The mechanism of depth accommodation in Nymphoides Peltata and other water plants

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THE MECHANISM OF DEPTH ACCOMMODATION IN

NYMPHOIDES PELTATA

AND OTHER WATER PLANTS

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ABSTRACT

Auxin and ethylene are shown to be important mediating factors in depth accommodation in *Nymphoides*, as in other water plants. Control of growth by both these regulators is shown to depend on their capacity to affect extensibility of the cell wall. Neutral sugar composition in the fraction of the cell wall most likely to be the site of the wall-loosening reactions is assayed, but no change in the proportions of the constituent monosaccharides was detected even where large increases in length were induced by auxin and ethylene. Thus no light was shed on this aspect of cell growth.

Using a variety of techniques, the effects of ethylene and auxin are assessed in the light of current theories on the mode of action of plant growth regulators; both auxin and ethylene are shown to stimulate proton excretion when they promote extension. Acid alone also stimulates extension. It is thus concluded that auxin and ethylene promote growth by an "acid-growth" mechanism i.e. they stimulate hydrogen ion efflux from the cytoplasm into the cell wall, the resultant pH drop in the wall causes an increase in WE and thereby leads to a substantial increase in the rate of cell expansion.

This is the first reported finding that ethylene works by an acid-growth mechanism when it promotes growth. The extension of "acid-growth" to this novel situation of rapid-growth promotion is important support for the theory. In a second species, *Regnellidium diphyllum*, predictions of

the "acid-growth" theory were tested but despite repeated attempts, were not fulfilled. This is the only known tissue which shows rapid growth in response to auxin, but not to acid. Implications for the "acid-growth" theory, of the findings on both *Nymphoides* and *Regnellidium* are discussed.

ABBREVIATIONS

ACC   amino-cyclopropane-1-carboxylic acid

AVG   aminoethoxyvinylglycine

ATP   adenosine triphosphate

CH   cycloheximide

CK   cytokinin

2,4-D   2,4-dichlorophenoxyacetic acid

ER   endoplasmic reticulum

FC   fusicoccin

GA   gibberellic acid

GC   gas chromatography

HR   high resolution

IAA   indol-3yl-acetic acid

αNA   αnaphthyl acetate

NC   non cellulosic

NCN   non cellulosic neutral

PCIB   p-chlorophenoxyisobutyric acid

RBA   rhizobitoxine analogue

SAM   S-adenosyl methionine

TFA   trifluoroacetic acid

WE   wall extensibility

WLF   wall-loosening factor

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PREFACE

The problem of how plants grow has long attracted interest. Apart from the fundamental importance of this question, it is of potentially great significance for those who attempt to manipulate plant growth, for example, in agriculture.

The form attained by a plant depends on the collective growth of the individual cells which constitute that plant. Thus plant growth and form is ultimately controlled by the factors which control the extent and orientation of cell growth.

Systems used for studies of plant growth should ideally show marked growth, triggered by a clearly defined event. The auxin response of some coleoptiles is such a system. A novel system showing spectacular growth promotion was recently identified: the submergence response (which leads to depth accommodation) in some aquatic and wetland species. This thesis presents a study of growth in such a system. I hope this work will be of particular interest to workers in three fields of research:

   1.   the mechanism of plant growth;

   2.   ethylene as a plant growth regulator;

   3.   depth accommodation in water plants.

CHAPTERS 1-5

INTRODUCTION

CHAPTER 1

DEPTH   ACCOMMODATION

CHAPTER 1

DEPTH ACCOMMODATION

Some aquatic plants are entirely free-floating (e.g. *Lemna* sp.) and are not troubled by fluctuation in the level of water in their habitat. Many·aquatic plants however, have floating organs but take root in the underlying substratum (e.g. *Nymphaea alba*). For such anchored plants, any considerable rise in water level may result in submergence of the ertswhile floating organs. Since these organs normally float, it seems likely that their being submerged for long periods would not be beneficial. Some of the reasons for this are obvious: for example, the (normally) floating flower of *Nymphaea alba* will not be accessible to its insect pollinators if submerged; in the case of leaves, less light will reach them when submerged, access to atmospheric gases (especially CO_2) will be restricted, and they will be subject to increased attack by phytophagous and epiphytic organisms.

Many plants combat these problems by resuming rapid elongation of a connecting structure, such as the petiole or the peduncle, so that an organ submerged by a rise in water level will be quickly returned to the surface.

This adjustment to changing water level is known as depth accommodation.

Depth accommodation is not peculiar to floating organs; it is also found among emergent organs of water plants (such as the coleoptiles of paddy rice), in aerial leaves of plants of marsh and water margin (Arber 1920), and in other situations where plants are liable to inter- mittent flooding.

Besides being a response of established leaves to flooding, depth accommodation also occurs to bring newly-developed leaves up to the water surface. In *Nymphoides peltata* for example, most leaves develop from the nodes of a creeping stem which grows along the substratum. However deep the water may be, within reason, petioles of such young leaves will continue to undergo rapid elongation until the leaf lamina arrives at the surface. Thus this is another example of depth accommodation. This type occurs in all rooted plants with floating leaves, and is irrespective of change in water level.

Depth accommodation can involve spectacularly rapid and large elongations (Sculthorpe 1967) and the phenomenon has long attracted serious botanical study (some of the earlier work is summarised by Arber 1920; Sculthorpe 1967). For example, Gessner (1959) showed that, regardless of the depth of water in which they were growing (this varied between 0 and 4 m), petioles of *Nymphaea alba* were always long enough to allow the leaf laminae to float freely. Indeed the petioles were always longer, by an approximately constant margin, than was strictly necessary to permit floating; the extra length may serve to allow the leaves to spread out on the water surface (see Figure 1).

For organs such as leaves to show depth accommodation, they must be able to:

1. distinguish between the submerged and floating conditions;

2. undergo rapid petiole elongation for however long immersion persists.
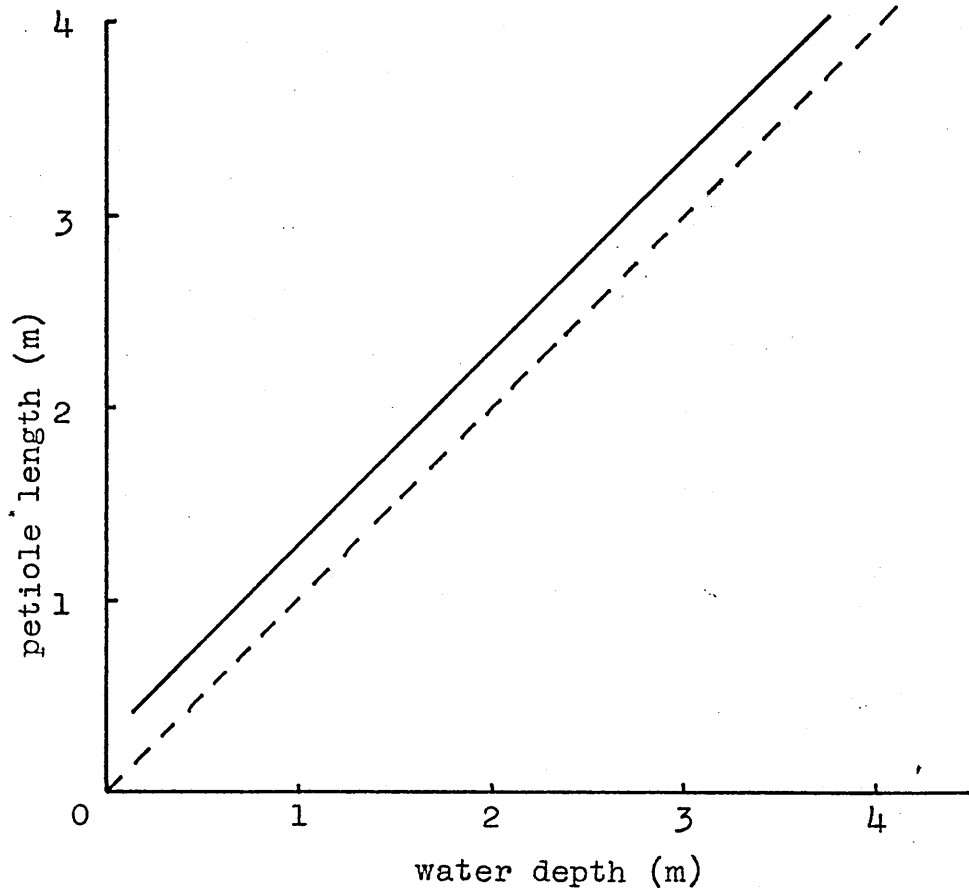
FIGURE 1 The relation between petiole length and water depth in
Nymphaea alba.

The dotted line shows the pattern if petiole length
precisely equalled water depth. The full line shows the
actual finding.

From Gessner 1959.

The possible mechanism by which plant organs recognise the submerged condition has been the subject of several ingenious proposals (see Arber 1920; Sculthorpe 1967). For example, Frank (1872) considered that pressure changes concomitant with submergence could be sensed by the plant. None of these proposals was, however, entirely satisfactory.

A solution was provided by Ku *et al.* (1970) who studied the submergence response in rice seedlings. They noted that non-submerged, untreated seedlings grown in a closed container, produced longer coleoptiles than similar seedlings grown in a stream of air. This suggested that a volatile product of the seedlings, if allowed to accumulate, could stimulate growth. These workers demonstrated that ethylene was a major volatile product of rice seedlings, and that exogenously added ethylene would markedly stimulate growth of the seedlings. It was concluded *(op. cit.)* that promotion of growth by ethylene gas is responsible for the submergence response *in vivo*, and that the system works because only during submergence do ethylene levels within the plant's air spaces rise significantly (see below).

Since 1970, several water plants besides rice have been reported to use an ethylene-dependent mechanism in their submergence response. These include:

Callitriche platycarpa	Musgrave *et al.* 1972
Ranunculus sceleratus	Musgrave and Walters 1973
Regnellidium diphyllum	Musgrave and Walters 1974
Sagittaria pygmaea	Suge and Kusanagi 1975
Potamogeton distinctus	Suge and Kusanagi 1975
Rorippa nasturtium-aquaticum	Cookson 1976
Hydrocharis morsus-ranae	Cookson 1976

It seems likely that ethylene accumulation and the growth response are closely linked in most or all plants with a similar depth-accommodating ability.

Submergence leads to high ethylene levels in organs of water plants because either:

1.   normal dissipation of endogenous ethylene, by diffusion from the tissue, is curtailed under submerged conditions due to the containing effect of the water; or

2.   submergence may impose a physical stress on the organs concerned, causing increased ethylene production by them and hence raising internal ethylene levels. Stresses are known to increase ethylene production in growing tissues of many land plants, for example, in the etiolated pea epicotyl (Saltveit and Dilley 1978) and it is conceivable that the stress of submergence would cause increased ethylene production in water plants. The resultant higher internal ethylene levels would persist for as long as the stress remained, i.e. until the leaf regains the surface.

For reasons stated below, the first of these possibilities now seems more likely:

1.   stresses appear to have no effect on ethylene production in several water plants, e.g. *Regnellidium diphyllum* (Walters and Osborne 1979), *Hydrocharis morsus-ranae* (Cookson 1976), and *Ranunculus sceleratus* (Cookson 1976). This absence of stress-induced ethylene is in complete contrast to the situation in many land plants. See, for example, Abeles (1973); Saltveit and Dilley (1978). Jaffe (1980) notes that mechanical stress retards growth in all of a large number of land plant species tested; since the link between mechanical stress and growth retardation has been shown to involve ethylene in all cases closely studied (see Jaffe 1973; Goeschl and Pratt 1968), one can assume that all

the plants tested by Jaffe (1980) show stress-induced ethylene. Stress-induced ethylene production in land plants is discussed by Yang and Pratt (1978). It should be noted that ethylene often inhibits extension in land-plant tissues, in contrast to its effect in water plants (Table 1).

2. besides the observed absence of stress-induced ethylene in water plants, the confining effect of water is such that ethylene accumulation would automatically occur under submerged conditions anyway, whether or not there was increased ethylene production. For example, Kawase (1976) concluded from ethylene measurements with *Helianthus* cuttings that ethylene accumulation during immersion was chiefly due to, "blockade of ethylene escape by water". Kawase also showed that even a very thin film of water, such as that provided by wet tissue paper, was sufficient to severely restrict escape of ethylene. In addition, Drew *et al.* (1979) indicate retardation of exodiffusion as the major cause of ethylene accumulation in flooded *Zea* roots, though they do not discount the possibility that oxygen deficiency (which may also occur in waterlogged roots) leads to increased ethylene production as well; this despite the finding of Kawase (1976) that ethylene production in submerged tissue of *Helianthus* was actually lower than in non-submerged controls.

Burg and Burg (1965a) note that the diffusion coefficient of ethylene in water at 20°C is about $1.68 \times 10^{-5} \text{ cm}^{-2} \text{ sec}^{-1}$, whereas that in air at the same temperature is about $0.15 \text{ cm}^{-2} \text{ sec}^{-1}$. This difference (almost 9000-fold) explains why ethylene cannot escape freely from the submerged plant.

An interesting additional factor may also contribute, in some plants, to the accumulation of ethylene under submerged conditions: Dacey (1981) showed that in *Nuphar luteum*, a water plant with floating leaves, there normally occurs during daylight, a through-flow of air from the atmosphere, into young leaves, down the petioles, through the rhizome, and out again

through older leaves. This "ventilation" is of appreciable magnitude with mass-flow rates exceeding 10 cm min^{-1}, and inevitably, it will tend to flush endogenous gases, including ethylene, out of the plant. Ventilation will be stopped if the leaves become submerged; because of this, the already marked difference in ethylene content between the submerged plant and that with floating leaves, will be accentuated, at least during the hours of daylight. Although ventilation of this type has not been reported from plants other than *Nuphar*, there is no obvious reason why it should not occur in plants of a similar habit, such as *Nymphoides*. Indeed, in the latter species, older leaves are easier to blow through than younger ones, indicating that younger leaves are more gas-tight; this is the chief requirement for ventilation (see Dacey 1981).

Thus there are firm grounds for supposing that accumulation of ethylene in submerged plants is automatic, being caused by reduced escape of the gas, rather than by production of stress-induced ethylene.

Since ethylene build-up appears to be an inevitable consequence of immersion, raised ethylene levels will serve as the indicator of submergence, as well as the trigger for any appropriate responses.

It is probable that in many situations besides depth accommodation, in which submergence is followed by a specific response, ethylene is the immediate effector (Jackson 1982). For example, development of cortical air spaces follows waterlogging of *Zea* roots, and ethylene has now been shown to be responsible for this (Drew *et al.* 1979). Extending the argument, it might be proposed that plants which habitually live entirely submerged, such as *Ranunculus fluitans* and *Potamogeton lucens*, either produce extremely low levels of ethylene so that accumulation does not occur, or they are constantly exposed to relatively high levels of the gas. In the latter case, they must be insensitive to ethylene, or their normal growth is ethylene-mediated.

In summary: endogenous ethylene will accumulate in submerged tissue. Ethylene-stimulated responses appear to account for depth accommodation and, possibly, for other responses to flooding in plants.

CHAPTER 2

E T H Y L E N E

CHAPTER 2

ETHYLENE

The recognition of ethylene as a plant growth regulator began with nineteenth century observations on the defoliating capacity of illuminating gas; trees situated near leaking mains were seen to shed their leaves prematurely. Ethylene was later shown to be the active constituent (Neljubow 1911, and see Abeles 1973). Today there is scarcely an aspect of plant growth and development in which a regulatory role for ethylene has not been demonstrated or inferred (Pratt and Goeschl 1969; Lieberman 1979).

Ethylene is produced by many pteridophytes, bryophytes, cyanophytes, bacteria, fungi, and by probably all tissues of higher plants (Osborne 1977; Considine and Patching 1975; Abeles 1973). Production rates are in the range 0.1-10 nl g (fr. wt)$^{-1}$ h^{-1} in many growing tissues, although this can vary markedly between species, between tissues, and with stage of development. Various external factors can also affect the rate of ethylene production (Abeles 1973; Osborne 1968, 1977).

There is no evidence that ethylene is a normal product of animal tissues, nor does it have any known effect on animal tissues when supplied at hormonal levels (i.e. about 1 µl l^{-1}). High levels of ethylene have anaesthetic properties in animals but preparations of 70-80% ethylene in oxygen are highly explosive and, according to Abeles (1973), ethylene was

abandoned as a hospital anaesthetic following several incidents in which patients or medical staff were killed. In view of the very different concentrations involved, the mode of ethylene action in animals is probably quite different to that in plants.

Synthesis of ethylene by plant tissues can raise internal concentrations of the gas to physiologically active levels; thus there can be no doubt that ethylene is a natural, endogenous growth regulator. There has been some discussion as to whether ethylene can be described as a "hormone" or not (e.g. Pratt and Goeschl 1969). By definition, this would require that the substance be produced at one site and transferred to another at which, in very small concentrations, it would exert some regulatory effect. Osborne (1968, 1977) argues that the term is appropriate, particularly in view of instances in which ethylene affects distinct "target cells", such as in some abscission zones (Wright and Osborne 1974). These arguments are interesting, but probably of little consequence: the concept of hormones was borrowed from another field of research, and whether ethylene (or the other plant growth regulators for that matter) happen to fit the description, seems unimportant (Lieberman 1979).

The boiling point of ethylene is -103°C, thus it is a gas under all physiological conditions. Ethylene is unique among growth regulators in this respect. With the possible exception of some of its structural analogues (such as acetylene) which are only 1% ($\frac{v}{v}$) as effective (Burg and Burg 1967), and some toxins (such as SO_2, Ferguson et $al.$ 1978; ozone, Ashmore et $al.$ 1978), ethylene is the only gas which, when present at hormonal levels, can affect plant growth.

Ethylene can be effective at extremely low levels: for example, concentrations of only 0.1 μl l^{-1} have marked effects in etiolated pea seedlings (Goeschl and Pratt 1968), and Zobel (1973) found that growth in

the "diageotropic" tomato mutant was completely normalised by as little as 5 nl 1^{-1} of ethylene. Crocker (1948, cited in Osborne 1968) describes curvature responses in African marigolds, to 1 nl 1^{-1} of the gas.

It is usually found that effects of ethylene are not increased by raising the level of the regulator beyond about 1-10 μl 1^{-1} and, perhaps in contrast to other plant growth hormones, even very high levels of ethylene may have little or no toxic effect (Miller *et al*. 1970, but see Loy and Pollard 1981). It should be noted that levels mentioned here are those applied to the atmosphere surrounding the treated tissue; the actual level inside the tissue will almost certainly be slightly higher, since it will consist not only of the applied level, but also of an extra quantity of endogenous ethylene (the precise amount of which depends on the rates of ethylene production in, and escape from, the tissue (Burg and Burg 1965a; Pratt and Goeschl 1968). Similarly, because a particular level of exogenous ethylene elicits a response, it is not necessarily correct to assume, as some workers appear to have done (e.g. Sacher *et al*. 1979), that the normal endogenous level is lower than that supplied. All that such results reveal is that the applied level plus an unknown endogenous level will induce the response, and that the endogenous level alone is insufficient.

The list of developmental processes in which ethylene is known to play a major regulatory role is long. Some examples are listed below:

Fruit ripening	Addicott 1970
Leaf abscission	Jackson and Osborne 1970
Flower senescence	Kende and Hanson 1977
Determination of flower sex in cucurbits	Byers *et al*. 1972
Retardation of cell division in some meristems	Burg 1973
Induction of seed germination	Negm and Smith 1978
Determination of cell expansion patterns	Lieberman and Kunishi 1972

Ethylene is involved in wound responses in many land plants (Yang and Pratt 1978) and in the closely allied "thigmomorphogenesis", i.e. response to mechanical stimuli (Jaffe 1980).

A persistent connection has emerged between some ethylene responses and geotropism; for example:

1. Among the three obvious responses of etiolated pea seedlings to ethylene is the "transverse geotropism" first noted as a characteristic response by Neljubov (see Goeschl and Pratt 1968). This involves the upper region of the seedling assuming a peculiar horizontal orientation.

2. In the "diageotropic" mutant of tomato, shoot growth is horizontal rather than vertical. However, the orientation of growth can be normalised by administering as little as 5 nl 1^{-1} of ethylene to the plant (Zobel 1973).

3. If seedlings of any of several species, including pea, are placed horizontally, their root begins to curve downwards (and the shoot upwards) within a few minutes. But if ethylene is present, geotropism will not occur, and the horizontal growth will be maintained (Burg and Burg 1966). Ethylene does not prevent geotropism in coleoptiles of the monocotyledonous plants *Avena* and *Zea*, and probably monocotyledons in general are less sensitive than dicotyledons in this respect.

4. Inhibitors of ethylene synthesis or action, especially the rhizobitoxine analogue AVG (aminoethoxyvinylglycine), markedly delay the geotropic response in horizontally-placed mature stems of several species of dicotyledon (Wheeler and Salisbury 1980; Salisbury and Wheeler 1981); also, unilateral application of ethephon (a compound which decomposes at the pH prevailing in the cytoplasm, to release ethylene, de Wilde 1971; Warner and Leopold 1967) can induce curvature under some conditions (Wheeler and Salisbury 1980). It is speculated that ethylene plays a very important

role in the mechanism of geotropic (or "gravitropic") bending in stems
(see Wheeler and Salisbury 1981).

In none of these examples does ethylene simply abolish geotropic
sensitivity, rather it reorientates the response. The plants are obviously
still "aware" of gravity otherwise the orientation of growth would be random
which is not the case. Ethylene appears to be an aid to normal geotropism,
essential in the case of the diageotropic mutant, but possibly only
expediting curvature in many stems. Precisely how important ethylene is
in geo-, and perhaps other tropisms and how its role here relates to other
ethylene-dependent growth phenomena, is not known, but the above examples
provide further confirmation of the total involvement of ethylene in plant
growth control.

In many cases in land plants, the effects of ethylene entail inhibi-
tion of extension, or acceleration of destructive processes such as
abscission and senescence. Thus ethylene is often thought of as being an
"inhibitory" hormone (e.g. Galston and Davies 1969; Jones 1980). However,
with the discovery in 1970 of ethylene-promoted growth (i.e. in the
submergence response) it became clear that, as with other plant growth
regulators, the nature of the effect varies depending on the tissue and
circumstances, so that the adjectives "inhibitory" or "promotory" cannot
be generally applied (see Miller *et al.* 1970). Although ethylene effects
on growth in land plants are indeed often inhibitory, several cases of
promotion have been reported. The major examples are listed below:

i.   ethylene promotes growth in oat mesocotyl (but not in the coleoptile)
(Suge 1971);

ii. ethylene-induced epinasty in leaves and flowers results from increased
growth of cells on the adaxial surface of the petiole or petal (Palmer 1972);

iii. ethylene can promote some elongation in fruit peduncle cells of
Ecballium elaterium (Jackson *et al.* 1972);

iv. ethylene will stimulate active radial growth in parenchyma cells of the stelar region of the stem of young bean plants (Poovaiah 1974). This effect is similar in some ways to the reorientation of cell growth which often takes place following ethylene treatment of dicotyledonous seedlings, however, in the bean stem, the increase in radial cell growth is particularly marked (i.e. 2-6 fold) and it would seem to be more than a simple reorientation of growth, in which cell volume would be virtually unchanged (Osborne 1974);

v. ethylene was shown by Poovaiah and Leopold (1973) to stimulate stem elongation in the grass *Poa pratensis*, by up to 20-fold over a period of six weeks;

vi. ethylene can promote growth in cells of the leaf abscission zone of, e.g. *Phaseolus vulgaris* (Osborne 1977);

vii. in some fruits with a biphasic pattern of enlargement, ethylene may promote cell growth during the later phase of enlargement (Osborne 1977), though it is inhibitory during the first phase;

viii. ethylene probably promotes asymmetric shoot extension when it causes hook closure in etiolated pea hypocotyl, and when it stimulates horizontal nutation of stems and plageotropism in roots (Burg *et al.* 1971);

ix. in the fern *Onoclea sensibilis*, the gametophyte stage shows ethylene-enhanced cell elongation (Miller *et al.* 1970); it has been suggested that this plant is similar to the water plants in that it inhabits very damp or "quasi-aquatic" situations (Walters and Osborne 1979);

x. although ethylene alone inhibits extension in *Avena* coleoptiles, an ethylene pretreatment of 24 h may increase auxin-induced growth (Marinos 1960). Similarly, Michener (1938, cited in Abeles 1973) reported that ethylene could increase the responses of pea and oat seedlings to auxin, and Burg *et al.* (1971) note that the duration of auxin-induced growth pea and oat seedlings is prolonged by ethylene.

xi. the presence of ethylene appears to be a prerequisite for benzyladenine-stimulated hypocotyl elongation in a dwarf strain of watermelon (*Citrullus lanatus*); thus, under selected conditions (i.e. in BA-treated tissue which has been pretreated with AVG to eliminate endogenous ethylene), ethylene addition is followed by marked elongation (Loy and Pollard 1981).

Unlike the situation in water plants, these instances of ethylene-promoted growth appear to have little in common with each other in terms of their occurrence in response to environmental variables.

In summary: ethylene is an endogenous plant growth regulator. Its effects on growth are often inhibitory, but in the water plants, and in a significant number of land plant tissues, ethylene actually promotes growth.

Interactions between ethylene and auxin.

A characteristic of ethylene is that its effects often depend on interactions with other growth regulators, notably auxin.

1. Effect of auxin on ethylene production. The rate of ethylene production in many tissues may be regulated by the amount of auxin present; for example, Burg ang Burg (1968) observed a correlation between endogenous auxin levels and ethylene production in pea tissue. Further, addition of auxin often results in a surge of ethylene production from plant tissues: in young cotton plants sprayed with 2,4-D or IAA (both are auxins) ethylene production is five-fold greater than in unsprayed controls (Morgan and Hall 1964); and in etiolated pea stem sections, ethylene production is clearly related to the applied auxin level (Burg and Burg 1966).

Time course studies have shown that the lag time between addition of auxin and increase in ethylene production, may be as little as 20 minutes

(Franklin and Morgan 1978). Auxin-induced ethylene has been detected in many different tissues, including roots, stems. flowers, fruits and leaves (Burg and Burg 1968; Abeles 1973).

There appears to be little or no relation between auxin levels and ethylene production in the water plants, as is the case in some ripe fruit and senescent tissue. In the latter, it may be that membrane degeneration has proceeded to such an extent that normal regulation of processes such as ethylene production is no longer possible (Roberts and Osborne 1981). Membrane degeneration cannot explain the detachment of ethylene production from auxin levels in water plants, since even mature, healthy leaves of *Regnellidium* and *Hydrocharis* show no auxin-induced ethylene (Cookson 1976). In *Ranunculus sceleratus* however, there may be some induction of ethylene by auxin (Cookson 1976).

Because auxin commonly stimulates ethylene production in tissues of land plants, it has been postulated that some responses to added auxin may, in fact, by caused by auxin-induced ethylene. For example, Chadwick and Burg (1967) proposed that inhibition of pea-root growth, by auxin, was actually a response to ethylene. They showed that auxin would promote ethylene production in the tissue, and that ethylene alone would inhibit root growth. In addition, when ethylene was present, application of low levels of auxin did not cause any further inhibition of growth. Finally, CO_2 - an inhibitor of ethylene action in many plants - decreased the inhibition of root growth by auxin, again implying a role of ethylene. There is however, some doubt as to whether the kinetics of auxin-induced ethylene production match those of auxin-induced root inhibition (Andraea *et al.* 1968, cited in Pratt and Goeschl 1969; Rauser and Horton 1975).

A mediating role for auxin-induced ethylene has also been postulated in the following cases:

inhibition of bud growth;

promotion of flowering in pineapples;

inhibition of cell division in some meristems;

inhibition of hypocotyl hook opening in pea;

See Burg 1973; Pratt and Goeschl 1969; Kang *et al.* 1971.

Auxin-induced ethylene is more pronounced in dicotyledonous seedlings than in monocotyledons, and this has been suggested as a possible basis for the greater sensitivity of the former to auxin-based herbicides (Burg and Burg 1968).

There have been no suggestions that growth promotion by auxin in land plants, involves ethylene as an intermediate messenger.

2. **Antagonism.** Auxin and ethylene often have antagonistic effects on land plant tissues. For example, in tissues of many stems and coleoptiles, auxin can promote extension, whereas ethylene inhibits it (Osborne 1974). Similarly, ethylene often promotes leaf senescence and abscission, whereas auxin inhibits them (Horton and Osborne 1967).

It is also observed that auxin can "shield" tissues against the effects of ethylene: i.e. in the presence of auxin, ethylene is much less active (Sargent *et al.* 1974). This may explain why their effects are sometimes apparently opposite, such as, possibly in abscission (Osborne 1968).

The observed pattern of growth and development in some tissues may depend on an endogenous balance between the levels of auxin and ethylene (Osborne 1976, 1975). This balance can be upset by factors such as wounding (which increase ethylene production), and can thus provide an essentially complete growth-regulatory system. Such a system may operate in the geotropic bending response of some grass nodes: when a flowering

stalk of *Echinocloa colonum* is placed horizontally, lateral redistribution of auxin in tissues of the node is observed. Thus there may be a gradient of auxin in the node, with highest levels on the lowermost side. Auxin-induced ethylene production will occur especially in the lower parts of the node (where auxin levels are highest) but because ethylene is freely diffusible, there will be only a slight gradient of this regulator across the stem. Because the lower part of the node has a relatively large amount of auxin, it will be shielded against the effects of ethylene, and will tend to show an auxin-type response, i.e. rapid extension. The upper side in contrast, because it does not have high auxin levels to buffer it against the ethylene, will show an ethylene-type response (i.e. swelling rather than elongation). Upward bending will result (Osborne 1975).

Further work on this system suggests that ethylene production patterns may be secondary, rather than causal, to bending (Wright *et al.* 1978). Nevertheless, this example illustrates how auxin and ethylene could interact to control plant growth.

Another example can be seen from the report of Sargent *et al.* (1974) who studied the pattern of growth in epidermal cells of intact, etiolated pea shoots. Ethylene alone caused an increase in wall thickness, with little change in cell volume. The response to added auxin was biphasic; initially, elongation was promoted, and there was an accompanying decrease in wall thickness; but after some 12 h, growth rate diminished and the wall began to thicken. Sargent *et al.* (op. cit.) interpret the first phase of this response as being a straightforward auxin-induced growth with, in the second phase, auxin-induced ethylene becoming the dominant factor. The transition occurs because although production of ethylene is induced soon after auxin application, it does not affect growth until later. This is because initially, the cells are buffered against ethylene by high auxin levels. The auxin levels decrease to a fairly low value by about 9 h after auxin application, but auxin-induced ethylene persists for much

longer, so that from 9 h or so, the cells will be responding primarily to ethylene. An interesting discussion of these and other possible cases of growth control through dual regulation by auxin and ethylene, is given by Osborne (1976).

Although the best known interactions of ethylene are with auxin, there are reports of antagonism between ethylene and other plant growth regulators. Since the work presented in this thesis pertains chiefly to growth effects of, and interactions between, auxin and ethylene, only brief examples of ethylene's interaction with other regulators are given:

i. GA and ethylene were found to have antagonistic effects on the later stages of cell growth in the pea subhook region: GA stimulates extension, whereas ethylene promotes swelling (Stewart *et al.* 1974).

ii. both onset of rib-rolling, and the surge in ethylene production in senescing flowers of *Ipomoea tricolor* were inhibited by the cytokinin, benzyladenine. As already noted, ethylene promotes rib-rolling in this tissue (Kende and Hanson 1976).

iii. Goren *et al.* (1979) showed that the ABA-promotion of callus development in *Citrus sinensis* bud culture was a secondary consequence of ABA-induced ethylene production.

3. <u>Dependence</u>. Development of some ethylene effects is contingent upon auxin being available: i.e. although the ethylene effect in a given tissue may be different from any auxin effect in that tissue, ethylene alone is incapable of producing the response. Further confirmation of the very close interaction which may occur between auxin and ethylene is seen in the phenomenon of dependence.

In the response of several of the semi-aquatic plants to ethylene, complete dependence on the presence of auxin has been shown (Walters and

Osborne 1979; Samarakoon *et al.* 1980; Imaseki and Pjon 1970). Dependence may be characteristic in water plants (see page 125). In land plants, dependence is apparently quite rare; however, ethylene-stimulated peduncle elongation in *Ecballium elaterium* cannot occur in the absence of some factor - possibly auxin - supplied by the fruit (Jackson *et al.* (1972). Also, as noted by Ridge and Osborne (1969) and Sargent *et al.* (1973), ethylene will induce swelling in pea epicotyls only if some factor supplied by the tip - again probably auxin - is present.

A few other cases of dependence besides that of ethylene on auxin are known. Some dependence of ethylene on the presence of CO_2 was reported when ethylene stimulated seed germination in osmotically-inhibited lettuce seeds (Negm and Smith 1978). There are scattered reports of dependence among other growth regulators e.g. Kefford 1962; Stuart and Jones 1977; Loy and Pollard 1981.

The existence of dependence may provide an insight into the mechanism of action of a growth regulator. For example, Masuda (1965) and Yanagishima (1965) (both cited by Shibaoka 1972) note no effect of GA in jerusalem artichoke and yeast cells (respectively) unless auxin was present. They proposed that GA "prepares" the tissue for a subsequent auxin action. How this might occur is not known.

Shibaoka (1972) reports dependence of a GA effect on the presence of auxin: i.e. GA promotes growth in azuki bean epicotyl only if IAA is present. This effect might be explained by postulating that GA simply inhibits auxin-induced ethylene production, or antagonises ethylene action: when auxin alone is applied to bean segments, it induces rapid extension but this eventually slows and is replaced by lateral expansion. When GA is present however, auxin-induced rapid-growth is maintained for much longer, and there is no development of lateral expansion. By

comparison with the findings of Sargent *et al.* (1974) (see page 22), it is probable that the second phase of growth (involving swelling) is mediated by auxin-induced ethylene. Any factor (such as GA) which inhibits auxin-induced ethylene production would delay onset of the second phase, and prolong the growth seen with auxin.

It is certain that more such cases of dependence in land plants await discovery, including further instances of dependence of ethylene on auxin. It is also possible that in the majority of cases, ethylene effects in land plants are dependent on the presence of auxin, as appears to be the case in the water plants.

Putative mechanisms of ethylene-induced growth effects in land plants.

Postulated mechanisms of ethylene-induced effects on growth in land plants deserve consideration since one or more may provide some indication of how ethylene operates in water plants. The major possibilities, together with their chief problems, are outlined below:

1. <u>Ethylene effects on auxin levels</u>. Because of the intimate interaction observed between ethylene and the very important growth regulator, auxin, it has been proposed that ethylene might affect growth indirectly, by modifying auxin levels. This could involve changes in auxin synthesis, transport, or breakdown. These possibilities are attractive in that, as well as accounting for the mechanism of the ethylene effect, they could also explain the commonly-observed dependence of ethylene on the presence of auxin (see page 23).

Various workers have demonstrated effects of ethylene on auxin transport (Morgan *et al.* 1968; Burg and Burg 1966; Osborne and Mullins 1969; Riov and Goren 1979) and metabolism (Morgan *et al.* 1968; Beyer and

Morgan 1970a; Riov 1982) but, in general, the kinetics and magnitude of the changes are not consistent with their being of major importance in the mechanism of the ethylene effect, especially where rapid changes are involved, such as ethylene-induced growth inhibition in pea (Warner and Leopold 1971) and in *Hordeum* (Hall *et al.* 1977).

2.   Glycoproteins.   Hydroxyproline-rich protein is of near universal occurrence in plant cell walls, moreso even than cellulose (Miller *et al.* 1974; Thompson and Preston 1967).   There is reason to believe that chemical binding of this component to other wall constituents plays a role in controlling extensibility of the cell wall which, in turn, determines growth rate.   (The connection between WE and growth rate is outlined and discussed in Chapter 3.)   Inverse correlations have been drawn between hydroxyproline content and wall extensibility (Cleland and Karlsnes 1967; Jotterand-Dolivo and Pilet 1975; Sadava *et al.* 1973; Holst *et al.* 1980).

Ethylene has been shown to cause marked increases in the levels of wall-bound hydroxyproline when it inhibits extension of pea epicotyls (Ridge and Osborne 1970; Nee *et al.* 1978); these two ethylene effects are reduced, in parallel, by α, α dipiridyl (Sadava and Chrispeels 1973), suggesting a close relationship between them.   On the other hand, some workers were unable to identify close correlations between growth rate and hydroxyproline levels (e.g. Winter *et al.* 1971, cited by Osborne 1976), and Eisinger and Burg (1972) could detect no promotion by ethylene, of hydroxyproline incorporation into wall protein in peas.   Further, given the kinetics of ethylene effects on growth, it seems unlikely that significant changes in hydroxyproline levels could occur during the time elapsing between ethylene addition and the inhibition of growth, which is less than 20 minutes (Warner and Leopold 1971; Nee *et al.* 1978; Hall *et al.* 1977).

3. __Microfibrils.__ The primary cell wall includes long cellulose micro-fibrils. Mechanical considerations lead to the belief that orientation of these will control the direction of cell expansion (this subject is considered in more detail in Chapter 5); consistent with this belief are observations that agents which alter the direction of cell expansion (inducing for example, swelling rather than extension) are also observed to alter the net orientation of wall microfibrils (Probine 1965; Burg and Burg 1968; Eisinger and Burg 1972; Burg *et al.* 1971). It has been suggested that ethylene-induced swelling in peas is caused by such a shift in the orientation of deposition of wall microfibrils, from predominantly transverse (with respect to the long axis of the cell), to longitudinal (Ridge 1973; Burg 1973). This is a very appealing hypothesis, but lateral swelling in this tissue is reported to begin within 15 minutes of exposure to ethylene (Eisinger *et al.* 1979). The re-orientation of microfibrils however, is not detected prior to 3 (-18) h from ethylene addition. In view of this, one cannot be certain that the latter causes the former.

4. __Acid-growth.__ Auxin and other regulators may control plant growth by regulating proton extrusion and cell wall pH (this is the "acid-growth" theory; see page 50). Very few studies of ethylene and acid growth in land plants have been made; in one such study, ethylene inhibited auxin-, and FC-, induced growth in pea tissue, but it had no effect on the H^+-excretion elicited by these compounds (De Michelis and Lado 1974).

Summary: The mechanism of ethylene-induced effects on growth in land plants is not known. Various postulations are poorly supported by evidence, while others are not relevant to the water plants since they are mechanisms of growth inhibition which cannot be inverted to cover situations of growth promotion (such as No. 3 above). No mechanisms for ethylene-promoted growth in land plants have been advanced. Some of the above possibilities

were considered further in relation to promotion of growth by ethylene.
(See Chapter 10).

Detection and Reception of ethylene.

Although very direct modes of ethylene action have been considered
(such as direct enzyme activation, Abeles 1973), it seems likely, by
analogy with other plant growth regulators and with animal hormones and
neurotransmitters, that the first step in a response to ethylene will be
some interaction at a specific "receptor".

Requirements of "receptors" are outlined by Rubery (1981). An
ethylene receptor should reversibly bind molecules of the gas with high
affinity, specificity and speed. There may also be some relationship
between the parameters of ethylene binding and the dose-response pattern
for biological activity in the same tissue.

