CALCIUM AND PHYTOCHROME-CONTROLLED LEAF UNROLLING IN BARLEY

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DEDICATION

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ABBREVIATIONS

ABA	Abscisic acid
ALA	5 Aminolevulinic acid
AMP	Adenosine-5-phosphate
cAMP	cyclic Adenosine monophosphate
ATP	Adenosine triphosphate
$[Ca^{2+}]_{cyt}$	Intracellular calcium ion activity
$[Ca^{2+}]_{ext}$	Extracellular calcium ion activity
CM	Calmodulin
CTC	Chlorotetracyclin
DG	Diacylglycerol
DiC8:0	1,2-dioctanoyl-rac-glycerol
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycolbis(aminoethylether)-N,N,N',N'-tetra -acetic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FR	Far-red
GA_3	Gibberellic acid
GABA	Gamma aminobutyrate
HIR	High irradiance response
5-HT	5-Hydroxytryptamine
IP_3	Inositol-1,4,5-trisphosphate
LFR	Low fluence response
MES	2-(N-Morpholino)ethanesulphonic acid
MOPS	3-(N-Morpholino) propanesulphonic acid
mRNA	Messenger ribonucleic acid
NAD(H)	Nicotinamide adeninedinucleotide-oxidized (-reduced)
NADP(H)	Nicotinamide adeninedinucleotidephosphate-oxidized (reduced)
NED	Naphthylethylenediamine dihydrochloride
NiR	Nitrite reductase
NR	Nitrate reductase
NRA	Nitrate reductase activity
Pfr	Far-red light absorbing form of phytochrome
PI	Phosphatidyl inositol
Pr	Red light absorbing form of phytochrome
P_{tot}	Total phytochrome
R	Red light
TFP	Trifluoroperazine
VLFR	Very low fluence response
W5	N-(6-aminohexyl)-1-naphthalenesulphonamide
W7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide
W12	N-(4-aminobutyl)-2-naphthalenesulphonamide
W13	N-(4-aminobutyl)-5-chloro-2-naphthalenesulphonamide

CHAPTER 1 : INTRODUCTION

The production of leaves in higher plants is a major component of growth, and the underlying processes which contribute to this are thus fundamental to the understanding of plant morphogenesis. The formation of a leaf commences with the separation of a leaf primordium of dividing cells from the shoot apex. After division and differentiation of the cells, there is a final expansion phase to produce the mature leaf blade. In graminaceous species, such as barley, this final stage is completed by the unrolling of the otherwise mature leaf blade from a spiral to a flattened form. When maintained in darkness, this process does not occur, the leaves remaining folded. From this, Virgin (1962) concluded that the presence and/or absence of light was essential to the triggering of this terminal process in grass leaf morphogenesis. This study aims to examine both the control of induction of leaf unrolling in barley and the mechanism of induction itself.

The control of induction.

The importance of light to leaf expansion in dicotyledonous species was demonstrated by Liverman et al (1955). The expansion of etiolated bean-leaf discs was found to be promoted by red light (R) and inhibited by far-red light (FR). Virgin (1962) demonstrated that a similar control process operated in wheat leaf unrolling. The action spectrum for brief light treatment (two minutes) showed a peak in the red region around 660 nm for the induction of unrolling in etiolated leaf sections. This induction was repeatedly reversible by FR. From this it was concluded

that the unrolling process was controlled by the phytochrome system first described by Borthwick et al (1952) for the control of seed germination in lettuce. This type of response involving the detection of presence or absence of light, where maximum sensitivity occurs around the 660 nm wavelength and, for brief sequential light treatments, FR can reverse the R effect is known as a low fluence response (LFR). This differentiates it from the other functional classes of response mediated by the phytochrome system in etiolated plant tissue; the very low fluence responses (VLFRs) and high irradiance responses (HIRs). Through these and R/FR modulated phenomena in green plants it appears that the phytochrome system can act to measure several parameters. These include the amount of light, light intensity, spectral quality and duration of a dark interval. As such it must be highly versatile.

The LFR mode of action is of primary importance in the de-etiolation of plants, where the function of the phytochrome system is to act as a quantum counter allowing the induction of a response on receipt of a required light dose. This requirement can be manifested as a strict threshold for full induction or as a graded response to increasing fluence upto a maximum. The unrolling response falls into this latter category (Virgin, 1962; Smith, 1975), although it has been argued that all graded responses may be the summation of many different individual threshold responses at the cell level (Kronenberg and Kendrick, 1986).

LFRs involving growth are the result of biochemical/biophysical changes within some or all cells of the responding tissue. From these changes more detailed information regarding the mode of action of the phytochrome system may be obtained.

At least 64 enzymes in a variety of species have been shown to exhibit activity regulation via the phytochrome system (Lamb and Lawton,

1983; Schäfer et al, 1986). The level at which this control operates varies. At least one enzyme, nitrate reductase, appears to show some activity regulation at the post-translational level (Johnson, 1976). However, many mRNAs also display regulation by the phytochrome system, indicating translational or pre-translational control (Tobin and Silverthorne, 1985). Both positive and negative regulation of different mRNA species can occur within the same sample in response to R (Mösinger et al, 1985). This indicates that the primary control operates at a level prior to this. Using in vitro transcription systems it has been shown that the positive and negative regulation described above, resides at the transcriptional level of control (Silverthorne and Tobin, 1984; Mösinger et al, 1985). This may be interpreted as placing control by light on a heirarchical level above transcription. Alternatively it could represent a coordinated, intrinsic programme of gene switching, an idea first suggested by Mohr (1972). The control by the phytochrome system of events in cells that are difficult to explain in terms of transcriptional control alone, argues against the latter explanation being wholly true, and indicates that transcriptional control is likely to be subservient to some other primary process. These events include electrical effects and changes including altered membrane properties that may be too rapid to arise from changes in gene expression. The 'Tanada effect' describes changes in the surface electrical charge of barley root tips in response to R and FR. These changes are repeatedly photoreversible and are complete within 30s (Tanada, 1968). Other more rapid electrical effects have since been measured. These include membrane depolarizations in Nitella with lag times as small as 0.4s (Weisenseel and Ruppert, 1977) and Avena coleoptile cells, with lag times less than 1s (Newman, 1981). Such rapid electrical effects at the plasma membrane

indicate a relatively close linkage between the phytochrome system and membrane properties. The basis of this connection is likely to reside in the control of ion movements across the membrane. H^+, K^+ and Ca^{2+} have been shown to be transported in response to R via the phytochrome system (Brownlee and Kendrick, 1979; Dreyer and Weisenseel, 1979).

Further evidence that the phytochrome system can operate independently of gene expression is furnished by Serlin et al (1984). This is a demonstration of R/FR photoreversible modulation of oat mitochondrial ATPase activity in vitro. The effect of light treatment was detectable within 5 min. These data may be criticised as being produced by a highly artificial system and thus of questionable physiological relevance. However, they provide evidence that functional responses to the phytochrome system do occur in the absence of an active genome.

From the above, it is clear that the phytochrome system orchestrates a wide variety of cellular activities that biochemically include changes in gene expression and physiologically, in the short term at least, include altered membrane properties that are independent of genetic control. The causal relationship between these dual spheres of activity, and of the phytochrome system itself to these spheres remains unresolved at present, although theoretical explanations with supporting evidence do exist. Some of these will be covered in this study.

