. and R. Hertel (1978). Planta 142, 1-10.

Jacobsen, J.V. (1977). Annu. Rev. Plant Physiol. 28, 537-564.

Jelsema, C.L., M. Ruddat, D.J. Morre and F.A. Williamson (1977). Plant and Cell Physiol. 18, 1009-1019.

Jones, A.M., L.L. Melhado, T.-H.D. Ho and N.J. Leonard (1984). Plant Physiol. 74, 295-301.

Keith, B. and L.M. Srivastava (1980). Plant Physiol. 66, 962-967.

Keith, B., R. Boal and L.M. Srivastava (1980). Plant Physiol. 66, 956-961.

Keith, B., S. Brown and L.M. Srivastava (1982). Proc. Natl. Acad. Sci. USA. 79, 1515-1519.

Keith, B., N.A. Foster, M. Bonettemaker and L.M. Srivastava (1981). Plant Physiol. 68, 344-348.

Kende, H. and G. Gardner (1976). Annu. Rev. Plant Physiol. 27, 267-290.

Kindel, P.K., D.L. Gustine and R.R. Watson (1970). Plant Physiol. 46 Suppl. 27.

Kindel, P.K., D.L. Gustine and R.R. Watson (1971). Fed. Proc. 30, Abs. 1117.

Kindel, P.K. and R.R. Watson (1973). Biochem. J. 133, 227-241.

Laskey, R.A. and A.D. Mills (1975). Eur. J. Biochem. 56, 335-341.

Le Page-Degivry, M.-Th., J.-N. Bidard, E. Rouvier, C. Bulard and M. Lazdunski (1986). Proc. Natl. Acad. Sci. 83, 1155-1158.

Lea, E. and J. Collins (1979). New Phytol. 82, 11-18.

Leach, H.W. and T.J. Schoch (1962). Cereal Chem. 193, 318-327.

Lobler, M. and D. Klambt (1985a). J. Biol. Chem. 260, 9848-9853.

Lobler, M. and D. Klambt (1985b). J. Biol. Chem. 260, 9854-9859.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J.Randell (1951). J. Biol. Chem. 193, 265-275.

Lubke, K., E. Schillinger and M.Topert (1976). Angew. Chem. Int. Ed. Eng. 15, 741-748.

Maan, A.C., D. Vreugdenhill, R.J. Rogers and K.R. Libbenga (1983). Planta 158, 10-15.

MacRobbie, E.A.C. (1981). J. Exp. Bot. 32, 563-572.

Mascaro, L.J. and P.K. Kindel (1977). Arch. Biochem. Biophys. 183, 139-148.

Matern, U. and H. Grisebach (1977). Eur. J. Biochem. 74, 303-312.

Milborrow, B.V. and J. Noddle (1970). J. Biochem. 119, 727-734.

Millborrow, B.V. and D.R. Robinson (1973). J. Exp. Bot. 24, 537-548.

Milligan, S. (1986). Ph.D. Thesis. Edinburgh University.

Moore, F.H. (1979). Plant Physiol. 64, 594-599.

Moore R. and J.D. Smith (1984). Planta 162, 342-344.

Mozer, T.J. (1980). Cell 20, 479-485.

Murphy, G.J.P. (1980). Planta 149, 417-426.

Norris, L., R.E. Norris and M. Calvin (1955). J. Exp. Bot. 6, 64-74.

Norstrom, C.G., T. Swain and A.J. Hamblin (1953). Chem. Ind. (London). p 85.

Oostrom, H., Z. Kulescha T.B. Van Vliet and K.R. Libbenga (1980). Planta 149, 44-47.

Ortmann, R., H. Sandermann and H. Grisebach (1970). F.E.B.S. Lett. 7, 164-166.

Ovodov, Yn.S., R.G. Ovodova, O.D. Bondarenko and I.N. Krasikova (1971). Carbohydr. Res. 18, 311-318.

Ovodova R.G., V.E. Varkowsky and Y.S. Ovodov (1968). Carbohydr. Res. 6, 328-332.

Pan, Y.T. and P.K. Kindel (1977). Arch. Biochem. Biophys. 183, 131-138.

Partridge, S.M. (1949). Nature 164, 443.

Perry, T.O. (1968). Plant Physiol. 43, 1866-1869.

Perry, T.O. and O.R. Byrne (1969). Plant Physiol. 44, 784-785.

Picken, J.M. and J. Mendicino (1967). J. Biol. Chem. 242, 1629-1634.

Pierce, M.P. and K. Raschke (1980). Planta 148, 174-182.

Polya, G.M. and A.W. Davies (1978). Planta 139, 139-147.

Quarrie, S.A. and H.G. Jones (1977). J. Exp. Bot. 28, 192-203.

Randerath, K. (1970). Anal. Biochem. 34, 188-205.

Ray, P.M. (1977). Plant Physiol. 59, 594-599.

Ray, P.M., U.C. Dohrmann and R. Hertel (1977a). Plant Physiol. 59, 357-364.

Ray, P.M., U.C. Dohrmann and R. Hertel (1977b). Plant Physiol. 60, 585-591.

Ray, P.M. and D.A. Rottenberg (1964). Biochem. J. 90, 646-655.

Rayle, D.L. and R. Cleland (1977). Curr. Topics Dev. Biol. 11, 187-214.

Reddy, A.S.N., S.K. Sopory and A. Datta (1983). Biochem. Int. 6, 181-190.

Ring, S.R. and R.R. Selvendran (1978). Phytochem. 17, 745-752.