Some other gases have a degree of ethylene-like activity (e.g.
acetylene and propylene); molecules of such compounds share several common
features with each other and with ethylene (Burg and Burg 1967). These
are:

1. an unsaturated bond adjacent to the terminal carbon atom;

2. low molecular weight;

3. few substitutions which lower electron density at the unsaturated
position.

In addition, Sisler (1977, cited in Sisler 1979) notes that all compounds
with ethylene-like activity are so called "π acceptors".

These characteristics are identical with those required for stability of olefin-silver complexes. Thus the receptor site probably contains a metal, possibly zinc (Burg and Burg 1967) or copper (Beyer and Blomstrom 1981).

Specific binding of ethylene has been demonstrated in *Phaseolus* cotyledons (Jerie *et al.* 1979; Bengochea *et al.* 1980a) and tobacco leaves (Sisler 1979). There is reason to believe that these binding systems are the postulated receptors involved in biological activity: for example, for a number of compounds (such as propylene and acetylene), capacity to compete with ethylene for the binding site correlates well with ability to induce ethylene-like physiological responses (Bengochea *et al.* 1980b).

The nature of the interaction between ethylene and the receptor site is not known. An ionic reaction seems unlikely in view of the finding that there is no exchange of H when deuterated ethylene (C_2D_4) interacts at the receptor (Beyer 1972). Despite several earlier reports, careful work by Beyer (1975a, b) has demonstrated incorporation (to ethylene glycol) and metabolism (to CO_2) of minute quantities of ethylene in tissue of pea seedlings. Similar incorporation and metabolism has since been found in several other tissues (such as flowers of *Ipomoea tricolor*, Beyer and Sundin 1978; cut carnation flowers, Beyer 1977; cotton and bean abscission explants, Beyer 1975c, cited in Beyer 1979; tomato fruit, Beyer and Blomstrom 1980).

Although known metabolites of ethylene are not super-efficient at inducing physiological responses (possibly with one exception; see Beyer and Blomstrom 1981), a connection between the metabolising system and the physiological receptor is strongly suggested by findings that the two are inhibited in parallel by substances such as silver ions (Beyer 1979). It is much less probable that the function of this metabolising system is to remove or deactivate the hormone. Because ethylene is volatile, it is

normally ephemeral in the plant anyway. Besides this, Beyer and Sundin (1978) note that the metabolic system in morning glory flowers is of such small capacity that no more than 0.5% of the ethylene synthesised in that tissue could be removed by this system.

A different and, as far as reported, unique metabolising system occurs in cotyledons of *Vicia faba* (Jerie and Hall 1978). In this case, ethylene is converted to ethylene oxide. The relationship, if any, between the system in *Vicia*, and that in other tissues is not clear, but the activity of the *Vicia* system is much greater than that of the other system, and it could have some hormone-deactivating function.

A link between ethylene action and metabolism seems very likely but the metabolism may occur at one or more biochemical steps remote from the initial receptor. This is because the binding, which is presumably activity of the immediate receptor, is displaceable (Sisler 1979) and may therefore involve a looser association than is implied by the covalent bonding involved in incorporation. Perhaps metabolism occurs when the receptor triggers a pathway leading to ethylene action.

Summary: Putative receptors of ethylene are known, as binding activity in whole tissue, and in cell-free extracts. The binding characteristics in many of these are close to those expected for the actual receptor. Incorporation of ethylene may occur following (or during) binding at the receptor. There are at least two different ethylene metabolising systems among the higher plants.

It is expected that the receptor for ethylene will be the same in water plants as it is in land plants. This is because the dose-response pattern is similar for most ethylene effects, including promotion of growth in water plants. Also, since the water plants considered here are thought to be comparatively recently evolved from land-dwelling ancestors, it seems unlikely that a completely new receptor complex has been adopted.

Further, Ag$^+$ can inhibit ethylene action in both land and water plants (Cookson 1976).

I consider it unlikely that a rapid metabolising system of the type present in *Vicia faba* cotyledons occurs in water plants. This is because: a) the appearance of large quantities of ethylene oxide in the air above water plant tissue would certainly not have gone unnoticed in the many studies of ethylene production which have been done, and which involve incubation of tissue in a confined space. (This argument requires that ethylene oxide give a visible peak when gas-chromatographed on the more commonly used "alumina" column, as opposed to the "poropak" column used by Jerie *et al.* (loc cit.). I see no reason why it should not; b) the progressive removal, by metabolism, of large quantities of ethylene from experiments involving treatment with the gas in closed containers, would likewise not go unnoticed.

Metabolism of the type found in peas may occur in water plants.

Ethylene biosynthesis.

Methionine is thought to be the precursor of ethylene in higher plant tissues (Adams and Yang 1981). There is one reported exception (Cookson 1976, see below) and in some other cases, a different precursor is thought possible (Lieberman 1979).

Carbon atoms 3 and 4 of the methionine molecule will form ethylene. The biosynthetic pathway probably involves the following steps (see Adams and Yang 1981):

methionine ⟶ S-adenosyl methionine ⟶ Aminocyclopropane ⟶ ethylene
                     (SAM)   -1-carboxylic acid
                                                     (ACC)
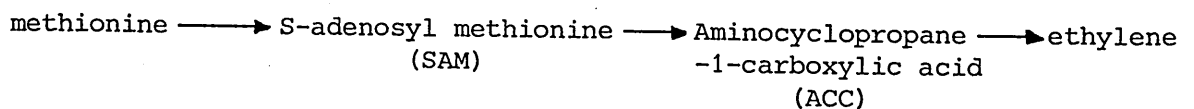
Anaerobic conditions, and some other inhibitory factors prevent conversion of ACC to ethylene. However, it appears that much of the regulation of

ethylene production occurs at the SAM-ACC stage: for example, both auxin and wounding can promote activity of the enzyme ACC-synthase (Yoshii *et al.* 1980; Yu *et al.* 1979; Boller and Kende 1980). Since availability of ACC is normally a limiting factor, greater ethylene production results from stimulation of this enzyme.

Aminoethoxyvinylglycine (AVG) is a potent inhibitor of ethylene synthesis (Lieberman *et al.* 1975, cited by Adams and Yang 1981). Boller *et al.* (1979) have shown that AVG inhibits ACC-synthase activity, so that this compound too appears to act at the SAM-ACC step.

The cellular location of ethylene-synthesising components has been investigated by a number of workers; subcellular fractions which synthesise ethylene have not been found (Anderson *et al.* 1979). Earlier reports that an intact cell wall was required for ethylene synthesis (e.g. Mattoo and Lieberman 1977) have been disproved.

The single exception in which methionine is apparently not a precursor of ethylene (noted on the previous page) is *Regnellidium diphyllum* (Cookson 1976). *Regnellidium* is a semi-aquatic plant with marked ethylene-promoted growth in the petiole. It is certain that *Regnellidium* is not representative of water plants as a whole, in this respect, since Cookson (1976) showed that in the water plants *Hydrocharis morsus-ranae* and *Ranunculus sceleratus*, labelled ethylene was produced following application of ^{14}C-labelled methionine. Consistent with the proposal that *Regnellidium* has a unique biosynthetic pathway, is the observation that whereas AVG inhibits ethylene production in *Ranunculus* and *Hydrocharis*, it has no effect in *Regnellidium* (Cookson 1976). A different explanation for this enigma is considered on page 283.

Summary: biosynthesis of ethylene in water plants is similar to that in land plants, with the possible exception of *Regnellidium*.

It is likely that many of the ideas and results from ethylene studies in land plants will be helpful in interpreting the position in water plants. However, it must be remembered that there are ways in which ethylene relations in water plants differ markedly to those in land plants; some of these are summarised in Table 1.

Feature	Water Plants	Land Plants
Auxin-induced ethylene	–	+
High ethylene production from senescent tissue	–	+
Wound-induced ethylene	–	+
Ethylene-promoted growth	+	+/–
CO_2 inhibits ethylene effects	–	+
Dependence of ethylene on auxin	+	+/–

TABLE 1 Generalised differences between land and water plants with respect to ethylene physiology.

CHAPTER 3

T H E   N A T U R E   O F   P L A N T   G R O W T H

CHAPTER 3

THE NATURE OF PLANT GROWTH

From Chapter 1 it should be evident that depth accommodation depends,
in those water plants so far studied, on enhanced growth of the petiole
(or other supporting structure) in the presence of ethylene. The next step
is to investigate how ethylene promotes growth. The present chapter
considers the nature of plant growth and the manner in which growth could
be controlled; the way should then be clear for a critical assessment of
how ethylene promotes growth in petioles of *Nymphoides* and other water
plants.

Growth, here defined as irreversible increase in volume, occurs in
plant organs mainly by entry of water into cell vacuoles, with accompanying
cell enlargement.

Cell division may also occur in elongating organs, but division alone,
without cell enlargement, will not contribute to growth. If there are
constraints on the degree to which individual cells may expand before
dividing, then cell division could become an important limiting factor in
long term growth responses. Where growth promotion by auxin and ethylene
in petioles of water plants is concerned, the kinetics are so rapid as to
exclude any requirement for cell division (see page 125).. In addition,
virtually all measurements so far reported on cell numbers in elongating
organs of water plants indicate no involvement of cell division: i.e. the

number of cells present remains roughly constant even where an organ increases in length by up to sixty-fold (Musgrave *et al.* 1972; Musgrave and Walters 1974; Cookson 1976).

Although localised wall growth is known from a small number of special cases (e.g. "tip growth" in pollen tubes) cell enlargement normally involves uniform expansion of the entire cell wall, or at least all that lying in the extending axis (Green 1954; Wardrop 1955, cited in Preston 1974).

The influx of water which drives growth is thought to occur passively, in accordance with gradients of the chemical potential of water. Steady-state growth of any cell can be described as in equation 1:

$$dV/dt = Lp \, \phi \, (\psi_o - \psi_{s,i} - Y)/(Lp + \phi) \quad \ldots\ldots \text{ equation 1}$$

where

dV/dt is growth rate,

Lp is hydraulic conductivity,

ϕ is wall extensibility,

ψ_o is water potential of the external solution,

$\psi_{s,i}$ is the osmotic potential inside the cell,

Y is the wall yield stress.

(Lockhart 1965; Ray *et al.* 1972).

A slightly modified version of equation 1, incorporating the reflection coefficient, has also been used (Cosgrove and Green 1981). It is not particularly pertinent to this discussion and will not be considered further.

From equation 1, it can be seen that any increase in growth rate must involve one or more of the following:

1.   increase in ψ_o

2.   increase in Lp

3.   decrease in $\psi_{s,i}$

4.   decrease in Y

5.   increase in ϕ

Many attempts have been made to discover which of these five parameters is affected when growth rate is altered by growth regulators, especially auxin. Taking these components in turn:

1. ψ_o; the source water potential. This is normally beyond the control of the plant and, although low ψ_o may severely limit growth, there is little that plants can do to alter it. Features such as the rapidly-growing root system found in many land plants may have the effect of keeping ψ_o up. For non-marine aquatic plants, the environment is literally awash with water, and ψ_o will not normally fall much below its maximum possible value. Growth promoters are not considered to exert their effect via this component.

2.   Lp; the hydraulic conductivity of the route of water flow from source to growing cell. The possible role of this component has attracted much debate in recent years. It has often been assumed that Lp is large compared to ϕ (Burstrom 1971; Cleland 1977) in which case, the bottom line of equation 1 (Lp + ϕ) approximates to Lp, and the Lp terms can be cancelled from equation 1, to produce:

$$dV/dt = \phi \ (\psi_o - \psi_{s,i} - Y) \qquad \ldots \ldots \text{ equation 2}$$

In addition, when Lp is large, cell turgor pressure (P) equates to $\psi_o - \psi_{s,i}$ and equation 2 can be expressed as:

$$dV/dt = \phi \ (P - Y) \qquad \ldots \ldots \text{ equation 3}$$

See Ray *et al.* 1972.

Equations 2 and 3 are applicable only if Lp is relatively large. However, in many intact land plants the path of water flow from the solution surrounding the root to cells of apical tissues, is of considerable length. It includes several regions of relatively high resistance to flow, such as the root endodermis and, especially, the interval between the end of the xylem and the growing cell (Boyer and Wu 1978, cited in Molz and Boyer 1978). Thus Lp probably imposes some restriction on growth rate in some tissues of intact plants, particularly rapidly-growing apical shoot tissues of tall plants. In such tissues, a gradient of water potential ($\Delta\psi$) will be required to drive water across these resistances thus, although water potential of the intracellular solution (ψ_i) tends towards that of the extracellular (ψ_o), in practice the latter will always be somewhat greater.

With short segments floating on water (as used in many growth experiments, including my own) Lp will be high, and it is less likely to be a major limiting factor (Green and Cummins 1974; Uhrström 1974; Green *et al.* 1977).

In *Nitella*, Lp is apparently not limiting to growth rate, even when elongation surpasses the normal rate by five-fold (Green 1968) thus equation 3 is applicable to this system. However, *Nitella* is a "giant" alga, and Green's work used single cells for which the flow path is certain to be much shorter than the mean flow path to cells in a segment. There are reports which suggest that Lp, although large, can be limiting even in floating segments, especially if growth is rapid (Cosgrove and Steudle 1981; Molz and Boyer 1978). Thus equation 3 may not be generally applicable even to segments.

Whether Lp can seriously limit growth or not, is of less relevance here, than the question of whether growth-promoting agents such as auxin, affect growth rate by altering Lp, or whether they affect one of the other

components of equation 1. Dowler *et al.* (1974), working from three separate laboratories, could detect no auxin enhancement of water permeability of pea segments, despite an earlier report to the contrary (Kang and Burg 1971). Since auxin does promote growth in this tissue, Dowler *et al.* (1974) concluded that effects on Lp "were not of special significance in relation to the mechanism of auxin action". Similarly, Van Volkenburgh and Cleland (1981) concluded that light-induced increases in growth rate of leaf cells did not depend on a shift in Lp. On the other hand, Boyer and Wu (1976, 1978) calculated that auxin increases Lp when it promotes growth in soybean hypocotyls.

A shift in Lp could be involved in increasing growth rate of cells which are already growing, but if one allows that Lp can never approach zero, then a shift in Lp can not be responsible for induction of growth in previously non-growing cells. In the latter, ϕ or (more likely) $\Delta\psi$ must be zero anyway, so that whatever value Lp assumes, dV/dt will remain at zero. Promotion of growth in such cases *must* involve change in a component other than Lp. In most cases, promotion of growth by auxin appears to occur in tissues in which the endogenous growth rate is not negligible (e.g. see Figure 12.i of Penny *et al.* 1978; Figure 2 of Cleland 1977; Table 5 of Lado *et al.* 1972). Thus this criterion for excluding an involvement of Lp, is not applicable. However, in some cases of auxin-promoted growth (e.g. Philipson *et al.* 1973a; and see page 176), and with fusicoccin (FC)-promoted growth in squash cotyledons (Marrè *et al.* 1974), the endogenous growth rate is virtually nil and, in these instances, the promotions can be regarded as being induction of new growth rather than a multiplication of old. These cannot reasonably be explained by an effect on Lp.

Lp affects growth rate by restricting water entry; in effect keeping turgor pressure lower than it "should" be (i.e. lower than it would be

if the system was at equilibrium, with $\psi_o = \psi_i$). If a shift in Lp is responsible for any growth-promotion, then a rise in cell turgor will automatically occur with the onset of that promotion. In a similar manner, a rise in ψ_o would also be associated with a rise in turgor pressure. Indeed, it is not surprising that changes in ψ_o have the same effect here as changes in Lp, since Lp and ψ_o are interrelated to some extent, depending on where ψ_o is measured, i.e. on what is taken as the "source water". For example, for cells in an intact plant, one would normally measure ψ_o in the solution surrounding the root. But one could measure ψ_o, for a given cell, in the solution immediately adjacent to that cell. In the latter case, a change in the value of Lp of the pathway of water flow between root and shoot would be seen as a change in ψ_o of the solution adjacent to the measured cell.

Conversely, if turgor increase is not associated with a particular growth promotion, changed Lp cannot be the cause of that promotion. There are a few reports showing measurement of P during a change in growth rate, e.g. that of Cosgrove and Steudle (1981) who monitored P by the resonance-frequency method, during light-induced inhibition of growth in cucumber and sunflower seedlings. From the above argument, if light affects growth rate via Lp, there should be a decrease in P as the inhibition occurs. In fact, Cosgrove and Steudle found precisely the opposite of this; P increased as growth was inhibited, indicating that the primary effect of light was not on Lp. They presented other evidence to confirm that light did not affect Lp when it inhibited growth. These and other considerations lead most workers to agree that Lp is not the component through which growth regulators normally act.

3. $\psi_{s,i}$; the intracellular osmotic potential ($= -1$ [osmotic pressure]). This is a measure of the solute concentration inside the cell. Turgor pressure provides the stress to extend the wall, and is an absolute require-ment for growth. Other factors being constant, $\psi_{s,i}$ controls turgor. In

a non-growing cell, water potential is the same inside and out. In this situation, turgor pressure equals $\Delta\psi_s$, i.e. the difference between the osmotic potentials of the solutions inside ($\psi_{s,i}$) and outside ($\psi_{s,o}$) the cell. If the cell is in pure water at atmospheric pressure, $\psi_{s,o}$ is zero, and $P = -\psi_{s,i}$. This also applies for growing cells to which equation 3 holds (where Lp is very large compared to ϕ). In other growing cells (those in which Lp is not very large relative to ϕ) P will lag behind $\Delta\psi_s$, but decrease in $\psi_{s,i}$ will still raise turgor pressure and thus promote growth.

Several reports have shown that $\psi_{s,i}$ is not decreased by growth-promoting factors (e.g. Ordin et al. 1965, cited by Nissl and Zenk 1969; Van Volkenburgh and Cleland 1981; Thomas et al. 1981). Indeed, $\psi_{s,i}$ may actually increase during growth, because of dilution by incoming water (Stevenson and Cleland 1981); in many growing cells however, osmoregulation appears to occur, and $\psi_{s,i}$ is maintained at the lower value. Osmoregulation may be very important for long-term growth; it involves raising the total number of solute molecules in the cell as growth occurs, and may be limited by the availability of a source of such solutes. Increases in the number of solute molecules in the cell, which take place concurrently with growth, may be induced by the growth-promoting factor itself or they may be response to growth in general. In the latter case, if the factor is supplied in the presence of growth inhibitors, there will be no alteration in the number of solutes in the cell; this is the situation with auxin-promoted growth in Avena coleoptiles (Stevenson and Cleland 1981). Light, on the other hand, raises the total number of solutes in bean leaves whether or not its effect on growth in that tissue is prevented by mannitol (Van Volkenburgh and Cleland 1981).

In any case, $\psi_{s,i}$ is not decreased during growth promotion; thus the faster growth must be caused by a shift in some other component of equation 1.

Auxin can cause movement of bean leaves by inducing asymmetrical extension in the pulvinus. It seems that in this case, auxin-induced decrease in $\psi_{s,i}$ *is* responsible for cell extension (Krieger 1978); however, the effect is reversible, and cannot therefore be described as "growth" according to most definitions. In addition, some long-term growth promotions by auxin, may involve decreases in $\psi_{s,i}$, for example, in Jerusalem artichoke tuber (Yamagata and Masuda 1975); however these latter promotions are apparently rare, and kinetically they are clearly distinct from rapid promotions of the type I have studied.

4. Y; the yield stress for wall extension. Growth rate might be expected to be directly proportional to turgor pressure, but this is not the case. Work with many species has shown that growth promotion by factors such as auxin does not occur unless turgor is substantially above zero (Cleland 1959). This indicates the existence of a "critical turgor", and demonstrates that the wall must be under appreciable stress (i.e. "yield stress") before growth can begin. Critical turgor is about 0.6 Mpa (6 atm.) in *Avena* coleoptiles (Cleland 1959). A yield stress has also been demonstrated for *in vitro* extension of *Avena* coleoptile tissue in response to acid treatment (Rayle and Cleland 1972).

The biochemical basis of Y is not known, but it has been suggested that Y is a minimum elastic extension required before wall loosening can occur, rather than a minimum stress for physical extension *per se* (Cleland 1971b). Consistent with this hypothesis are observations that wall-loosening events do indeed not occur unless the walls are under tension: no potential for later extension is accumulated in auxin-treated segments whose growth is inhibited by plasmolysing solutions of mannitol; i.e. "stored growth" does not occur (Cleland and Rayle 1972; but see Cookson 1976 and page 184 of this thesis).

Possible explanations for the existence of Y are:

a.   the bond breakage which constitutes wall loosening is reversible, and unless the wall is under tension, cleaved bonds will re-form in their original configurations so that no increase in extensibility results.

b.   the relevant bonds are inaccessible ordinarily; only when walls are elastically extended do these bonds become exposed to wall-loosening agents.

c.   the bonds involved in wall loosening are cleaved much more readily when under tension.   Tension is known to affect the rate of cleavage in some polymers, e.g. that of elastin by elastase (Rayle and Cleland 1972).

A decrease in Y would increase the amount of turgor "available" in excess of Y, to drive extension, and would thus promote growth.   There is evidence that Y can change in growing *Nitella* cells, so that P-Y remains constant (over the longer term) despite change in P (Green 1968).   This may also be true of *Avena* coleoptile sections (Green 1972; but see Cleland 1971b).   Several groups have searched for changes in Y induced by growth-promoting factors, but Y has usually been found to remain constant as growth promotion begins (Cleland 1959, 1977; Van Volkenburgh and Cleland 1981; and see Cleland 1971b).   It seems unlikely, therefore, that Y is the term in equation 1 by which regulators control growth.

5. ϕ; wall extensibility.   Cell growth rate will tend to rise if the restraining wall becomes more extensible.   Endogenous variation in growth rate between different parts of the same plant has been shown to correlate with variation in ϕ (Yamamoto *et al.* 1974a, cited by Cookson 1976) and several growth factors have been shown to increase ϕ when they promote growth; for example, auxin (Preston and Hepton 1960; Cleland 1971b, 1977; Cookson and Osborne 1978, and others); light (Cosgrove and Green 1981; Van Volkenburgh and Cleland 1981); cytokinin (Thomas *et al.* 1981); GA (Adams *et al.* 1975); ethylene (Cookson and Osborne 1978).   There is now little doubt that when growth regulators affect growth rate over the short term, they do so primarily or exclusively by inducing change in ϕ.

Summary: several variables could theoretically influence growth rate in plant tissue. Generally, however, growth regulators appear to control growth rate by altering wall extensibility.

CHAPTER 4

T H E   M E C H A N I S M   O F   W A L L   L O O S E N I N G

CHAPTER 4

THE MECHANISM OF WALL LOOSENING

In the previous Chapter it was concluded that growth regulators normally control plant growth by influencing wall extensibility. This is especially true where rapid changes in growth rate are involved.

The process(es) by which wall extensibility is increased can be termed wall loosening. The possible mechanism of wall loosening has attracted considerable attention, and there has been significant progress in recent years. A brief discussion of wall loosening is relevant here.

Because of present ignorance of the detailed structure of the plant cell wall (discussed in Chapter 5) the nature of the bond breakage which must be responsible for wall loosening at the biochemical level, is not understood. Several ways in which wall extensibility might be controlled have been considered; the chief examples are considered below. Since most relevant research on this subject has concerned auxin-induced effects, much of the following discussion will centre on this growth regulator.

1. Wall synthesis. It has been proposed that wall extensibility (WE) is directly related to the rate of wall synthesis, and that auxin influences WE via wall synthesis. It is conceivable that synthetic activity at important stress-bearing bonds would increase the fluidity of the whole wall. In agreement with this proposal are observations that auxin can increase the rate of wall synthesis when it stimulates growth (Ray 1962), and that wall

thickness often remains fairly constant despite large increments of growth (Preston 1974; Albersheim 1974), suggesting that the pace of growth is linked to that of synthesis. It appears that promotion of wall synthesis by auxin is not simply a secondary response to elongation, since it still occurs when elongation is inhibited by Ca^{2+} (Baker and Ray 1965). Also, for various tissues, endogenous activity of some wall synthesis enzymes is found to correlate with growth rate (e.g. Bachman and Zetsche 1979) and is promoted by auxin (Hall and Ordin 1968, cited by Cleland 1971b), and by other growth promoters such as GA (Montague and Ikuma 1978), and FC (Ray 1980), when they stimulate growth.

However, for several reasons, it now seems unlikely that extensibility is regulated simply through wall synthesis:

i.   Ray (1962) showed that in sugar-starved coleoptile tissue, auxin-induced elongation occurs in the virtual absence of wall synthesis.

ii. Rapid elongation can be induced in frozen-thawed tissue, in which wall synthesis does not occur (Rayle *et al.* 1970).

iii. Kinetic studies show rapid promotion of elongation, by auxin, within twenty minutes of its application, whereas no effect on wall synthesis has been detected prior to 1 h (see Cleland 1971b).

It is concluded that promotion of wall loosening by auxin is independent of wall synthesis. Over the longer term, wall synthesis will almost certainly contribute to growth, by maintaining the wall's capacity for further extension.

2.   Gene Activation. The kinetics of auxin-induced elongation, being very rapid, have been used to refute suggestions that auxin acts via gene activation (Ray 1969).

Concept of the Wall-Loosening Factor.

Although wall-loosening must occur at bonds in the wall, it is

probable that auxin has its primary effect within the cell (or at the plasma membrane) rather than in the wall; this is because:

a.   Auxin has no effect on isolated walls unless live, intact cells are also present (Rayle *et al.* 1970);

b.   Auxin action requires cellular metabolism, as shown by experiments with inhibitors (see Ray 1969). Such metabolism does not occur in the cell wall;

c.   Auxin receptor sites have been found in various cellular fractions, but not in the wall (Rubery 1981; Rayle and Cleland 1977).

It follows that there must be communication between the site of auxin's initial action, and the site of wall loosening (Ray 1969). It is proposed that some factor which can cause loosening in the wall, is transported from the cell in response to auxin (Cleland 1977; Rayle and Cleland 1977). Several putative "wall-loosening factors" (WLF) have been considered, including various polysaccharide-hydrolase enzymes, which could cause wall loosening by cleaving stress-bearing bonds in the wall.

3.   Hydrolase release. The proposal that the WLF is a hydrolase is supported by findings that auxin increases the activity of various such enzymes when it promotes growth (Datko and Maclachlan 1968; Fan and Maclachlan 1966; Tanimoto and Igari 1976; Katz and Ordin 1967; Johnson *et al.* 1974; Evans 1974b). In addition, hydrolase activity correlates well with growth rate in some systems (Nevins 1970; Keegstra and Albersheim 1970, both cited in Cookson 1976).

Reports that continued protein synthesis is a requirement for auxin-induced growth were also considered consistent with the hypothesis that the WLF is a hydrolase. However, although cycloheximide (CH) prevents auxin-induced growth within 30 minutes, it does this regardless of the prevailing extension rate, suggesting that any growth-limiting proteins

are highly unstable (Cleland 1971c). Polysaccharide hydrolases, on the contrary, are noted for their stability; thus it seems unlikely that CH stops auxin-induced growth by blocking supply of these enzymes to the wall. In addition, inhibition of growth by CH can be explained without recourse to a proteinaceous WLF (Marrè *et al.* 1973b).

There is evidence to suggest that the WLF is not a hydrolase: kinetic studies of auxin-induced elongation show an effect within about 10 minutes (Evans and Ray 1969; Warner and Leopold 1971; Philipson *et al.* 1973b) but auxin-increased hydrolase activity is not usually detectable prior to 1 h. Even where hydrolase activity is increased at about the correct time (Masuda and Yamamoto 1970), the increase seems too small (10%) to account for the large changes in growth rate which ensue. A further problem with hydrolases is that their effects are often irreversible, whereas wall-loosening may be rapidly reversible (Cleland 1968; see page 43).

Finally, there is no known enzyme which, when added to isolated walls, promotes extension with kinetics similar to, or faster than, those of auxin (Cleland 1977).

In conclusion, no known hydrolase is likely to be the WLF. This does not necessarily mean that such enzymes are not involved in growth promotion; for example, wall-bound enzymes may be activated by some other WLF, see page 57).

An alternative WLF is the hydrogen ion. This is expressed in the so-called "acid-growth" theory (Hager *et al.* 1971; Cleland 1971b; Rayle and Cleland 1972a, 1972b).

4. <u>Acid Growth</u>. This theory postulates that auxin in some way stimulates the cell to cause a pH drop in its cell wall (i.e. to increase the level of protons in the wall). The resultant raised proton levels somehow cause wall loosening. There is a large body of evidence to support the acid-growth theory.

That acidic solutions can promote elongation of growing tissue has been known since the 1930's (Strugger 1932, cited by Rayle and Cleland 1972b; Bonner 1934; Brecht 1936, cited by Barkley and Leopold 1973). Rayle and Cleland (1970) and Rayle *et al.* (1970b) studied acid-induced growth, and showed that the acid effect is on WE, that the promotion can be large, occurs with a very short lag, and is found even in isolated walls. Acid-induced growth was compared with auxin-induced growth, and several similarities were noted. A possible involvement of acid in the auxin effect was discussed. For example, Rayle *et al.* (1970b) state that "The action of auxin may be to allow the release into the wall of some agent capable of causing rupture of acid-labile bonds".

Compatible with the acid growth theory are the following observations:

a.   the elongation rate with low pH is at least as rapid as that with auxin (Rayle and Cleland 1970);

b.   both promotions have a similar, peculiar temperature dependence (Rayle and Cleland 1972b);

c.   the lag time which elapses between addition and response, is shorter with acid than with auxin, as expected if the latter promotes growth via excretion of the former (Rayle and Cleland 1972b);

d.   growth promotion by acid and auxin are not additive, indicating similar mechanisms (Rayle 1973);

e.   acid can stimulate growth in the presence of metabolic inhibitors, such as KCN, which prevent auxin-induced growth (see Rayle and Cleland 1977);

f.   both promote growth only if the stress on the wall exceeds a minimum value (Rayle and Cleland 1972b).

It is sometimes stated that the observed absence of "stored growth" in each of the two promotions is a further similarity (e.g. Rayle and Cleland 1972b) however, since stored growth requires wall loosening in the absence of stress, I consider this to follow automatically from f. (above).

Soon after proposal of the acid growth theory, several groups demonstrated that auxin would induce H^+-excretion from growing cells (Cleland 1973; Rayle 1973; Marre et $al.$ 1973a; Ilan 1973). This is very strong support for the "Acid-growth" theory, particularly since:

i. the amount of H^+ excretion is found to correlate with the amount of growth induced (Cleland 1975); and

ii. .the onset of H^+ excretion coincides with the onset of growth (Jacobs and Ray 1976; Cleland 1976c).

Auxin-induced H^+ excretion has now been found in many different tissues (Mentze et $al.$ 1974) and, according to Tepfer and Cleland (1979), "The walls of all rapidly growing plant tissues which have been tested so far are loosened by acid treatment." (my italics). Thus acid fits both requirements of the postulated WLF:

1. It promotes wall loosening;

2. It is transported from the cell to the wall, in response to auxin.

Additional evidence that H^+ is the WLF involved in auxin-induced growth, comes from studies which show that neutral buffer can inhibit auxin-induced extension, presumably by precluding wall acidification (Durand and Rayle 1973). Further, with the exception of calcium, chemicals which inhibit growth non-osmotically also inhibit wall acidification (Rayle and Cleland 1977; Cleland and Rayle 1977).

There have been many objections to the acid growth theory. However, a large proportion of the contrary evidence (i.e. examples of dissimilarity

between auxin-, and acid-induced growth) can be explained within the framework of the acid growth theory on either of the two following points:

1. <u>Impenetrable cuticle</u>. A waxy cuticle coats most plant organs, indeed, Cleland and Rayle (1978) go so far as to state that all tissues which undergo rapid elongation in response to auxin possess such a barrier. This barrier retards the entry and exit of H^+ from the tissue; consequently it should be abraded in many types of acid growth experiment. Failure to realise this has resulted in some erroneous conclusions. For example:

a. The pH optimum for acid-promoted growth in *Avena* coleoptiles was originally thought to be about pH 3 (Rayle and Cleland 1970) but with lightly abraded or peeled segments, the true optimum emerges, at around pH 5 (Rayle 1973; but see Soll and Böttger 1982).

b. It was concluded that some auxin-sensitive tissue will not undergo acid growth (Barkley and Leopold 1973), but if the same tissue is first peeled, acid growth is readily demonstrable (Cleland and Rayle 1975).

c. Vanderhoef *et al.* (1977) concluded that auxin does not stimulate H^+ excretion when it promotes growth in soybean tissue. However, Rayle and Cleland (1980) showed that auxin-induced H^+ excretion, from the same tissue, could be detected if the tissue was first abraded.

Potency of the cuticle as a barrier to H^+ has been repeatedly demonstrated (e.g. Dreyer *et al.* 1981), and this factor must be taken into account in acid growth experiments. Problems of cuticular penetration are considered further on page 138.

2. <u>Kinetics</u>. One difference between acid-, and auxin-induced promotions lies in their durations: it has been found that acid-induced growth is generally not as long-lived as auxin-induced growth in the same tissue.

This can be explained in part, by turgor loss following acid damage to membranes (Rayle and Cleland 1972b). A further portion of the extra longevity seen with auxin may be due to osmoregulatory factors: as growth occurs, ψ_s will tend to rise because of dilution, causing turgor to fall and growth rate to decrease. If auxin causes ψ_s to be maintained at its original value, but H^+ has no such effect, then longer-term differences in growth rate could develop even if the wall-loosening mechanism was the same for both promoters. It seems unlikely that this latter factor is of great importance since there is evidence that osmoregulation with auxin is a response to growth rather than a direct response to auxin (Stevenson and Cleland 1981); one would thus predict that osmoregulation should be the same whatever the cause of growth, be it auxin or H^+. In addition, even when external loading is applied to supplement or replace turgor, the duration of acid-induced growth, though extended (Cline 1979), cannot equal that of auxin-induced growth. It seems an inescapable conclusion that auxin is more efficient at promoting wall loosening over the longer term than are protons alone, and this is borne out in experiment: it has been shown that auxin maintains and even increases the capacity of the wall to be loosened by H^+, presumably by a process involving wall synthesis and repair (Cleland 1980). This is probably the most important cause of the difference in longevity between the responses, and it does not indicate separate mechanisms of wall loosening.

In summary: there is strong evidence that H^+ is the WLF in auxin-induced rapid growth.

The evidence discussed so far is concerned with the involvement of H^+ as a WLF in auxin-induced growth. However, rapid growth can be promoted by several factors besides auxin. There is evidence that in many of these other cases, H^+ could be the WLF (Table 2); this is particularly clear in the case of the fungal toxin fusicoccin (FC), which stimulates both growth

Growth Promoter:	Tissue:	Reference:
auxin	various	Cleland 1975
FC	various	Cleland 1976a.
CK	squash leaf	Marre et al. 1974
GA	Avena stem segment	Hebard et al. 1976
PCIB	Zea root	Moloney et al. 1980
light	bean leaf	Van Volkenburgh and Cleland 1979
CO_2	Avena coleoptiles	Evans 1974b
αNA	Zea coleoptiles	Vesper and Evans 1979
tropic stimuli	Zea root and shoot	Mulkey et al. 1981
acid	various	Rayle and Cleland 1970

TABLE 2   Some cases in which, for various growth regulators, growth promotion has been associated with wall acidification.

and H^+ excretion, in many tissues, with a lag even shorter than that seen with auxin (Cleland 1976a).

Some workers have speculated that H^+ could be a universal WLF in all cases of rapid growth promotion in plants (e.g. Tepfer and Cleland 1979), though there are a few reports which disagree with this proposal (e.g. Stuart and Jones 1978).

The acid growth hypothesis is reviewed by Rayle and Cleland 1977; Cleland and Rayle 1978; and Cleland 1980).

Mechanism of H^+-induced wall loosening.

Many workers agree that H^+ is a WLF, but how H^+ causes wall loosening is not known. Part of the problem results from incomplete knowledge of cell wall structure (see page 65).

Several possibilities have been discussed, these include:

1. Direct cleavage. H^+ could directly cleave acid-labile covalent bonds (Rayle and Cleland 1972b); such bonds are present in the wall, e.g. the hydroxyproline-arabinose link (Rayle et al. 1970). Significant later developments make this possibility seem unlikely. Thus, the pH optimum for acid growth has been shown to be nearer pH 5 than pH 3, and such slight acidity is unlikely to cleave covalent bonds directly.

2. Cation displacement. H^+ could displace divalent cations (mainly Ca^{2+}) from stabilising positions in the wall - possibly in pectic gels - and it has been shown that Ca^{2+} does inhibit elongation in coleoptiles (Thimann and Schneider 1938, cited by Cleland and Rayle 1977), though it does not prevent H^+ excretion (Cohen and Nadler 1976). On the other hand, Ca^{2+} can not decrease extensibility of isolated walls. Also K^+, which is physically similar to H^+ and should also displace Ca^{2+}, does not promote extension (Rayle and Cleland 1972b). It appears that Ca^{2+} ions may inhibit growth

by interfering with H^+-induced wall loosening (Cleland and Rayle 1977), possibly by competing with H^+ for attachment sites.

If wall loosening is due to cation displacement, the cation chelator EDTA, at neutral pH, should be as effective as low pH at promoting growth. In oat-coleoptile segments, and in epidermal strips of *Helianthus* hypocotyl, EDTA does promote some extension of isolated walls, but it is much less effective than low pH (Tepfer and Cleland 1979; Soll and Böttger 1982). In the alga *Valonia ventricosa*, which also shows acid growth, EDTA is as effective as low pH, and it has been concluded that Ca displacement plays a central role in wall loosening in *Valonia*, but a much lesser role in coleoptiles (Tepfer and Cleland 1979). EDTA also promotes growth in the alga *Nitella axillaris* (Métraux and Taiz 1977), and in some higher plant roots (Kuzmanoff and Evans 1980).

3.   Hydrogen bonding. Wall acidification would result in changes in the pattern of hydrogen bonding, which may in turn alter WE. In the model of wall structure proposed by Keegstra *et al.* (1973), hydrogen bonding between xyloglucan and cellulose is a potential site for wall loosening. This proposal is supported by observations that auxin, or low pH, can increase solubilisation of xyloglucan from the cell wall (Labavitch and Ray 1974a, 1974b; Jacobs and Ray 1975; Terry *et al.* 1981).

On the other hand, agents which disrupt hydrogen bonds, such as urea, do not promote extension in isolated coleoptile walls (see Rayle and Cleland 1977); also, Valent and Albersheim (1974) were unable to detect any effect of pH on the *in vitro* binding of xyloglucan to cellulose. These findings would seem to dispute the importance of hydrogen bonds in controlling WE.

4.   Enzyme activation. A drop in wall pH could activate a wall-bound, wall-loosening enzyme (Hager *et al.* 1971); hydrolase enzymes are present in the wall (Huber and Nevins 1979), and they commonly have acid pH optima (Johnson *et al.* 1974). In addition, a role for wall proteins is indicated

by the finding that proteolytic treatment (Yamagata *et al.* 1974) and Cu^+ ions (Hager *et al.* 1971) severely inhibit acid-induced growth in isolated walls, presumably by inactivating enzymes (see Rayle and Cleland 1977). Finally, Yamagata *et al.* (1974) report that low temperatures (2-4°C) can markedly diminish acid-induced growth, again suggesting enzymic mediation.