Information pertaining to the mode of action of the phytochrome system may also be obtained from a description of the nature of the system itself.

Phytochrome is a blue/green pigment ubiquitous in green plants. It is a biliprotein, the characteristic feature of which is the possession of an open chain tetrapyrrole conjugated to the protein moiety. The tetrapyrrole acts as a chromophore, absorbing certain wavelengths of

visible light. Phytochrome is a photochromic pigment, existing in two forms, one with maximal absorption in the red (Pr) and another with maximal absorption in the far-red region of the spectrum (Pfr). These forms interconvert when supplied with the appropriate irradiation. However, due to overlapping bands in their absorbance spectra, light of wavelength less than about 750 nm generates a photoequilibrium mixture of the two forms. The maximum proportion of Pfr that can be generated in a sample is approximately 86% (Vierstra and Quail, 1983). Both photoconversions are first order processes with intermediate species (Rudiger, 1986). Photoconversion of one form to the other results from an alteration in the structure of the chromophore and an associated change in the interaction of the chromophore and apoprotein.

De novo synthesis of phytochrome occurs exclusively as Pr. Thus, in etiolated tissue, Pfr only appears following exposure to light, and thereafter the Pfr level will be a function of the spectral composition of light, the light intensity and the rates of synthesis and degradation of phytochrome. Pfr from etiolated tissue is unstable undergoing degradation at a much greater rate than Pr. Further autoregulation occurs in oats, where negative transcriptional control of the phytochrome gene is detectable, but this is not seen in all species (Colbert et al, 1985; Quail, 1987; Lagarias, 1987). These properties show that phytochrome can adequately detect the necessary information to initiate the LFR, HIR measure spectral quality of light, and duration after light/dark transitions.

The instability of Pfr may appear to rule out a useful function in de-etiolated plants, however, green tissue contains at least two distinct stable species of phytochrome (Tokuhisa et al, 1985) one of which appears similar or identical to the phytochrome accumulated in etiolated plants,

but present at a much lower level. The existence of stable distinct green tissue phytochrome presumably allows for the detection of spectral composition in elongated responses of plants to shading (Morgan et al, 1980; Child and Smith, 1987) where the R/FR ratio is a measure of canopy density (R is absorbed by a canopy much more than FR).

Although the molecular properties of phytochrome, as stated, can account for all phenomena attributed to 'the phytochrome system', this does not elucidate any linkage to the observed changes in gene expression or membrane properties. It may be deduced that the physiological function of phytochrome derives from the differences between Pr and Pfr and thus any differences may contain information regarding the nature of putative links to the response. At least two regions of the apoprotein are differentially susceptible to proteolysis in the Pfr and Pr forms, indicating conformational differences do exist between Pr and Pfr (Vierstra and Quail, 1982; 1983; Lagarias and Mercurio, 1985). The chromophore is more reactive chemically with tetranitromethane in the Pfr form in vitro (Hahn et al, 1984) and, in vitro at least appears to undergo a reversible 31^{o} rotation relative to the apoprotein (Eckelund et al, 1985). These differences indicate that on photoconversion, a change in the chromophore leads to a new interaction with the apoprotein which in turn leads to a conformational change. The nature of this conformational change is not clear. However, an extra thiol group on the apoprotein becomes reactive to chemical modification in the Pfr form (Eilfeld et al, 1987). Another change is a reduction in in vitro autophosphorylation activity on conversion to Pfr (Wong et al, 1987; 1988). This ability to undergo self-phosphorylation, phosphorylate other proteins and the apparent presence of an ATP binding site has led to the suggestion that the primary action of phytochrome may involve altered phosphorylation;

however, the lack of *in vivo* data precludes further speculation at present. Changes in protein phosphorylation would provide a feasible mechanism to regulate gene expression, and, if associated with membranes, could explain changes in membrane properties.

The cellular distribution of phytochrome as revealed by immunocytochemistry does not support the notion of phytochrome/membrane associations (McCurdy and Pratt, 1986), nor do the molecular properties of phytochrome, all of which indicate that it is a soluble globular protein (Vierstra and Quail, 1986). Against this must be set the fact that on isolation, most membrane-bound organelles have associated phytochrome, some of which, in mitochondria at least, is intrinsic (Roux, 1986). Haupt et al (1969) have also demonstrated that in the alga *Mougeotia* phytochrome molecules are 'fixed' relative to the cell structure.

In conclusion it may be stated that the precise steps of control of induction in the LFR are not known at present. Neither is it clear whether the changes in gene expression are dependent upon changes in membrane properties and/or ion movement, or whether these two groupings of effects are separate aspects of short-term and long-term response coordination. The next section examines what is known of the induction mechanism in the unrolling response itself and draws upon recent work on the transduction of other LFRs to develop a hypothesis of phytochrome action in unrolling.

The mechanism of induction.

Early studies of unrolling in cereal leaves concentrated on the response in green tissue. In many graminaeceous species it was found to be a reversible process under conditions of varying humidity and thus not a true growth response (Kerner and Oliver, 1896). However, Burström (1942), in studying emergent primary leaves rather than mature tissue,

found that the unrolling of green leaves as they emerged from the coleoptile resulted from differential growth in the mesophyll. It was also deduced that this growth resulted primarily from an increase in wall extensibility. Virgin (1962) reached a similar conclusion in relation to the unrolling of etiolated leaf tissue, as induced by R. However, it is only recently (Virgin, 1988), that the demonstration that unrolling of etiolated tissue results from differential cell expansion has been performed. Shortly after phytochrome induced unrolling was demonstrated in etiolated wheat, the effect was demonstrated in maize and barley (Klein et al, 1963; Carr and Reid, 1966). The latter study also used actinomycin D to inhibit unrolling indicating the possible necessity of transcription in the unrolling process. However, both this study and that of Poulson and Beevers (1970) indicated that escape from actinomycin D inhibition occurred a considerable period before unrolling was detectable indicating the possibility that the effect was indirect.

Using radiolabelled leucine, Kang (1971) demonstrated a correlation between R-induced unrolling and incorporation of label into a cell supernatant fraction. From this it was suggested that unrolling was due to the increased synthesis of a soluble protein in the tissue. Rajagopal and Masden (1981) extended this work. Drawing on current knowledge of cell wall biochemistry, they proposed that the change in cell wall properties detected by Virgin (1962) might be due to the secretion of an extensin-like protein altering wall extensibility. In support of this it was demonstrated that the proline analogue azetidine carboxylic acid inhibited unrolling. This inhibition was partially reversed by proline. Extensin and related molecules are known to be rich in the proline derivative hydroxyproline (Lamport, 1970). Against this, it must be noted that Kang (1971) specifically excluded label incorporation into wall

proteins as being involved in unrolling. Overall, given the time course of unrolling (Poulson and Beevers, 1970), and the above data, it appears likely that synthesis of a protein or proteins is significant in the change in wall properties that accompanies unrolling. It is the transduction of such a change that concerns this study.

Early attempts to explore this problem focused on the apparent transmission of an unrolling signal from irradiated to non-irradiated regions of a leaf section (Wagner, 1964; 1965). However, such studies overlooked the existence of light piping, a phenomenon whereby the inducing light would be conducted from light-treated to untreated areas of a leaf section. Such an explanation removes any requirement for any physical signal beyond the light itself.