Roberts, R.M., R.H. Shah and F. Loewus (1967). Plant Physiol. 42, 659-666.

Rosen, O.M., R. Herrera, Y. Olowe, L.M. Petruzzelli and M.H. Cobb (1983). Proc. Natl. Acad. Sci. 80, 3237-3240.

Rubery, P.H. (1981). Annu. Rev. Plant Physiol. 32, 569-596.

Saks, Y., M. Negby and I. Ilan (1980). Aust. J. Plant Physiol. 7, 73-79.

Sandermann, H. (1969). Phytochem. 8, 1571-1575.

Sandermann, H. and H. Grisebach (1970). Biochim. Biophys. Acta 208, 173-180.

Santi, D.V., C.H. Sibley, E.R. Perriard, G.M.Tomkins and J.D. Baxter (1973). Biochemistry. 12, 2412-2416.

Scatchard, G. (1949). Ann. N.Y. Acad. Sci. 51, 660-672.

Schmidt, O.Th. (1930). Liebigs Ann. Chem. 483, 115-123.

Schweiger, R.G. (1967). Arch. Biochem. Biophys. 118, 383-387.

Smart, C.C. (1981). Ph.D. Thesis. Edinburgh University.

Smart, C.C. and A.J. Trewavas (1983a). Plant, Cell and Environment 6, 507-514.

Smart, C.C. and A.J. Trewavas (1983b). Plant, Cell and Environment 6, 515-522.

Smart, C.C. and A.J. Trewavas (1984). Plant, Cell and Environment 7, 121-132.

Spellman, M.W., M. McNeil, A.G. Darvill, P. Albersheim and K. Henrick (1983). Carbohydr. Res. 122, 115-129.

Stewart, G.R. (1969). Nature 221, 61-62.

Stoddart J., W. Breidenbach, R. Nadeau and L. Rappaport (1974). Proc. Natl. Acad. Sci. USA. 71, 2355-2359.

Stoddart, J.L. and M.A. Venis (1980). *In* J. Macmillan, ed., Encyclo. Plant Physiol. New Series Vol. 10. Springer-Verlag, Berlin. pp. 445-510.

Sussman, M.R. and A. Kende (1978). Planta 140, 251-259.

Taiz, L. (1984). Annu. Rev. Plant Physiol. 35, 585-657.

Taiz, L. and J.-P. Metraux (1979). Planta 146, 171-178.

Thomas, C.J.R., A.R. Smith and M.A. Hall (1984). Planta 160, 474-479.

Thomas, C.J.R., A.R. Smith and M.A. Hall (1985). Planta 164, 272-277.

Tilberg, E. (1975). Physiol. Plant. 34, 192-195.

Trewavas, A. (1979). Trends Biochem. Sci. 4, 199-202.

Trewavas, A. (1981). Plant, Cell and Environment. 4, 203-228.

Updegraff, D.M. (1969). Anal. Biochem. 32, 420-424.

Van Beusekom, C.F. (1967). Phytochem. 6, 573-576.

Van Der Linde, P.L.G., H. Bouman, A.M. Mennes and K.R. Libbenga (1984). Planta 160, 102-108.

Van Scherpenberg, H., W. Grobner and O. Kandler (1965). *In* Beitrage zur Biochemie und Physiologie von Naturstoffen. Fischer, Jena. pp. 387-406.

Van Steveninck, R.F.M. (1974). *In* Membrane Transport in Plants, eds., U. Zimmermann and J. Dainty. Springer-Verlag, Berlin. pp. 450-456.

Van Steveninck, R.F.M. and M.E. Van Steveninck (1983). *In* F.T. Addicott, ed., Abscisic Acid, Chap. 6. Praeger Puplishers, N.Y. pp 171-238.

Van Volkenburgh, E. and W.J. Davies (1983). J. Exp. Bot. 34, 835-845.

Van Volkenburgh, E., S. Hunt and W.J. Davies (1983). Ann. Bot. 51, 669-672.

Van Volkenburgh, E., M.G. Schmidt and R.E. Cleland (1985). Planta 163, 500-505.

Veldstra, H. (1944). Enzymologia 11, 137-163.

Venis, M.A. (1977a). Adv. Bot. Res. 5, 53-88.

Venis, M.A. (1977b). Nature (London) 66, 268-269.

Venis, M. (1985). *In* Hormone binding sites in plants. Research Notes in Plant Science. Longman, N.Y. pp 134-137.

Vongerichten, E. (1901). Liebigs Ann. Chemie 318, 121-136.

Vongerichten, E. (1902). Liebigs Ann. Chemie 321, 71-83.

Wardrop, A.J. and G.M. Polya (1977). Plant Sci. Lett. 8, 155-163.

Watson, R.R. (1971). Ph.D. Thesis, Michigan State University. University Microfilms Inc., Ann Arbor. Order no. 71-31,330.

Watson, R.R. and N.S. Orenstein (1975). Adv. in Carbohydr. Chem. and Biochem. 31, 135-184.

Weiler, E.W. (1979). Planta 144, 255-263.

Wellmann, E. and H. Grisebach (1971). Biochim. Biophys. Acta 235, 389-397.

Wellmann, E., D. Baron and H. Grisebach (1971). Biochim. Biophys. Acta 244, 1-6.

Wickerberg, B. (1957). Acta Chem. Scand. 11, 506-511.

Yoshida, K. and T. Takegami (1977). J. Biochem. 81, 791-799.