At the time of writing however, there is no known enzyme which fits the requirements of the postulated acid-activated, wall-loosening enzyme (e.g. Rayle and Cleland 1977). Also, Soll and Böttger (1982) report that in epidermal tissue of *Helianthus* hypocotyl, acid-induced extension shows none of the characteristics of enzyme-mediated processes, such as pH and temperature optima. Similarly, formaldehyde pretreatment, which should disrupt enzymes, had little effect on acid-induced growth at least over the short term.

In *Nitella*, although short boiling (15 minutes) halves the rate of acid-induced growth, even prolonged boiling (12 h) does not entirely prevent it. This suggests the possibility of a non-enzymic mechanism (Métraux and Taiz 1977).

5. Soluble pectin binding. pH-dependent binding between soluble pectin and a heat-labile wall component was shown by Bates and Ray (1981); this could be involved in WE changes in some, as yet, unknown manner; however, unlike the acid effect on WE, this acid effect appeared to be irreversible. Thus the two effects may be unrelated.

6. Lectin-binding. It has been proposed that lectins in the wall control wall extensibility (Kauss and Glaser 1974; Kauss and Bowles 1976). These substances are found in the walls of at least some plants, and their binding potential has been shown to vary with pH. A situation can be envisaged in which lectins, linked covalently to one group of polysaccharides, could simultaneously (but reversibly) form crosslinks to specific sugar groups (probably galactose) on other polysaccharides, and thus keep the

wall rigid. Reduction in the tenacity of this lectin binding (caused by a pH drop) could cause the wall to become more fluid. Cell extension would then ensue.

Many of the problems associated with the wall-loosening enzyme possibility (No. 4 above) also apply here since lectins, like enzymes, are proteinaceous.

Summary: the mechanism by which H^+ ions influence wall loosening is not known, but cation-displacement or an acid-activated wall-loosening enzyme seems likely. It may be unwise to favour a particular theory at this early stage, but recent work by Soll and Böttger (1982) places the balance of evidence towards the "calcium displacement" possibility.

The mechanism of H^+ excretion

Although wall acidification could be brought about in several ways (e.g. the cell could selectively absorb OH^- ions, see Penny 1977; Thomas 1976), the process is commonly referred to as H^+ excretion (or H^+ secretion).

Some possible mechanisms are outlined below (this subject is reviewed in Cleland 1982). A membrane-bound ATP-ase might be responsible for pumping out protons (Hager *et al.* 1971), electrical neutrality would be partially restored by increased passive entry of cations such as K^+, or by efflux of some anion. This proposal is supported by observations that addition of ATP can stimulate auxin-induced elongation under anaerobic conditions.

Although ATP-ases are present in plant cell membranes (Hodges *et al.* 1972), large-scale promotion of their activity by auxin has yet to be demonstrated (Tepfer and Cleland 1975; see also Rayle and Cleland 1977). Small promotions have been noted with FC (Beffagna *et al.* 1977; Marrè 1979). H^+ excretion has been postulated to be more or less tightly coupled to K^+

uptake (Marrè 1977; Cleland and Lomax (1977), but both FC and auxin induce membrane hyperpolarisation (Etherton 1970; Cleland *et al*. 1977; Marrè *et al*. 1974), showing that counter-ion flux is not complete.

Bicarbonate uptake could contribute to wall acidification (Raven and Smith 1974). Also increased CO_2 production has been proposed as a means of wall acidification (Sloane and Sadava 1975; but see Oberbauer *et al*. 1978) and many growth regulators do increase respiration rate. On the other hand, the acidity developed from auxin-treated cells cannot be removed by bubbling N_2 through the medium (see Cleland and Rayle 1977), and cannot therefore be due to CO_2 alone.

Since medium-acidification induced from segments treated with growth promoters has very little pH-buffering capacity, it is unlikely that efflux of organic acids or other acid-buffering compounds is responsible (Johnson and Rayle 1976). ·

Another possibility is the "bucket-brigade" hypothesis of Ray (1977) which states that, in response to auxin, acid-filled vesicles are budded off the endoplasmic reticulum (ER), these migrate to, and fuse with the plasmalemma, releasing their contents into the wall. This fits with findings of auxin binding sites on the ER and would also explain why auxin-induced H^+ excretion shows a ten-minute lag, while other types of acidification can occur almost immediately.

Summary: the mechanism of H^+ excretion is not known, though several proposals are currently under consideration. Excretion may not occur by the same mechanism for each growth promoter.

CHAPTER 5

S T R U C T U R E   A N D
C O M P O S I T I O N   O F   T H E   C E L L   W A L L

CHAPTER 5

STRUCTURE AND COMPOSITION OF THE CELL WALL

A discussion of plant growth would not be complete without some consideration of the structure and composition of the cell wall, since it is this organelle which limits cell growth, and it is here that growth-controlling messengers from the cell (WLF's - see page 48) exert their ultimate effect.

Consider growth promotion by auxin: rapid elongation induced by auxin results from an increase in wall extensibility (WE). On this there is general agreement (see Hall 1976; Monro *et al.* 1976: and see Chapter 3). Extensibility and other mechanical properties of the cell wall, indeed of any polymeric substance, are governed by the arrangement of chemical bonds between the constituents. To increase WE, a growth promoter must induce cleavage at critical bonds. Complete characterisation of the mechanism of auxin-induced growth would thus include a description of how auxin brings about this cleavage, and at which bonds. In the absence of detailed information about the chemical nature of the cell wall, it is difficult to envisage which bonds might be critical in controlling extensibility. Conversely, it is also difficult to predict what the effect of cleavage of any particular set of bonds would be, in terms of change in WE.

It might be predicted that in most or all of the plants investigated to date, WE must be controlled and regulated in a very similar manner: for

example, the kinetics of auxin-induced growth are similar in many species, and there is evidence that the wall-loosening factor induced by auxin and by many other growth promoters, is the same in many tissues (see page 54). It might, therefore, also be expected that a similar bond or set of bonds is present at critical extensibility-controlling positions in the walls of all plants so far reported.

The Cell Wall.

It is convenient to distinguish between "primary" and "secondary" walls. Secondary walls are simply primary walls modified by the addition of various layers and incrusting materials. The cytoplasm of cells with secondary walls is often dead, and these cells have usually ceased to grow. For the purposes of this discussion, only potentially growing cells (those with primary cell walls) need be considered.

The primary cell wall is constructed of cellulose microfibrils embedded in a matrix of polysaccharide and glycoprotein. Water is a major element *in vivo*, making up some 70-80% of the wall's fresh weight. Exactly how water contributes to the wall's rheological behaviour is not clear, but it must play an important role (Northcote 1972).

The microfibrils are conglomerates of very long cellulose molecules, degree of polymerisation up to about 14,000 (Hall 1976). These in turn consist almost exclusively of straight chains of β-1,4-linked glucose molecules. Many such chains can become closely packed and great strength is imparted to the whole structure because of hydrogen bonding between groups on adjacent molecules within the chain, and between groups on molecules of neighbouring chains (Albersheim 1975). There is still controversy over the exact arrangement of these chains, e.g. it is not certain whether neighbouring cellulose molecules lie parallel (with their reducing ends pointing the same way), or antiparallel (see Albersheim 1975).

Microfibrils are visible under the electron microscope, being some 7-10 nm across. In cross section, microfibrils are believed to consist of some 80-100 individual cellulose molecules each lying parallel to each other, and to the body of the microfibril. In the central core of the microfibril, packing of cellulose molecules is often so ordered as to be crystalline. This was first shown using polarising microscopy, and later confirmed by X-ray diffraction (Preston 1974). Between adjacent crystalline regions (micelles) along the fibril, and towards the periphery of the fibril, the arrangement is less regular, and is said to be paracrystalline. In para-crystalline regions there is thought to be some mixing of cellulose with matrix materials. The latter can also be so ordered as to be partly crystalline.

The matrix is composed mainly of polysaccharides. Until recently, matrix polysaccharides were considered to be subdivided into "hemicellulosic", and "pectic" fractions. However, it has been pointed out that these distinctions reflect solubilisation procedures used in extraction, rather than chemical entities within the wall, and several reports (including at least one general text, Hall (1976) now consider only two subdivisions of the entire . wall; cellulosic and non-cellulosic. The matrix is all the latter.

Nine monosaccharides are found as members of the matrix in primary walls of higher plants. The main ones are: rhamnose, arabinose, xylose, galacturonic acid, galactose and glucose. There are lesser amounts of glucuronic acid, mannose and sometimes fucose. Several sugars common in other parts of the cell are not found as structural elements of the wall, e.g. fructose.

The nature of the non-cellulosic (NC) polymers varies between species, between tissues within the plant, and with age within a tissue, but studies on various materials have revealed substantial amounts of xyloglucan, arabinogalactan, and rhamnogalacturonan.

Glycoprotein is an apparently universal feature of primary wall matrix material, making up some 10% or more of the wall's dry weight (Lamport 1970). Its role is not clear, but some is present as a structural glycoprotein. There could also be an enzymic function. Particularly remarkable is the frequent occurrence of hydroxyproline residues in wall glycoprotein. This imino acid is very rare inside the cell.

Although under normal conditions, the cellulose reinforcing material probably bears most of the stress on the wall, it is thought to be changes in bonding among matrix components which govern wall extensibility and thus growth rate (e.g. Hall 1976; Monro et al. 1976).

In primary walls of higher plants, new microfibrils are usually laid down in a helix of low pitch around the cell, with few new microfibrils running parallel to the extending axis of the cell (usually the longer axis). It is this feature which is thought to be responsible for the wall's relatively high resistance to lateral expansion, and which ensures that most cell growth in elongating zones of both shoot and root takes place along the long axis of the organ. If the wall was isotropic, cells would tend to be spherical, and in some algae with randomly orientated microfibrils, elongation is indeed isodiametric, e.g. *Valonia* (see Hall 1976).

There are now several models which seek to explain how matrix polymers are arranged. The first detailed model emerged from work on suspension cultured sycamore cells (Keegstra et al. 1973); see Figure 2. These workers used methylation analysis to show the precise bonding pattern between monosaccharide constituents of the NC fraction. From that they were able to make reconstructions of the original polymers (see Albersheim 1975). In this model, the matrix polymers are covalently interlinked to form a single gigantic molecule which would presumably extend beyond the individual cell and include all the matrix material in the entire plant. Attachment
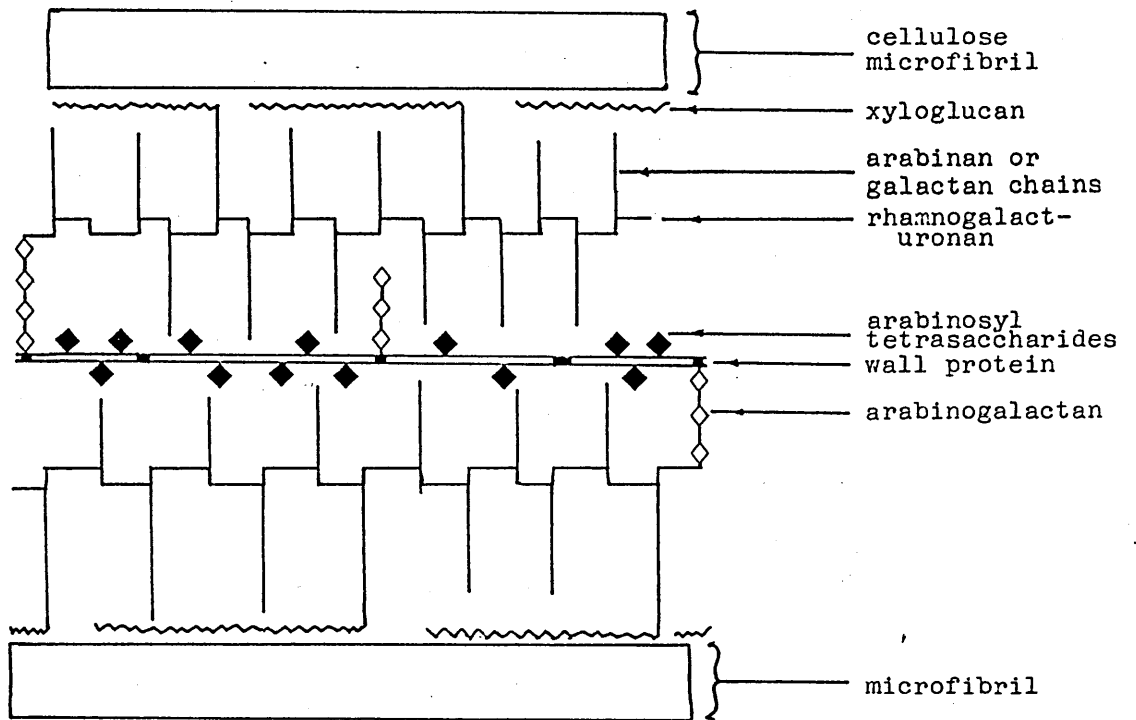
FIGURE 2 Two-dimensional representation of postulated structure
of the primary wall in suspension-cultured cells of
Acer pseudoplatanus.

From Keegstra *et al*. 1973.

of matrix to microfibrils is by hydrogen bonding between the fibril and adjacent xyloglucan molecules.

There is evidence that this model holds for a number of tissues besides the suspension-cultured *Acer* cells (Wilder and Albersheim 1973; Albersheim 1974), but it is not universally applicable. For example, Monro *et al.* (1976) working with lupin and mung-bean hypocotyl tissue found several results which are not compatible with the model of Keegstra *et al.* and postulate a substantially different, though less detailed, model. Wall composition in the monocotyledons differs in several ways from that in dicotyledons (e.g. Burke *et al.* 1974), and further models have been proposed for the monocotyledon cell wall (Darvill *et al.* 1977); see Figure 3.

These models should serve to illustrate the great advances made in the past decade towards the understanding of wall structure. However, the contradictions between them show that the picture is far from complete, and that there may be important differences between the wall structures of different species.

A very important consideration for models of wall structure is the directionality of growth. As Monro *et al.* (1976) have pointed out, a sliding of microfibrils along one another would lead mainly to radial expansion of the cell. Such creep of parallel microfibrils, with the distance between them not changing, is postulated as the basis of growth in the model of Keegstra *et al.* Since predominantly radial expansion is not normally observed, it would seem that sliding of fibrils along each other is not the principal way in which growth occurs. By the same reasoning continuous breaking up of the fibrils, with separation into smaller pieces (e.g. involving a cellulase), is not the principal process in growth. It too would lead to radial or spherical expansion.
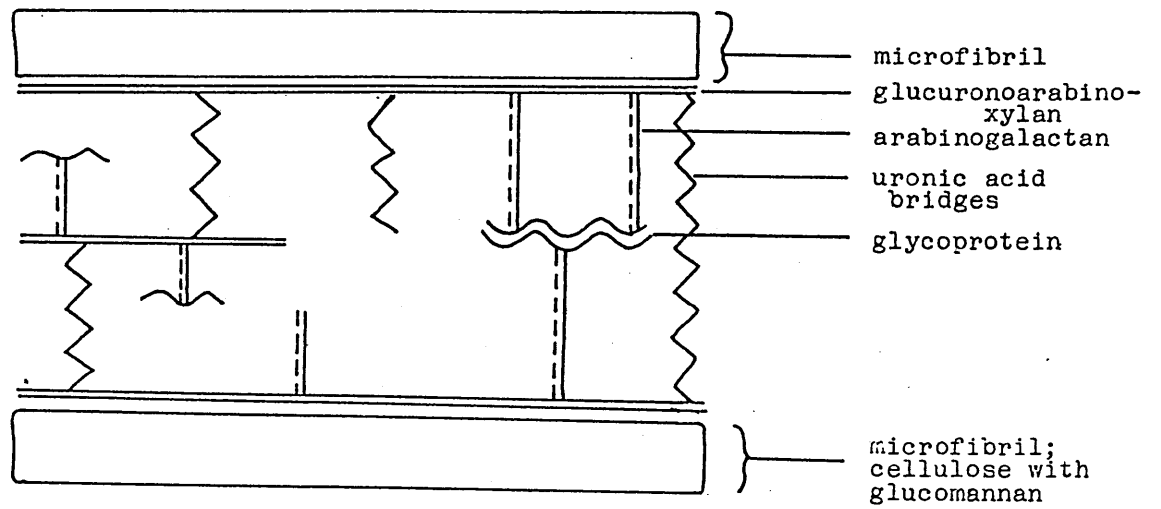
FIGURE 3 Two-dimensional representation of postulated
structure of the cell wall in maize
coleoptiles.

From Darvill *et al*. 1977.

There must be at least some increase in the distance separating neighbouring microfibrils. This confirms that growth-controlling bonds are likely to be between matrix components. To further complicate the picture, realignment of microfibrils is observed as the cell extends: i.e. when laid down on the inner surface of the wall, the microfibrils are arranged in a flat helix around the cell. As extension occurs, this angle is observed to increase (Veen 1970a; Gertel and Green 1977). However, new material continues to be laid down transversely at the inner surface of the wall, so that gradients occur of age, angle subtended to the transverse plane, and amount of extension undergone, from the inside of the cell wall outwards. This is in accordance with the "multi-net" growth hypothesis (Roelofson and Houwink 1953; Gertel and Green 1977). If extension involved a simple parallel separation of fibrils, such multinet growth would not occur, fibrils would not be reorientated, and would remain at the same angle to the transverse plane throughout their existence.

Extension involving only realignment of fibrils is theoretically conceivable, but it would impose additional problems; e.g. it would cause a decrease in the diameter of the cell. It would also impart a twist to the cell, so that the top of the cell would appear to rotate with respect to the base. Such a rotation can be observed in the growing sporangiophore of *Phycomyces* (Ortega *et al.* 1974), but for cells in a tissue, twisting of this sort would not be constructive, since adjacent cell walls could rotate in opposite directions, causing shear stresses between cells, and weakening the whole organ. On the other hand, the observed realignment of microfibrils, with growth, does imply some autonomy of movement between adjacent cell walls, since realignment of fibrils in contiguous walls will often be in opposite directions (Figure 4). Strong bonds between matrix components of adjacent cell walls (possibly compatible with the "giant molecule" model of Keegstra *et al.* 1973; Figure 2) can be inferred, otherwise primary tissues would fall apart under the shearing stresses associated with realignment.
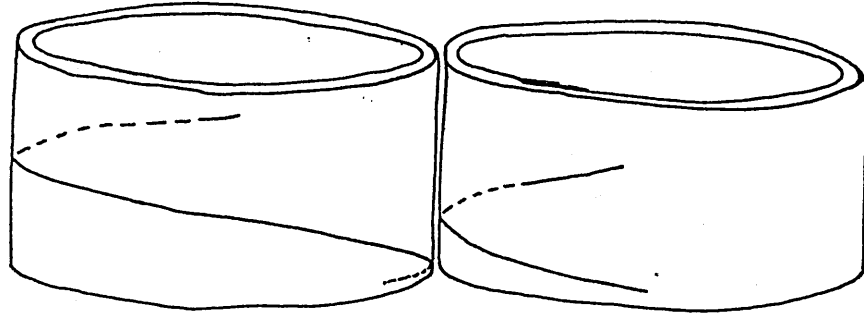
Two adjacent cells with microfibril orientation represented.

Microfibril orientation of the above viewed from the inside of the right hand cell.
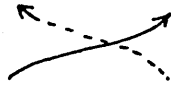
The same, after elongation:

FIGURE 4   Diagrams showing that realignment of fibrils in adjacent walls of similar cells during growth, is in opposite directions.

Microfibrils in the further wall are shown as dotted lines.

Given realignment of fibrils during growth, it follows that there must be a shearing slippage of fibrils along each other to counteract the rotation effect. Some parallel separation probably also occurs during growth. Creep along circumferentially orientated fibrils (as envisaged in the model of Keegstra *et al.* 1973) would not alone produce longitudinal extension, nor would it be compatible with fibril realignment during growth. As stated by Monro *et al.* (1976) "an accurate cell wall model must eventually take into account stresses on the wall (and) orientation of wall components", as well as "detailed structure of wall polymers and the nature of the bonds between components".

The models of wall structure so far proposed are useful working guides, but they cannot be considered complete.

A brief analysis was carried out, of composition of one fraction of the cell wall in *Nymphoides* and *Regnellidium*. The fraction was selected in the light of previous work, as the one most likely to be involved in control of WE. Composition of this wall fraction from tissue treated with combinations of auxin and ethylene was followed, in an attempt to identify changes associated (possibly causally) with growth (see Chapter 11).

CHAPTERS 6 & 7

M A T E R I A L S   &   M E T H O D S

CHAPTER 6

P L A N T   M A T E R I A L   A N D

C U L T U R E   M E T H O D S

CHAPTER 6

PLANT MATERIAL AND CULTURE METHODS

1.   *Nymphoides peltata* (S.G.Gmel.) O. Kuntze (formerly *Limnanthemum nymphoides* (L) Hoffmans and Link; *L. peltatum* (S.G.Gmel.) is the yellow-fringed water lily.   It was collected by Dr. I. Ridge from a pond in the field (near Clitheroe, Lancashire, UK.).   The plant was grown and allowed to propagate asexually (which it does readily by means of rhizomes) in glass tanks 38 x 38 x 120 cm long (Plate 1).   The tanks contained about 5 cm of "Levingtons" compost (Fisons Ltd., UK) overlain by 1 cm of John Innes No. 1. compost, and covered with tap water to a depth of some 30 cm. The JI compost was added because it prevents the lighter, soil-less compost from becoming stirred up during filling of the tank.

The culture tanks were kept in a greenhouse with supplementary heating (supplied by "Humex" fan heaters) whenever the temperature fell below about 18°C.   Supplementary lighting was tried, using 250 watt mercury-vapour lamps (Thorn MBF Ballast lamps) during the 5 h after dawn and 5 h preceding dusk; this appeared to have very little effect on the vigour of cultures, especially during the summer months when most of the experiments contributing to this thesis were carried out.   Thus the supplementary lighting was discontinued.

Water level in the tanks was maintained by topping up as necessary with distilled water.   Sequestered iron and nutrients (Bio plant food,
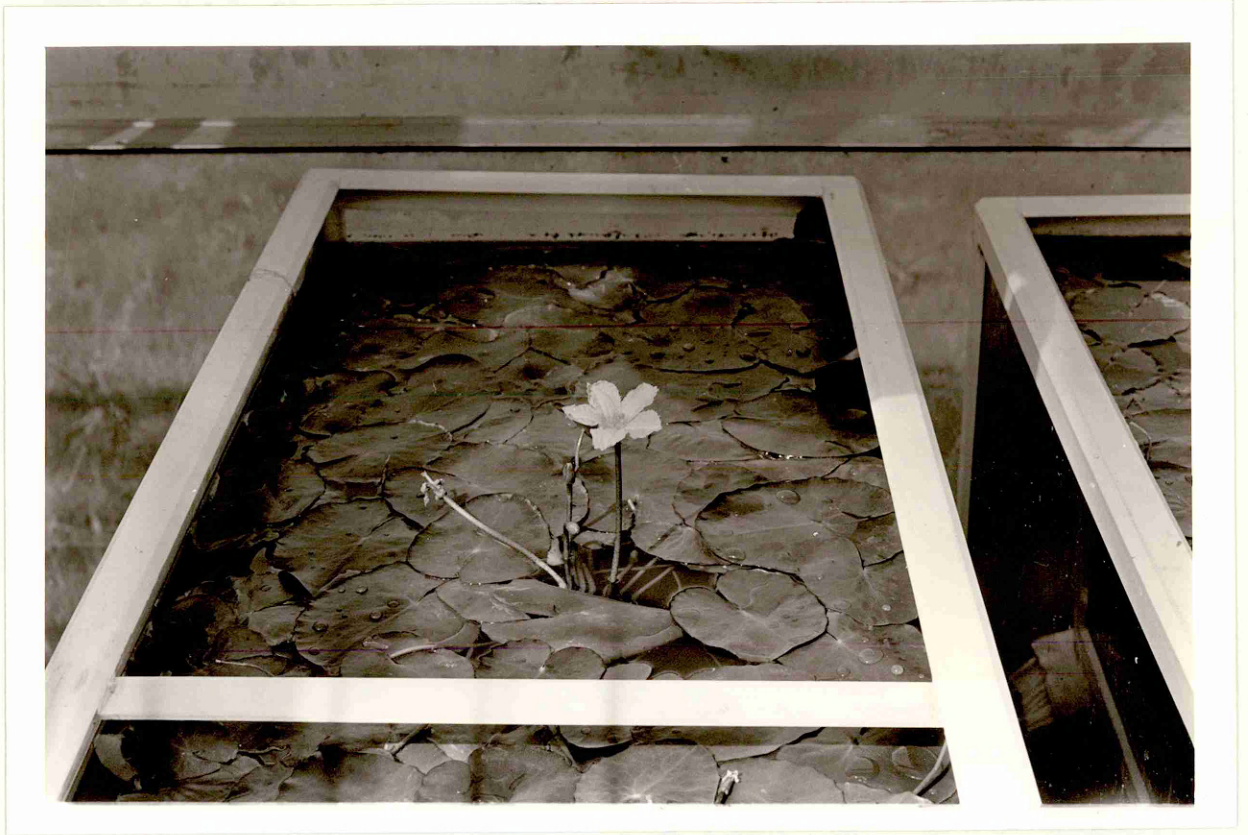
PLATE 1   *Nymphoides peltata* in a culture tank.

Scale: the flower shown is about 40 mm
in diameter.

Pan Brittanica Industries, UK) were also added to the water occasionally. Cultures of *Nymphoides* were largely pest free, though occasionally numbers of greenfly attacked the surface of the floating leaves. To repel these, a proprietory insecticide was tried (Murphy's systemic) however, this caused severe damage to the leaves (probably because although the minimum insecticide was added to serve the entire volume of the tank, the insecticide floated quite well, at least initially, so that floating leaves were exposed to levels far in excess of the manufacturer's recommended dosage). Thereafter, insecticide was not used, and aphids were removed manually as necessary. Large numbers of copepods populated the *Nymphoides* tanks. These were not discouraged since they removed algae from the water, from submerged surfaces of the plants, and from the glass walls of the tanks. When harvesting material, healthy leaves of approximately 3-5 cm wide were chosen; the leaf, together with the apical part (about 10 cm) of the petiole, was excised. Petiole segments could then be cut as required.

Left-over pieces of petiole, together with leaf laminae not used in experiments and any leaves which had grown too large for experiments, were dried, powdered, and returned to the tank to aid in maintenance of the nutrient status.

2.   *Regnellidium diphyllum* Lind. was kindly supplied by Dr. D.J. Osborne (then of the ARC unit of developmental botany, Huntingdon Road, Cambridge). This plant is a tropical fern of the *Marsiliaceae*. It was growth in perspex chambers 51 (deep) x 46 (wide) x 71 cm (long) containing 4-5 cm of water-saturated "Levingtons" compost (Fisons Ltd., UK) (Plate 2).

As with *Nymphoides*, vegetative propagation of *Regnellidium* occurs freely by means of rhizomes which grow along the surface of the substratum. The cultures of *Regnellidium* were kept in a greenhouse (conditions as for *Nymphoides*). The culture chambers were high sided to maintain humidity around the plants; even so, the use of air-humidifiers (model 505, Defensor,

PLATE 2 *Regnellidium diphyllum* in a culture tank.

Scale: a pen is included in the picture.

Zurich) was necessary to prevent dessication of the plants. This was especially so in winter, when the electric fan heaters (which can greatly decrease relative humidity) were employed most.

The soil was kept wet by adding distilled water as necessary. As with *Nymphoides*, sequestered iron and other nutrients were added occasionally (and/or whenever the leaves began to show signs of defficiency). To harvest material, the entire leaf was excised just above the rhizome.

Since cultures of both *Regnellidium* and *Nymphoides* are continually growing and expanding, young leaves are always available. This is very convenient; it eliminates the need for careful planting schedules.

3. Etiolated pea plants were occasionally used. Seeds of *Pisum sativum* L. var. "Alaska" (Sharpe and Co., Sleaford, Lincs.) were placed in shallow dishes and partly covered with distilled water at 25°C for about 12 h. The seeds were then planted in about 3 cm of wet vermiculite in plastic trays and placed in total darkness at 25°C. Seedlings were periodically inspected using a green safelight, and watered as necessary. When the third internode of the etiolated epicotyl reached a length of about 2 cm, the material was harvested for use in experiments.

Segment Handling.

Immediately after harvesting, segments were prepared from the plant material. With peas, 1 cm segments were cut, in ordinary room lighting, from just below the plumular hook, using a dual-bladed cutter with accurately-spaced blades. With *Nymphoides*, the region which can show rapid growth is much more extensive than in peas (Figure 5), and segments can be obtained from a considerable length of the apical region of the petiole. Usually 2-cm segments were used, either from the apical 2 cm of the petiole, or from the subapical 2 cm as well. Material from further down the petiole
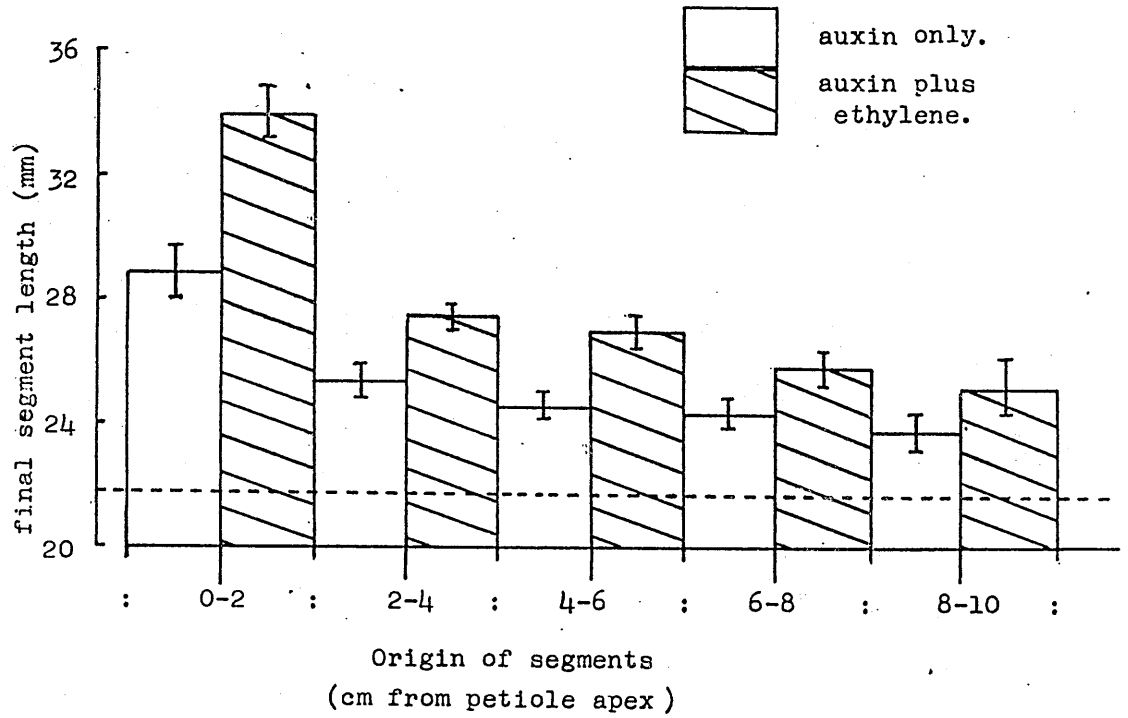
FIGURE 5 Elongation in response to auxin (open bars) or to auxin plus ethylene (hatched bars), in segments from various portions along the *Nymphoides* petiole.

2 cm segments were excised at various distances from the apical (leaf) end of the petiole, and incubated for 9 h in auxin (10^{-5} M IAA) with or without ethylene (25 µl l^{-1}). Elongation of untreated apical segments is shown for comparison (dashed line). Each bar is a mean for 25 segments ± SE.

was not normally used; neither were apical and subapical segments used in
the same experiment. "2-cm segments" were excised using a dual-bladed
cutter which cut to a length of 21.4 mm.

Two distinct morphs of *Nymphoides* petiole were sometimes apparent;
in the extreme case, one morph was thick, waxy, green and fleshy, while
the other morph was pale and thin. Other differences between the morphs
are listed in Table 3.. Since the differences between the morphs in terms
of their sensitivity to auxin and acid were not significant (Figure 6), no
attempt was made to separate them in experiments.

Aging. For reasons explained in Chapter 8 (page 125) segments were usually
"aged" prior to treatment. Aging involves leaving the segments to float
on distilled water, under room lighting and temperature, for a few hours
between excision and experimental treatment. Because significant growth
may occur in control segments in the hours immediately following excision
(presumably because of residual endogenous auxin), it was most convenient
to cut segments to their stated initial length after the aging period
rather than at the time of excision. Thus the procedure adopted was:

a.　harvest material;

b.　remove leaves and cut segments to about 25 mm;

c.　age for 4-5 h floating on distilled water in ordinary room lighting
and temperature;

d.　cut to starting length, i.e. usually 21.4 mm;

e.　carry out experiment.

Incubation during experiments was normally carried out with 10 segments per
treatment, floating on 25 ml of solution in a 250 ml beaker.

Thick type	Thin type
petiole thick	thinner
petiole dark green	paler
petiole oval	rounded
petiole surface visibly waxy	waxy layer less apparent, lenticel-like dots appear.
leaf large	smaller
aerenchyma regular	more scattered

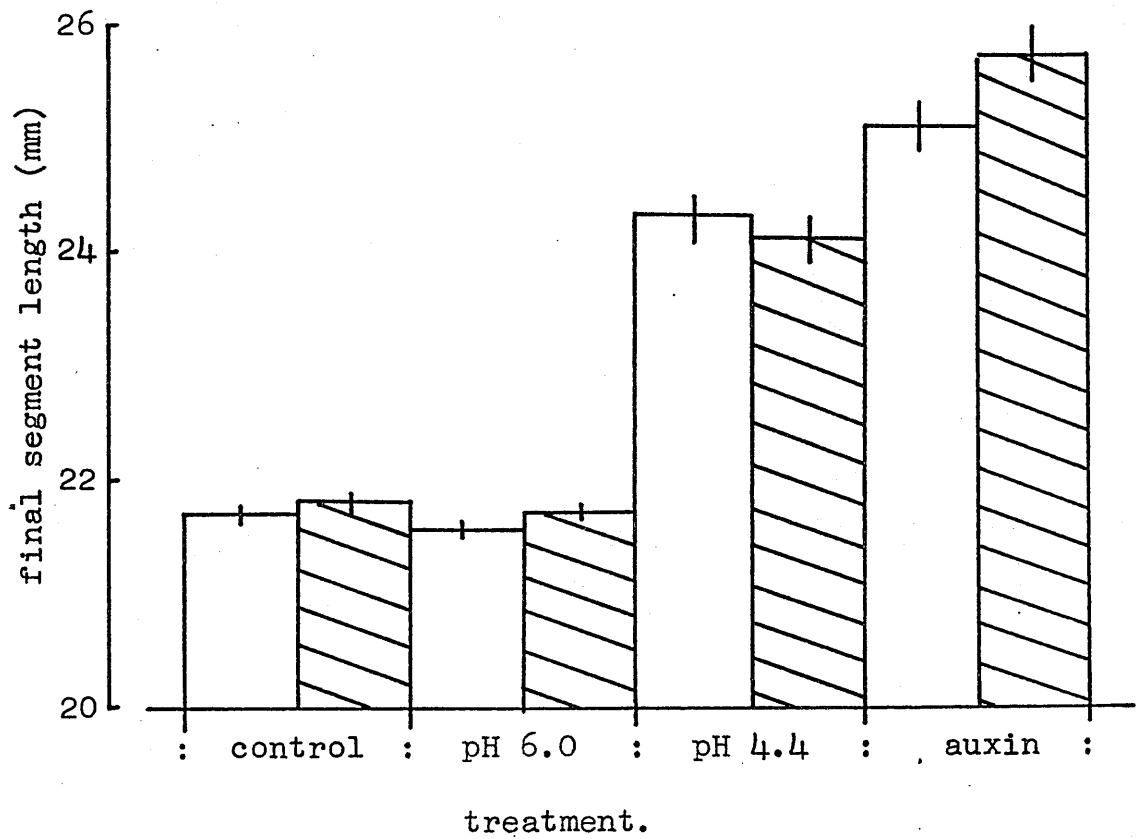TABLE 3   Characteristics of the two 'morphs' sometimes found in *Nymphoides* cultures.

FIGURE 6 Elongation with auxin and acid, in segments from
the thin (open bars) and thick morphs (hatched bars)
of *Nymphoides* leaf.

Apical segments, initial length 21.4 mm, were
incubated for 7 h in water $\pm$ 10^{-5} M IAA, or in
50 mM K-citrate/phosphate buffer. Bars are means
for 15 segments $\pm$ SE.

Further Manipulations of Segments.

1.   Carborundum abrasion. For some experiments, segments were abraded by

gently rubbing them between a dampened thumb and forefinger dipped in

120 mesh carborundum powder (The Carborundum Co., Manchester, UK). After

abrading, excess carborundum powder was rinsed from the segments using

distilled water.

2.   Other Abrading Techniques. A small number of abrading techniques were

tried for particular experiments; these methods are described in the accounts

of the relevant experiments in the "Results and Discussion" (particularly

Chapter 10).

3.   Preparation of split segments. 3 or 4 cm petiolar segments were split

by a single central longitudinal cut, made with a scalpel blade, from the

apical end of the segment, to within 1 cm of the basal end. This forms a

Y-shaped structure.

4.   Preparation of Cored segments and Cores. A stainless-steel tube

(diameter 2 mm) was pushed, with a small amount of rotation, up through

the *Regnellidium* petiole segment so that the tube destroyed the radial

partitions separating the air canals (Plate 3). When a useful length had

been cored (1 cm or more) the tube was withdrawn and the cored piece cut

off. The central core could then be pulled from inside the outer sleeve.

Coring was not used with *Nymphoides* segments.

Application of hormones.