As early as 1957, Scott and Liverman noted that gibberellic acid (GA_3) could stimulate R-induced bean-leaf disc expansion. Poulson and Beevers (1970) demonstrated that GA_3 could increase the unrolling of both R-treated and untreated etiolated barley leaf sections. Taken with the observation of Reid et al (1968), that R (30 min) could induce a transient increase in gibberellin content of etiolated barley leaves, it was considered that the effect of R might operate via an increase in gibberellin levels. Similar observations were made for etiolated wheat (Beevers et al, 1970). However, the two systems differed in their response to the gibberellin synthesis inhibitors AMO-1618 and CCC (Loveys and Wareing, 1971a). In wheat, the R-induced increase in gibberellin was unaffected by the inhibitors, but in barley a significant reduction was observed. This was interpreted as indicating the presence of two distinct effects of R, one involving interconversions of existing pools of conjugated and free gibberellin, and another including de novo synthesis of gibberellin. The possible importance of the latter process to unrolling was indicated by

the inhibition of unrolling in the presence of amo 1618 or CCC (Loveys and Wareing, 1971b). However, this demonstration was for etiolated wheat tissue, which did not show inhibition of the R-stimulated increase in gibberellin in the presence of inhibitors. Clearly, non-specific effects are not excluded by this experiment.

Evans and Smith (1976a) demonstrated R-induced rises in gibberellin levels in isolated barley etioplasts and went on to detect phytochrome at the etioplast envelope (Evans and Smith, 1976b; Hilton and Smith, 1980). It was suggested that R leads to gibberellin interconversion within the etioplast, increasing free gibberellin levels. Similar results were obtained with wheat etioplasts (Cooke and Saunders, 1975a) and subsequently wheat etioplast envelopes (Cooke and Kendrick, 1976). To support the notion that an R-induced pulse of increased gibberellin activity was responsible for the induction of leaf unrolling, Cooke and Saunders (1975b) compared the timecourse of gibberellin increase with the escape from photoreversibility of the unrolling process in wheat. Both were measured as lying between 5 and 10 minutes. This would be expected given a causal relationship between the gibberellin pulse and unrolling. However, there are several problems with this interpretation. Firstly, when the above comparison is made for barley, the gibberellin peak is measured at about 10 min after R (Beevers et al, 1968), but the escape from FR reversibility does not occur until 1½ to 2 h after R (Smith, 1975; Sundquist and Briggs, 1982). Secondly, in measuring increases in GA activity, only a fraction of the gibberellin-active substances are active in inducing unrolling. This makes interpretation of the effects of en bloc increases in gibberellin content difficult.

In addition to the above problems, there are other reasons to doubt the relevance of gibberellins to R-stimulated unrolling. Most studies

that have used exogeneous GA_3 to substitute for light in the induction of unrolling have used unphysiologically high concentrations. Kang (1971) found GA_3 to be ineffective in the induction of unrolling in barley. This and the apparent additive nature of GA_3 and R-effects on unrolling (Poulson and Beevers, 1970; Sundquist and Briggs, 1982) indicates that the effect of gibberellins, if any, may be quite separate from that of R.

Several other plant growth regulators have been evaluated for effects on the unrolling process, with and without R. Kinetin stimulates unrolling, often to greater effect that GA_3, in both wheat and barley independently of R-treatment (Beevers et al, 1970; Kang, 1971). Full studies of the interaction of cytokinin and R have not been performed, and data on any putative changes in in vivo cytokinin levels in response to R are very limited (O'Brien et al, 1985). Against there being any likelihood of a causative link between phytochrome and cytokinin actions in the finding that the effects of Cis-4-cyclohexene-1,2-dicarboxamide (CHDC), an inhibitor of phytochrome mediated cell elongation (and barley leaf unrolling) can be overcome by application of benzyladenine (Stewart and Bewley, 1988). However, this could also be interpreted as being the result of disrupted cytokinin metabolism by CHDC interfering with an effect of R of cytokinin levels. Until the relevant work has been done, further comment is not possible.

Of the other plant growth substances tested, ethylene was without effect and abscisic acid (ABA) and auxin were inhibitory (Kang, 1971; Loveys and Wareing, 1971b). In the case of ABA at least, the inhibition was probably non-specific as both dark and R-stimulated unrolling were inhibited.

From the above it may be concluded that there is little direct evidence concerning the nature of the processes linking phytochrome and

the changes in gene expression that are proposed to be important in unrolling. However, there is evidence regarding the transduction of other phytochrome responses that may be relevant here.

One of the most completely characterised phytochrome responses is that of the rotation of the Mougeotia chloroplast in response to unidirectional light. In this response, unilaterally applied low fluence R treatment stimulates the single flattened chloroplast of the Mougeotia cell to rotate until it is perpendicular to the direction of the light source (Haupt, 1959). The first element of what is now thought to be the transduction chain leading to rotation was characterised by Dreyer and Weisenseel (1979). This was an R/FR reversible stimulation of $^{45}Ca^{2+}$ uptake into cells, preincubated in darkness and subjected low fluence light treatments. Subsequently, intracellular Ca^{2+} in Mougeotia was shown to be concentrated in membrane bound vesicles (Rossbacher et al, 1984). Using the calcium sensitive stain chlorotetracyclin (CTC), Wagner et al (1987) detected changes in vesicle fluorescence, and suggested that R treatment could also cause release of Ca^{2+} from intracellular vesicles. These changes were detectable within 5 minutes and thus appear to precede the uptake from extracellular sources which according to the data of Weisenseel (1986) exhibits a lag time of approximately 10 minutes. Whatever the temporal/functional relationship of these two phenomena, the effect of R treatment is to alter the net flux of Ca^{2+} between compartments in Mougeotia and, by inference, the Ca^{2+} activity in those compartments.

Measurements of intracellular Ca^{2+} activities are difficult to perform. However, measurements in the algae Chara and Nitella by a variety of techniques suggest a free Ca^{2+} concentration of 0.1 - 10 µM in the cytoplasm (Williamson and Ashley, 1982; Miller and Sanders, 1986ab).

If this represents a general figure for plant cells [indirect measurements by Gilroy et al (1986) in higher plant cells give similar results], then the cytoplasmic Ca^{2+} activity in Mougeotia will be several orders of magnitude below that of the extracellular compartment and the Ca^{2+}-containing vesicles (Rossbacher et al, 1984). Relatively small changes in the Ca^{2+} flux at the boundary of the cytoplasmic compartment will cause large changes in Ca^{2+} activity in the cytoplasm.

The potential significance of this conclusion is illustrated by Serlin and Roux (1984). The Ca^{2+}-ionophore A23187 was used to induce an artificial increase in intracellular Ca^{2+} in Mougeotia. There are distinct problems in the use of A23187 which will be addressed later in this study. However, by applying A23187 to cells adjacent to the chloroplast edge, rotation could be induced in the absence of a unidirectional light treatment, but only in the presence of Ca^{2+} in the bathing medium. This infers that localized increases in intracellular Ca^{2+} 'repel' the chloroplast which moves down a bilateral Ca^{2+} gradient in the cell. This parallels the bilateral gradient of Pfr that is set up in the cell during unilateral irradiation (Haupt, 1982). It is suggested by Serlin and Roux (1984) that the bilateral gradient of Pfr leads to localized Ca^{2+} flux changes and a bilateral Ca^{2+} gradient in the cell. The chloroplast orientating mechanism then responds to this.