1.   Auxins. Two auxins were used, indol-3yl-acetic acid (IAA), and

2,4-dichlorophenoxyacetic acid (2,4-D). These were purchased from the

Sigma Chemical Co., Poole, Dorset, UK. Stock solutions of the K^+ salt

of each auxin were made at 10^{-3} M and stored at 4°C; they were discarded

when 6 weeks old. No appreciable deterioration of the stock solutions
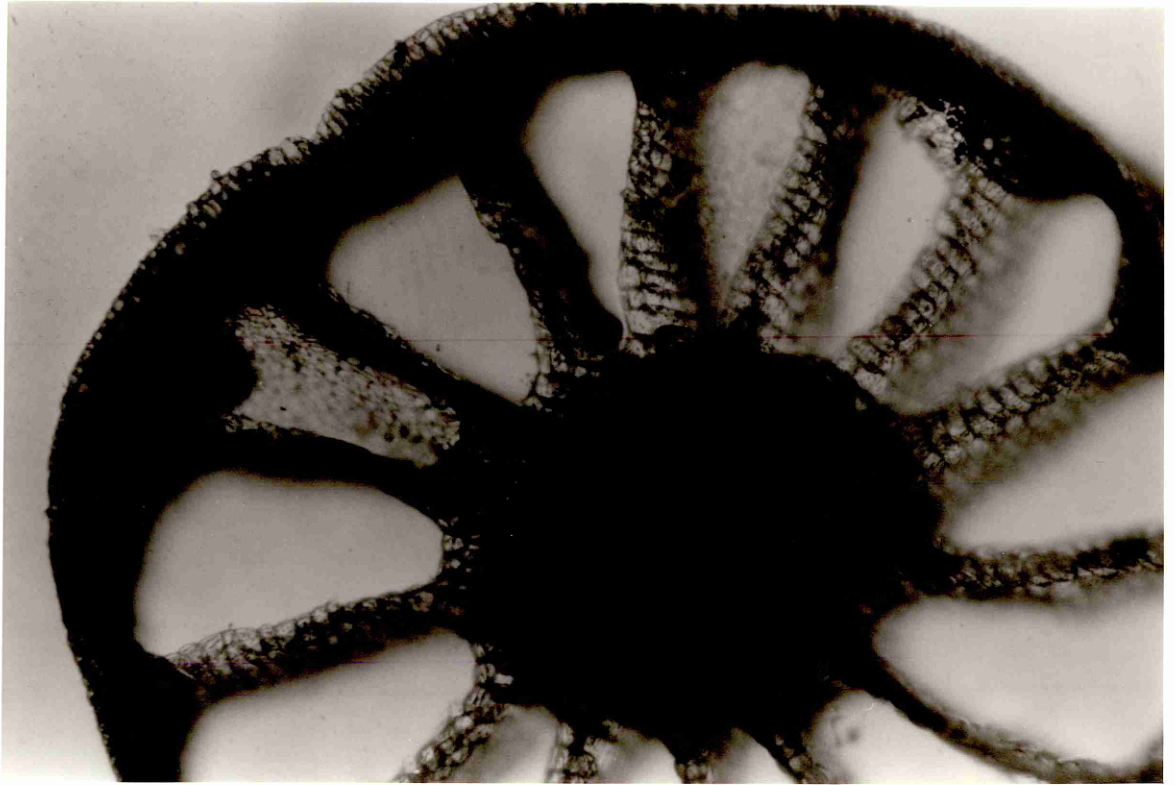
PLATE 3   Petiole of *Regnellidium* seen in TS, to show radial
partitions.

Scale: the petiole shown is about 3 mm in diameter.

occurred over 3 months storage, as estimated by a simple bioassay (Figure 7). Appropriate quantities of the stock solutions were added directly to treatment solutions to produce the stated auxin concentrations for each experiment.

2.   Gibberellin. GA was purchased from Sigma. It was dissolved in water to form a 10^{-3} M stock solution and appropriate amounts were added to the treatment media to produce the required hormone concentrations.

3.   Abscisic Acid. ABA was purchased from Sigma and made up separately for each of the relevant experiments. A stock solution 25-fold more concentrated than the level to be used in the experiments was prepared. To aid in solution of the ABA, pH of the medium was adjusted to 11 with NaOH, then returned to pH 7 with dilute HCl. An appropriate quantity of stock solution was added to the treatment media as required.

4.   Fusicoccin. FC was kindly given, through Prof. E. Marrè, by Dr. G. Michieli of Montedison S.p.a., Diag/CRA Valutazione Biolice, Via Bonafidini, 148, Italia. Following the procedure of Lado et al. (1972) somewhat freely, a stock solution of 10^{-3} M was made up by dissolving 6.8 mg of FC in ethanol and making up to 10 ml with water. An appropriate quantity of this stock solution was added directly to treatment solutions to provide the required level of FC. A small quantity of ethanol is also thus applied, but even at the highest level of FC used (10^{-5} M), the alcohol level reached only 0.001%. This level when applied without FC under experimental conditions, was found to have no appreciable effect on either control-, or auxin-induced growth (Figure 46). Consequently, the alcohol content of FC solutions was ignored in subsequent experiments.

5.   Ethylene. In experiments involving ethylene treatment, beakers containing treatment solutions and segments were placed in airtight, 10 l glass desiccators. Using a gas-tight syringe (normally 1 ml Gillette "Sabre"), an appropriate quantity of pure ethylene was withdrawn, through a "suba-seal"
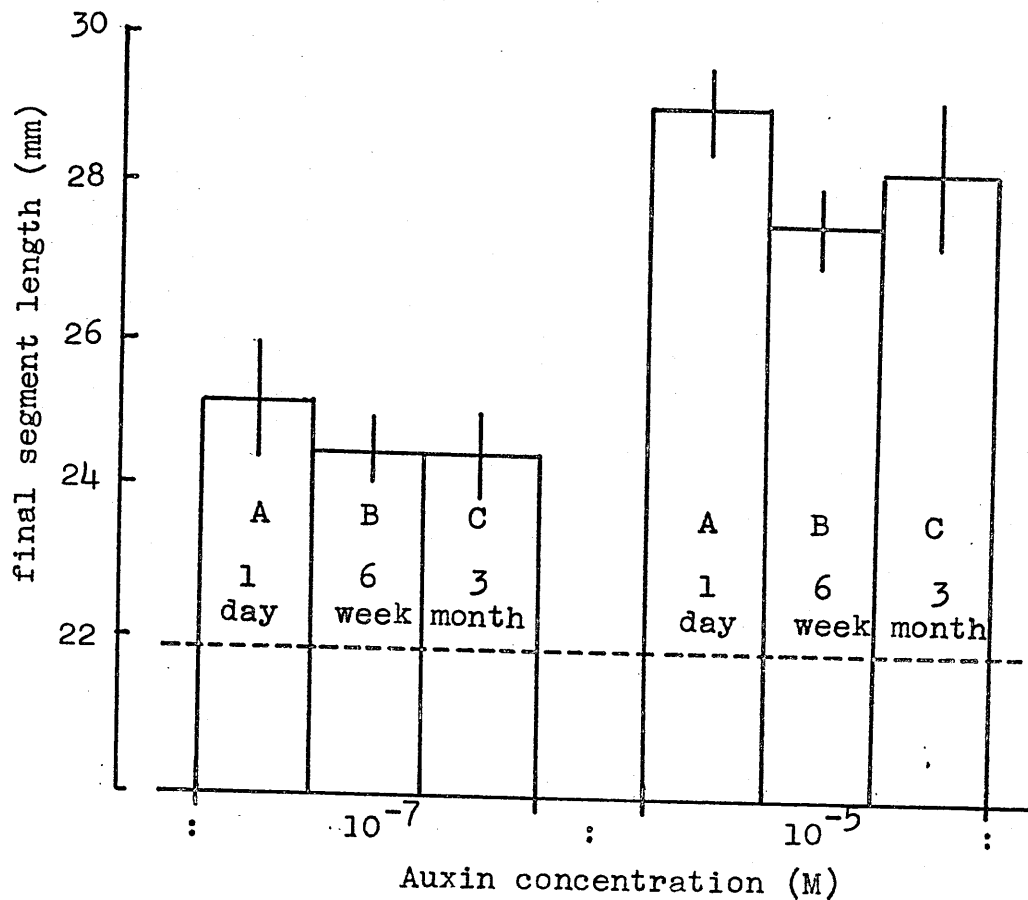
FIGURE 7 Growth-promoting capability of IAA solutions
(10^{-7} and 10^{-5} M) after storage for various lengths
of time.

Apical *Regnellidium* segments, initial length 21.4 mm,
were incubated for 22 h in the stated level of auxin.

IAA solutions of the stated dilution were prepared
before the experiment from 10^{-3} M stock solutions of
the stated age. Bars show means for 8 segments ± SE.
Control growth (no auxin) is also shown (dashed line).

injection septum, from a cylinder of ethylene (purity: 99.8%, BDH Ltd., Poole, UK). The ethylene was then introduced into the desiccator through an injection septum set into a hole in the lid.

To facilitate thorough mixing of injected ethylene with air in the desiccator, the syringe plunger was forced up and down a number of times before the syringe was withdrawn from the injection septum. This flushes ethylene through the inlet tube and into the desiccator proper.

The standard desiccator stopper was not used although a suba-seal could be fitted to it: this was because it incorporates a dead volume of air which is connected to the body of the desiccator by a sharply-curved tube (Figure 8). This constriction severely retards the efficient mixing of injected ethylene with air in the desiccator proper, as shown in tests in which a small quantity of ethylene was injected into desiccators fitted with various types of stopper. The progress of mixing of injected ethylene with the air in the desiccator was followed by successive assay of the air in the stopper, using GC (methods given on page 108). When the standard desiccator stopper (with constriction) was fitted, the ethylene level in the samples remained very high until several hours after ethylene injection (Figure 8), indicating that large quantities of ethylene had remained in the stopper, and had not mixed with the air surrounding the segments. This means that a considerable delay would occur between ethylene injection and exposure of segments to the stated level of ethylene, with unknown consequences for growth responses. Clearly, this is not acceptable, and home-made stoppers consisting of a bung pierced by a straight, short, wide glass tube capped with a suba-seal, were used. With these, the ethylene level in withdrawn samples approximated to the intended level within a few minutes of injection, and mixing was complete within 60 min. (Figure 8). Ethylene levels were checked periodically during ethylene treatments using a GC assay.

Time (h) after injection	Standard stopper	wide glass tube stopper
1	− (200+)	57
$2\frac{1}{2}$	− (200+)	56
$3\frac{1}{2}$	− (200+)	56
21	− (200+)	56
24	− (200+)	55
48	53	51

FIGURE 8   Dissipation of injected ethylene into desiccators, through two types of stopper.

The table gives level of ethylene (μl l^{-1}) remaining in the stopper at various times after injection. If injected ethylene has diffused evenly throughout the vessel, approximately 50 μl l^{-1} should remain in the stopper.

The diagrams (top) show cross sections of each type of stopper. The route of entry of ethylene is also shown (dashed line). GC sensitivity was such that ethylene levels in excess of 200 μl l^{-1} produced an off-scale response.

CHAPTER 7

EXPERIMENTAL   TECHNIQUES

CHAPTER 7

EXPERIMENTAL TECHNIQUES

Measurement of Growth.

1. Low-Resolution measurement. For routine estimation of growth, segment

length was measured, at the stated time, to the nearest 0.5 mm using a

fine rule. Curved segments were simply pressed straight along the edge of

the rule so that their lengths could be gauged.

In a small number of experiments, higher resolution of segment length

was obtained using a photographic enlarger. The segments were laid out on

the glass plate of an enlarger, and the transmitted images was later measured

using a ruler or, for curved images, a centrally-placed "flexicurve".

Similar methods have been used by several workers (e.g. Pope *et al.* 1979;

Pilet 1976; Brummell and Hall 1980).

In one experiment, the lengths of segments growing in sealed glass

desiccators was estimated without opening the desiccators. This allowed

continuous exposure of segments to gases (ethylene) in the chamber, without

the normal interruption of gas treatment during measurement. This method

also reduces the danger of contamination of control chambers with ethylene,

which may arise when treatment desiccators are opened alternately in a

limited space. In this experiment, estimation of length was carried out by

placing the sealed desiccator on the base of an overhead projector. An

enlarged image of the desiccator, plus plastic petri dish contained within, and segments floating in the dish, was then focussed on a wall. Length of the segment images could be measured using a ruler and flexicurve. Two refinements greatly facilitated this method: first, the rounded desiccator lid was replaced by a flat perspex sheet (with a hole drilled in it to fit a rubber injection septum). This greatly improved light transmission and image clarity. Second, the petri dishes (containing incubation medium and floating segments) were each provided with a ring of filter paper which fitted around the inside of the dish and remained suspended at the surface of the incubation medium. These rings prevented contact of any segment with the plastic side walls of the petri dish. Such contact could otherwise occur, with associated obscuration of the transmitted image of the edges of the segment, because of: a). the shadow of the petri dish wall; b). refraction and diffraction of the transmitted light at the point of contact between the segment and the side of the dish, where the water surface becomes distorted by capillary forces.

For convenience and accuracy, this method is greatly surpassed by transducer methods (see page 90); I describe it because it may be of interest to those without access to a transducer with chamber, but who wish to make time-course measurements of length without repeatedly disturbing a gaseous treatment. Useful results can be obtained (Figure 9).

2.   High-resolution measurement.   Two methods were used to make relatively high resolution measurement:

a.   Lever/projection System.   A length of plant material was weighted to the bottom of a large beaker of water (or other incubation medium). The free end of this specimen was attached to the shorter of two arms of a pivotted lever (the lever is weighted so that the 2 sides balance across the pivot despite their different lengths: see Figure 10). From the longer arm of the lever, a weight is suspended so that it hangs in front of the
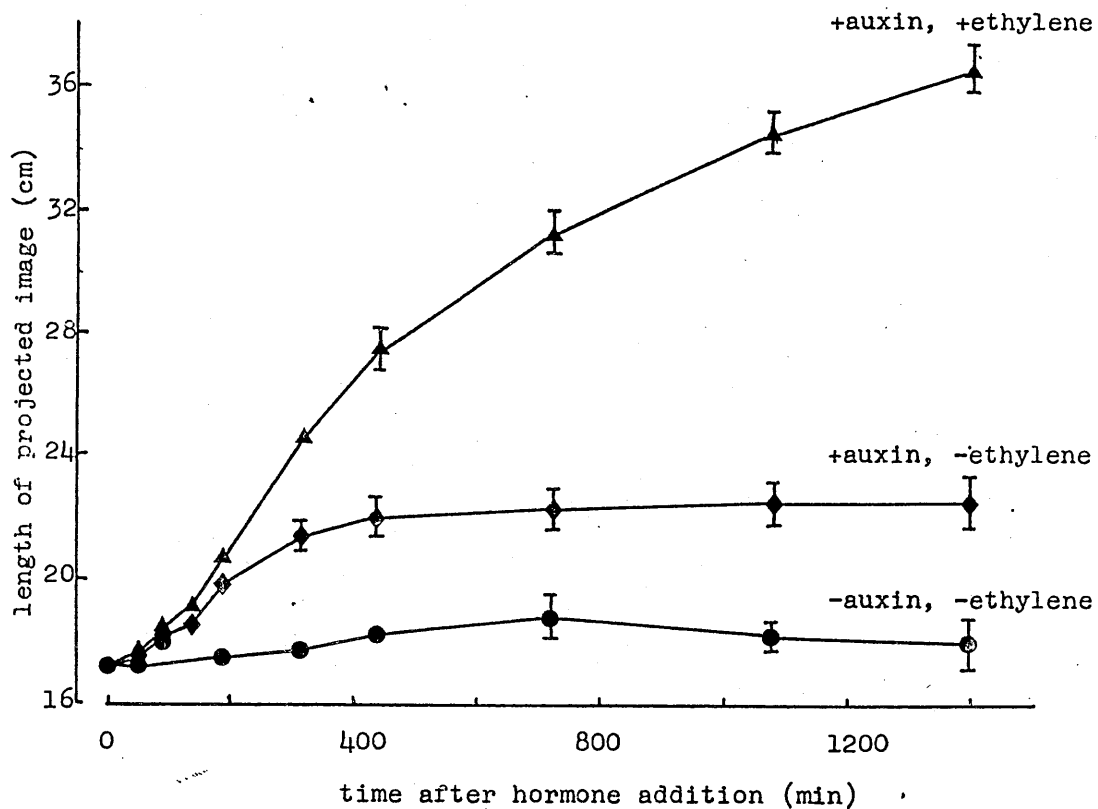
FIGURE 9   Use of the through-projection system to measure elongation
with time, of *Regnellidium* segments treated in auxin (♦),
or auxin plus ethylene (▲). Control growth is also shown
(●).

Apical petiole segments, initial length 21.4 mm, were used.
Auxin was as 10^{-5} M IAA, ethylene was supplied at 25 µl 1^{-1}.
Each point is a mean of 10 segments ± SE.

Length of the projected images is shown on the axis, actual
length of the segments at the end of the experiment (1400 min)
were:

> ● 21.9 ± 0.1 mm
> ♦ 28.0 ± 0.7 mm
> ▲ 46.1 ± 1.0 mm
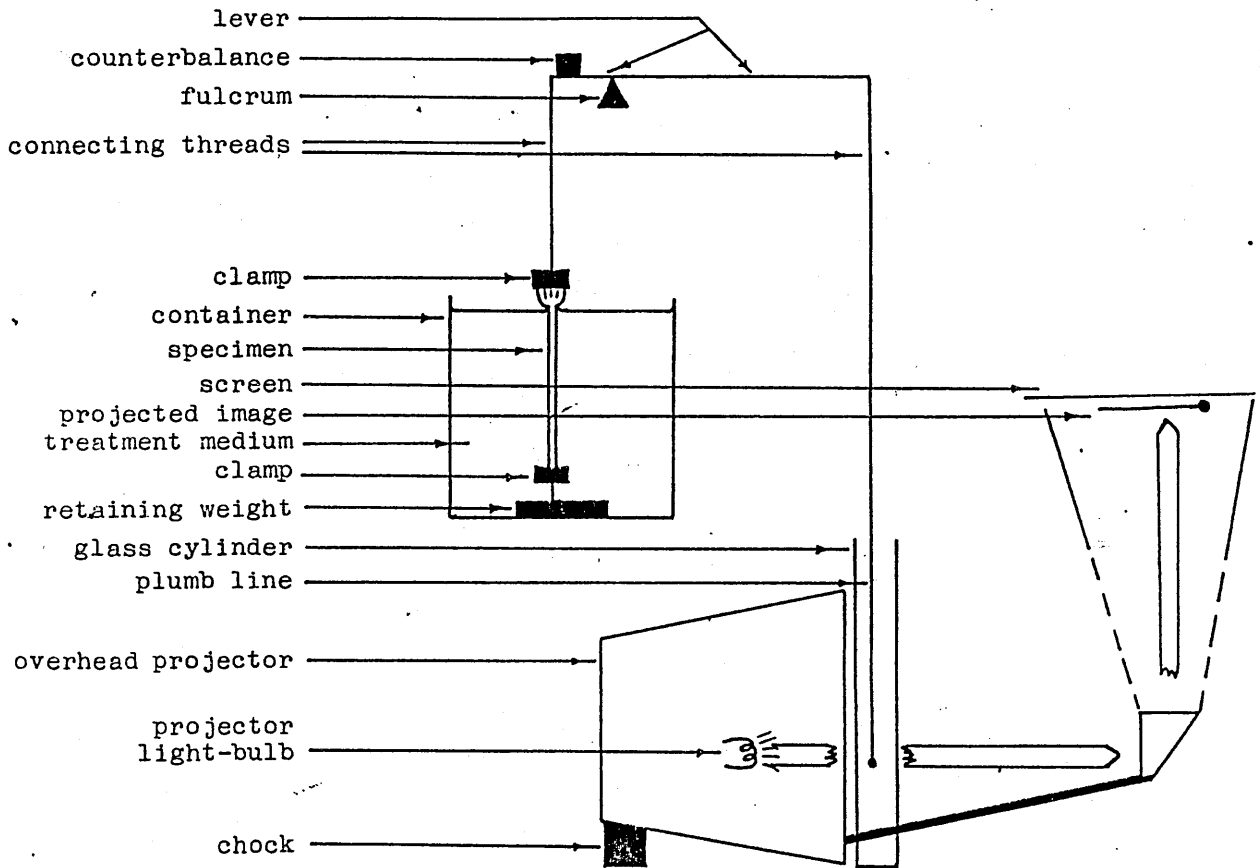
FIGURE 10   Diagram of Lever/Projection system as used for some high resolution measurements.

A tension could be applied to the specimen by adjusting the counterbalance weight (top). Extension is followed by measuring movement of the projected image across the screen (right).

glass base-plate of a horizontally-placed overhead projector. A projected image of the suspended weight (greatly magnified) is then focussed on a nearby wall. As the plant grows, the longer arm of the lever falls, and the weight suspended from it moves across the face of the base plate causing the projected image to track along the wall. This last movement is monitored at intervals with a ruler, to provide a measurement of growth rate. Magnification is the product of 2 components:

i. dependent on the ratio of the lengths of the 2 arms of the pivotted lever

ii. dependent on the distance between the projector and its screen (i.e. the wall).

Magnification of about 60x was normally used here.

Further refinements to this system include enclosing the suspended weight in a tall glass measuring cylinder to isolate it from air movements in the room. To obtain relatively friction-free pivotting of the lever arm, the lever was glued across the balance wheel of a clock so that movement at the fulcrum occurred on the bearings of the balance wheel.

Again, the transducer used (see below) was much more convenient than this light-projection system. However, with the latter one can apply tension to the specimen (by de-equalising the balance of the lever). This could not be done on the transducer as used here. Consequently, the light-projection method was used in some later experiments involving applied "pulls", e.g. those testing the effect of pH on extension of frozen-thawed material.

b. <u>Transducer</u>. Most of the high-resolution measurements reported here were done using position-sensing transducers. The relevant methods are discussed in two sections.

Section I - Electrical equipment.

Two independent transducers were used so that comparisons between growth

responses could be made simultaneously: for example, ethylene promotes

growth only when auxin is present; but auxin also promotes growth; thus

to estimate an effect of ethylene, one must monitor the difference between

growth of an auxin-plus-ethylene "experimental" treatment, and that of an

auxin-only "control".

The units used were Sangamo (North Bersted, Bognor Regis, Sussex, UK)

model DF/5.0/S displacement transducers. Power supply and output demodula-

tion for each transducer was provided by a Sangamo DCU-1B conditioner unit.

Outputs from the 2 transducers were recorded side by side on a single chart

using a dual-pen recorder (Philips model PM 8252).

The transducers were positioned so that as petiole segments grew,

they pushed the soft iron core of the transducer up through the centre of

the unit. Live coils within the transducer develop a voltage dependent on

the vertical position of the soft iron core within the unit; change in

output voltage is thus dependent on growth of the specimen.

The iron core of the transducer was found to be too short (3 cm) for

convenient use in the apparatus; an extension was therefore fitted by

inserting a 7 cm length of fine steel wire into a small hole drilled in

the lower end of the existing extension (see Figure 11). At the lower end

of this wire extension, where contact was made with the specimen, a small

blob of sealing wax was affixed so that the wire would not stick into the

specimen.

Particular attributes of the transducer used are:

1. Light weight of the core. Even with the added extension the entire

core assembly (which rested on the specimen) weighed only 0.9 g (cf. up to

soft iron core

transducer

core extension

treatment medium

syphon tube

container

drip needle

wax rim

wax blob

segments

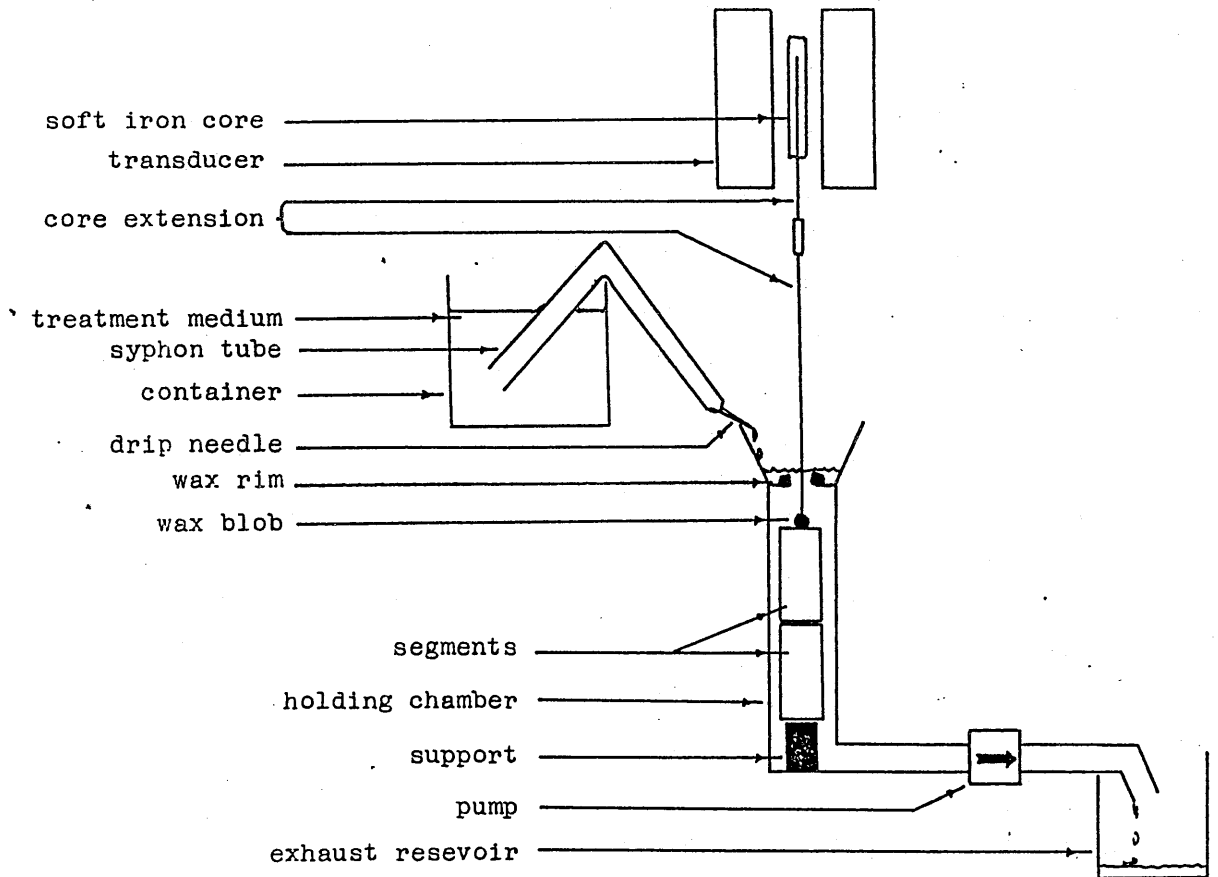holding chamber

support

pump

exhaust resevoir

FIGURE 11 Diagram of the arrangement used for high resolution growth measurement with displacement transducers.

Transducer output voltage, being proportional to extension, is fed to a chart recorder. Note that the pump is used to draw fluid out of the chamber.

8 g with some types of transducer). Counterbalancing devices were thus considered unnecessary.

2. Long operating stroke. The transducers used gave linear responses over a 1-cm displacement of the core. This means that necessity for resetting the transducer, even over longer-term experiments with rapidly-growing material, was minimal. Some transducers have a linear response range of only 2 mm or less, and may require frequent resetting, which is inconvenient since it requires:

a. presence of the operator;

b. interruption of the recordings;

c. disturbance of the specimen.

Such problems can however, be greatly alleviated by fitting an automatic resetting device (Green and Cummins 1974; Cosgrove and Green 1981). Such equipment was not available here, and resetting was done manually by raising a clamp which held the body of the transducer.

For convenience, a "plus 100%" offset facility on the conditioner units was used. This causes the transducer to output zero when the core is at its lower limit rather than when at centre.

Section II - Transducers.

Arrangements for holding and treating segments.

Several different arrangements were tried; most were suitable for treating the segments with liquid media and with solutions of growth substances. However, most were not suitable for exposure of material to both liquid and gaseous treatments. There are few published reports covering high resolution measurement of the effect of gases on plant growth; of these,

most use intact seedlings growing in an enclosed chamber containing the desired gas mixture (Rauser and Horton 1975; Warner and Leopold 1971; Penny *et al.* 1974; Hall *et al.* 1977). Such methods are not amenable to segments, or do not allow concurrent exposure of the same areas of tissue to both auxin and ethylene. Musgrave and Walters (1974) used a travelling microscope to obtain high-resolution measurement of ethylene-induced growth in *Regnellidium* leaves. However, their system utilised intact fronds with the leaf blades growing in air; such an arrangement is of limited value for investigations of auxin effects because of uncertainty about the degree to which auxin will penetrate the petiole in the absence of apical cut surfaces (see page 140). In addition, Musgrave and Walters' apparatus did not allow direct treatment of the entire specimen with both auxin and ethylene. Thus their system was here rejected for investigations of the kinetics of auxin-, and ethylene-induced growth.

Some workers have made transducer-type measurements of the influence of gaseous compounds, especially CO_2 (Harrison 1965; Barkley and Leopold 1973; Bown *et al.* 1974), but including ethylene (Nee *et al.* 1978), on growth of excised segments. These have invariably involved applying the gaseous substance by flushing the segments with aqueous medium containing the dissolved gas at a predetermined level. This procedure is less than ideal for studies of kinetics because it incorporates a time delay while the dissolved gas diffuses into the specimen. This delay may not be insignificant since even narrow layers of water are known to be effective barriers to ethylene movement (Kawase 1976).

Another problem associated with keeping segments continually immersed is that control treatments could accumulate appreciable quantities of endogenously-synthesised gases, including ethylene. Ethylene accumulation is an automatic consequence of immersion in many tissues, given the slow diffusion rate of ethylene in water (see page 9).

Ideally then, the experimental set-up would allow, i) direct exposure of the tissue to ethylene in the gas phase; and ii) complete immersion in auxin solutions so that auxin can contact all areas of the segments, not least the cut surfaces. The set-up described below allows a satisfactory resolution of these paradoxical requirements. Segments were held vertically in a glass tube 9 cm long, and of inside diameter c. 2.5 mm (i.e, the holding chamber, Figure 11). This was just wide enough to contain all but the widest segments without restricting their elongation. I refer to this as 'sleeve mount'. Similar types of mount have been used by some other workers (e.g. Nee *et al.* 1978; Edwards and Scott 1974; Barkley and Leopold 1973). The commonly-used method of mounting segments by threading them on a long needle, wire, or thread (e.g. Phillipson *et al.* 1973a; Evans and Ray 1969), while admirable for use with hollow segments such as those of some coleoptiles, was rejected for the present study since it caused excessive tissue damage. The technique used by, for example, Penny *et al.* (1974) of impaling the base of the segment on the point of a pin was also unsuitable for the present work, since columns of segments were usually used in preference to single segments, in order to obtain results from a larger population.

Plastic tubing connected the base of the glass holding chamber to a peristaltic "mini-pump" (Schuco Scientific Ltd., Woodhouse Road, London) which was arranged to draw medium *out* of the holding chamber (Figure 11). A plastic cup was glued to the top of the holding tube; medium was dripped into this cup from a syringe needle placed on the end of a glass tube which, in turn, siphoned fluid from a 1 l reservoir. The rate of medium entry into the cup could be adjusted by altering either:

i.   the gauge of the syringe needle, or

ii. the level of medium in the reservoir relative to the level of the
     drip needle.

The apparatus was arranged so that the rate of medium entry into the cup was only 50%-80% that of liquid removal from the holding chamber by the pump. Air was free to enter the cup to make up the deficit in volume. If the apparatus was used as stated so far, an unbroken trickle of medium continually entered the holding chamber from the cup, and ran down one side of the tube (contacting only some parts of any segments contained therein); the rest of the holding chamber remained air filled. This is not what was required. It was found that if a rim of paraffin wax was applied to the upper end of the glass holding chamber, surface forces developed at the entrance to the chamber, between the aqueous medium and the hydrophobic wax, caused alternate blocks of air, then water to move *en masse* down the holding chamber, and over the segments contained within it: this is because an appreciable depth of aqueous medium must accumulate in the cup before the medium can close in over the waxed entrance hole of the holding chamber. Until this critical depth is reached and surpassed, air alone will enter the holding chamber. Prior to achievement of the critical depth, medium does not flow across the wax from any one side, and a large reversed meniscus builds up until medium suddenly closes in over the entrance hole, completely excluding further entry of air; water alone is then drawn into the holding chamber for a time. Because pumping out of the holding chamber is set faster than dripping into the cup, the level of medium in the cup falls until a new critical depth is reached (much lower than the first), when the medium film breaks away completely from the waxed entrance hole. Once again allowing only air to flow in. The cycle thus repeats.

The duration of each part of the cycle could be varied as desired by altering:

i.   the rate of medium entry to the cup,

ii. the rate of medium efflux from the holding chamber.

I usually used a cycle of about 1 min in each of water and air, however, the lengths of these periods was not considered critical. The pumps most frequently used had 10 rpm motors which, with large bore tubes fitted to the drive cam, provided a flow rate of about 1 ml min^{-1}.

Hard-grade embedding wax was used to form the hydrophobic rim; before the start of each run, the old wax rim was detached from the holding chamber, melted down, and reapplied using a hot glass rod. This was necessary since the hydrophobic properties of each rim appeared to diminish after about 18-24 h, possibly because of colonisation of the surface of the wax by microbes. Petroleum jelly was not tried on the rim, since it was feared that the jelly might absorb auxin in some experiments, then release it to the ruin of subsequent experiments.

The transducer unit and holding chamber were clamped in position on a clamp stand which was placed inside a 35 1, glass chromatography tank. Plastic leads from the holding chamber to the pump, and electric leads from the transducer to the conditioner unit, passed through a hole in the lid of the chromatography tank. The tank was made virtually air-tight by pouring glue around the leads where they pierced the lid. A second hole in the lid bore a gas-tight injection septum.

Exhaust gas from the "plus-ethylene" chamber was led well away from the "minus-ethylene" chamber, to avoid the risk of contaminating the latter. Occasional checks of the ethylene level in each tank, using GC (see page 108), confirmed that the levels remained as intended.

A small amount of water was usually placed on the floor of each chromatography tank at the start of a run; this helped to keep the humidity high throughout. Lighting was by ordinary fluorescent room lights which remained on throughout experimental runs. No special temperature-control equipment was used, however the experiments were done in a small, isolated

room with double doors and no windows, so that temperature was fairly consistent at 23-25 °C during the winter months, and slightly higher during summer. In initial trials, the pumps were fitted inside the tanks but overheating was then a problem; this was overcome by fitting the pumps outside the tanks.

The two tanks were placed side by side and as close together as possible so that any fluctuation in environmental conditions would affect control and experimental runs equally and should therefore cancel out within each pair of runs. Growth measurements were normally taken using a column of 4 x 1 cm apical *Nymphoides* petiole segments.

For experiments in which the immediate kinetics were to be observed, auxin "addition" was taken as the time that auxin solution first began to bathe the top-most segment of a column. Ethylene, in this type of experiment, was injected near the opening of the glass holding tube and time of "addition" was taken as the time that ethylene-laden air was first drawn down past the segment. In these kinetics experiments, the segments would have been initially exposed to a very high level of ethylene; this was to ensure that ethylene reached the cells as soon as possible after "addition".

The limits to resolution of the transducer system were imposed largely by the stability of the tanks (which were mounted on rubber bungs). The transducers were set to give a 10V output for 1-cm displacement of their cores; the chart recorder could be set to show full scale deflection (25 cm) for a 1 mV change in input voltage. Thus 1 cm movement of the transducer core could theoretically produce a movement of up to 2,500 m on the chart; this is a magnification factor of 250,000. Growth increments as small as 0.5 μm could easily be followed if required. Normally, much less sensitive settings were used so that the chart pen would not go repeatedly off-scale. For example, most runs with 4 x 1 cm segments were set so that the chart recorder would show full-scale deflection for 1 cm of growth of the column of segments.

In a small number of cases, rate of growth was measured rather than segment length; this was done by the method of Penny *et al.* (1974): the transducer outputs were plugged not into the chart recorder, but into digital voltmeters, and the digital read-out was noted, to the nearest 0.1 mV (= 0.1 µm), every 20 or 30 sec. Rate was calculated from the difference between successive readings, and expressed graphically as a five-point moving average.

Measurement of Medium Acidification.

This was done using small pH electrodes to sample incubation medium containing abraded segments. Similar methods have been used by Evans and Vesper (1980); Bates and Cleland (1979); Rayle (1973) and others.

A large number of abraded segments (usually 26 x 1 cm segments) were incubated in a small volume (usually 4 ml) of lightly-buffered medium in a 10 ml beaker. 1 mM K^+-citrate/phosphate buffer, pH 6.4, including 0.1 mM $CaCl_2$ was the usual medium. Incubation media were vigorously oxygenated for 30 min prior to, and during experiments, through a syringe needle connected to a cylinder of pure oxygen (Air Products Ltd.). The medium was adequately circulated by the rising oxygen bubbles.

To take a pH measurement, about 3 ml of incubation medium (but no segments) were decanted into a 5 ml beaker containing a 1 cm length of stainless steel wire to serve as a spin bar. Electrodes were dipped into the vigorously-stirred 3 ml of medium, and pH noted when it had stabilised (within 40 sec). The medium was then tipped back into the 10 ml treatment beaker from which it had come, and incubation continued; pH measurement thus took about 1 min per treatment. The electrodes used were: 1) glass needle pH electrode with bevelled tip, model MI-408, purchased from Microelectrodes Inc., Londonderry, N.H., USA; 2) small Ag/AgCl glass reference electrode filled with a solution of 3M KCl saturated with AgCl.

This was also purchased from Microelectrodes Inc. The two electrodes were connected to a 10^{12} ohm input impedance research voltmeter (Model P.H.M. 63, Radiometer, Copenhagen).

Auxin was simply added to the incubation medium as required. Ethylene was applied, in these studies, by bubbling the gas through the incubation medium, slowly but continuously for 30 min prior to segment addition, and for the duration of the experiment. The precise level of ethylene perceived by the segments is not known, but it is virtually certain to have been saturating; at any rate, a large ethylene-induced growth response could be induced (Figure 45).

Microelectrodes.

i. Manufacture and testing.

For more definitive measurements of H^+ excretion than can be provided by monitoring medium acidification, insertable pH micro-electrodes were used. Microelectrodes are probably the ideal tools for studies of change in wall pH. They can penetrate to the very site of the important change, and can thus give accurate information about kinetics. Other techniques have certain disadvantages in this respect (see page 222) though good results are obtained by some workers despite these (e.g. Evans and Vesper 1980). Another advantage of insertable microelectrodes is that they can identify pH change in limited areas of tissue. This may be more difficult with other techniques; for example, with the medium-acidification technique described above, a mean value is obtained for the entire segment. Significant differences in wall acidification, which might occur between the various regions of the segment, would thus go undetected; this may be important (page 211). Such differences can be studied with microelectrodes (see page 219).

For the present study, glass pH microelectrodes were considered superior, in terms of stability and fidelity to pH change, to other types of electrode such as antimony-coated or tungsten electrodes (see Thomas 1978; Roos and Boron 1981; Brown and Owen 1979). Antimony-coated electrodes in particular, have frequently been used in previous studies of extra-cellular pH in plants (e.g. Jacobs and Ray 1975; Penny et al. 1975).

Microelectrodes were made from pH-glass tubing (Corning code 0150, purchased from Clark's Electromedical Instruments, Pangbourne, Reading, UK). The glass was insulated with "Shellac" purchased locally.

Extreme miniaturisation of electrodes was not attempted here; indeed, in this study it would be undesirable for several reasons:

a.   the electrodes should be robust enough to pierce into plant tissue. Extremely small electrodes are very fragile;

b.   extracellular recording was required in the present study, consequently the electrodes should be so large that there is no danger of them entering a cell and recording intracellular pH (plus membrane potential);

c.   extremely small electrodes have proportionately higher electrical impedances, which can be a problem.

By "extremely small" electrodes I refer to designs with tip diameters of, say, 5 μm or less, and with exposed-tip lengths of about 50 μm or less. Electrodes with tip diameters down to 0.06 μm have been reported (Hollander 1976, cited in Roos and Boron 1981) as well as those with exposed tip lengths of virtually nil (Pucacco and Carter 1976; Thomas 1974). Such electrodes are intended for intracellular work and, for the reasons noted above, are too small for the present purpose.

Commercially available "microelectrodes" on the other hand, are much too large for this work. For example, the smallest glass pH electrode

currently available from "Microelectrodes Inc." of Londonderry, N.H.,
USA, has a tip diameter of some 0.8 mm, and requires immersion to a depth
of 1.0 mm.

Ideally, for work on *Nymphoides* petiole, and probably many other
tissues, the electrodes should pierce easily into the peripheral region of
compact tissue, which is some 0.1-0.3 mm deep. The electrodes used here
were of appropriate size.

Several different types of electrode were tried, such as glass/glass-
sealed recessed-tip (Thomas 1974), and protruding tip electrodes (Hinke 1961);
also membrane glass/glass-sealed electrodes (Pucacco and Carter 1976). These
were found to be extremely difficult to make, especially in the larger size
required here: ironically perhaps, slightly larger electrodes are probably
more difficult to manufacture in some designs, than are extremely small
electrodes. This is because the insulating glasses commonly used (boro-
silicate, Corning code 7740; and aluminosilicate, Corning code 1720) have
expansion coefficients greatly below that of pH glass. Consequently if
relatively thick glasses of the two types are fused together, there is a
high probability of cracks developing as the joint cools. This is less
of a problem when the glasses are very thin (Thomas 1978).

There is an alternative insulating glass which has expansion
coefficient much closer to that of pH glass; this is lead glass (Corning
code 0120). Although lead glass has some disadvantages (Thomas 1978), it
has been used for electrode construction by several workers (for example,
Harrison and Walker 1977; Khuri 1969; Hinke 1969). Unfortunately I was
unable to obtain lead glass. I suspect it may have been suitable for the
larger electrodes used here.

Electrodes used here were made as follows: pH glass tubing was pulled
out on a conventional glass puller (Model 2001, SRI Ltd.), to form micro-

pipettes. Each micropipette was mounted on a micromanipulator and its tip sealed by approaching it to the heated platinum element of a home-made micro-forge (Hinke 1969; Thomas 1978) while observing through a binocular microscope. As soon as the tip had sealed the pipettes were withdrawn. Shellac was applied around the shank of the pH-glass micropipette by placing flakes of the material on the glass, then heating with a soldering iron. Using the tip of the iron, shellac was worked evenly over the glass surface and down towards the tip, again observing through the binocular microscope, until only a small piece of glass remained visible at the tip of the electrode. Alternatively, the tip can be completely covered in shellac then re-exposed to the desired extent by approaching it towards the hot element of the microforge, observing as the shellac melts slightly and recedes from the tip.

Electrodes are placed on a holder of the type shown in Thomas (1978) or Khuri (1969), and immersed in a beaker of distilled water at 60°C. The beaker is then put in a vacuum desiccator and the water boiled for 1-2 min. by reducing the pressure. This fills the electrode with water. Larger air bubbles remaining inside the electrode can be dislodged with fine steel wire ("cat's whisker"). Using a microsyringe, water inside the electrodes is replaced with the desired filling solution. Microsyringes were made by melting and drawing out the barrel of a 1 ml plastic syringe (Thomas 1978). The filling solution used here was 0.1 M acetate buffer, pH 7, with 1% v/v of 3 M KCl added.

A chlorided silver wire was inserted into the open end of the electrode and sealed in with a drop of dental wax (kindly supplied by Dr. I. Tikam of the Eaglestone Dental Clinic, Milton Keynes). The silver wire was chlorided either by dipping in molten silver chloride (Thomas 1978), or electrolytically (Morath and Hertel 1978; Janz and Ives 1968). The silver wire protruding from the electrode was soldered onto screened cable

and plugged in to a battery powered electrometer with input impedance variable up to 10^{14} ohms (Keithley model 504).

Earlier versions of these electrodes were found to work well for about 12-24 h, but would often then undergo a voltage collapse (such an event was recorded on chart paper and is reproduced in Figure 12). After this collapse, the electrode impedance would be found to have dropped to perhaps 10^6 ohms or even less. Clearly, there had been a breakdown in insulation. The problem was eventually found to stem from partial hydration of the pH glass, incurred during storage at the supplier's depot. A hydrated surface film extended up the outside of the glass electrode and back down the inside providing a short circuit. pH glass should be stored desiccated (Baucke 1976) or immersed in methanol, but this was not done at the supplier's (Clark, of Clark's Electromedical, pers. comm.). The problem could be countered by heating the shaft of the electrode, until melting of its surface began, with consequent destruction of the hydrated surface film. With all subsequent electrodes, this step was carried out prior to application of the shellac coat. The partial hydration of the pH glass did have the advantage that completed electrodes would work immediately after construction; they did not require hydration before use, as glass electrodes usually do (Thomas 1978). Some workers hydrate completed electrodes for up to a week or more, depending on the conditions (Carter et $al.$ 1967).

The pH-measuring circuit was completed by an Ag/AgCl glass reference electrode, consisting of a borosilicate-glass micropipette containing 3 M KCl in 1% wt/v agar. (For a detailed consideration of reference electrodes, see Covington 1969; Bates 1964).

Resistance of the pH electrode was estimated by placing the pH-, and reference electrodes in a solution of constant pH and observing change in measured voltage as the input impedance of the electrometer was altered. Resistance is given from equation 4
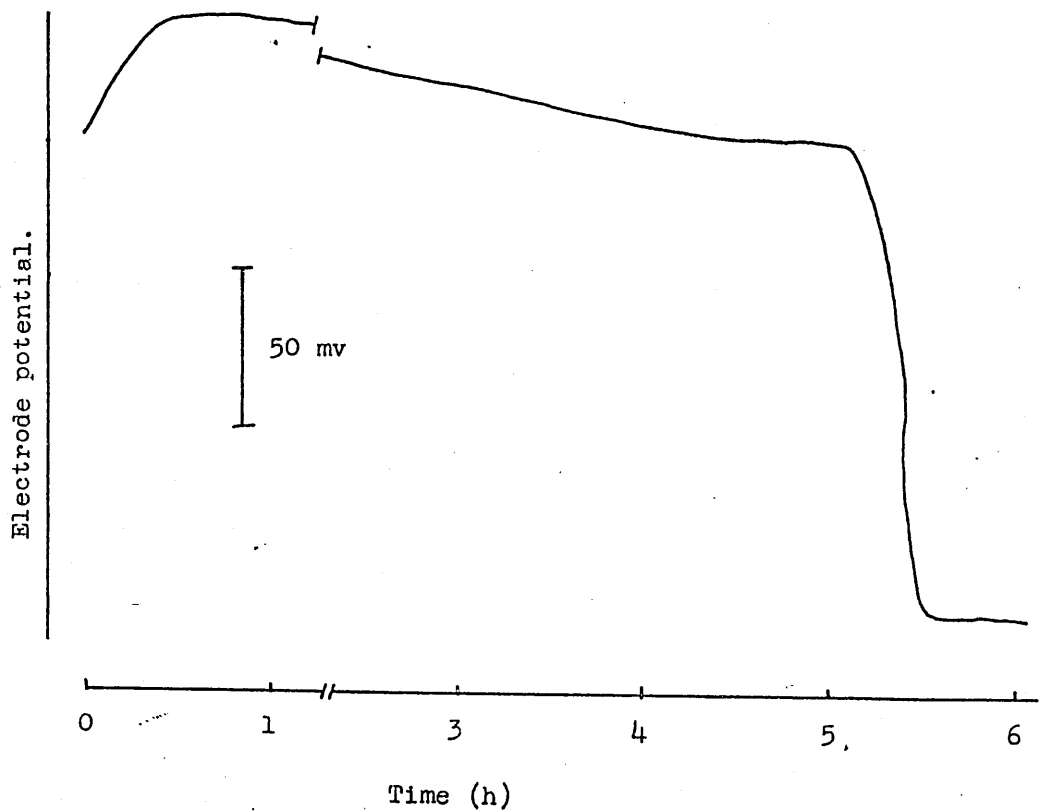
FIGURE 12   Recording of electrode voltage with time, from a pH
electrode.

Towards the end of the recording, the electrode voltage
collapses rapidly, probably because of a breakdown in
electrode insulation.

$$R = \frac{VM}{V'} - M \qquad \cdots\cdots\cdots \text{ equation 4}$$

where R = electrode resistance,

V = measured potential at very high voltmeter impedance (i.e. 10^{14} ohms)

M = meter impedance when the second reading of potential (V') was taken,

V' = apparent (measured) potential when meter impedance is set to M.

For accurate calculation M should be about the same as R. Typically, my electrodes had resistances of 10^{10}-10^{11} ohms.

ii. Recording from plants with the microelectrodes.

The pH electrode was fixed to a micromanipulator and advanced into the segment (see Figure 13). A careful watch was kept during this stage, through a viewing microscope. When the electrode had been positioned the voltmeter was switched on and its output recorded on a chart recorder (Phillips model 8251). An equilibriation period of at least 2 h was allowed before addition of any test substances.

Very little electrical "shielding" was used since the apparatus was situated in a peaceful corner of the room. However, earthing leads to the microscope and micromanipulator were necessary to remove 50 Hz interference.

Prior to insertion into a segment, electrode output was calibrated against proprietary buffers of pH 7 and pH 4. An ideal electrode would give the full potential predicted by the Nernst equation (equation 5):

$$E = RT \ln \frac{a_o H^+}{a_i H^+} \qquad \cdots\cdots\cdots \text{ equation 5}$$

where E = the electrical potential (mv),
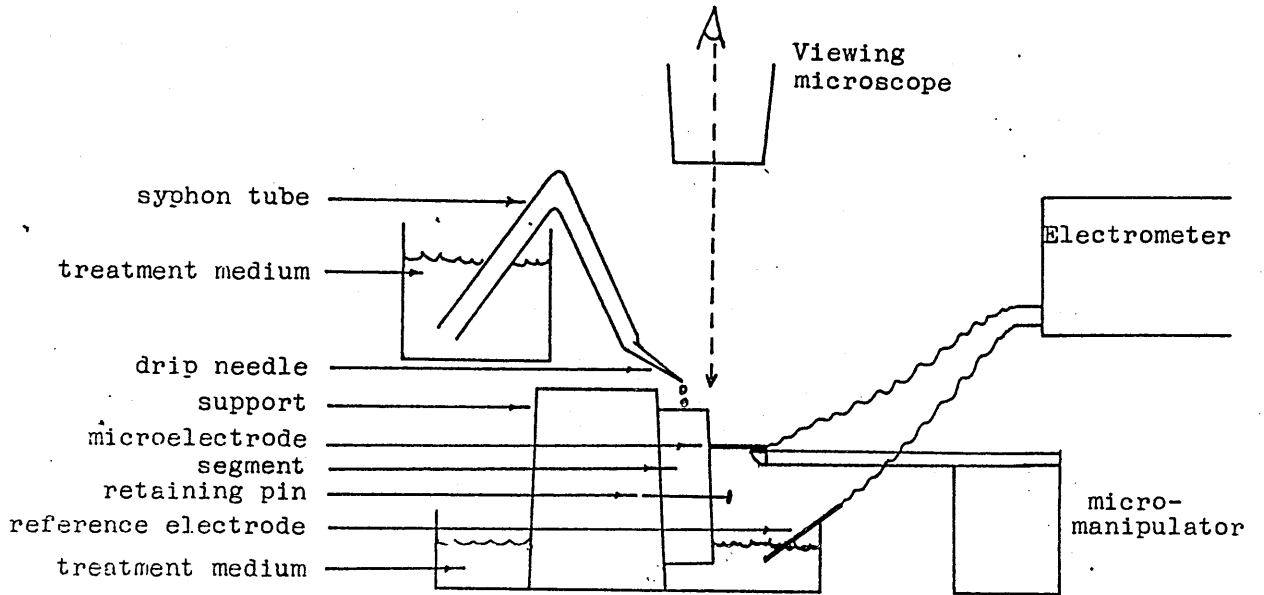
R = the gas constant,

FIGURE 13 Diagram of arrangement commonly used for microelectrode
recording from plant segments.

F = the Faraday

$a_o H^+$ = activity of protons outside the electrode,

$a_i H^+$ = activity of protons inside the electrode.

Converting the natural logarithms to base 10, and applying numerical values for the constants;

$$E = 59 \log_{10} \frac{a_o H^+}{a_i H^+} \quad \text{at } 25°C.$$

For a pH difference of 1 (outside lower), $\dfrac{a_o H^+}{a_i H^+}$ = 10,

Log_{10} of this is 1, thus E = 59 mv.

Any electrode which gave a potential of less than 55 mv/pH unit was discarded.

Segments were usually held vertically, being pinned to a rubber bung. They were gravity-fed with medium from a dripping needle. The base of the segment was in contact with a small reservoir of medium into which the reference electrode was placed (Figure 13). Auxin or FC were added to the dripping medium as necessary. Ethylene was not used in these experiments.

Determination of Ethylene.

Low levels of ethylene in air were detected and quantified by Gas-Solid Chromatography, largely following the methods of Ward *et al.* (1978). A Pye GCD Gas Chromatograph with flame ionisation detectors was used. The column was 4' x $\frac{1}{4}$" Fl Alumina (J.J. Chromatography, Kings Lynn, Norfolk, UK). Gas samples, usually 1 ml, were injected on-column from Gillette gas-tight syringes with push-on hypodermic needles. Determinations were done isothermally at a column temperature of about 135°C with injector at about

140°C and detector at about 150°C. The carrier gas was high purity nitrogen

(Air Products Ltd.) flowing at about 40 ml min^{-1}. Flame gases were standard

purity hydrogen (Air Products Ltd.) at 45 ml min^{-1}). The hydrogen flow rate

was most critical; small adjustments in it greatly affected the response of

the GC to ethylene. Output from the detector was fed to a chart recorder

(Phillips model 8251).

The GC response was found to be such that peak height on the chart

was approximately proportional to the quantity of ethylene injected over the

important range (0.01-100 nl ethylene in 0.2-2.0 ml injection volume). Thus

the ethylene level in any sample was estimated by comparing the peak height

of the ethylene response for that sample, with the peak height for standards

of known ethylene concentration. Standards normally used were:

1.   a 10 μl l^{-1} mixture of ethylene in air made up in the laboratory

in a 50 l carboy;

2.   a 1% mixture of ethylene in nitrogen, supplied commercially (Phase

Sep. Ltd.) was also used to check column performance.

For routine determinations, ethylene was quantified with a retention

time of 45-60 seconds, depending on the precise column conditions (see

Figure 14).

The only major problem encountered with GC assay of ethylene was that

of leaking injection septa. The rubber or plastic discs contained within

the injection port became badly worn when repeated injections were made

with the relatively large needles used here, with the result that injected

gas samples could be back-flushed out of the column via the injection port.

Frequent replacement of the septum can be done, but it is inconvenient,

since the carrier gas pressure must be allowed to fall substantially before

the injection port is opened. An alternative method is to place an extra

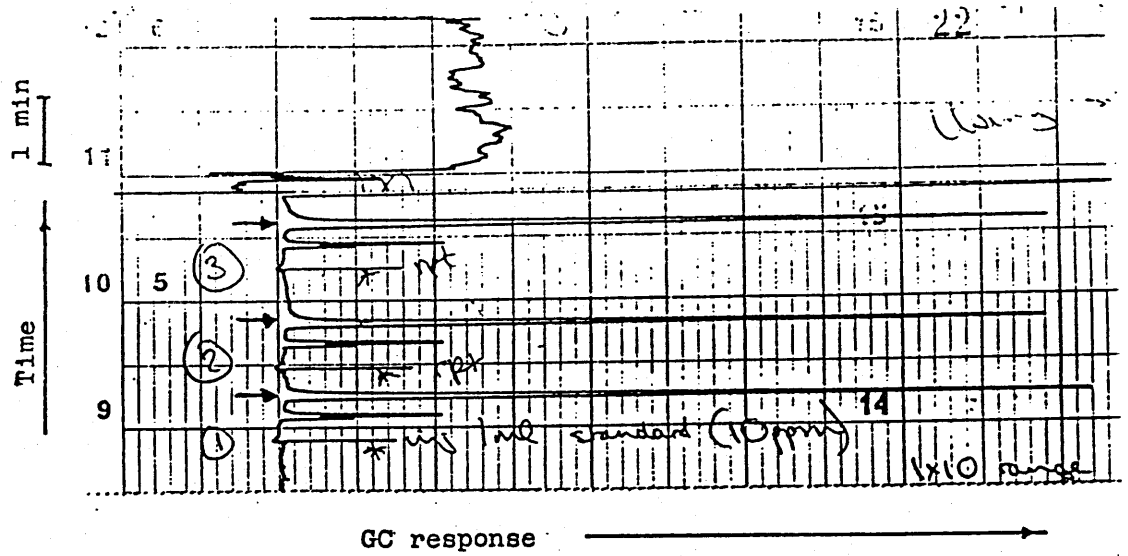injection septum over the syringe needle prior to injection, and to press

GC response ⟶

FIGURE 14   Gas chromatogram showing the response to three
successive injections of 1 ml each, of a mixture
of 10 µl 1^{-1} ethylene in air.

The time of injection is marked by asterisks,
and the ethylene peaks are arrowed. Chart speed
was 10 mm min^{-1}.

down firmly on the syringe during, and immediately after injection. The extra "collaring" septum forms a firm seal with the injection port and back-flushing is prevented.

When monitoring ethylene production by plant tissue, the material (petiole segments, whole leaves, or leaf discs) was incubated in "25 ml" (actual volume about 30 ml) ehrlenmeyer flasks, sealed with suba-seal injection septa. Gas samples were withdrawn periodically using gas tight syringes (usually 2 ml). After sampling, the flasks were flushed with air for a few minutes, then resealed for a further incubation period.

Vacuum extraction.

In some cases, gases were extracted from petiolar tissue using a vacuum treatment. Reasons for this were:

1. to gain samples of the internal air of the petiole for assay of ethylene levels etc.;

2. to remove air prior to sectioning for microscopy;

3. so that, on release of the vacuum, infiltration of treatment solutions (which might not otherwise enter the segments) would be induced.

Tissue was submerged in beakers of water (or treatment solution) during vacuum treatment. Intact *Regnellidium* fronds were held under water in a perspex grid through which their petioles protruded. Petiole segments of *Regnellidium* and *Nymphoides* were weighted down with glass rods. The treatment beakers were placed in 10 l or 15 l desiccators which were connected to a vacuum pump. Pressure was reduced to 0.5 atm. for 1 minute then returned slowly. The pressure was again reduced to 0.5 atm. for 1 minute, and returned slowly. It was found that such successive pressure reductions extract more gas than does a single one.

For experiments involving collection of extracted gases, a large funnel, capped with a rubber septum, was filled with water and inverted over the tissue prior to application of the vacuum. Evolved gas was collected from the funnel using a hypodermic syringe. In the few minutes required to complete this procedure, loss of ethylene by solution in the water will be negligible; however, salt solutions will dissolve even less ethylene than water, and have been used by some workers (Beyer and Morgan 1970b).

To enable predetermined reductions in pressure (in the absence of a reliable vacuum gauge), a volumetric flask was half filled with water and inverted in a beaker of water in the treatment desiccator. As the pressure is reduced, air in the volumetric flask expands, expelling water from the flask. When the air just fills the volumetric flask, one knows from Boyle's law that the pressure has been reduced to 0.5 atm.

Vacuum extraction has been used by several workers (e.g. Lyons *et al.* 1962; Kawase 1976; Mudge and Swanson 1978). In many cases, the pressure reduction was very large compared to that used here. For my experiments however, the smaller vacuums were more appropriate since often live tissue was treated and used in further growth experiments.

Wall Analysis Procedures.

Treatment of Material.

1. *Regnellidium* petiolar segments of 4 cm in length were excised from the apical region of freshly harvested fronds. Preincubation and subsequent treatment were as for *Nymphoides* (see below).

2. *Nymphoides*; 8 cm petiolar portions were excised from the region adjacent to the leaf. Each of these was subdivided into 2 x 4 cm segments, and these 4 cm segments were pooled.

Segment incubation.

Segments were preincubated for 10 h floating on either distilled water, or on a solution of 1% glucose in distilled water. Preincubation in water only, is believed to starve segments of sugars, thus inhibiting wall synthesis. This is important if changes connected with the initial action of auxin are not to be obscured by subsequent wall synthesis (Ray 1962). Loescher and Nevins (1972) could detect a marked auxin-induced change in non-cellulosic (NC) glucose levels in the walls of *Avena* coleoptile only if segments were depleted of sugars in the manner described, prior to growth promotion.

Preincubation was under ordinary fluorescent lighting, at a temperature of approximately 22°C. Immediately after the pre-incubation period, a sample of the segments incubated without glucose was homogenised. These provided the "zero time" wall samples. Their preincubation was without glucose. The remainder of the segments were distributed to one of three hormone treatments. Whichever sugar regime had obtained during preincubation was maintained during hormone treatment. Thus a total of six treatments were used (Table 4). Segments were floated on these treatment media (made up in distilled water) for 12 h, after which time segment length was estimated using a randomly chosen subsample of 25 from the 36 segments in each treatment. The segments were then frozen at -20°C prior to homogenisation and wall analysis.

Isolation of Wall Material.

1. Homogenisation of tissue.

Preliminary work on *Regnellidium* showed that several of the commonly-used homogenising techniques were unsatisfactory for this material, even though it does not seem to be particularly tough. Methods used in similar

Auxin $10^{-5}M$	Ethylene 50 ppm	Glucose 1 %
-	-	-
-	-	+
+	-	-
+	-	+
+	+	-
+	+	+

TABLE 4 Combinations of treatments for the wall analysis experiments.

work vary from simply pressing the segments between glass plates, to blending with sonication. These were apparently satisfactory where used (e.g. Ray 1963; Lamport *et al.* 1973). Even after prolonged maceration using pestle and mortar, ground glass homogeniser, or polytron homogeniser (Northern Media Supplies, Humberside), many unbroken cells remained in the water-plant material. Satisfactory homogenates were achieved only when using a Wiley Mill (Pascall Engineering, Crawley, Sussex); with this device, the plant tissue is ground by rotating in an unglazed ceramic chamber containing balls of various sizes, also of unglazed ceramic.

The plant material was given a preliminary grinding in a few millilitres of 0.1 M Na phosphate buffer, pH 7, using a pestle and mortar. The resultant partial homogenate was washed into the Wiley mill together with a further 25 ml of buffer. A few drops of octanol were also added as surfactant, to prevent excessive foaming. After milling at 0°C for 30 min (rotation rate of 100 rpm; chamber inside diameter: c. 8 cm), the chamber contents were rinsed out and the homogenate centrifuged at 500 x g for 5 min in a Mistral 4L centrifuge (M.S.E., Crawley, Sussex). The supernatant was discarded, and the centrifugate returned to the mill with 25 ml of buffer and octanol, as before. The purpose of this early centrifugation step was 2-fold; first, to check on the progress of the milling. This was important since occasionally portions of segments entered a rim formed at the junction of the mill chamber with its lid, where they were protected from the full grinding action. The pre-milling mortar and pestle step also helped to reduce this problem; secondly, the early centrifugation step helped to remove soluble enzymes which might otherwise have caused some hydrolysis or autolysis (Huber and Nevins 1979).

Homogenisation was completed by a further 10-15 h milling at 0°C. The homogenate was then rinsed from the mill chamber, and centrifuged at 500 x g for 5 min. The centrifugate was stored, frozen at -20°C, until

material from all six treatments (plus the zero time sample) had been homogenised. The homogenates were then washed.

2. Washing of Wall Material.

The pelleted wall material was cleaned by successive cycles of resuspension and centrifugation using a sequence of:

3 washes in buffer (0.1 M phosphate buffer, pH 7),

3 washes in 1% Triton X100 detergent,

3 washes in 1.0 M NaCl,

3 washes in distilled water.

Microscopical examination of this cleaned wall material showed a featureless paste, with no intact cells. The wall preparations were lyophilised for 12 h, by which time their weight was constant, and stored, frozen at -20°C, pending hydrolysis.

Fresh material as well as the wall preparations were checked for the presence of starch using the standard I_2Kl test (see page 293).

3. Hydrolysis.

The procedure of Albersheim et al. (1967) was followed (see also Jones and Albersheim 1972). Frozen wall samples were relyophilised, and a small amount of each (usually 25 mg) was weighed into screw-topped hydrolysis tubes. The tubes were marked by engraving on the caps prior to weighing. These tubes were of pyrex, with teflon-lined plastic caps (V.A. Howe Ltd., London). They proved much more convenient than flame-sealed glass tubes, and similar screw-topped tubes are now routinely used by several groups for work of this nature (Y. Masuda, pers. comm.; A. Darvill, pers. comm.; F. Klis, pers. comm.).

To each wall sample was added 2 ml of a solution of 2 N tri-fluoro acetic acid (TFA) containing 1 mg of myo-inositol. The tubes were then capped and heated at 121°C in an autoclave for 1 h. This hydrolysis procedure has been shown to provide maximum recovery of NC sugar in other materials (Albersheim *et al.* 1967). After cooling, the tubes were centrifuged at 1000 x g for 10 min, and the supernatants were pipetted off. The residue was resuspended in 3 ml water and re-centrifuged as before. Again the supernatant was carefully pipetted off. The water wash was repeated twice more, and the washings were combined with the original supernatant.

TFA solution was removed from these hydrolysates by directing a jet of nitrogen gas through syringe needles and onto the surface of the hydrolysate fluid. This allowed rapid drying. The residue thus produced contained hydrolysed sugars of the NC fraction. These liberated NC sugars were then treated to produce derivatives quantifiable by gas chromatography (GC).

A direct analysis of the hydrolysed sugars themselves was attempted using high pressure liquid chromatography (HPLC). This technique has the advantage of being extremely fast, and requiring no derivatisation of sugars prior to separation. Unfortunately, satisfactory separation of two chemically very similar sugars, mannose and glucose, was not achieved. Since one of these is of particular interest in this study (in view of the findings of Loescher and Nevins 1972) further HPLC had to be abandoned. However, perhaps with recent innovations (e.g. Palmer 1975), resolution of all sugars could possibly be achieved if an intensive study was carried out. Even if not, for work not requiring precise separation of glucose from mannose, HPLC should certainly be considered.

Derivation of Wall Hydrolysates.

The method of Jones and Albersheim (1972) was followed except that no attempt was made to quantify uronic acid components. Monosaccharides of

the dried hydrolysates (approx. 10 mg in each) were first reduced to the corresponding alditols by treatment of each sample with 2 ml of N ammonium hydroxide containing 3 mg $NaBH_4$. This treatment was continued for 1 h at room temperature with occasional shaking. Excess borohydride was then decomposed by adding several drops of glacial acetic acid (with shaking) until effervescence ceased.

At this point the mixtures were transferred to 5 ml beakers and evaporated to dryness in a stream of warmed, filtered air from an electric fan heater. Air filtration was contrived by mounting the entire apparatus in a sterile handling cabinet. A further six successive evaporations were then carried out, each with 2 ml of a 10% ($^V/_V$) solution of acetic acid in methanol. This serves to remove boric acid. For the last evaporation, the mixtures (now containing alditols of the NC sugars) were transferred once again to the screw-topped hydrolysis tubes. Finally the alditols were acetylated; 2 ml of acetic anhydride were added to each sample, then the capped tubes were heated at 121°C for 3 h, in an autoclave.

Gas Chromatography.

Alditol acetates derived from the wall sugars were quantified by GLC against standard mixtures prepared in the same way. The machine used for this was a Pye GCD, the basic model, but with gas-flow controller units, and a temperature-programming module (Plate 4). Detection was by flame ionisation. Output was recorded on a chart recorder (Varian Aerograph) fitted with a disc integrator, from which peak areas were obtained. The carrier gas was nitrogen.

Initially, work was done using columns packed with the phase specified by Jones and Albersheim (1972) i.e.

    2% polyethyleneglycol succinate,
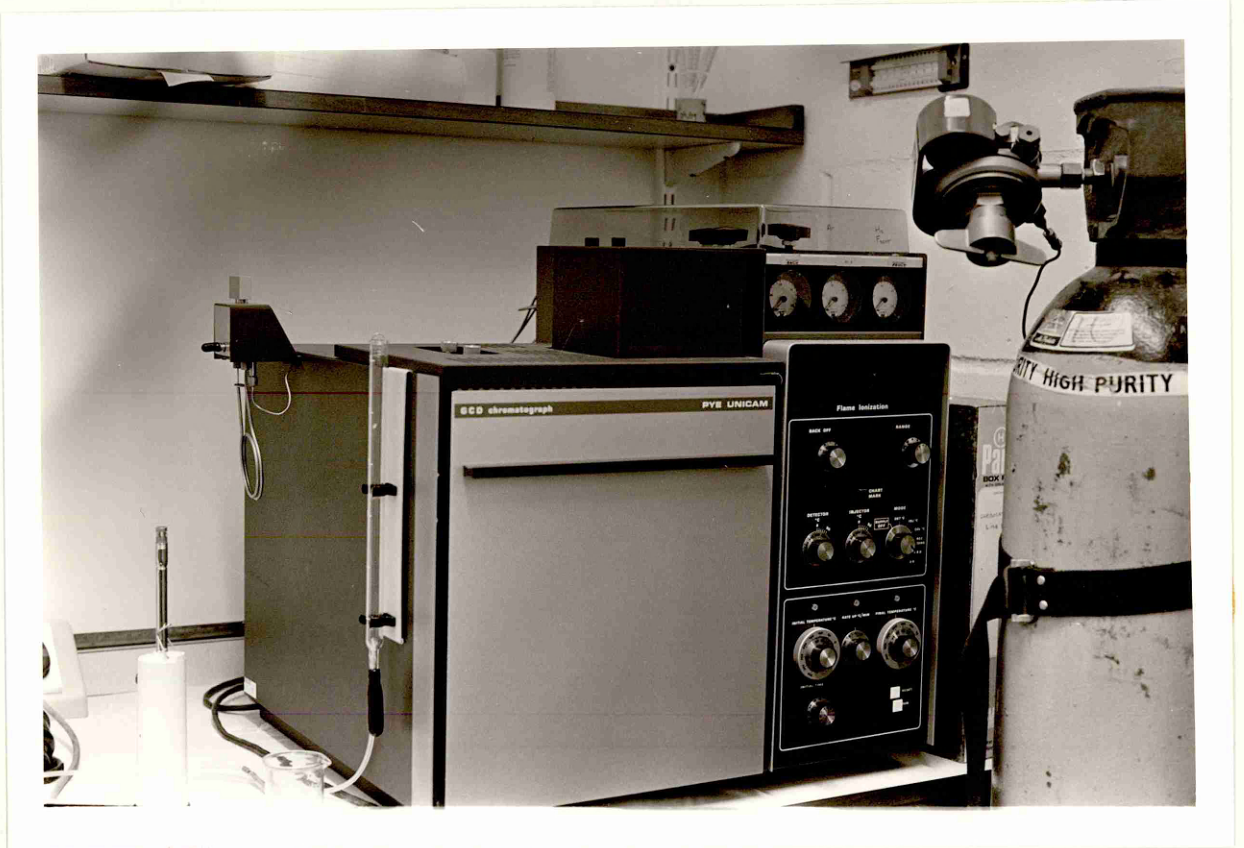   2% polyethyleneglycol adipate,
    0.4% XF 1150 silicone oil.

PLATE 4   Gas Chromatograph used in this work.

coated on Gas Chrom P (100/120 mesh). This phase has been used successfully by many workers, e.g. Loescher and Nevins (1972). However, using this phase, only poor separations were here obtained, even on 7 ft x $\frac{1}{8}$ in i.d. columns, and with various temperature programmes. The reason for the poor separations obtained with this column was not clear, though it is possible that the column packing was not sufficiently tight, since it was done manually (albeit with suction and with vibration supplied at 50 Hz from an engraving pencil) rather than with a "fluidiser", as used by Jones and Albersheim (1972). Several different phases were tried with little success (e.g. 10% PEGA on celite 100/120 mesh, see Harris and Northcote, 1970). Satisfactory separations were eventually obtained using a phase consisting of Supelco GP 3% SP 2340 coated on 100/120 mesh Supelcoport (Supelco materials supplied by Atlas Bioscan, Canvey Island, UK). This was packed as before, with suction and vibration, into a 7 ft x 2 mm i.d. glass column, and conditioned at 245°C for 12 h.

All the hydrolysates were re-run on this column, and only results from separations on this column are presented.

GC running conditions.

1. Gas flows; N_2 40 ml min^{-1}, Air 650 ml min^{-1} (approx), H_2 45 ml min^{-1}.

2. Temperatures; Detector 300°C, Injector 265°C. The column temperature was programmed usually with a post-injection hold of 5 min at 185°C, then a rise to the maximum of 230°C (occasionally up to 245°C) at a rate of 4°C min^{-1}.

3. The injections (normally 1 μl) were on-column, using Hamilton micro-syringes.

Comments: Under these conditions the longest retention time (that for inositol hexa-acetate) was about 20 min (cf. 65 min in Jones and Albersheim 1972).

Resolution of the various alditol acetates under these conditions was good

(see Figure 15), except that the rhamnitol hexa-acetate peak in cell wall

samples was partly overlapped by a later, smaller peak which, judging by

the retention time, was probably the derivative of fucose. It might have

been possible to separate these two peaks using column temperatures

programmed from a lower starting point, but I did not attempt this since:

1.   Detection of change in NC glucose was the primary objective of this

work.

2.   The fucose shoulder was small and seemed of constant relative

size.

3.   An estimate of the two individual peak areas in the combined peak

could be made, if necessary, from the present charts (see Figure 15).

4.   Further separation would necessitate a lower column temperature, with

consequently longer retention times, which are undesirable.

The fucose shoulder was thus ignored and is incorporated in results

for rhamnose.

Because of its higher temperature tolerance (up to 275°C) the phase

used here is an improvement on those in previous published work on plant

cell-wall sugars. (Since this work was done, a report has appeared of

experiments in which a similar phase was used for separation of derivatives

of neutral sugars from the plant cell wall; Terry *et al.* 1981).

To obtain peak areas, a disc integrator attached to the Varian chart

recorder was used. It was not found necessary to use conditions of

strictly constant baseline; satisfactory peak areas could be obtained by

subtracting the base area from the total area under any peak. Thus lengthy

column conditioning, and use of a dual column arrangement to counteract

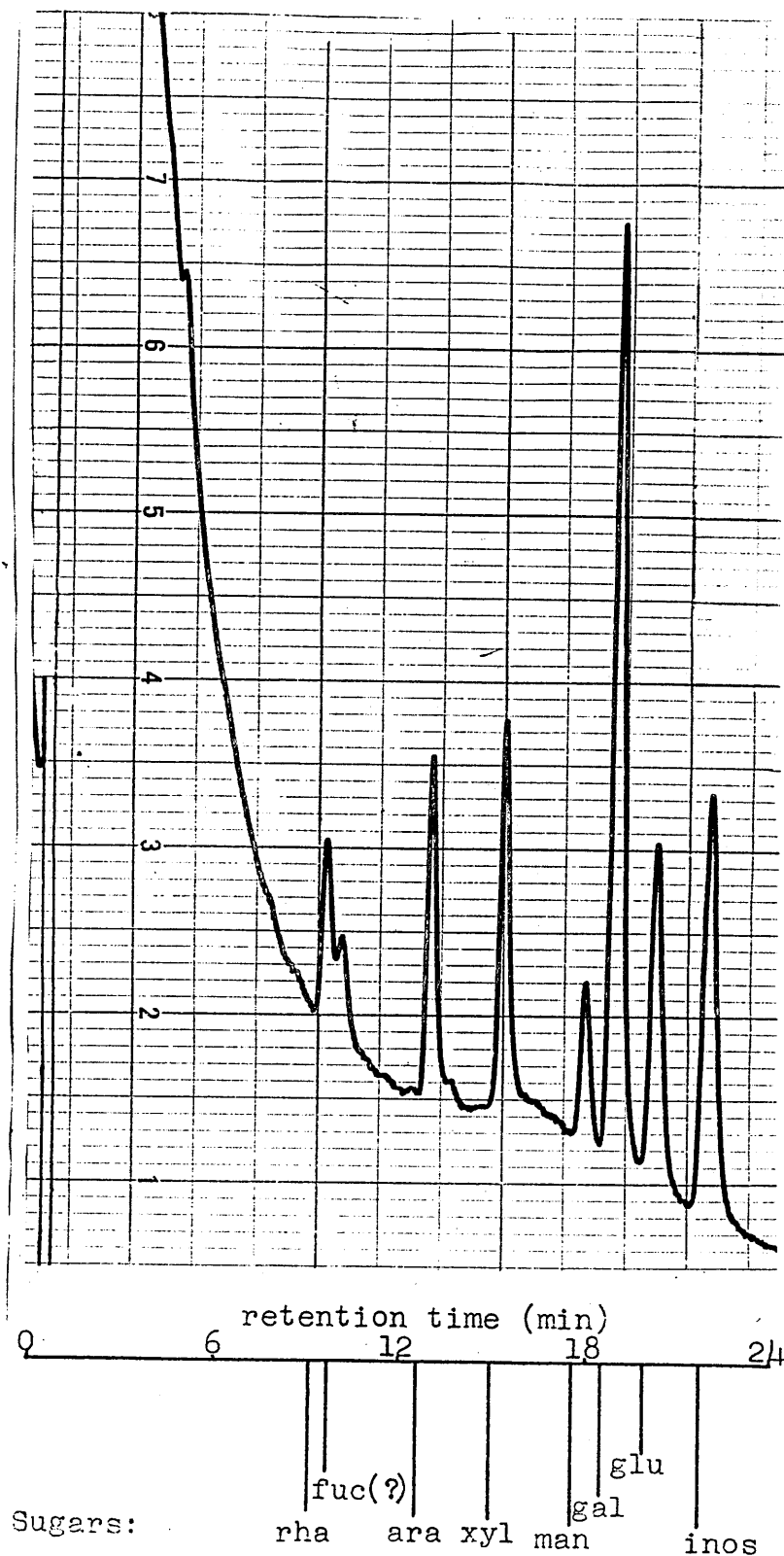column bleed, was unnecessary. Some loss of precision was undoubtedly

FIGURE 15 Example of separations obtained in GLC analysis of cell wall sugars.

2 µl of alditol acetates derived from *Nymphoides* wall material was injected at time zero. GC operating conditions are given in the 'methods' section.

Sugars: rha = rhamnose, fuc = fucose, ara = arabinose, xyl = xylose, man = mannose, gal = galactose, glu = glucose, inos = inositol.

incurred, but accuracy was very high in comparison to differences between treatments which have been found in previous work (for example, Loescher and Nevins, 1972, find a 75% decrease in NC glucose under some conditions).

CHAPTERS 8-11

RESULTS   AND   DISCUSSION

CHAPTER 8

THE SUBMERGENCE RESPONSE IN
NYMPHOIDES: COMPARISON WITH *REGNELLIDIUM*

CHAPTER 8

THE . SUBMERGENCE . RESPONSE . IN .
NYMPHOIDES: COMPARISON WITH *REGNELLIDIUM* .