The mechanism of rotational sensitivity to Ca^{2+} is proposed to involve the ubiquitous Ca^{2+}-receptor protein calmodulin. This has been identified in Mougeotia (Wagner et al, 1984). The important of this receptor is indicated by the effectiveness of calmodulin-binding drugs such as Trifluoroperazine (TFP) and the W-compound series of naphthalene-sulphonamide derivatives in inhibiting rotation at relatively low concentrations (Wagner et al, 1984; Serlin and Roux, 1984). From work in a

variety of systems, calmodulin is known to respond to changes in Ca^{2+} activity by modulating the activity of a variety of enzymes of which it comprises a (dissociable) subunit. These enzymes include ATPases and a variety of kinases (Means and Dedman, 1980; Marmé, 1985). It is known that Mougeotia has the capacity to phosphorylate rabbit myosin light chain kinase in a Ca^{2+}-dependent manner (Wagner et al, 1987). Thus, the presence of actin in Mougeotia and the inhibition of chloroplast rotation by cytochalasin B has led to the suggestion that the rotation mechanism resembles muscle construction with Ca^{2+}/calmodulin dependent phosphorylation of an endogeneous myosin light chain kinase which interacts with an actomyosin complex that comprises the motor apparatus for rotation. Much of this scheme remains hypothetical, but it does provide a plausible explanation of the data.

The above system represents a model demonstration of a more general hypothesis of phytochrome signal transduction first proposed by Haupt and Weisenseel (1976), with the central concept that phytochrome-induced changes in Ca^{2+} distribution are fundamental to signal transduction. This notion has been developed by a variety of workers into a generalized version of the hypothesis described for Mougeotia chloroplast rotation in which phytochrome-induced changes in cytoplasmic Ca^{2+}-activity are detected by Ca^{2+} receptor-proteins, including calmodulin, which alter the phosphorylation state of a variety of cellular proteins, leading to modified cellular activity and the response. The argument for the importance of Ca^{2+} in signal transduction derives from studies on animal cell physiology, in which there is good evidence that similar systems are operational. This, and the presence of many elements of the putative system in plant cells offers the possibility of a quantum leap in the understanding of plant cell signalling.

Cytoplasmic Ca^{2+} is tightly regulated in plant cells by a variety of pumps at the plasma membrane, endoplasmic reticulum (FR) tonoplast and mitochondrial and chloroplast membranes (Hepler and Wayne, 1985). The ER pump appears to be responsible for setting the lower limit of cytoplasmic Ca^{2+} (Buckhout, 1984). This is similar to the situation in animal cells. Also similar is the apparently low cytoplasmic Ca^{2+} activity relative to the extracellular millieu and other cellular components. The presence of calmodulin and calmodulin-sensitive enzymes completes the necessary machinery to permit a Ca^{2+}-based transduction system to exist. However, for such a system to operate, a mechanism to change cytoplasmic Ca^{2+} levels must exist. In animal cells this is achieved through receptor-mediated hydrolysis of polyphosphoinositides in the plasma membrane to yield inositol-1,4,5-tris phosphate (IP_3). IP_3 then induces Ca^{2+} release from the ER, prior to recycling back to polyphosphoinositide. The necessary components of such a cycle have been identified in plant systems (Heim and Wagner, 1987a and b; Heim et al, 1987; Pfaffman et al, 1987). Furthermore, turnover of components of the cycle has been shown to increase in response to both cytokinin (Connett and Hanke, 1987) and light (Morse et al, 1985; 1987). IP_3 has also been shown to stimulate Ca^{2+} release from Cucurbita ER microsomes in vitro (Drøback and Ferguson, 1985). All of the necessary components of a system to parallel that seen in animal cell signalling are thus identifiable in plants. However, a functional interaction in the transduction of a response has not been demonstrated to date.

There are two types of evidence in support of the notion that changes in cytoplasmic Ca^{2+} are central to the transduction of phytochrome responses. The first concerns the widespread occurrence of phytochrome-modulated Ca^{2+} fluxes. In addition to the photomodulation of Ca^{2+} move-

ment in Mougeotia, there is evidence for phytochrome-controlled Ca^{2+} movements in maize protoplasts (Das and Sopory, 1985), oat coleoptile cells (Hale and Roux, 1980), red cabbage leaves (Hatout-Bassim and Peckett, 1975), Nitella (Weisenseel and Ruppert, 1977) and spores of Onoclea (Wayne and Hepler, 1985). At the subcellular level photomodulation of Ca^{2+} movement has been demonstrated in oat mitochondria (Roux et al, 1981) and corn ER-enriched microsomes (Dieter and Marmé, 1983).

The other body of evidence concerns the use of agents to disturb the Ca^{2+} homeostasis of the cell and the observed effects this has on phytochrome responses. By use of techniques that induce chelation of Ca^{2+} to reduce the level available in a system a wide variety of phytochrome responses have been shown to be Ca^{2+}-dependent. These include Onoclea spore germination (Wayne and Hepler, 1984), the 'Tanada effect' in mung beans (Tanada, 1968), membrane depolarization in Nitella (Weisenseel and Ruppert, 1977), NAD kinase activation (Anderson and Cormier, 1978), Oat mitochondrial APTase activity (Serlin, 1984), nuclear protein phosphorylation in Pisum (Datta et al, 1985) and swelling of wheat protoplasts (Bossen et al, 1988).

However, the fact of Ca^{2+}-dependency reveals little of the actual role of Ca^{2+} in these responses. Only in a few systems, other than the Mougeotia rotation response has further work been done to clarify the role of Ca^{2+}. In the Pisum nuclear phosphorylation system, calmodulin inhibitions were shown to inhibit R-stimulated phosphorylation. However, it is difficult to separate calmodulin specific from non-specific detergent effects in these circumstances, thus ambiguity remains. Calmodulin inhibitors were also used to inhibit the germination of Onoclea spores. The same questions apply here as with the above system, and will remain

without the use of rationally designed antagonists to quantify side-effects such as the W-compounds used by Serlin and Roux for Mougeotia (1984). With A23187 a small promotion of germination was obtained in the absence of R treatment. This may imply that elevating intracellular Ca^{2+} in Onoclea does lead to spore germination, and given the evidence of R-induced Ca^{2+} uptake by spores, apparently resembles the situation in Mougeotia. However, there are problems with this interpretation. Firstly the nature of the Ca^{2+} uptake is not clear, only a net increase in spore Ca^{2+} content being measureable. Secondly, to demonstrate the Ca^{2+}-dependency of germination and the A23187 effect, a 24h preincubation with a Ca^{2+}-chelating agent was used. It could be argued that this places the cells in an unnaturally Ca^{2+}-stressed state. This is also likely to be the case with the studies on uptake into spores, in which the chelating agent ethyleneglycol bis (βaminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was used at 100 mM concentration. This is more than two orders of magnitude greater than that suggested by the same workers for physiological experiments (Hepler and Wayne, 1985). The relevance of findings under such artificial conditions is debateable.