Effects of auxin and ethylene.

The mechanism of the submergence response in *Nymphoides peltata* was

here investigated. By analogy with the situation in *Regnellidium diphyllum*

(Walters and Osborne 1979), *Ranunculus sceleratus* (Samarakoon *et al.* 1980)

and *Oryza sativa* (Imaseki and Pjon 1971), one would expect depth accommoda-

tion in *Nymphoides* to depend on promotion of growth by ethylene in the

presence of auxin. Consequently, petiolar tissue of *Nymphoides* was tested

for sensitivity to auxin and ethylene.

Auxin alone will promote elongation in segments of *Nymphoides*

(Figure 16). Ethylene will increase growth when supplied together with

auxin, but when supplied alone to aged segments ethylene has little or no

effect on growth (Figure 16); a presence of auxin is thus necessary for

development of ethylene-induced growth in *Nymphoides*. This is shown again,

in high resolution, in Figure 17. *Nymphoides* is, in this respect, similar

to the three water-plant species noted above.

Ethylene treatment of freshly-excised *Regnellidium* petiole segments

may promote growth, apparently in response to ethylene alone (Walters and

Osborne 1979; Cookson 1976). However, this is probably due to the presence

of residual endogenous auxin supplied to the segments from cells of the leaf
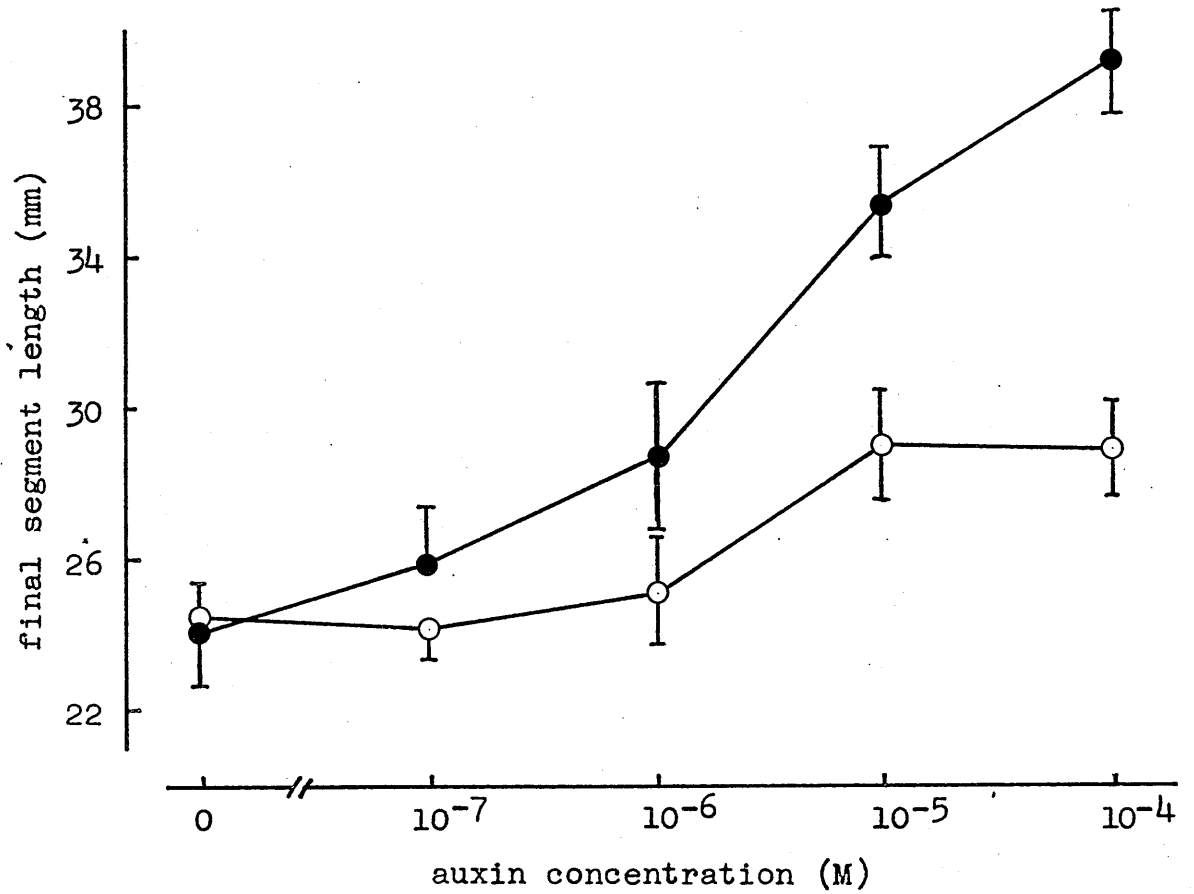
FIGURE 16 Effect of auxin (IAA) at various concentrations, supplied alone (o) or with ethylene (●), on elongation of *Nymphoides* petiole.

Subapical segments, initial length 21.5 mm, were treated as stated for 24 h. Ethylene was supplied at 25 μl l^{-1}. Each point is a mean for 12 segments ± SE.
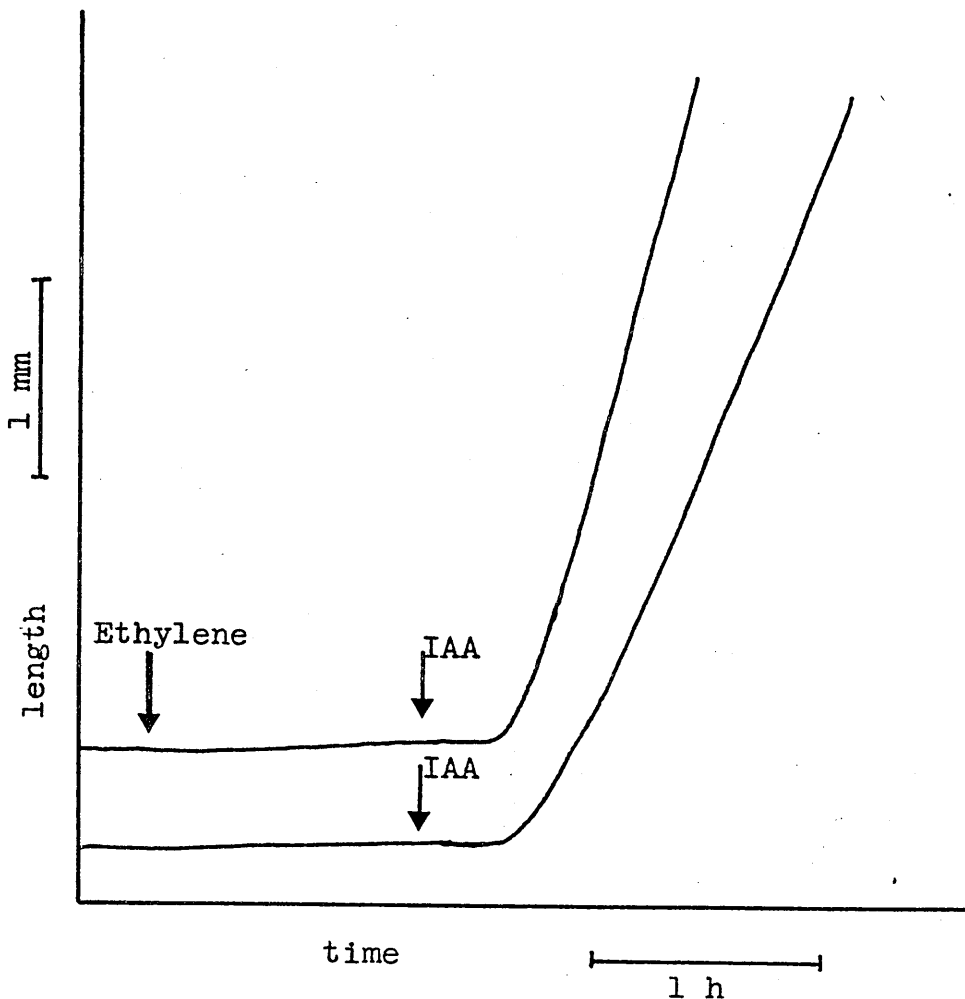
FIGURE 17 Effect of ethylene on auxin-induced elongation in
Nymphoides petiole.

Transducer recording from columns of 4 x 1 cm apical
segments. A saturating level of ethylene was applied
at the first arrow (upper curve only). 10^{-5} M IAA was
applied at the second arrow (both curves).

Redrawn from original.

lamina, prior to excision. This explanation is supported by experiments showing that if segments are allowed to age for a few hours after excision, by simply floating them on distilled water, they will not respond to ethylene unless auxin is added (Walters and Osborne 1979). Presumably, residual auxin dissipates during the aging period, by loss to the medium or catabolism. The same explanation probably accounts for a similar phenomenon in *Nymphoides* (see Figure 18, where a small response to ethylene alone is visible in fresh *Nymphoides* segments).

The use of freshly-excised *Nymphoides* (or *Regnellidium* or *Ranunculus sceleratus*) segments for work on ethylene-induced growth is not recommended because the level of residual auxin present could vary markedly between segments. Such variation would be reflected and magnified in any ethylene-induced growth responses, since these responses appear to be dependent on the amount of auxin present (Figure 16). Marked variability could be introduced if fresh segments are used. It is preferable, under normal circumstances, to age the segments, then supply a uniform level of exogenous auxin.

A related problem is found in *Nymphoides*; at about 20-24 h from excision, the segments regain apparent sensitivity to ethylene alone (Figures 18 and 19). It is probable that this is caused by a resumption of auxin synthesis within the segment, so that responses to ethylene once again become possible. Excised segments of many tissues (e.g. *Avena* coleoptile) when isolated from their normal auxin supply, are thought to generate "physiological tips"; these are centres of auxin synthesis and export (Went and Thimann 1937, cited by Iino and Carr 1982; Vesper and Evans 1978). Removal of the usual auxin supply may itself be the signal for depression of those genes responsible for auxin synthesis in such "physiological tips". A development of this sort would explain the resumption of sensitivity to ethylene observed in older segments of *Nymphoides*. A similar phenomenon could well occur in *Regnellidium*, though
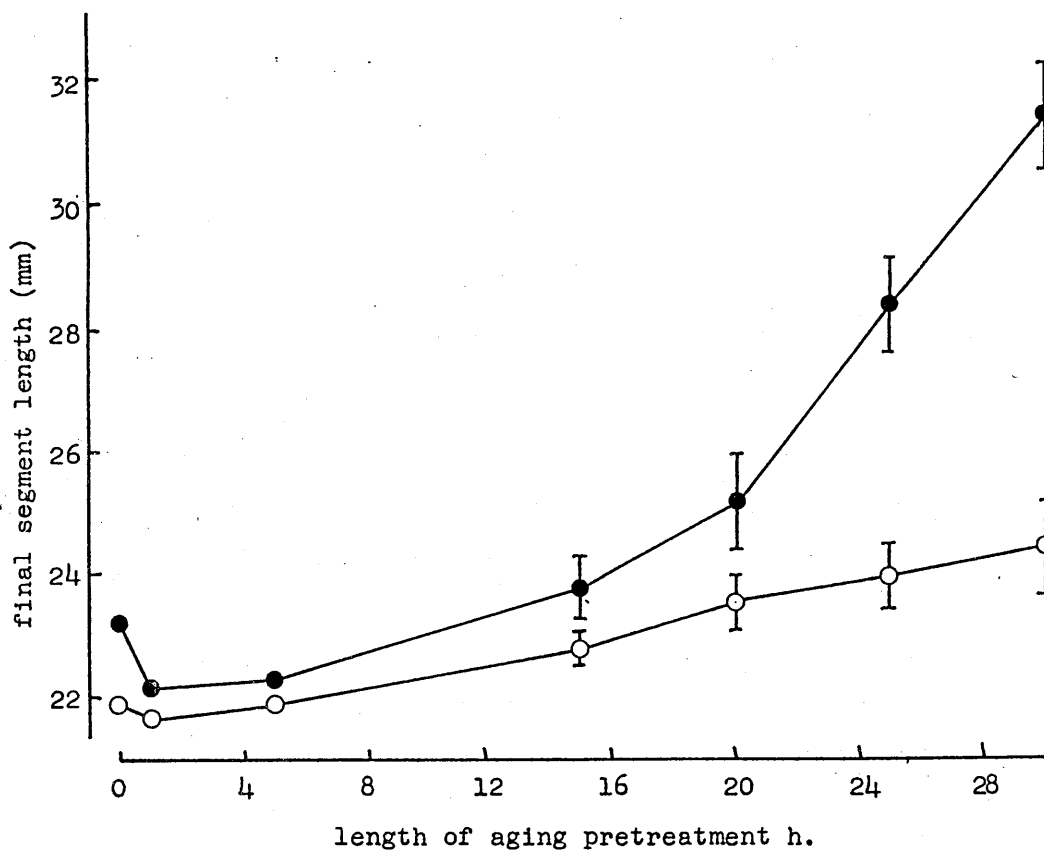
FIGURE 18   Elongation of *Nymphoides* petiole segments aged for various times prior to treatment for 10 h with (●), or without (o) ethylene.

Apical segments, initial length 21.4 mm, were excised and allowed to age, floating on distilled water, for the time period shown on the horizontal axis. Segments were then exposed to air only, or to air plus 75 µl l^{-1} ethylene, for 10 h. Their lengths were then measured.

Means (± SE) are for 10 segments.
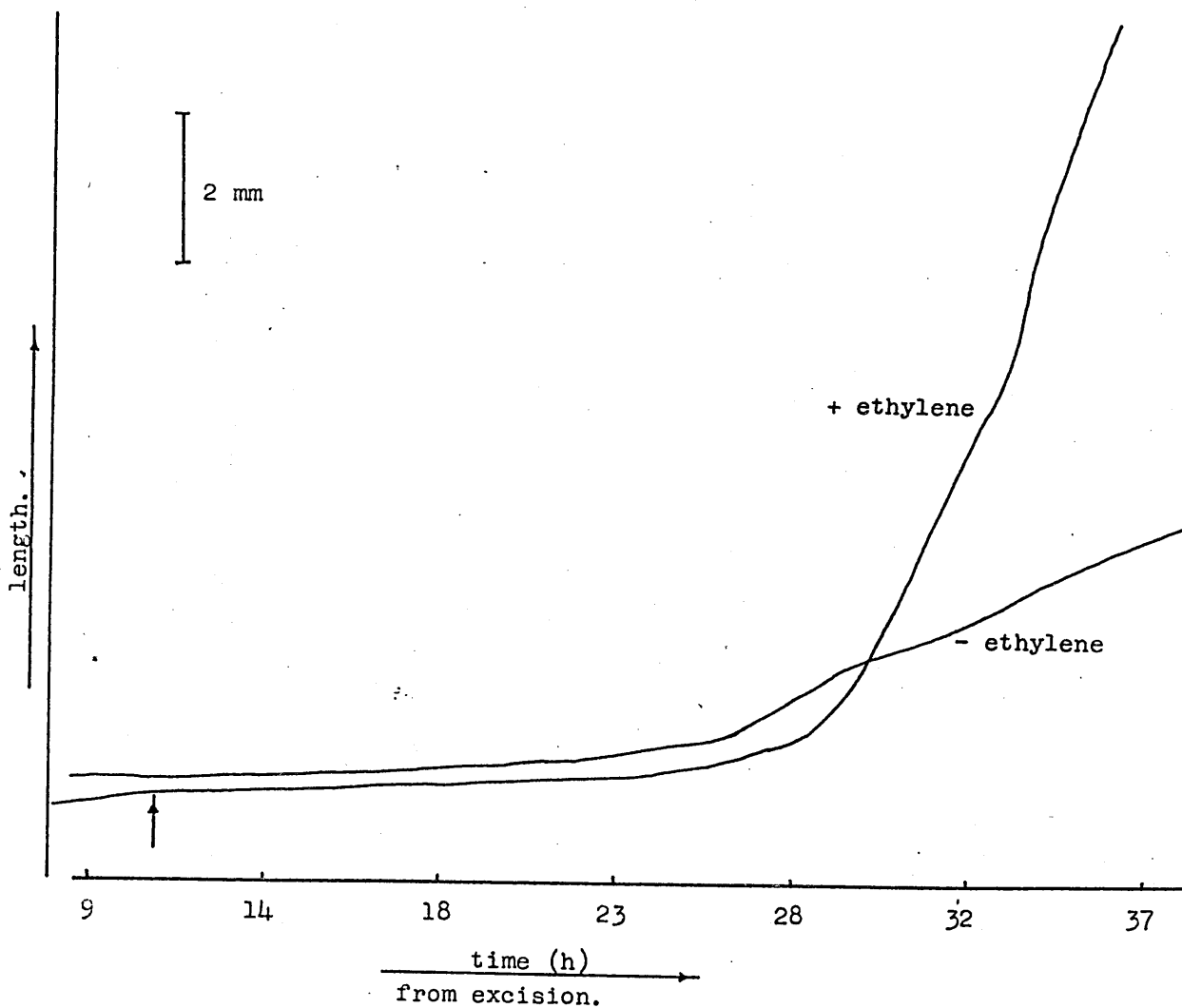
FIGURE 19 Pattern of growth, with time, of *Nymphoides* segments exposed to ethylene and allowed to age.

Transducer recording from columns, each of 4 x 1 cm subapical *Nymphoides* petiole segments. At the first arrow (approx. 10 h after excision) a saturating level of ethylene was applied to one of the segment columns (lower curve). No exogenous auxin was supplied.

Redrawn from original.

this has not been conclusively demonstrated. This effect should be borne in mind when carrying out longer term experiments with segments of these tissues; such experiments should not be extended beyond about 20-24 h after excision, without some consideration of the possible implications of a renewed presence of endogenous auxin.

These results confirm that rapid growth is promoted in *Nymphoides* petioles, by auxin, and by ethylene in the presence of auxin.

Ethylene accumulation

If elongation in petioles of a plant is stimulated by ethylene, then leaves of that plant will automatically show depth accommodation (Ku *et al.* 1970). This is because: (i) probably all higher plants produce ethylene (Osborne 1977) - this is certainly true of *Nymphoides* (Table 6) and *Regnellidium* (Table 5; Cookson 1976); (ii) whenever immersion occurs, endogenous ethylene is trapped within the plant, largely a consequence of the relatively slow diffusion of ethylene in water (see page 9).

Some workers have attempted to demonstrate, experimentally, that ethylene does indeed accumulate in submerged tissues. For example, Musgrave *et al.* (1972) collected the air bubbles given off by submerged *Callitriche platycarpa* plants and assayed them for ethylene. They showed that the ethylene level present in these bubbles was sufficient to cause rapid growth of stem internodes of this species.

The time course of ethylene accumulation following immersion, was here determined using sequential vacuum extraction. *Regnellidium* leaves were preferred over *Nymphoides*, for this experiment, because the more open structure and larger aerenchyma of the former tissue make it more amenable to vacuum extraction. The results show that ethylene does accumulate in these plants following submergence, and that the build-up is both marked and rapid (Figure 20).
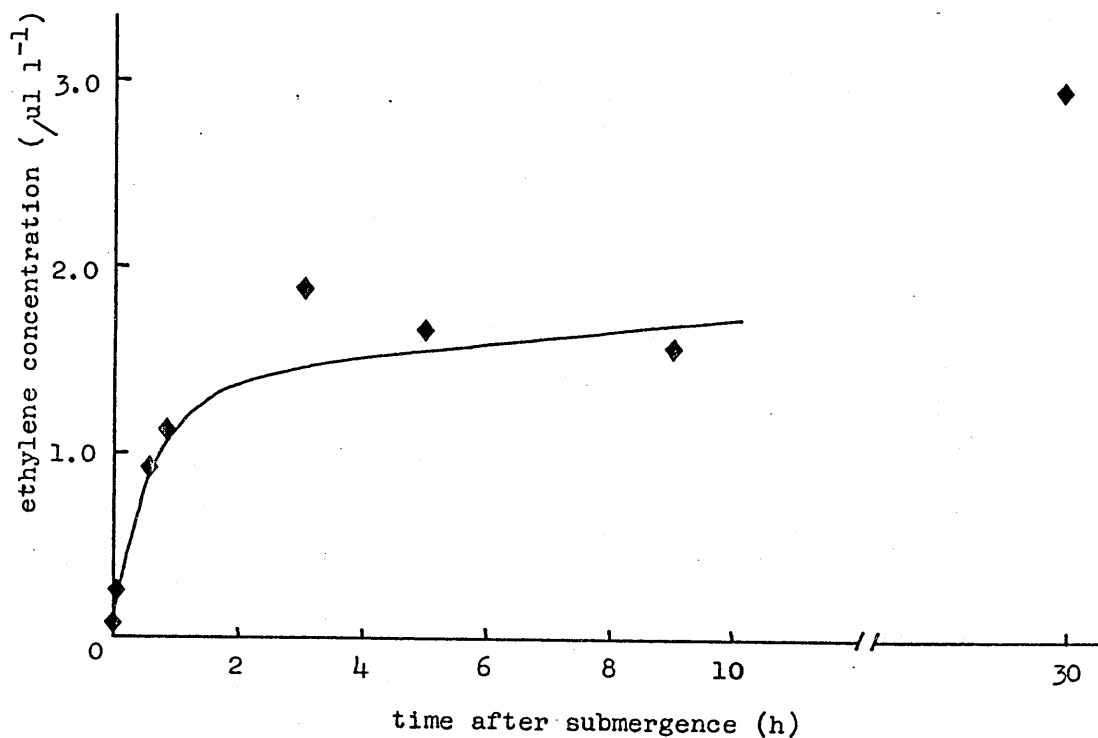
FIGURE 20   Time course of ethylene accumulation in submerged
            Regnellidium leaves.

At the stated times, air was vacuum-extracted from
batches of 9 *Regnellidium* leaves (petioles trimmed to
3 cm each) submerged in tap water under conditions of
normal room lighting and temperature. Extracted gases
were assayed by gas chromatography.

Thus, for various species that show depth accommodation, it has been found that (i) ethylene can promote rapid elongation; and (ii) ethylene will accumulate in submerged tissue. These two observations alone strongly suggest that ethylene-promoted growth is the main cause of depth accommodation. Further confirmation of this was provided by Cookson (1976) who showed that depth accommodation of submerged leaves of *Ranunculus sceleratus* is prevented by aminoethoxyvinylglycine (AVG) an inhibitor of ethylene biosynthesis; the inhibition was relieved by addition of ethylene.

There have been suggestions that ethylene production may be increased in submerged tissue, and that this greater production could be partly responsible for the build-up of ethylene which follows submergence (Ku *et al.* 1970). Stimulation of ethylene production following various types of stress has been observed in many land-plant tissues (Abeles 1973; Saltveit and Dilley 1978a), and it is conceivable that submergence would impose an ethylene-inducing stress in the water plants. There is, however, evidence that the water plants do not show stress-induced ethylene production. For example, ethylene evolution from leaves is not increased by various mechanical stresses in *Regnellidium* (Walters and Osborne 1979) nor in *Ranunculus sceleratus*, nor *Hydrocharis morsus-ranae* (Cookson 1976).

Thus stresses, including submergence, may not induce increased ethylene synthesis in the water plants. Certainly, for the model of depth accommodation discussed above (and summarised overleaf) to work, increased ethylene production during submergence is not essential.

Although stress-induced ethylene appears almost ubiquitous in land plants, its absence from some of the water plants should not be surprising bearing in mind that petiolar extension is promoted by ethylene in these plants. It could be less than helpful if petioles commenced rapid growth whenever the plant suffered stress from insect attack or stormy weather conditions.

Similarly, one might predict absence from the water plants, of the massive rise in ethylene production associated with onset of senescence in some land-plant tissues (Burg and Burg 1965b; Blanpeid 1972; Beyer and Sundin 1978). This prediction is confirmed by experiment; Table 5 shows that there is little or no increase in ethylene production on senescence of *Regnellidium*.

Similarly, stimulation of ethylene synthesis by auxin, seen in many land-plant tissues (see page 20) is also much less evident in the water 'plants, e.g. *Regnellidium* (Cookson 1976) and *Nymphoides* (Table 6).

Synopsis of the submergence response.

The following scenario is envisaged when a mature, healthy *Nymphoides* leaf accommodates to a rise in water level.

1. <u>Prior to the rise in water level</u>: the petiole contains a low background level of auxin (probably about 10^{-7}M IAA, judging from growth rates of petioles of leaves in air, and from the short aging period required to render excised segments unresponsive to ethylene alone). This level of auxin is continuously supplied to the petiole, by basipetal transport from sites of synthesis in the leaf. Where studied, basipetal transport of auxin has been demonstrated in water-plant petioles (Musgrave and Walters 1973; Walters and Osborne 1979) as in those of land plants, and there is no reason to doubt its ubiquity.

Ethylene is produced in the floating leaf, but quickly diffuses to the surrounding air without significant accumulation. Production of ethylene in the petioles is slight (Table 6). Thus, elongation of the petiole is not influenced by ethylene at this stage, and is relatively slow.

2. <u>Subsequent to a rise in water level</u>: auxin and ethylene production are unchanged, but ethylene escape from the submerged leaf is minimal, so that

| Tissue | ethylene production (nl g^{-1} h^{-1}) | | | | | |
	1-5 h	x	s	5-20 h	x	s
Healthy	5, 4, 4, 4, 7	4.8	1.3	16, 10, 10, 10, 14	12	2.8
Senescent	3, 2, 1, 3, 3	2.5	1.1	8, 3, 1, 3, 6	4	2.5

TABLE 5   Ethylene emanation from senescent, and from healthy
Regnellidium leaves.

At two times after the start of incubation, gas was
extracted from each of the ten incubation vessels
(five for each treatment) and its ethylene content
assayed by gas chromatography.

For "senescent" tissue, leaflets were chosen in which
necrosis was visible over 10-20% of the leaf area (usually
around the periphery). Incubation was carried out under
conditions of normal room lighting and temperature.

Tissue	Treatment	ethylene production $(nl.g\ fr.wt^{-1}\ h^{-1})$	
		0-6 h	6-18 h
Leaf discs	control	10.3	11.1
Leaf discs	auxin	6.7	5.5
Leaf discs	fusicoccin	12.2	7.4
Petiole segs.	control	0.03	0.05
Petiole segs.	auxin	0.05	0.05
Petiole segs.	fusicoccin	0.04	0.14

TABLE 6 Effect of auxin (10^{-4} IAA) and of fusicoccin (10^{-4} M) on ethylene production by leaf and segment material of *Nymphoides*.

Ethylene production at two times after the start of incubation is shown.

ethylene enters the petiole from the leaf lamina. There, by some process involving auxin, ethylene promotes elongation.

3. Lastly: petiole growth returns the leaf to the surface, ethylene escape resumes, and its concentration soon returns to a sub-stimulatory level. Soon after that, growth rate will return to normal.

Comparability of segments to intact material.

Much of the work on water-plant growth responses has utilised petiolar segments. These are very convenient for experiments, but results obtained from them will contribute little to an understanding of the mechanism of depth accommodation *in vivo* unless the segment growth response is seen to be similar to that of the intact plant; apparently, this has not been the case. For *Regnellidium*, the literature shows a marked difference between the response to auxin of intact fronds, and that of excised segments. For example, in petioles with the leaf present (intact fronds) there is at best, only a small response to 10^{-5} M IAA (Musgrave and Walters 1974; Cookson 1976; Walters and Osborne 1979) whereas in petioles with the leaf excised (including segments) this level of auxin induces a strong growth promotion (Walters and Osborne 1979).

I investigated this enigma in *Regnellidium*, and confirmed the difference in apparent sensitivity to auxin: although significant growth promotion usually occurred in intact leaves treated with auxin, the effect was clearly smaller than that in debladed material (Table 7).

An attempt was made to determine the cause of this difference. Four possible explanations were considered:

1. Petiole tissue with the leaf attached might already be supplied with an optimal level of auxin, so that further addition has no effect on growth. In debladed material, on the other hand, the supply of endogenous auxin is cut off, so that exogenous auxin will promote growth.

Tissue	auxin	final lengths mm		
		X̄	s	n
segments	−	23.7	1.83	10
	+	30.1	3.07	10
intact leaves	−	22.4	1.78	10
	+	22.9	1.19	10

TABLE 7   Comparison between the response to auxin in
Regnellidium petiole segments, and that in
petioles of intact leaves.

Apical petiole segments (initial length 21.4 mm)
were incubated floating on water (± 10^{-5} M IAA) for
20 h. Petioles of intact leaves were marked at
20 mm from their apex and immersed in water
(± 10^{-5} IAA) through holes in a perspex grid. A
weight (1 g) was attached to the base of each
petiole to secure it in position. Length of
the marked apical region was measured after 20 h.

That some auxin is supplied to the petioles by leaf blades is not doubted. It is however, much less than the optimum for growth. This can be seen by comparing growth rate in untreated, intact leaves with that in debladed, auxin-treated segments; the intact leaves grow much more slowly (Table 7), showing that their auxin supply is not sufficient to produce maximal growth (i.e. it is not saturating). Thus this explanation is untenable.

2. Deblading might be so traumatic as to alter in some way the entire physiology of the subject, rendering it unlike intact material.

One might, however, expect damage to reduce growth responses rather than increase them, but in this case it is the "damaged" tissue which grow most rapidly. In addition, the trauma of deblading can scarcely be worse than that of excision of the whole leaf from the parent plant, yet the latter does not cause any obvious physiological change. For these reasons it is considered unlikely that trauma alone introduces the change in sensitivity to auxin.

3. An inhibitor might be produced in the leaf, which travels to the petiole and there reduces the effect of auxin. In debladed petioles, the supply of inhibitor would be cut off, and these would consequently show a full response to added auxin.

Petioles respond to auxin immediately after deblading. Thus, one would have to postulate extraordinarily rapid breakdown of the putative inhibitor in the petiole, and consequently also very rapid synthesis (and export) of it in the leaf. The existence of an inhibitor with these unusual characteristics seems unlikely, nevertheless this possibility cannot be eliminated *a priori*.

4. Auxin penetration to cells in petioles of intact leaves might be restricted by the cuticle, so that auxin in the external solution has little effect on growth in this tissue. Debladed petioles would, in

contrast, be freely accessible to auxin in the medium, via the apical cut surface where there is no cuticle. Thus, these would show a marked response.

A basal cut surface is present in both intact leaves and debladed petioles, however it is not a major route of entry because the polar transport system tends to reject auxin at the basal end, and absorb it only at the apical end (Davies and Rubery 1978). In addition, any auxin which does gain entry at the basal end will be less important for growth than would a similar quantity at the apical end because, with the exception of the extreme apex, the potential for growth diminishes with distance from the petiole apex in both *Regnellidium* and *Nymphoides* (Figures 21 and 22).

In many land-plant tissues, the outer cuticle is known to present an effective barrier to general passage of substances (Darlington and Cirulis 1963; Dreyer *et al.* 1981), including auxins (Osborne 1976; Nissl and Zenk 1969; Kenney *et al.* 1969).

In water plants, a cuticle is almost universally present (Arber 1920) though it is often said to be "reduced". In the *Nymphoides* petiole, a cuticle is clearly visible under the microscope, as an outer layer which stains positively with sudan IV (data not shown), and there is strong evidence that it is an effective barrier to some substances (Figure 41). It seems possible then, that the apparent difference in sensitivity to auxin between debladed petioles and intact leaves, occurs because auxin does not easily gain entry across the general surface of the water-plant leaf, and must circumvent the cuticle by entering at a (apical) cut surface, if it is to promote marked elongation.

Of the four explanations considered, the last two have not been rejected. To identify the correct one, the following test was carried out: the effect of auxin was observed in debladed petioles which were either completely immersed in the treatment solution, or were immersed save for
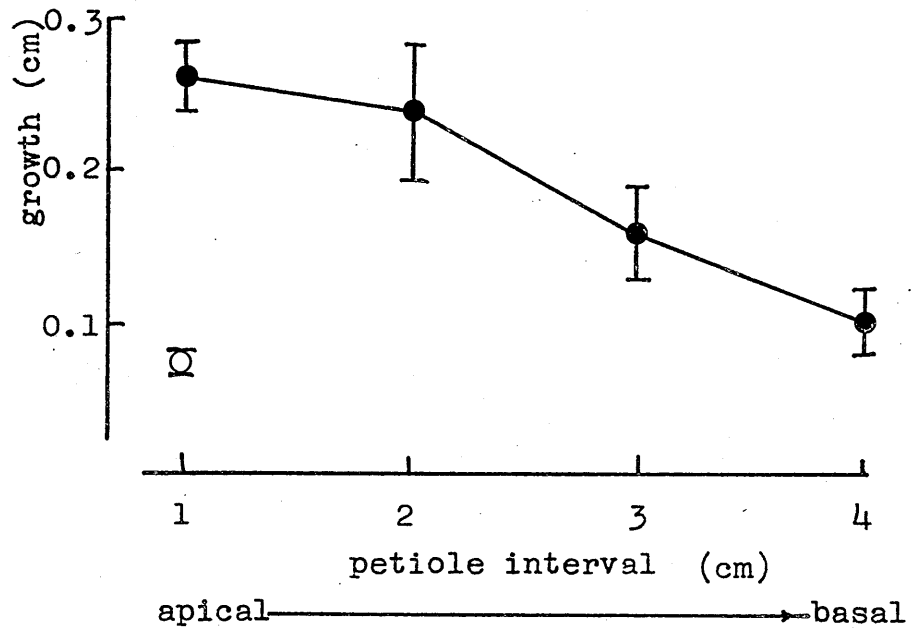
FIGURE 21 Sensitivity to ethylene in tissue from regions along
the *Regnellidium* petiole.

Intact leaves with petioles marked at 1 cm intervals
were exposed to air (o) or to 25 µl 1^{-1} ethylene in
air (●) for 24 h. Growth of the intervals was then
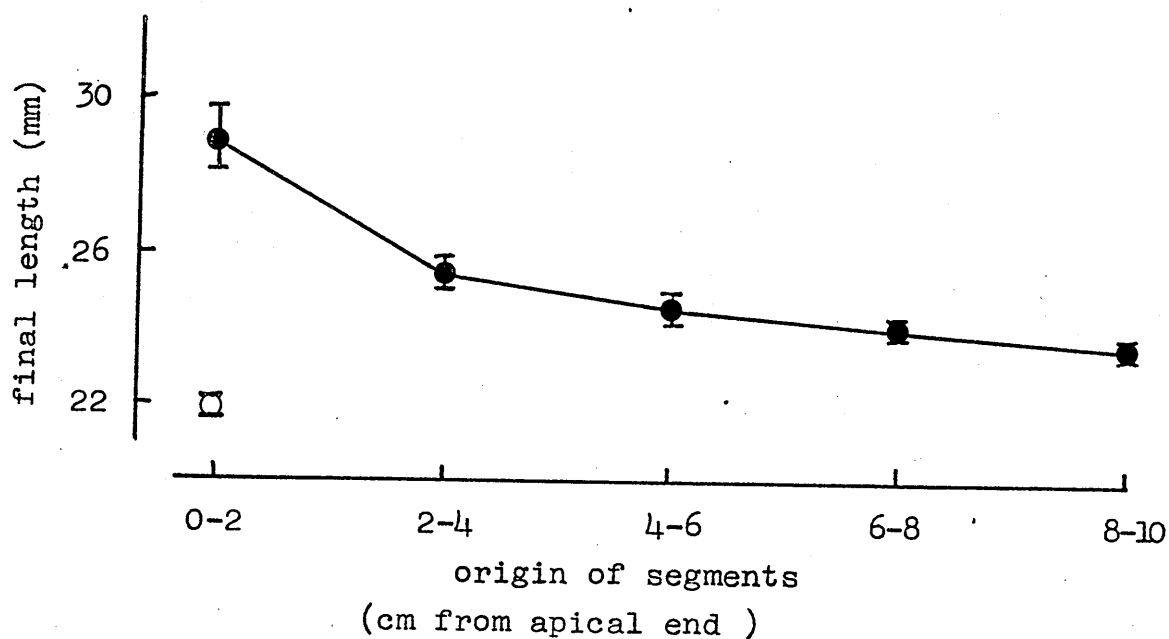measured. Each point is a mean of 8 segments ± SE.

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FIGURE 22 Sensitivity to 10^{-5} M IAA in tissue from regions along the *Nymphoides* petiole.

Segments, initial length 21.4 mm, were cut from various regions along the petiole and incubated with (●), or without (○) 10^{-5} M IAA for 9 h.

The horizontal axis shows where, in terms of distance in cm from the apical (leaf) end, the petiole segments were cut.

Each point is a mean for 25 segments ± SE.

their apical tip (see Figure 23). One would predict that if auxin entry
is indeed restricted to the apical cut surface (as in possibility 4), the
greatest growth response would occur in the completely-immersed tissue,
in which auxin gains direct access to the apical cut surface. One would
also predict that tissue exposed to auxin but not immersed entirely,
should grow little more than controls. If, on the other hand, an inhibitor
originating in the leaf, is responsible for this difference between
segments' and leaves' responses to auxin (as in possibility 3), there should
be little difference between growth of the two treatments in this test;
they should grow well since neither has a supply of the inhibitor. With
both *Regnellidium* (Figure 23) and *Nymphoides* (Table 8), results showed that
in the "tip-out" treatment there was little or no response to auxin, while
in the "tip-in" treatment there was a marked response. These experiments
also showed that "tip-out" treatments, although they do not respond to auxin,
are capable of such a response in that, if later submerged, they will
respond vigorously (Figure 23, Table 8).

This result allows rejection of the "inhibitor" possibility in favour
of the "barrier" possibility, and it is concluded that the difference
between the auxin-response of petiole cells in segments, and those in
intact leaves, is an artefact due simply to restricted auxin entry in the
latter case. Thus, there is no reason why segments should not be used to
provide information about auxin-induced, and ethylene-induced growth
responses in petioles of the water plants. If anything, the segment
response is more representative of the tissue's reaction to auxin.

Unlike the situation in many land plants, the cuticle of *Nymphoides*
petioles is not required to prevent desiccation; this tissue is
habitually surrounded by water. However, the presence of a significant
cuticle is not altogether surprising: as well as fulfilling a defensive
role, it probably serves to prevent loss of ions and other solutes,
including auxin, from the plant into the surrounding medium.

	tip out	
	tip in	medium level
		petiole
		perspex grid
		steel pin
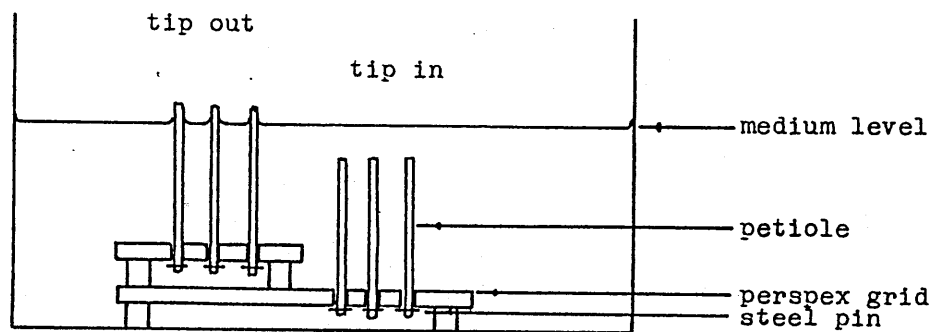
Auxin	position of tip	final length mm	
		x	s
none	out	21	0.5
	in	22	1.1
2 x 10⁻⁶M IAA	out	23	2.1
	in	30	2.0

the "tip-out" treatments were incubated for a further 20 h -this time with their tips in the medium.

-auxin	22	0.5
+auxin	34.6	6.4

FIGURE 23   Response to auxin in *Regnellidium* petioles treated with their apical tip just in, or just out of the medium.

The diagram (top) shows how the experiment was set up. A 20 mm apical portion of each frond was marked off at the start of incubation. The table (middle) shows lengths after 20 h incubation, of these regions. Each mean is for 6 measurements.

Auxin	position of tip	final length mm	
		$\overline{x}$	s
none	out	21.5	0.55
	in	21.9	0.69
10^{-5}M IAA	out	21.4	0.79
	in	34.1	2.48

-auxin	22.3	0.82
+auxin	34.4	1.13

TABLE 8   Response to auxin in *Nymphoides* petioles treated with their tips just in, or just out of the medium.

The experimental set-up was similar to that shown for *Regnellidium* (see Fig. 23).

An apical region 20 mm in length was marked on each *Nymphoides* petiole at the start of incubation. After 12 h, length of the marked zone was recorded. Petioles of the "tip-out" treatment were incubated for a further 12 h, this time with their tips in the medium; length of the marked zone was again recorded. The latter results are shown in the lower part of the table. Each mean is for 7 measurements.

The cuticle also enables petiolar cells to control the apoplast solution as a micro-environment, and this may be extremely important. For example, if growth promotion occurs by an "acid-growth" mechanism (see page 50), the cells must be able to lower wall pH to about 5. In the absence of a cuticle, excreted protons would leak away into the surrounding medium and it would be extremely difficult for the cell to lower wall pH sufficiently. Presence of a cuticle restricts the apoplast to a manageable volume.

Regnellidium fronds sometimes grow in air, and the cuticle will then serve to reduce desiccation. The presence of a cuticle does not necessarily mean that these water-plant leaf tissues are unable to absorb ions and other solutes directly from the environment. For example, there could be specific sites at which the cuticle is absent, analogous to stomata; these sites could house energy-requiring devices for accumulating ions, possibly involving "transfer cells", which are found in some water-plant leaves (Pate and Gunning 1972). In many aquatic macrophytes however, uptake through roots in the substratum is normally much more important than through leaves in the water (Haslam 1978).

Summary: Petioles of *Regnellidium* and *Nymphoides* are relatively impenetrable. This restricts solute leakage, but auxin entry is also restricted so that petioles of intact leaves do not show a full growth response when incubated in a medium containing auxin. This accounts for the difference in auxin-induced growth between debladed segments, and petioles of intact leaves.

On the optimum concentration for auxin-induced growth in water plants.

The optimum auxin concentration for promotion of elongation in *Nymphoides* petioles is 10^{-6}-10^{-5} M IAA for short-term growth (less than about 2 h; Figure 24), rising to about 10^{-5} M if measured over the longer term (24 h; Figure 16). This shift in the auxin optimum with time, may
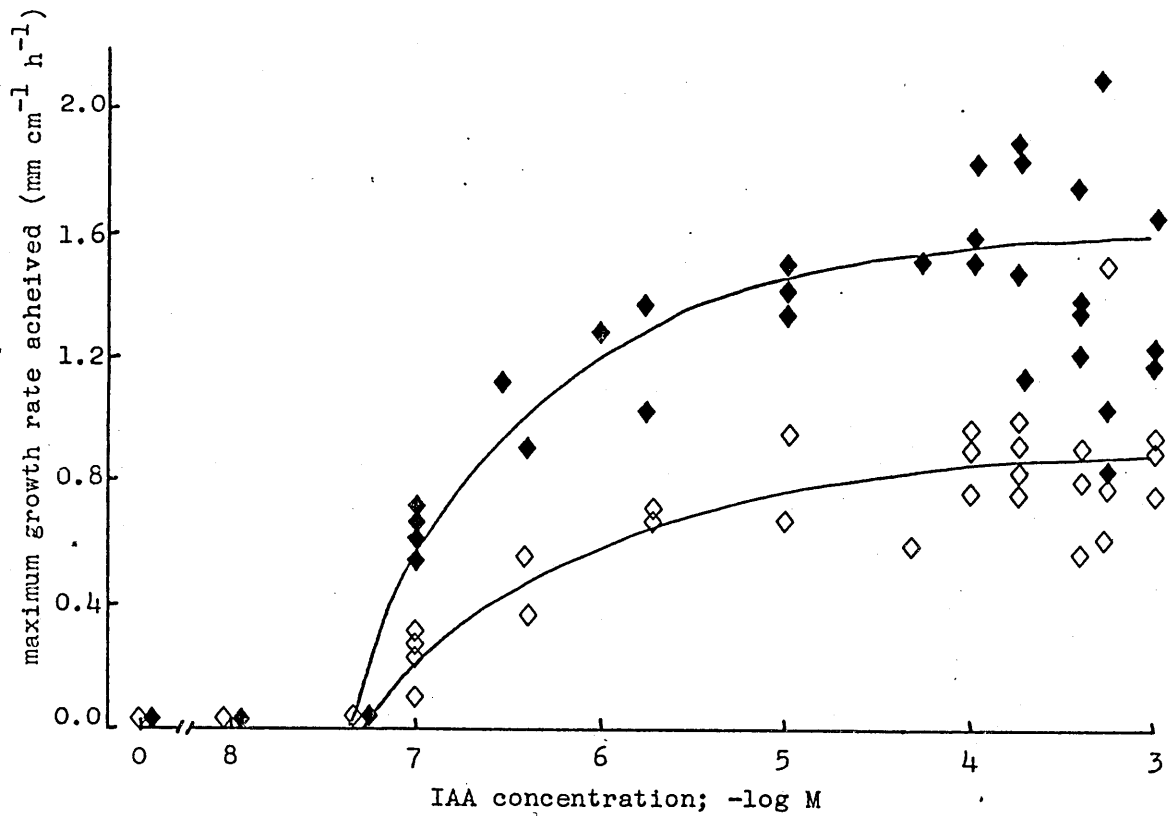
FIGURE 24   Effect of ethylene on the maximum rate of growth achieved by segments exposed to various levels of auxin.

Paired columns, each of 4 x 1 cm apical *Nymphoides* petiole segments, were simultaneously exposed to the same level of auxin. One of the pair was pretreated with ethylene (◆), the other (◇) was not. Pretreatment ethylene regime was continued throughout the experiment.

Maximum growth rate was estimated from the slope of transducer recordings of length.

The lines were fitted by eye.

be an artefact caused by auxin breakdown during longer incubations. A similar pattern of auxin optimum, including the shift with time, is also found in growing tissues of land plants (Cleland 1972).

High levels of auxin (up to 10^{-3} M) do not cause inhibition of elongation in *Nymphoides* (Figures 16 and 24) or *Regnellidium* (Figure 25) even over the longer term. This is unlike the situation in land-plant tissues, where supra-optimal auxin levels can be severely inhibitory over the longer term, producing bell-shaped dose-response curves (Cleland 1972). It has been proposed that auxin-induced ethylene is responsible for the growth inhibition in land plants at high levels of auxin (Cleland 1972). The results shown here are consistent with this proposal since, in water plants, where there will be no inhibition by auxin-induced ethylene, high levels of auxin are not inhibitory.

The optimum levels of auxin reported for elongation of some water-plant petioles (measured over 24 h), are extraordinarily high; viz. 10^{-3} M in *Ranunculus sceleratus* and 10^{-4} M in *Hydrocharis morsus-ranae* (Cookson 1976). However, these values were obtained using intact leaves and, as already discussed (page 143), auxin penetration into such intact organs is probably limited. It seems likely that the true auxin optimum in *Ranunculus sceleratus* and *Hydrocharis morsus-ranae* may be much closer to that found in *Nymphoides*, and that this would be revealed if auxin entry was facilitated by using debladed segments. This is a prediction I have not tested.

In *Callitriche platycarpa*, another plant showing a submergence response, ethylene-promoted internode elongation has been said to be GA-dependent rather than auxin-dependent (Musgrave *et al.* 1972). In view of findings that in all other water plants closely studied, the ethylene effect is auxin-dependent, perhaps the *Callitriche* system deserves reinvestigation. It may be that the ethylene response in this plant is
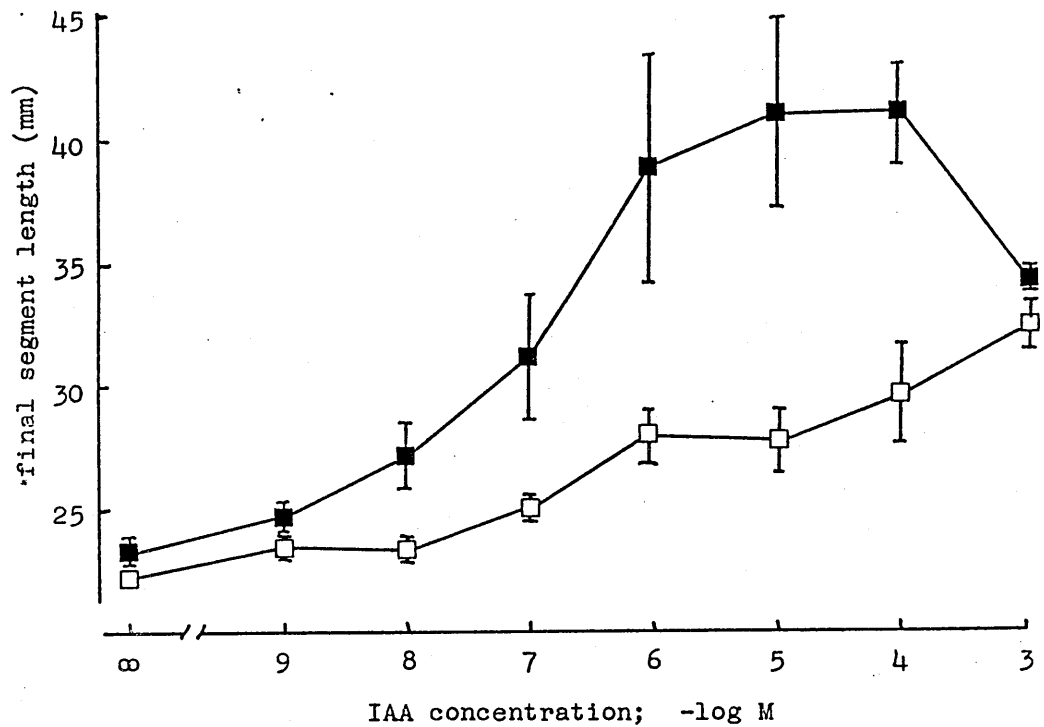
FIGURE 25 Effect of auxin and ethylene on elongation of *Regnellidium* segments.

Subapical petiole segments, initial length 21.4 mm, were incubated for 22 h in various levels of IAA either with (■), or without (□) ethylene at 20 μl l^{-1}.

Each point is a mean for 4 segments ± SE.

indeed auxin-dependent, as in other species. Ethylene would still have promoted growth in the experiments of Musgrave *et al.* because auxin would be present in the plants they used, being synthesised in the attached rosette of leaves. The effect of GA could be completely separate from the submergence response in *Callitriche*. Evidence that *Callitriche* is indeed similar to the other water plants comes from Musgrave *et al.*'s own data showing an ethylene effect in "under 30 min" (as in "auxin-dependent" water plants); the GA effect is not noted until some 8 h. Musgrave *et al.*'s findings with a GA-synthesis inhibitor (AMO 1618) do not fit easily with this concept, but it may be that the effect of this inhibitor is not as specific as has been thought and perhaps during the 8 h required for AMO to take effect, there is some alteration leading to changed auxin levels which, in turn, actually cause the change in growth.

I strongly suspect that a broadly similar auxin-dependent ethylene response is the cause of submergence-associated rapid growth in all those species so far reported, and perhaps in many others not yet closely studied.

Involvement of factors besides Ethylene in the submergence response.

There is little doubt that ethylene-induced growth is the principal element in depth accommodation. However, it has sometimes been found that elongation of ethylene-treated *Regnellidium* fronds is not as great as elongation of submerged fronds (e.g. Musgrave and Walters 1974). This suggests that an additional factor besides ethylene, contributes to growth under submerged conditions. Several possible factors have been considered:

1. Other gases. Just as ethylene accumulates under submerged conditions, gases such as CO_2 and O_2 might also accumulate, and these too could affect growth. Raised CO_2 levels are usually antagonistic to ethylene effects

in land plants (Burg and Burg 1967) but there is evidence that CO_2 can promote ethylene effects in water plants (Suge and Kusangi 1975; Suge 1971). It has been speculated that, as a general rule, CO_2 antagonises inhibitory ethylene effects, but increases promotive ones (Suge 1972).

I think it unlikely that marked CO_2 accumulation will occur in submerged *Regnellidium* tissue, especially under lighted conditions, when there is net consumption of CO_2. Even during periods of net CO_2 production the gas is unlikely to reach appreciable levels in the plant's air spaces, because CO_2 dissociates in water at near neutral pH, and is therefore very soluble. Nevertheless, a test was carried out to identify any possible involvement of altered CO_2 levels in the submergence response of *Regnellidium*. Results showed that raised CO_2 levels had no effect on either control, or ethylene-induced growth in *Regnellidium* petioles (Table 9). Changes in CO_2 level, therefore, are probably not involved in the submergence growth response.

Build-up of O_2 will occur in *Regnellidium* fronds submerged under lighted conditions. This can be monitored as an increase in buoyancy of submerged fronds, with time of submergence (Figure 26). It can be so marked that bubbles are evolved from the plant (Musgrave and Walters 1974). However, it seems unlikely that such build-up promotes growth, because high O_2 inhibits, rather than promotes, ethylene-induced growth in some other water plants (Ohwaki 1967, cited by Ku *et al.* 1970; Suge and Kusanagi 1975). In addition, fronds grow equally well whether submerged in light or dark conditions (Figure 27), though O_2 is likely to accumulate only under light conditions.

2. <u>Buoyant pull</u>. The interior of the petioles of many water plants is fenestrated to a remarkable degree by air canals and air spaces (Plates 3 and 5); the submerged leaf is buoyed up by air contained in these structures. This creates a significant tension in the petiole under
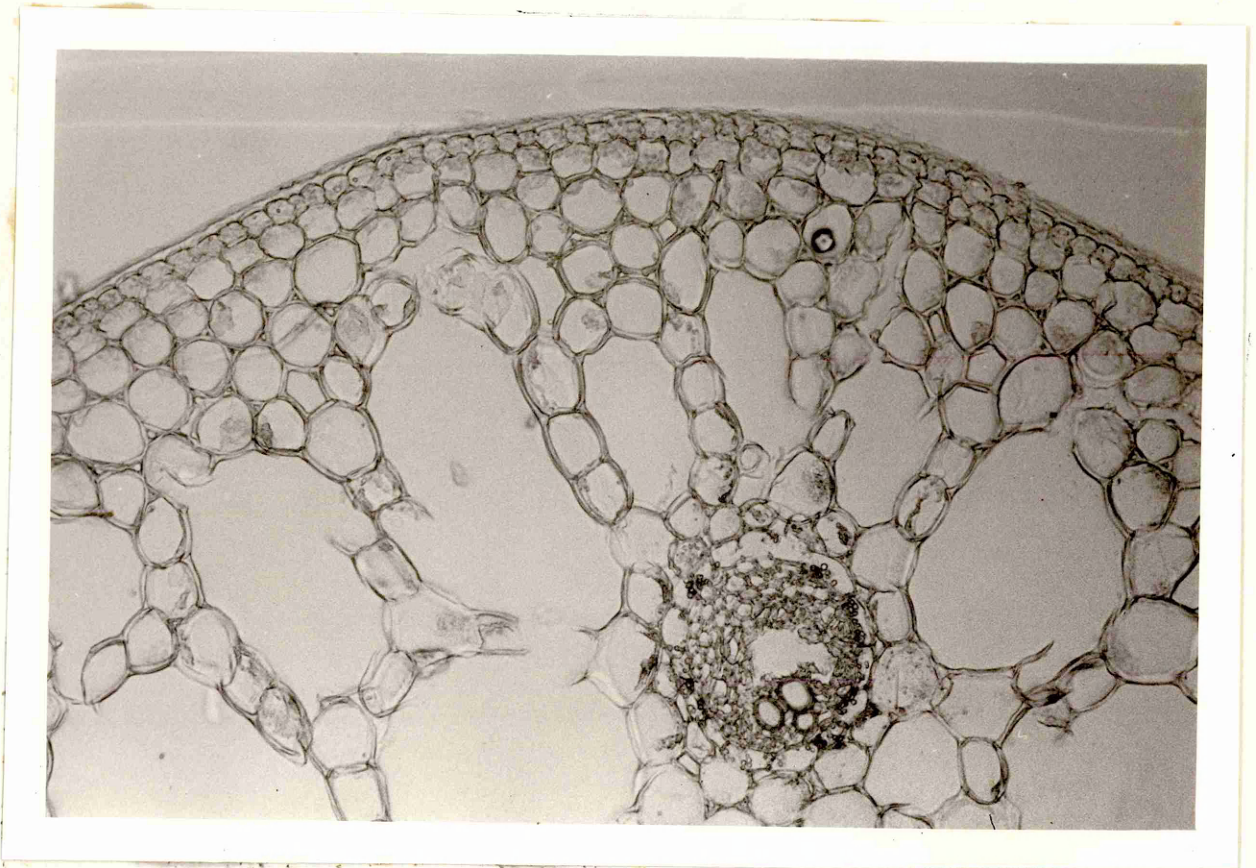
<u>PLATE 5</u> Petiole of *Nymphoides* seen in TS, to show the relatively
dense peripheral region.

Scale: the petiole shown is about 2.5 mm in diameter.

Treatment	final (mm) segment length	
	$\bar{x}$	s
Air	14.7	3.7
Air + CO_2	12.8	2.6
Air + eth.	20.5	6.4
CO_2 + eth.	18.2	3.8
Submerged	20.7	4.4

TABLE 9   Effect of raised CO_2 levels on ethylene-induced
elongation in *Regnellidium* petioles.

Intact leaves were used. The apical 10 mm of each
petiole was marked off at the start of incubation.
After 3 days in the stated treatments, in continuous
light, length of the marked portion was recorded.

CO_2 treatment involved exposure to about 5% CO_2
throughout incubation. Ethylene treatment was at
10 µl 1^{-1}.

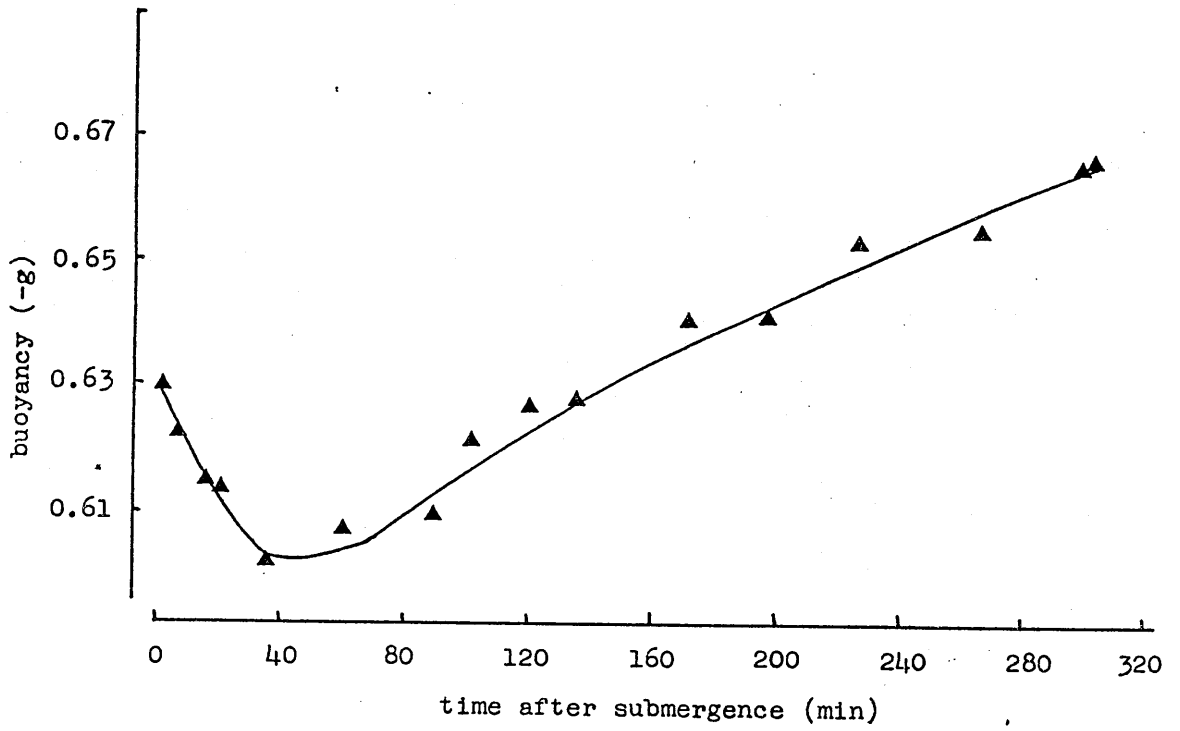Each mean is for 11 measurements.

FIGURE 26 Change in buoyancy, with time, of a submerged
Regnellidium leaf.

A single *Regnellidium* leaf with an 8 cm petiole
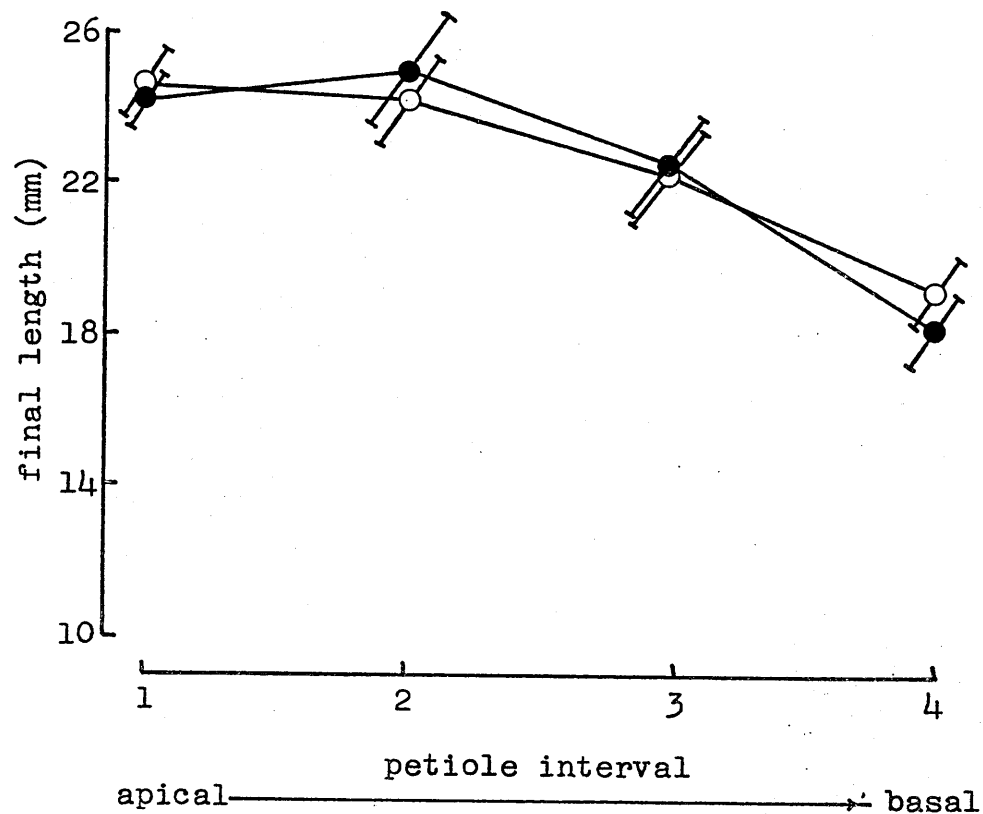was suspended in water and its "weight" monitored.

FIGURE 27   Comparison between elongation of submerged
Regnellidium fronds incubated in light, or dark
conditions.

At the start of incubation, the first four 1 cm
intervals of the petioles of intact *Regnellidium*
leaves (counting from the apical end) were marked
off. The tissue was then submerged for 24 h in a
tank of tap water, one half of which was blacked
out, so that some leaves were submerged in room
lights (o), and some in darkness ($\bullet$).

Each point is a mean for 9 segments ± SE.

submerged conditions, and it has been proposed that this tension may increase growth rate (Musgrave and Walters 1974). Tension in the petiole will certainly increase stress on the cell walls. Stress (such as that due to turgor) is necessary for growth (see page 41) and, under some circumstances, the rate of extension of a tissue increases linearly with the applied stress (Cleland 1971a; Rayle and Cleland 1972b).

Musgrave and Walters (1974) estimated the magnitude of "buoyant pull" in *Regnellidium* fronds and claimed that, in ethylene-treated fronds growing in air, the full submergence response is mimicked if a tension equal to that of buoyant pull, is applied to the petiole by attaching weights.

The question of buoyant pull was further investigated here. I found that the method used by Musgrave and Walters (op. cit.) to calculate buoyant pull, did not closely estimate the pull which contributes to growth: their method entailed noting the minimum weight required to sink a petiole ("rachis") of *Regnellidium*, with attached piece of rhizome (as used in their growth experiments). This value, which can be termed the "sink-equivalent", was reckoned to represent the buoyant pull which could affect growth. However, this presumes two things:

1. Musgrave and Walters calculated buoyant pull on debladed petioles of leaf blades, yet their measurements of growth under submerged conditions utilised tissue *with* leaf blades. This inherently assumes that leaf blades are not important in buoyant pull;

2. Musgrave and Walters assume that buoyant pull of the entire petiole (including the small piece of attached rhizome) is important in determining growth rate.

As outlined below, neither of these assumptions is entirely correct. Consider assumption 1: using a balance modified from the apparatus shown

in Figure 10, I measured buoyancy of the leaf blades of typical *Regnellidium* fronds (as used in my experiments) and obtained a value of about 0.2 g. This is a significant amount relative to the total buoyant pull of an entire leaf, including the petiole (about 0.5 g).

Concerning assumption 2; although all parts of the tissue are buoyant, growth will be influenced only by that buoyancy which contributes a tension to the elongating region of the petiole. In the submergence response of *Regnellidium*, most elongation takes place in the upper portion of the petiole (Figure 21). Buoyancy which does not impose a tension in this region will not be relevant to growth.

A buoyant body will impose stress only on that which prevents it from rising i.e. its anchor. In the context of a submerged *Regnellidium* frond, buoyant pull at any point along the petiole will be that supplied by all tissue *above* that point. Buoyancy of tissue lower down will not be important.

Thus to calculate the buoyant pull effective in growth for a *Regnellidium* frond, one should discount buoyancy of the middle and lower regions of the petiole (and that of any pieces of rhizome which may be present). Measurements indicate that this growth-effective buoyant pull (that contributed by the top part of the petiole, and by the leaf blades) is about 0.3 g. Fortunately, the two erroneous assumptions discussed above work in opposite ways, and cancel out to some extent. Nevertheless, the 0.5 g value for buoyant pull used by Musgrave and Walters (1974) is probably an overestimate by some 60%.

To test the effect on growth, of artificially-applied tension, Musgrave and Walters mimicked buoyant pull in *Regnellidium* fronds by attaching weights to the base of the petiole. They used double the calculated sink-equivalent weight because, "when 0.5 g was applied the

buoyant force was only neutralised, and another 0.5 g had to be added to apply a downward force equivalent to the buoyant pull". Again, this reasoning shows no regard to where the growth was occurring: if 1.0 g was attached, then pull experienced by tissues just above the point of attachment of the weight must have been 1.0 g! Higher up the petiole, the tension imposed at any point would be 1 g, less buoyancy of all tissue between that point and the point of attachment. In the growing region this would be some 0.6 g, or about double the *in vivo* growth-effective buoyant pull. Another factor tending to overestimate the absolute value of buoyant pull in Musgrave and Walters' data is their failure to take account of the change in apparent weight of their "weights" which occurs on submergence, due to displacement of water. Since this factor affected all their measurements equally, it does not significantly detract from their conclusions, and will not be considered further here, except to note that the error would probably be in the region of 20%, depending on the density of their "weights".

I calculate that approximately 0.2-0.3 g would have to be attached, in the manner shown by Musgrave and Walters, to "neutralise" buoyant pull in the growing zone. A further 0.2-0.3 g would then approximately mimic the *in vivo* buoyant pull, so that 0.6 g applied weight, plus ethylene, ought to be sufficient to mimic the entire submergence response, if that depends exclusively on ethylene and buoyant pull.

Musgrave and Walters' data show very little effect of 0.6 g applied weight, though they did observe promotion by larger weights. My results show little effect of any weight up to 1 g, on ethylene-induced, auxin-induced, or control growth of *Regnellidium* fronds (Table 10) though in some cases, promotion was observed with 3 g (data not shown).

A further difference between my results and those of Musgrave and Walters (1974) is that in many cases, I find ethylene alone almost as

Treatment	- Weight		+ Weight	
	$\bar{x}$	se.	$\bar{x}$	se.
Control	- 67.7	0.76	68.7	1.49
Auxin $10^{-5}M$	75.6	2.59	75.9	1.11
Ethylene $25 \ \mu l \ l^{-1}$	89.9	4.71	88.2	5.28

TABLE 10 The effect of external pull on control, auxin-induced, and ethylene-induced elongation in intact *Regnellidium* leaves.

Pull was applied by attaching a 1 g weight to the base of each leaf (starting petiole length: 60 mm). Figures are mean lengths (mm) for 10 leaves (± SE) after 20 h incubation.

effective as submergence at promoting elongation in intact fronds (Table 9). Thus the ethylene effect, plus a small contribution from buoyant pull may account for the entire submergence response.

I have no complete explanation for the reduced effect of pull in my data compared to those of Musgrave and Walters. One contributory factor could be the longer incubation times used in the latter's experiments. It may be that pull becomes more effective after some days of growth; indeed, from Figure 3 of Musgrave and Walters (1974) it is clear that the relative importance of pull is greater after 2 or 3 days of submergence. Thus it seems that pull may serve to maintain rapid growth rather than to enhance the rate *per se*. This could occur if, as growth proceeds, $\psi_{s,i}$, rises by dilution) causing turgor to drop. Eventually, turgor alone may not be sufficient to encourage maximum growth rate, and it is then that artificial tension becomes important.

Summary: tension equivalent to that normally occurring in submerged *Regnellidium* fronds has only a limited effect on growth, especially over the short term. The submergence response may be completely explained in terms of ethylene-induced growth plus this small pull effect.

CHAPTER 9

ON THE MECHANISM OF ETHYLENE- (AND AUXIN-)
INDUCED GROWTH IN WATER PLANTS

CHAPTER 9

ON THE MECHANISM OF ETHYLENE- (AND AUXIN-) INDUCED GROWTH IN WATER PLANTS

In the preceding chapter, it was shown that ethylene can promote elongation in petioles of *Nymphoides peltata*. This is in agreement with findings from several other water plants. These results support the view that ethylene-induced growth is part of a general mechanism by which semi-aquatic plants contend with fluctuating water levels.

The remainder of this thesis is concerned with the question of how ethylene promotes growth in *Nymphoides* and in other water plants.

Broad categories of ways by which plant growth may be regulated are considered in Chapter 3. One might begin an investigation of how ethylene promotes growth by considering which components of equation 1 (page 37) are influenced by ethylene. However, in the case of ethylene, there is a special circumstance: in several water plants, including *Nymphoides*, ethylene has little effect on growth unless auxin is present (see page 125). One could postulate therefore that, rather than having an independent effect on growth, ethylene alters elongation indirectly, by interfering with the metabolism or transport of auxin. These possibilities are considered below.

A.   Possibility that ethylene promotes growth in water plants via changed
auxin breakdown.

Some effects of ethylene in land plants may be due to alterations in
auxin breakdown. For example, ethylene alters activity of IAA-decarboxylase
enzymes in stem sections of several species, including *Pisum sativum*
(Morgan *et al.* 1968). The possibility that ethylene promotes growth in
water plants by a mechanism of this sort is appealing in that it offers a
ready explanation for the dependence of ethylene on the presence of auxin
in these plants. Conceivably, in tissue such as *Nymphoides* petiole,
ethylene might inhibit the processes by which auxin is normally inactivated.
These processes can be very efficient (Nissl and Zenk 1969) and ethylene-
induced changes therein could perhaps account for the observed promotion of
water-plant growth by ethylene, especially over the longer term. To test
this possibility, the effect of ethylene on growth in the presence of IAA,
was compared with that in the presence of 2,4-D. If ethylene acts simply
by delaying auxin breakdown and thus raising auxin levels, one might
predict that the gas would have little or no effect on growth when 2,4-D
was present rather than the natural auxin, IAA. This is because 2,4-D
is comparatively stable in most tissues, at least over the time period used
here, so that any factor which delays its breakdown should have virtually
no effect on growth. Figure 28 shows that in *Nymphoides*, ethylene has a
marked promotive effect even when 2,4-D is the auxin present. This indicates
that inhibition of auxin removal is not the major mechanism of ethylene's
action on growth.

In support of this conclusion are the data in Figure 29 which demon-
strate that frequent renewal of the IAA solutions bathing *Nymphoides*
segments has practically no effect on growth (in the presence or absence
of ethylene) up to 24 h, even though substantial ethylene-promoted growth
had already occurred by that time. If IAA removal in the absence of
ethylene is sufficiently rapid to account for the observed difference
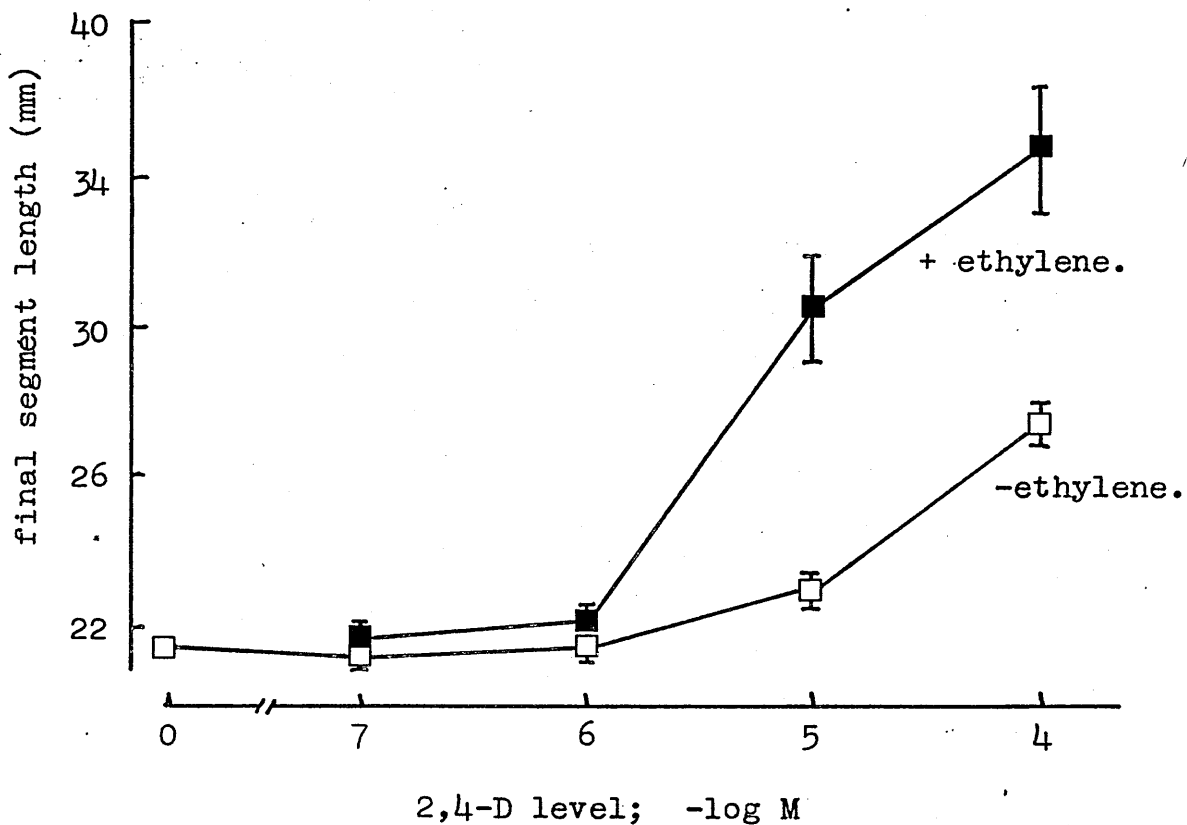
FIGURE 28   Effect of ethylene, in the presence of the synthetic
auxin 2,4-D, on elongation of *Nymphoides* segments.

Apical petiole segments, initial length 21.4 mm,
were incubated for 12 h with the stated auxin level.
Ethylene was supplied at 50 μl l^{-1}.
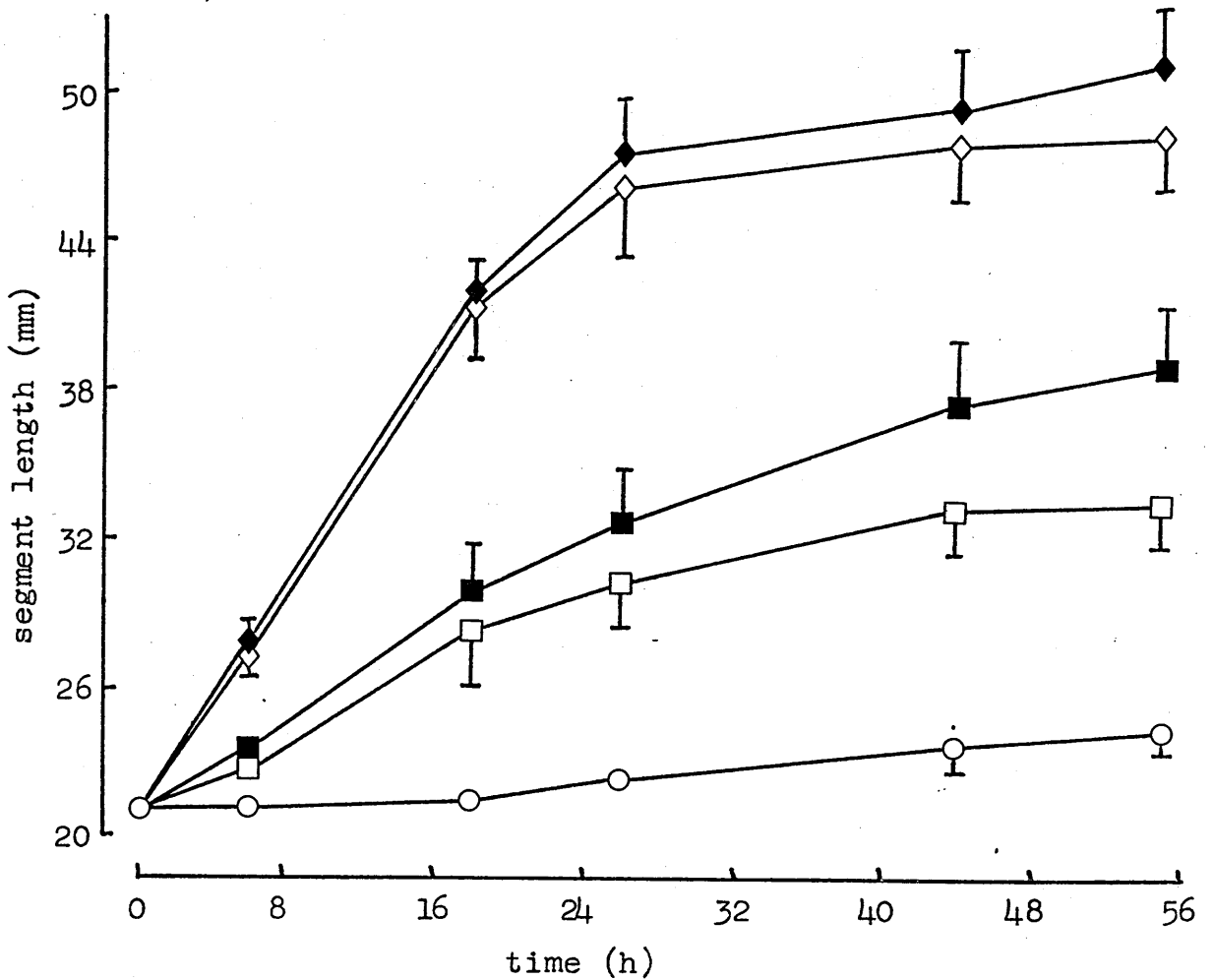
Each point is a mean for 10 segments $\pm$ SE.