Bossen et al (1988) have also used A23187 to replace a light treatment in inducing the protoplast swelling response. Promotional effects equivalent to that of R-treatment are obtained. However, the swelling response is rapid (c10 minutes) and the protoplast could also be considered an artificial system prone to artefacts. The absence of combinational studies with light and A23187 together in this study, makes it impossible to assess whether the two treatments operate in the same mechanism at present.

To conclude: with the possible exception of the Mougeotia model system, the evidence for Ca^{2+} having a transducing role in phytochrome

signalling is incomplete and inconclusive. Until a concerted, unified assessment, similar to that performed for Mougeotia, is made upon a response, deductions as to the role of Ca^{2+}-based hypothesis of action is currently insufficient for generalised predictions to be made as to the probable nature of the transducing system. In performing such an assessment, it is clear that the current techniques using ionophores, chelating agents, etc. are ambiguous. A highly critical evaluation of their usage is necessary to obtain unequivocal results. This has not been carried out in sufficient depth for any phytochrome responses to date.

The aim of this study is, therefore, to evaluate critically R-stimulated leaf unrolling in terms of a Ca^{2+}-based transduction hypothesis. This is justified in the absence of any firm evidence as to the nature of the early stages of the response pathway, and circumstantial evidence indicating the importance of Ca^{2+} in a variety of phytochrome responses. In effecting the above, the techniques used to obtain such information will be experimentally refined to reduce the ambiguity of the results. Through this, it is hoped to acquire a clearer insight into the control of leaf unrolling.

CHAPTER 2 : MATERIALS AND METHODS

Sources of Material.

Except where stated barley seeds (Hordeum Vulgare var. 'Golden Promise') obtained from Miln Masters Ltd. (Norwich, England) were used throughout.

Growth of Material.

Seeds were soaked overnight in tap water at $5^{o}C$ in darkness. At the same time, Vermiculite was saturated with tap water overnight at room temperature. The imbibed seeds were sown in darkness in the vermiculite in seed trays at a depth of approximately 10 mm. Inverted seed trays were placed over the sown seed to reduce drying of the vermiculite, and the whole placed on shelves enclosed by black polythene sheeting in a dark room maintained at $25^{o}C$. These growth conditions were maintained for 6d, by which time a large population of seedlings with primary leaves c 90 mm long had developed. These leaves constituted the source material for all experiments.

The following variations in the growth regime were applied:

1. For experiments involving the assay of nitrate reductase activity, the vermiculite was saturated with potassium nitrate solution (KNO_3, 20 mM);
2. The growth of seedlings in gabaculin was effected by placing the imbibed seeds on Whatman 501 filter papers (ashless) soaked in gabaculin solution (10 ml) in 50 mm plastic petri dishes. The petri dishes were placed on tap water-saturated paper towels

(Kimwipe) in seed trays, an inverted tray being placed over them, and the whole treated as for vermiculite grown seeds, described above.

Experimental Media.

All experimental solutions were made up in distilled water (Ca^{2+}<10μM) unless otherwise stated. For experiments involving the control of Ca^{2+} levels 'low-Ca^{2+}' media were prepared using double deionized water (no detectable Ca^{2+} by atomic absorption spectrophotometry, conductivity < 0.1 μScm^{-1}).

The standard buffer used throughout contained 2-(N-morpholino) ethanesulphonic acid (MES, 35 mm), all experimental solutions incorporating this. The pH was adjusted to 6.5 with NaOH (1 M) prior to use. 'EGTA buffer' was made up in an identical fashion with the additional inclusion of ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetracetic acid (EGTA, 2mM). EGTA was obtained from the Sigma Chemical Co., England.

In Chapter 4, experiments to assay Nitrate reductase activity (NRA) were performed. Except where stated potassium nitrate (KNO_3, 5mM) was incorporated into the incubation buffer for these experiments.

Treatments.

Light sources.

Where necessary experimental manipulations were performed under dim green light (2.5 nmol photons m^{-2} - s^{-1} at sample surface).

For induction experiments and photoreversibility studies, red and far-red light sources were utilised.

The red light (R) source was constructed from 24 'Deluxe Natural' fluorescent tubes (approx. 1.2m; Thorn Electric, Leicester, U.K.) with the

end 4 cm of each tube covered with black tape. The radiation was filtered through a 2 cm perspex envelope of 1.5% (W/V) copper (II) sulphate solution and a layer of No. 14 (ruby) Cinemoid (Rank Strand, Brentford, Middlesex, U.K.) sandwiched between glass. R treatment lasted 5 min at a photonfluence rate of 13.6 μmol m^{-2} s^{-1} at the sample surface unless otherwise stated.

The far-red (FR) source was constructed from ten 150W clear-bulb single-coil tungsten lamps semi-immersed in running water, filtered through a single layer of FR perspex (Plexiglas type FRF 700; Westlakes Plastics Co., Lem, Pa., U.S.A.). FR treatments lasted 10 min at a fluence rate of 480 μmol m^{-2} s^{-1} at the sample surface unless otherwise stated. Spectral photon fluence rate scans of the light sources were performed using a 'Li-Cor 1800' spectroradiometer and are shown in Fig. 2.1 a and b.

For phytochrome photoequilibrium measurements, and experiments to determine the effect of photoequilibrium states, a 'Halight 300' projector (T.H.D. Ltd., Peacehaven, England) was used as the light source. The experimental light was passed through a variety of interference filters (Balzar and Schott) to obtain a variety of transmitted spectra. The transmission characteristics of the filters used were measured with a Perkin-Elmer lambda-5 spectrophotometer and are shown in Fig. 2.2 a-f. Light treatments were given to experimental material in a closed chamber 100 mm from the light source, and were of 15 min duration, preliminary experiments having demonstrated that this was sufficient time to achieve photoequilibrium under these conditions.

Chemical treatments.

Inorganic ions.

Calcium, magnesium and potassium ions were added to the buffering

Fig. 2.1 Spectral photon fluence rate scans of experimental light sources. a. Red light; b. far-red light.