FIGURE 29 Effect of renewing the IAA solutions on elongation of
Nymphoides segments in auxin alone, and in auxin plus
ethylene.

Subapical petiole segments, initial length 21.4 mm,
were incubated for the stated time in 10^{-5} M IAA
alone ($\square$,$\blacksquare$) or in 10^{-5} M IAA plus 20 μl l^{-1} ethylene
($\diamondsuit$,$\blacklozenge$). Control growth is also shown (o).

Filled symbols represent treatments in which the IAA
solution was replaced by a fresh one each time the
segments were measured. No such replacement was done
in other treatments.

Each point is a mean for 12 segments ± SE.

between elongation in IAA alone, and that in IAA plus ethylene, then frequent replacement of IAA should substantially raise the effect of IAA alone. It did not (Figure 29). These data cogently suggest that ethylene's effect in *Nymphoides* is not a simple inhibition of auxin removal.

Evidence from kinetic studies is in complete agreement with this conclusion. For example, if ethylene is assumed to act by delaying auxin breakdown, several predictions about kinetics of the ethylene effect could be made. Three such predictions were put to the test:

Prediction 1 When ethylene is added to segments which are already growing in response to a low (suboptimal) level of auxin, there should be a gentle, gradual rise in growth rate to a new faster level. This is because, following the inhibition of auxin removal, there will be a steady accumulation of auxin, which should be reflected in kinetics of the response.

Results show however that, under these conditions, the response to ethylene is marked rather than gentle (Figure 30) and that the promotion quickly reaches its maximum rate. This is contrary to the prediction.

Prediction 2. When ethylene is added to segments which are already growing in response to optimal or supraoptimal levels of auxin, there should be no immediate effect on growth because, although ethylene would increase the level of available auxin, the tissue is already responding maximally to auxin, and no further increase in growth rate could take place. Over the longer term, ethylene would still increase elongation under these circumstances since any decline in auxin levels would be delayed. Thus ethylene would prolong the *duration* of the highest auxin-induced growth rates rather than itself directly increasing growth rate (see Figures 31a and 31b). As can be seen from Figure 17, ethylene induces, right from the start, a substantially higher rate of growth than does auxin alone, even at very high (supraoptimal) levels of auxin.
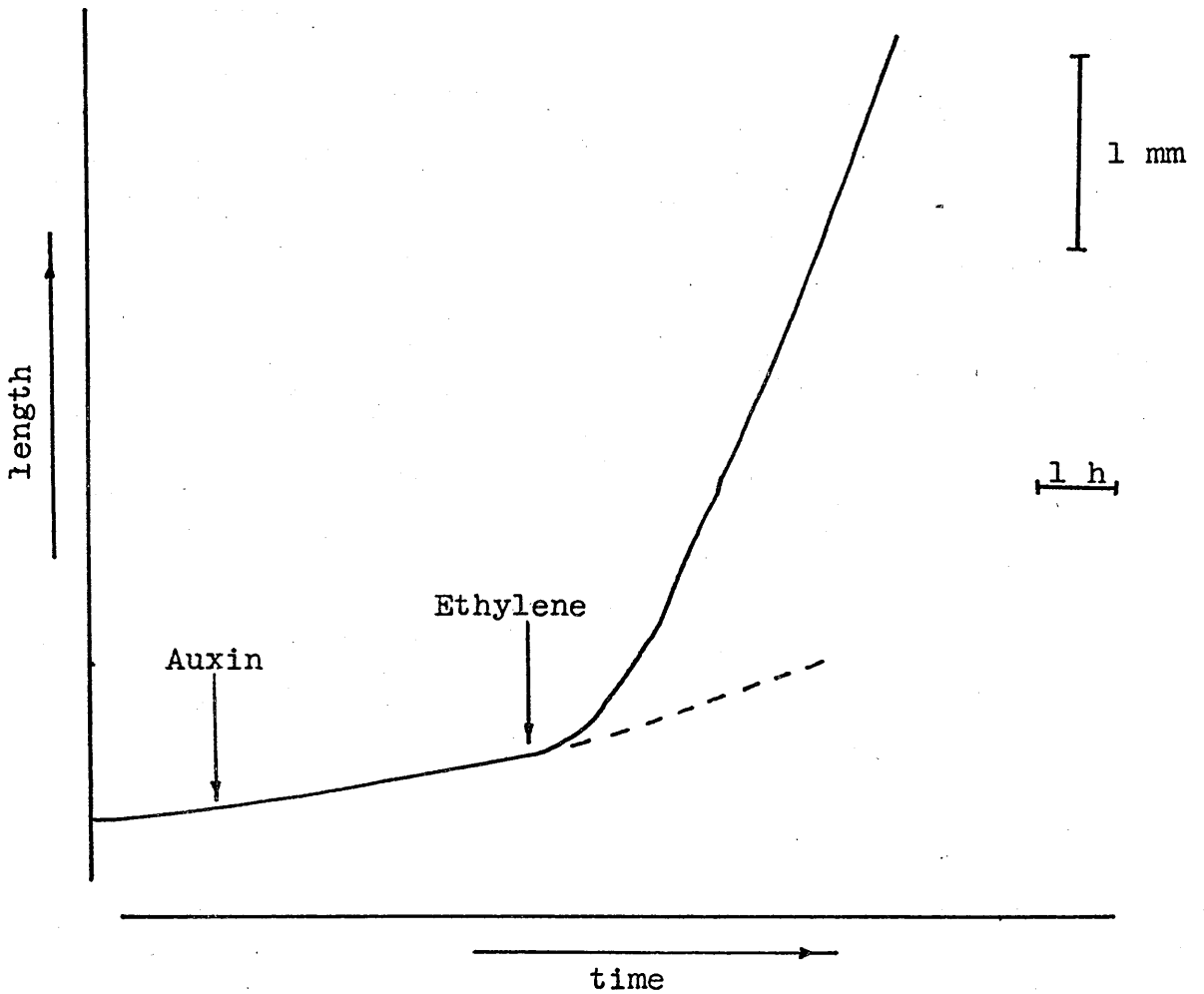
FIGURE 30 Kinetics of ethylene-promoted growth at low auxin levels, in *Nymphoides* segments.

Transducer recording from a column of 4 x 1 cm apical *Nymphoides* petiole segments. At the first arrow, 10^{-6} M 2,4-D was added. At the second arrow, saturating levels of ethylene were added. The dotted line shows growth in the absence of ethylene.
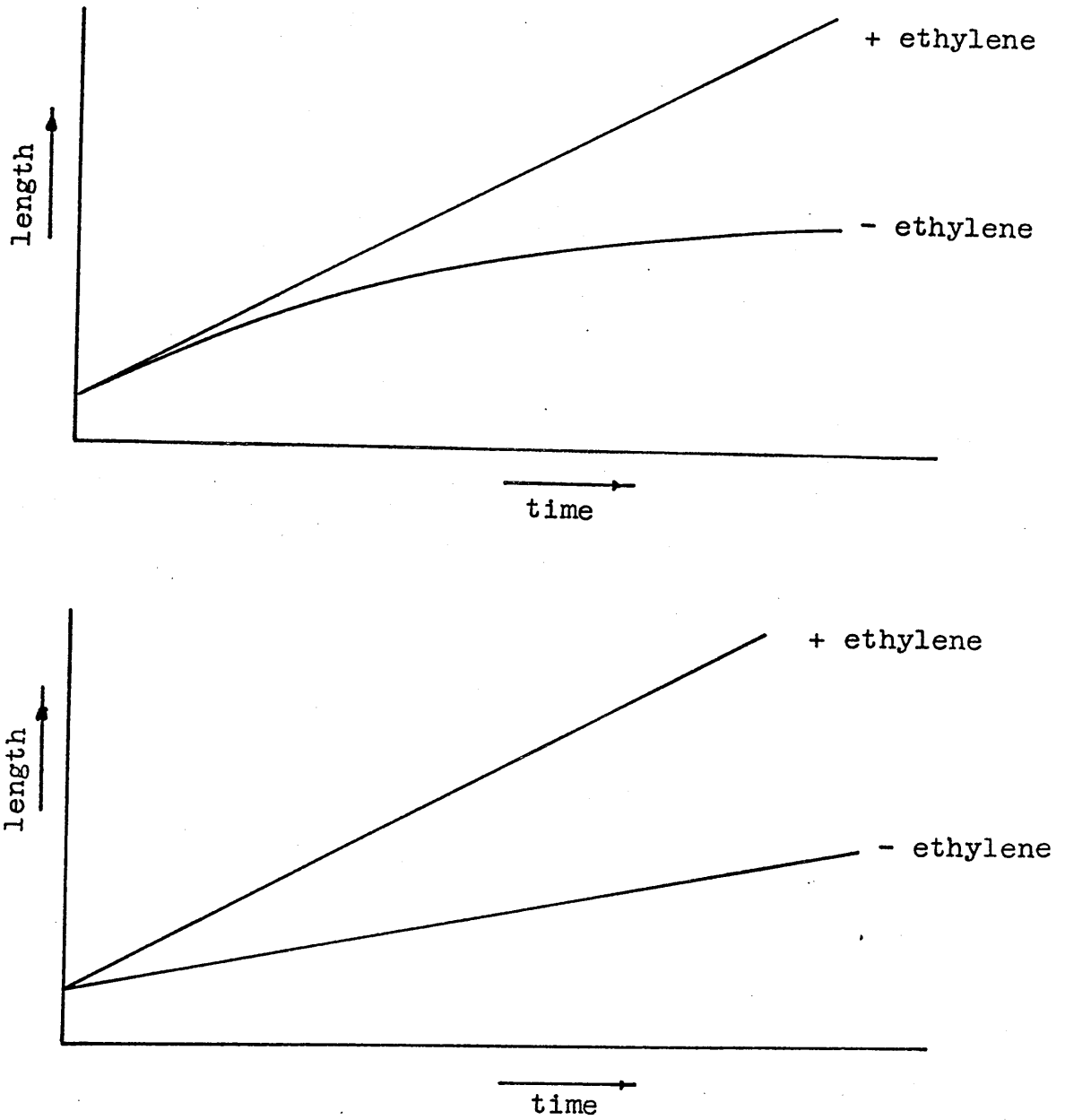
Redrawn from original.

Predicted patterns of elongation with time, for growth induced by auxin alone, and by auxin plus ethylene.

a. upper graph: pattern of ethylene simply prolongs the duration of rapid, auxin-induced rates.

b. lower graph: pattern if ethylene induces an increase in growth rate *per se*.

Prediction 3. From the proposal under consideration, the highest rates of growth possible with auxin *and* ethylene, should not exceed those attainable with optimal auxin applied alone. This is because in each case, only auxin would be directly promoting growth. Figure 24 shows that rates attained in response to ethylene, at many levels of auxin, are markedly higher than those possible with auxin alone, even at optimal levels.

For each of these three predictions then, the results are not compatible with the thesis that ethylene acts by delaying auxin breakdown; consequently that proposition must be abandoned.

The results noted above were gained using *Nymphoides*, but it seems likely that the same conclusions apply in *Regnellidium*; i.e. ethylene does not act by inhibiting auxin breakdown. This was ascertained by incubating segments in auxin solution (in the presence or absence of ethylene) then removing the segments and comparing the amount of auxin remaining in the solutions, using a crude bioassay. The results (Figure 32) show that as much auxin remained in the "auxin-only" treatment as did in the "auxin-plus-ethylene" treatment, even after 7 h incubation (by which time differences in elongation between the two treatments are pronounced). Thus, ethylene does not markedly delay auxin breakdown in *Regnellidium* petiole segments.

Further support for the conclusion reached above are observations that in *Regnellidium* (again as in *Nymphoides*) segment elongation is markedly promoted by ethylene even if 2,4-D is the auxin present rather than IAA (Figure 33).

Finally, Walters and Osborne (1979) have measured IAA levels in *Regnellidium*, using spectrofluorimetry; they detected no significant ethylene-induced changes.
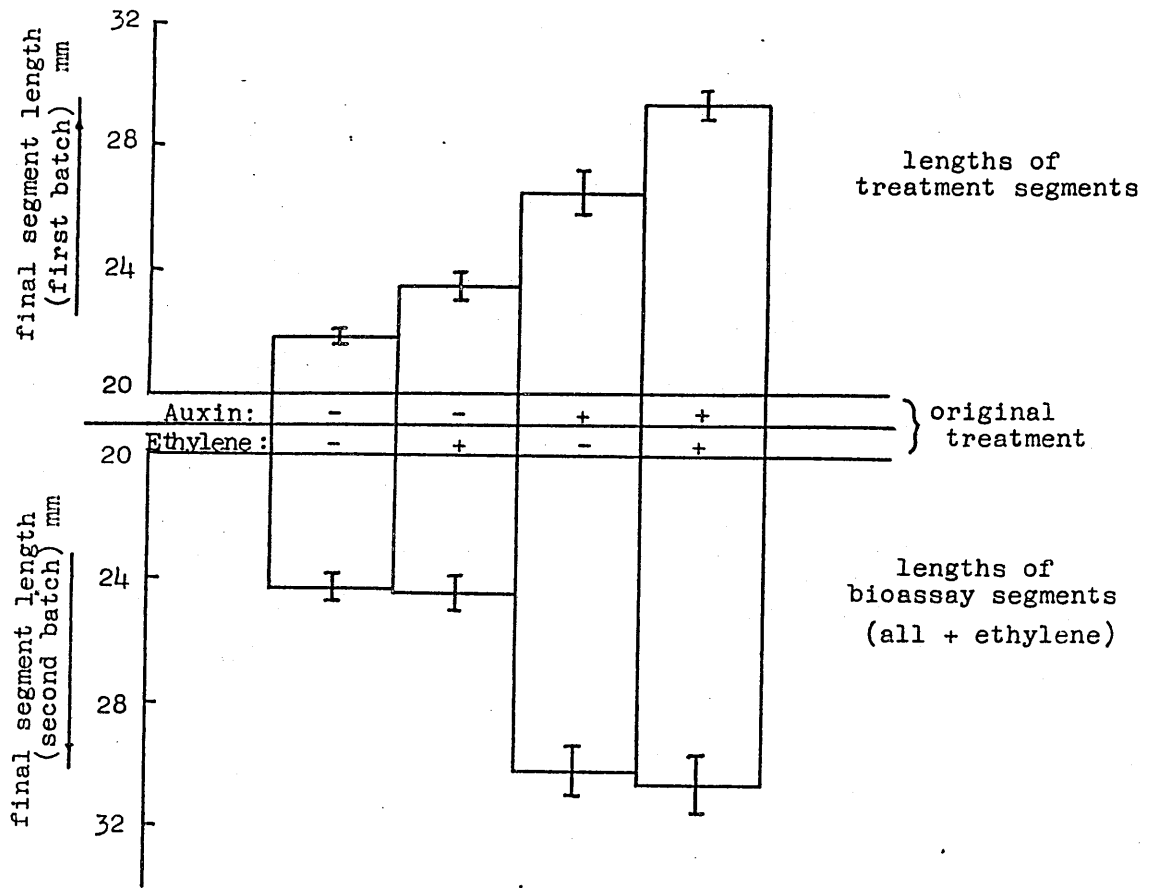
FIGURE 32 Effect of ethylene on the longevity of IAA in the medium
bathing *Regnellidium* segments.

Apical petiole segments, initial length 21.4 mm, were
incubated in 10^{-5} M IAA, with or without ethylene, for
8 h. Their lengths were then measured; these are shown
in the top part of the above graph ("treatment segments").
A second batch of segments ("bioassay") was then placed
in each of the original treatment solutions and incubated,
this time all with ethylene, for 8 h. Their lengths are
shown in the lower part of the graph.
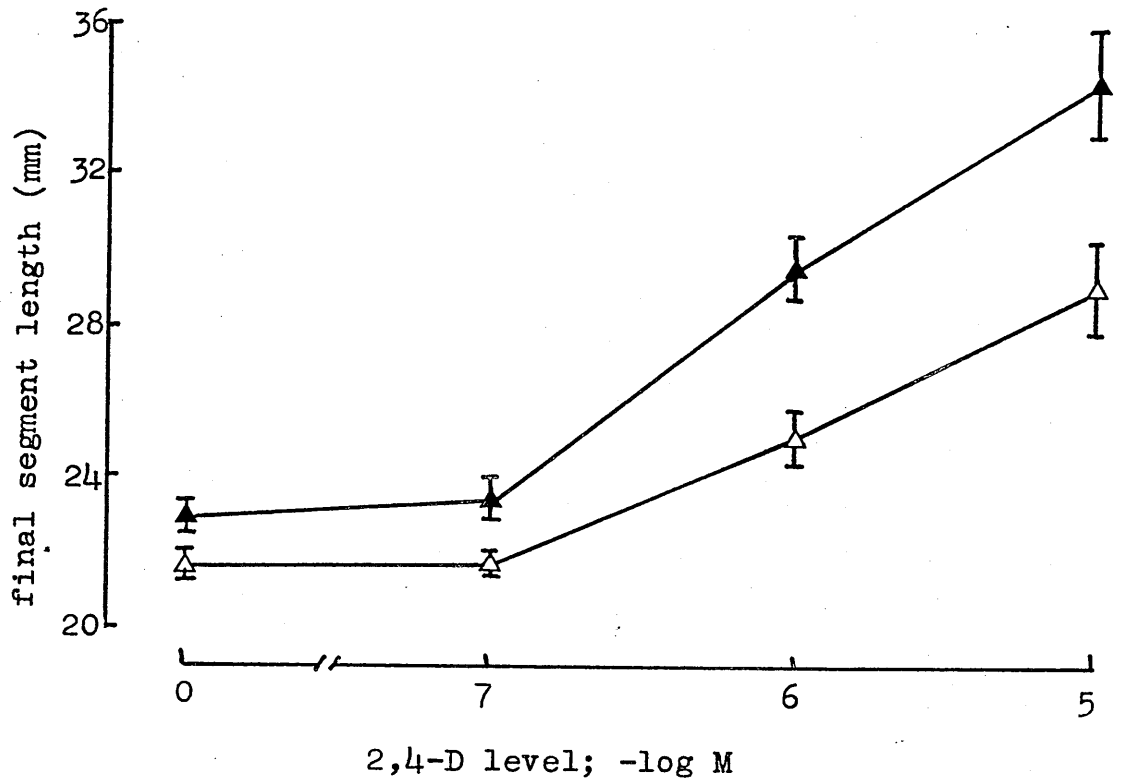
Bars give means of 10 segments ± SE.

FIGURE 33 Effect of ethylene in the presence of the synthetic auxin 2,4-D, on elongation of *Regnellidium* segments.

Apical petiole segments, initial length 21.4 mm, were incubated for 18 h at the stated level of auxin with (▲), or without (△) ethylene at 25 µl l^{-1}.

Each symbol shows a mean of 10 segments ± SE.

B. Possibility that ethylene acts via changed auxin transport.

Ethylene has been shown to affect auxin transport in some land-plant tissues (Beyer and Morgan 1970a). Consequently, it should be considered whether a mechanism of this sort could account for the ethylene effect on growth of water plants, especially since an effect through auxin transport would explain ethylene's dependence on the presence of auxin. Two papers have reported effects of ethylene on basipetal auxin transport in water plants: Musgrave and Walters (1973) claimed that ethylene rapidly enhances auxin transport through petiole segments of *Ranunculus sceleratus*, while Walters and Osborne (1979) noted that ethylene decreases transport in *Regnellidium* rachis segments. Attempts to reproduce the *Ranunculus* result have not met with success (Walters, unpub., cited by Cookson 1976). I have not attempted to discern effects of ethylene on auxin transport in *Nymphoides*, because it is not conceivable that any change in transport could increase auxin levels to the point where "ethylene-type" growth results, because there is no such point (Figure 24). The same three predictions would arise with this possibility as did with the previous one; these have already been rejected. Thus alteration in auxin transport is not a plausible explanation of ethylene's effect on growth.

C. Possibility that ethylene affects growth via altered auxin synthesis.

Effects via auxin synthesis can be summarily rejected on the same grounds as were the above two possibilities. The "synthesis" possibility does not even have the advantage of accounting for ethylene's dependence on the presence of auxin. Walters and Osborne's demonstration that ethylene has no effect on auxin levels (referred to above), also tends to belie this possibility.

Summary: 1. Although ethylene-promoted growth is dependent on the presence of auxin, and although the magnitude of the ethylene effect depends to some

extent on the amount of auxin present (Figure 24), it is extremely unlikely that ethylene acts simply by regulating the level of auxin in the tissue, whether by altering synthesis, transport or breakdown.

2. Ethylene's growth promotion, at all levels of auxin, involves a definite increase in growth rate. It is not simply a prolongation of the response to auxin.

Effect of auxin on ethylene levels.

Just as it is extremely improbable that ethylene promotes growth by raising auxin levels, the converse is also true; auxin does not promote growth in water plants by raising ethylene levels. The evidence for this is two-fold:

a. Application of large amounts of exogenous ethylene (even in the presence of low levels of auxin) does not saturate the growth response in *Nymphoides* (Figure 24). It is difficult to conceive therefore, how increased endogenous production (stimulated by auxin) could achieve the same thing.

b. Direct measurement of ethylene production in *Nymphoides* leaves reveals little or no effect of auxin (Table 6). Similar results are found with *Regnellidium* (Walters and Osborne 1979). Thus in *Nymphoides* and *Regnellidium*, neither ethylene nor auxin promotes growth by raising the level of the other.

In other water plants the situation is probably similar. Cookson (1976) notes that auxin does not promote ethylene production in *Hydrocharis morsus-ranae* and that, although there may be some auxin-induced ethylene in *Ranunculus sceleratus*, RBA (an analogue of the fungal metabolite rhizobitoxin) inhibites ethylene production in this plant without affecting IAA-induced growth. This shows that IAA-induced growth is not dependent on increased ethylene production. Cookson also shows that Ag^+ inhibits the ethylene

effect in *Ranunculus sceleratus* but it does not inhibit the auxin response in that species. This reinforces the view that auxin does not promote growth by raising ethylene levels.

Since neither auxin nor ethylene operates in these plants by raising the level of the other, and neither is, on its own, able to saturate the growth response, the mechanism of ethylene-promoted growth must be different in type, or degree, from the mechanism of auxin-promoted growth.

The effects of auxin and ethylene on elongation in *Regnellidium* petiole have been said to be additive (Cookson 1976). Additivity has often been taken as a criterion indicating separate mechanisms of action. For example, applying the converse: (i) non-additivity between the effects of FC and low pH, and of IAA and low pH, on growth, was taken to reflect the operation of identical mechanisms (Lado *et al.* 1976; Rayle 1973); and (ii) as one would predict, the effects of two auxins (IAA and 2,4-D) on elongation in *Nymphoides* are found not to be additive (Figure 34).

However, "additive" is not an accurate description of the relationship between auxin and ethylene in *Regnellidium* and *Nymphoides*, because ethylene has no independent effect. Rather, as can be seen from Figures 16, 24, 28 and 33, ethylene has a synergistic or multiplicative influence on auxin-induced growth. From Figure 24 it can be seen that for all levels of auxin, growth rate in the presence of ethylene is approximately twice that found with auxin alone. But in the sense that both hormones must be present for development of the maximum growth rate, their effects can be said to be additive.

It has been concluded that ethylene affects growth directly rather than by altering auxin levels. Thus it must alter a component(s) of equation 1 (page 37). Attention is next given to the problem of which component of this equation is affected when ethylene stimulates growth in water plants.
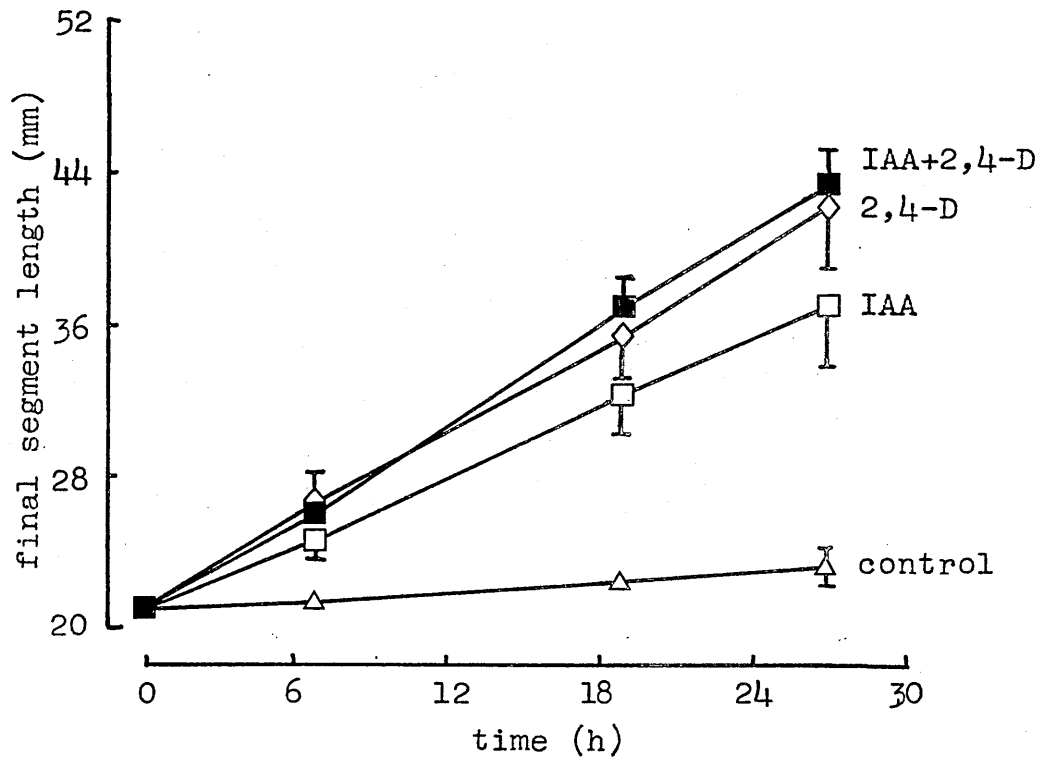
FIGURE 34 Effect of IAA and 2,4-D applied singly or together, on elongation of *Nymphoides* segments.

Apical petiole segments, initial length 21.5 mm, were incubated with 10^{-5} M IAA (□), 10^{-5} M 2,4-D (◊), or 10^{-5} M IAA + 10^{-5} M 2,4-D (■). Segment length was measured at various times after the start of incubation. Control growth is also shown (△).

Each point is a mean for 10 segments ± SE.

Ethylene, auxin and components of the growth equation.

Equation 1. $dV/dt = Lp \, \phi \, (\psi_o - \psi_{s,i} - Y)/(Lp + \phi)$

As discussed in Chapter 3 change in growth rate must result from change in one or more components of this equation.

Because promotions by auxin and ethylene are inseparable experimentally (and probably mechanistically) attention will here be given to both hormones.

There have been many demonstrations that auxin-promoted growth in land plants involves a change in the wall-extensibility component of equation 1. Because the kinetics of auxin responses in water plants are similar to those in land plants, it seems reasonable to suppose that a similar process is involved in both cases; i.e. auxin probably promotes water-plant growth by an effect on WE. This is later confirmed for *Regnellidium*; see page 184. It was thought likely that ethylene would affect a component other than WE, since this would provide an explanation for the "additivity" observed between the effects of auxin and ethylene on growth.

Components of equation 1 are considered in turn:

Water permeability (Lp) Whenever ethylene promotes growth in the wild, the tissue is submerged, i.e. completely surrounded by water. It might be thought that under these circumstances, hydraulic conductivity would be comparatively large, since the flow path for water entering growing cells will be short. If Lp is large, it is unlikely to be growth limiting; therefore promoters of growth are not likely to function by increasing it further.

However, in the *Nymphoides* petiole, there is a well-developed outer cuticle (see page 194) which will present a barrier to direct entry of water. In addition, the xylem in *Nymphoides* petioles in clearly developed, and bears

lignified annuli, suggesting that it is by no means vestigial, and that the living plant has a substantially normal transpiration stream. These two features indicate that the flow path for most water entering cells of a growing segment of *Nymphoides* petiole, will traverse the cut ends, and will be of significant length.

As discussed on page 40 , change in Lp can be invoked as a cause only when an existing growth rate is increased, and not when new growth is induced in a previously static tissue. Auxin can induce marked growth in segments of *Nymphoides* in which growth had virtually ceased, so that the ratio "auxin-induced rate/prevailing endogenous rate", can exceed 200. It seems extremely unlikely that such a promotion originates from a shift in Lp.

Ethylene, on the other hand, serves only to multiply an existing (auxin-induced) rate, normally by a factor of about 2 (Figure 24). This is entirely consistent with a postulated effect through Lp. Indeed, the proposal that auxin promotes growth via an effect on say, WE, and ethylene on Lp, would offer a ready explanation for the dependence of ethylene on auxin, since an ethylene-induced increase in Lp would lead to increased growth only if the segment was already growing, i.e. only if auxin was also present. This proposal would also account for their additive effects on growth.

Despite the above points, there are several reasons to suspect that ethylene does not promote growth via effects on Lp. For example:

a.   In spite of some reports (reference in Chapter 3), it seems unlikely that hydraulic conductivity could ever severely limit growth in segments floating on pure water. Consequently, it seems unlikely that large growth promotions could be occasioned in such material via this component;

b.   If ethylene acts simply by increasing Lp, it should increase (double) other types of growth besides that induced by auxin. Low pH will induce growth in *Nymphoides* (Figures 41 and 43) however, ethylene does not increase this promotion (Table 11). Thus it seems unlikely that ethylene has a direct effect on Lp.

These data do not necessarily exclude the possibility that ethylene promotes auxin-induced growth via Lp (for example, ethylene might require the presence of auxin before it can affect Lp); but the simplest possibility - that ethylene directly alters Lp - is untenable.

c.   If ethylene is postulated to promote growth by changing Lp, it must be assumed that Lp is normally (in the absence of ethylene) so low that it severely restricts growth. In that case, one would predict that abrasion of the outer cuticle should greatly facilitate water entry to segments, and thus should greatly promote auxin-induced growth. By the same logic, ethylene should not promote growth in abraded segments, where Lp is surely high already. The latter of these two predictions was tested. It was shown that ethylene can promote marked growth even in abraded segments (see Figure 45). It can thus be concluded that ethylene's effect on growth is not via altered water permeability.

Having reached this conclusion, we can use equation 3, equating growth rate to   WE (P-Y)   see page 38.

Possible ethylene (and auxin) effects on turgor pressure, yield stress and wall extensibility.

The three terms of equation 2, WE, P and Y, are here considered collectively. Experiments are often designed to distinguish between them rather than to assay them individually. Indeed, from the experiments discussed below, it is not possible to distinguish between effects on P, and effects on Y. In retrospect, this is of little consequence.

pH treatment.	Ethylene	Final segment length (mm)	
		$\overline{x}$	s
6.0	–	21.8	0.26
	+	22.2	0.26
4.0	–	24.1	0.66
	+	24.8	1.15

TABLE 11   Effect of ethylene on acid-induced growth
in *Nymphoides*.

Subapical petiole segments, initial length
21.4 mm, were pre-incubated for 3 h on
distilled water in the presence or absence
of 20 μl l^{-1} ethylene. The segments were then
placed in 20 mM citrate/phosphate buffer of the
stated pH for a further 3 h, and their length
was measured.

The pretreatment ethylene regime was continued
during treatment in buffer.

Means are each for 10 segments.

Statistical significance (estimated using the 't'
test): effect of ethylene at pH 4 Not Significant
(5% level).

Ethylene (or auxin) could increase growth rate by lowering cell osmotic potential (hence raising turgor). Cookson (1976) states that the minimum level of mannitol required to prevent elongation of *Regnellidium* segments is higher for ethylene-induced, than for auxin-induced growth. This suggests that osmotic potential is lower in the ethylene-treated tissue or that Y is lower (see equation 1: the minimum level of mannitol required to prevent growth occurs at point x in Figure 35, at which P = Y). If the cells are to raise P (their turgor pressure) in response to ethylene, extra solutes must be mobilised. One possible source of such solutes is the stock of iodine-positive granules (presumably of starch) which can be seen in profusion in many cells of the *Nymphoides* and *Regnellidium* petiole (see page 293).

However, in *Nymphoides*, kinetics of the responses to both auxin and ethylene (Figures 17 and 30) are rather more rapid than one would expect of a mechanism, such as that discussed above, dependent on raising turgor by gradual catalysis of insoluble reserves.

To investigate further the problem of which component of equation 2 is altered by auxin and ethylene when they promote growth, the following experiment was carried out: segments were incubated in mannitol solutions of various strengths, and exposed to combinations of auxin and ethylene.

Regnellidium segments were used rather than *Nymphoides*; this choice was dictated primarily by availability of material, but it has the advantage that the results are comparable with those of some other workers.

The results of this experiment should allow, for any treatment, determination of what proportion of the increased growth rate is due to shift in WE, and what proportion is due to shift in (P-Y); see equation 2. To illustrate, consider two hypothetical growth promoters A and B, whose effects are additive. Let A promote growth by increasing WE. The pattern
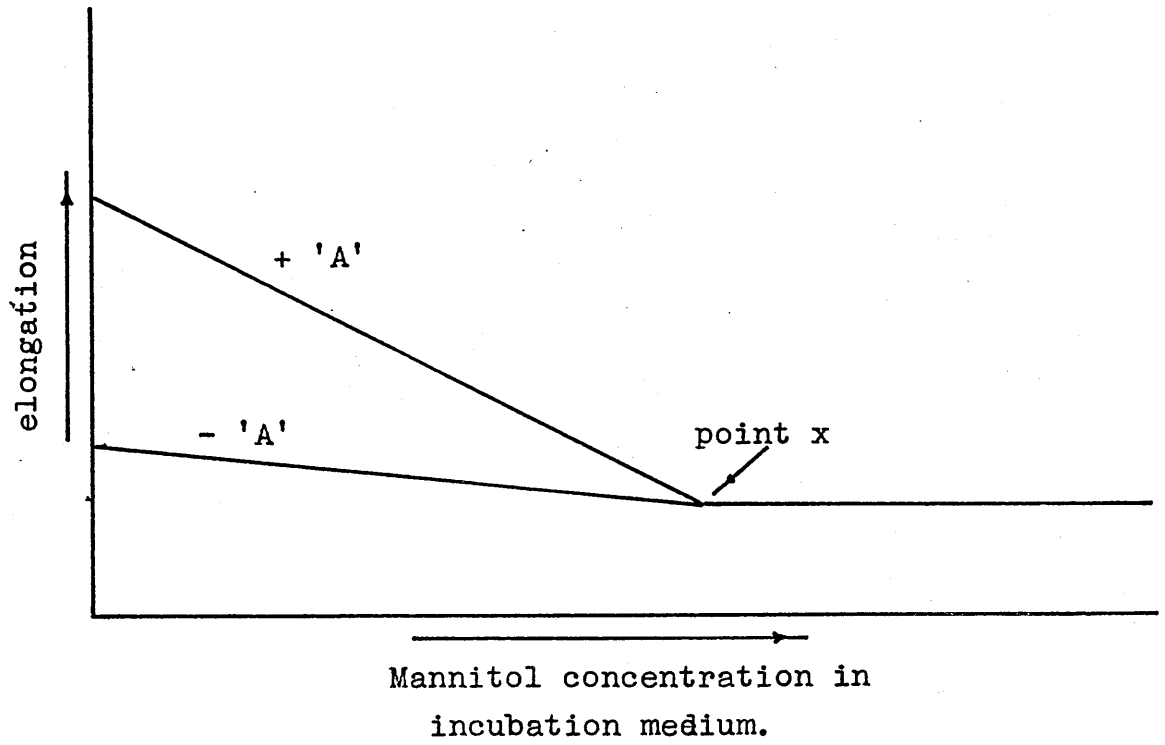
FIGURE 35   Diagram showing the predicted pattern of elongation
in segments incubated in various mannitol concen-
trations, and treated with or without growth promoter
'A', which affects wall extensibility.

At point x growth is just prevented.

of growth in segments treated with A, and incubated in a series of mannitol solutions, will be as shown in Figure 35. At point x in Figure 35, the osmotic potential of the external solution ($\psi_{s,o}$) is so low that water is withdrawn from the cell, reducing P to the point where P = Y, and leaving no P "available" in excess of Y, to drive growth.

When growth promoter B is added, as well as A, the pattern in Figure 35 could change in either of two ways, depending on whether B acted through change in P, Y or WE. Taking these in turn:

1. (a) If B acted solely by increasing cell turgor pressure, P, then (P-Y) would be greater in the presence of B, and a greater level of mannitol would be required, in the external solution, to prevent growth; i.e. point x in Figure 35, would be shifted to the right. The slope of the growth curve would remain as before because, for a given increase in turgor pressure, the wall will extend only as much as before since wall extensibility is unchanged. This pattern of growth in shown in Figure 36.

1. (b) If B decreases the wall yield stress, Y, the pattern would be as for increased P (Figure 36). This is because when Y decreases. P-Y increases, exactly as in 1(a); i.e. point x is shifted to the right. Slope is unchanged as for 1(a).

2. If B acts by increasing WE, the pattern will be as shown in Figure 37. P and Y are unchanged, so that growth will be stopped by the same concentration of mannitol as before, and point x occurs at the same place. But, for all values of P in excess of Y, the wall extends more than previously since, by definition, it is more extensible. Thus the slope of the growth curve is increased.

If B promotes growth by a combination of effects, any intermediate stage between the two extremes shown in Figures 36 and 37 could occur.