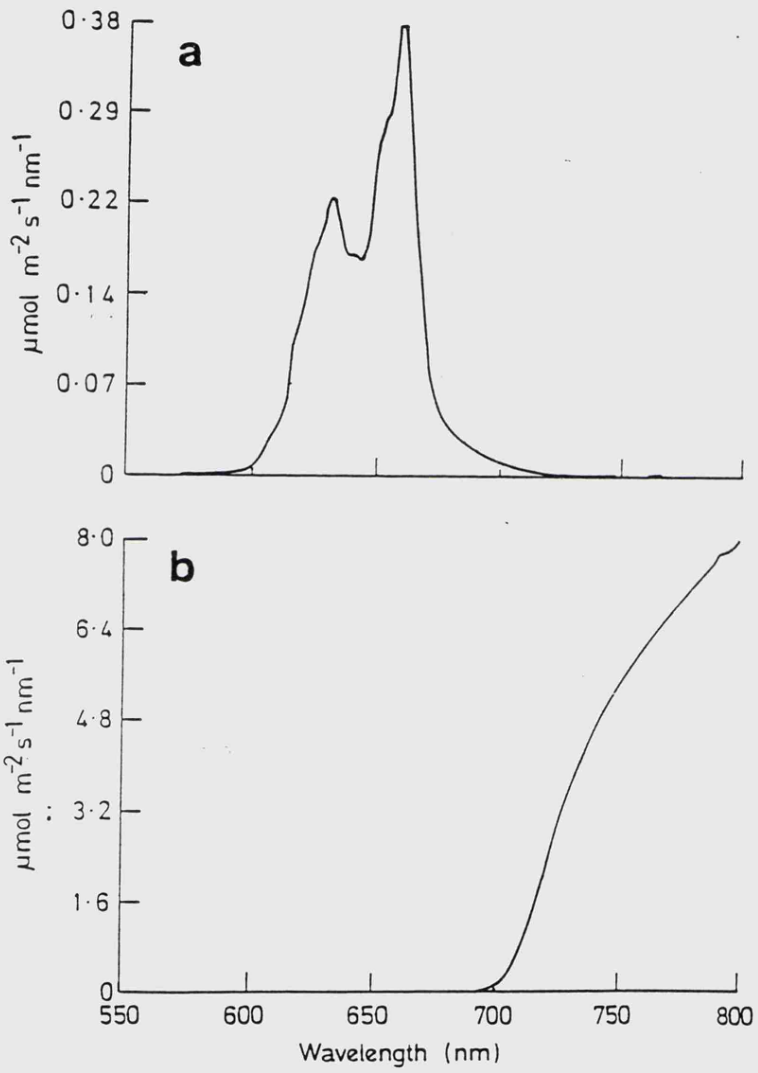
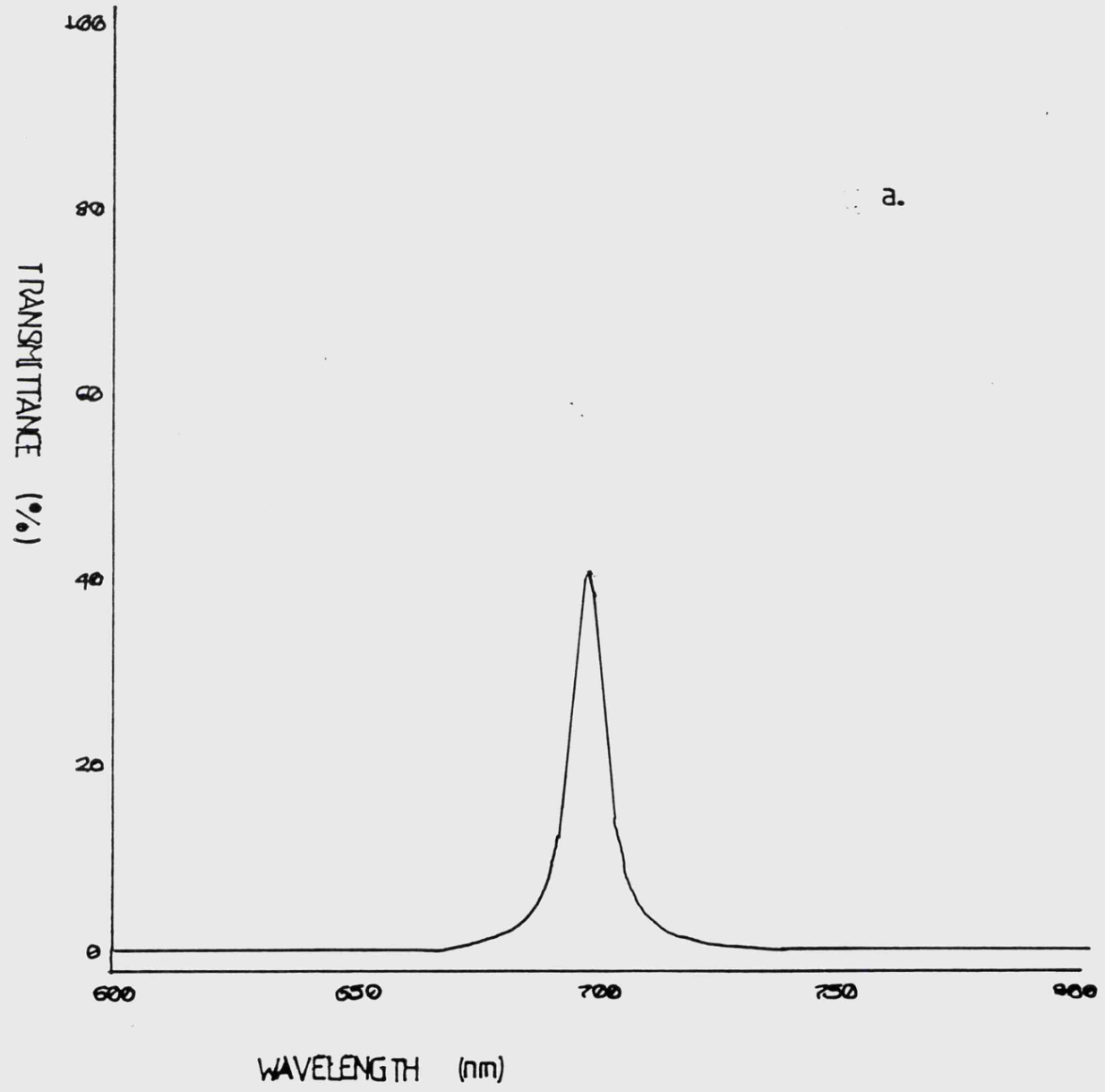
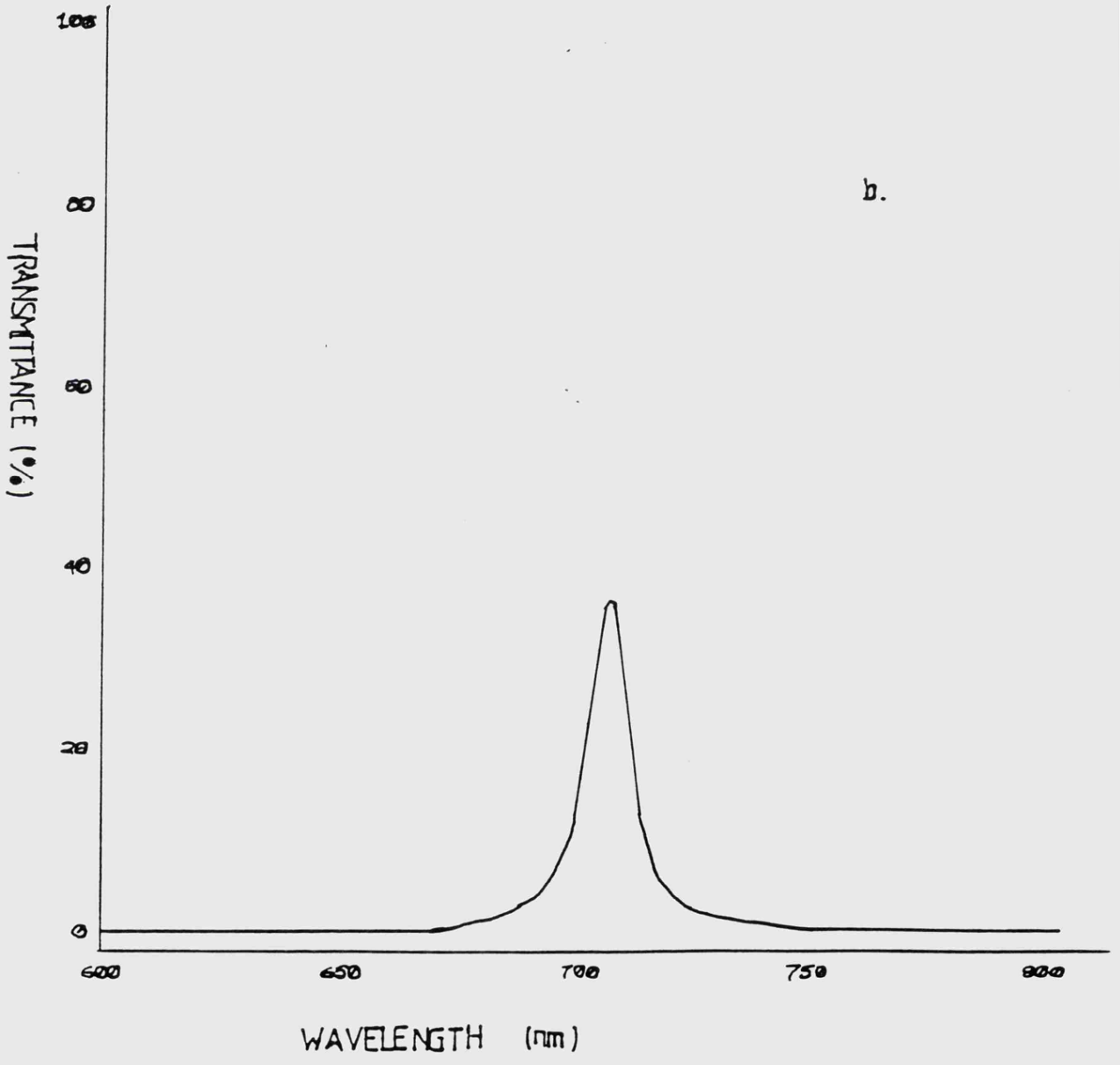

a
0·38
0·29
0·22
0·14
0·07
0
µmol m-2 s-1 nm-1
b
8·0
6·4
4·8
3·2
1·6
0
µmol m-2 s-1 nm-1
550
600
650
700
750
800
Wavelength (nm)

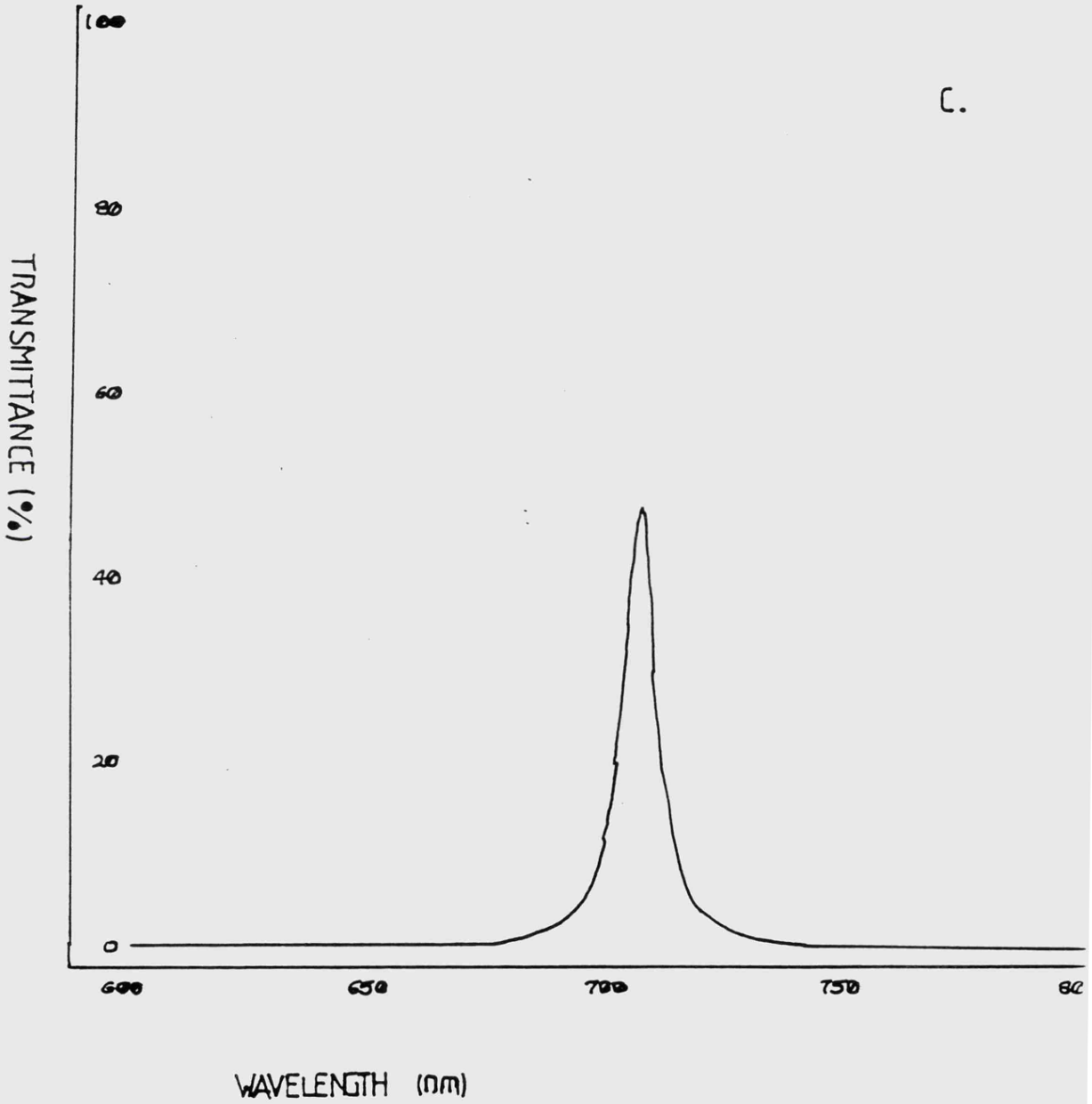
Fig. 2.2, a - f Transmission scans of interference filters performed on the λ5 spectrophotometer (Perkin-Elmer).

a. Balzar '697'
b. Balzar '700'
c. Balzar '707'
d. Balzar B-40 '712'
e. Schott '725'
f. Balzar '730'

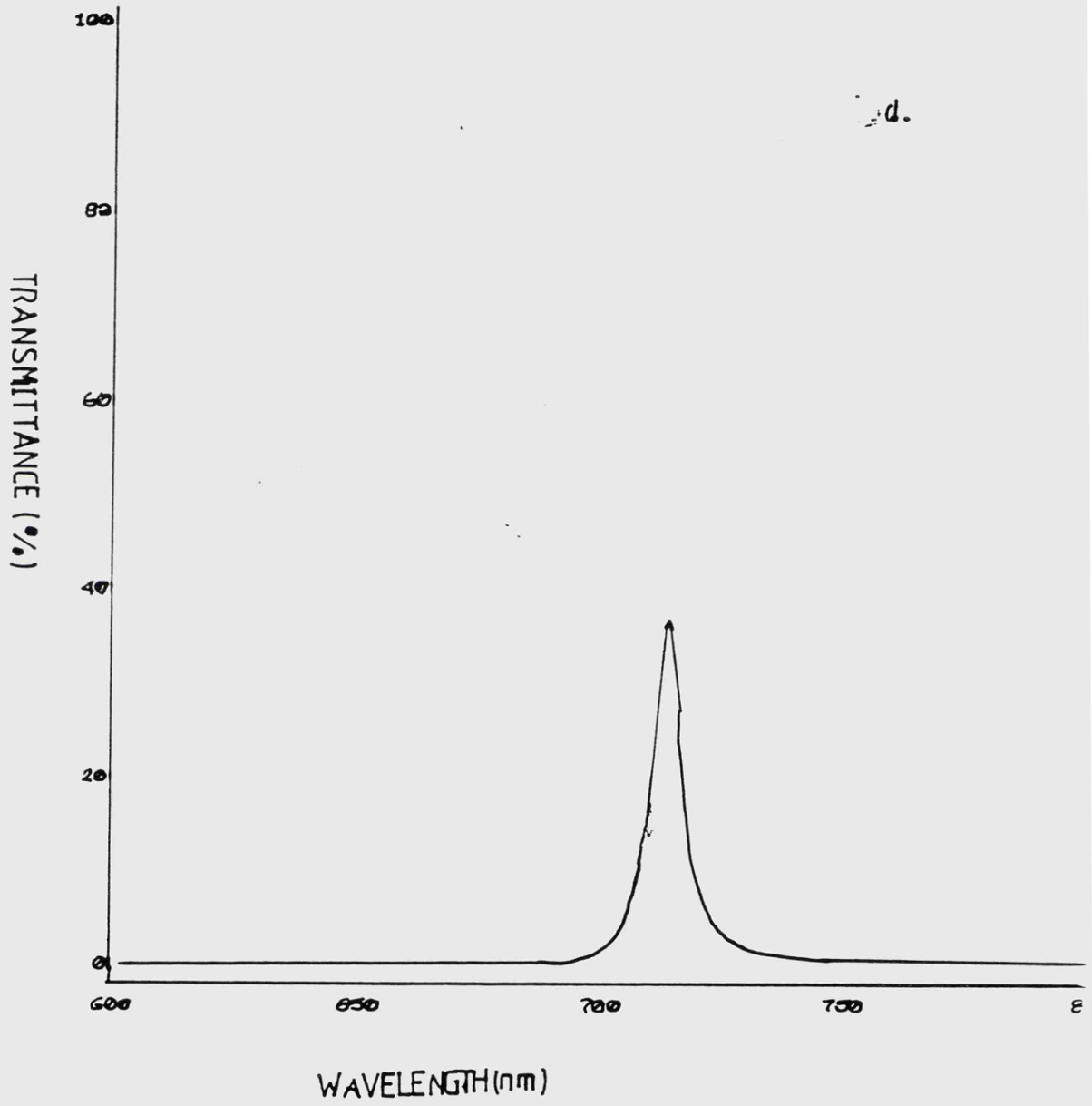

a.
100
80
60
40
20
0
TRANSMITTANCE (%)
600
650
700
750
800
WAVELENGTH (nm)


b.
TRANSMITTANCE (%)
100
80
60
40
20
0
600
650
700
750
800
WAVELENGTH (nm)

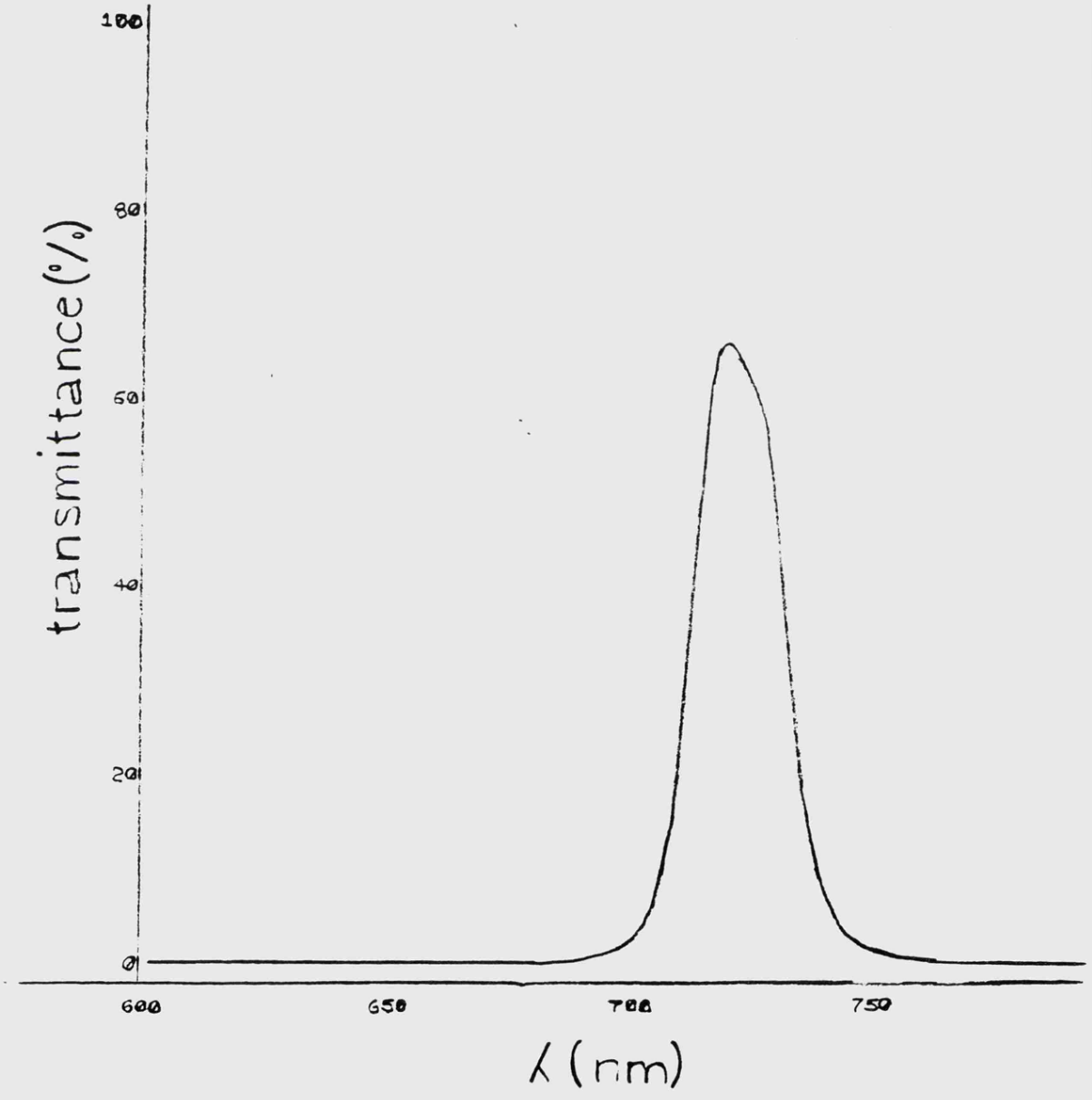
C.



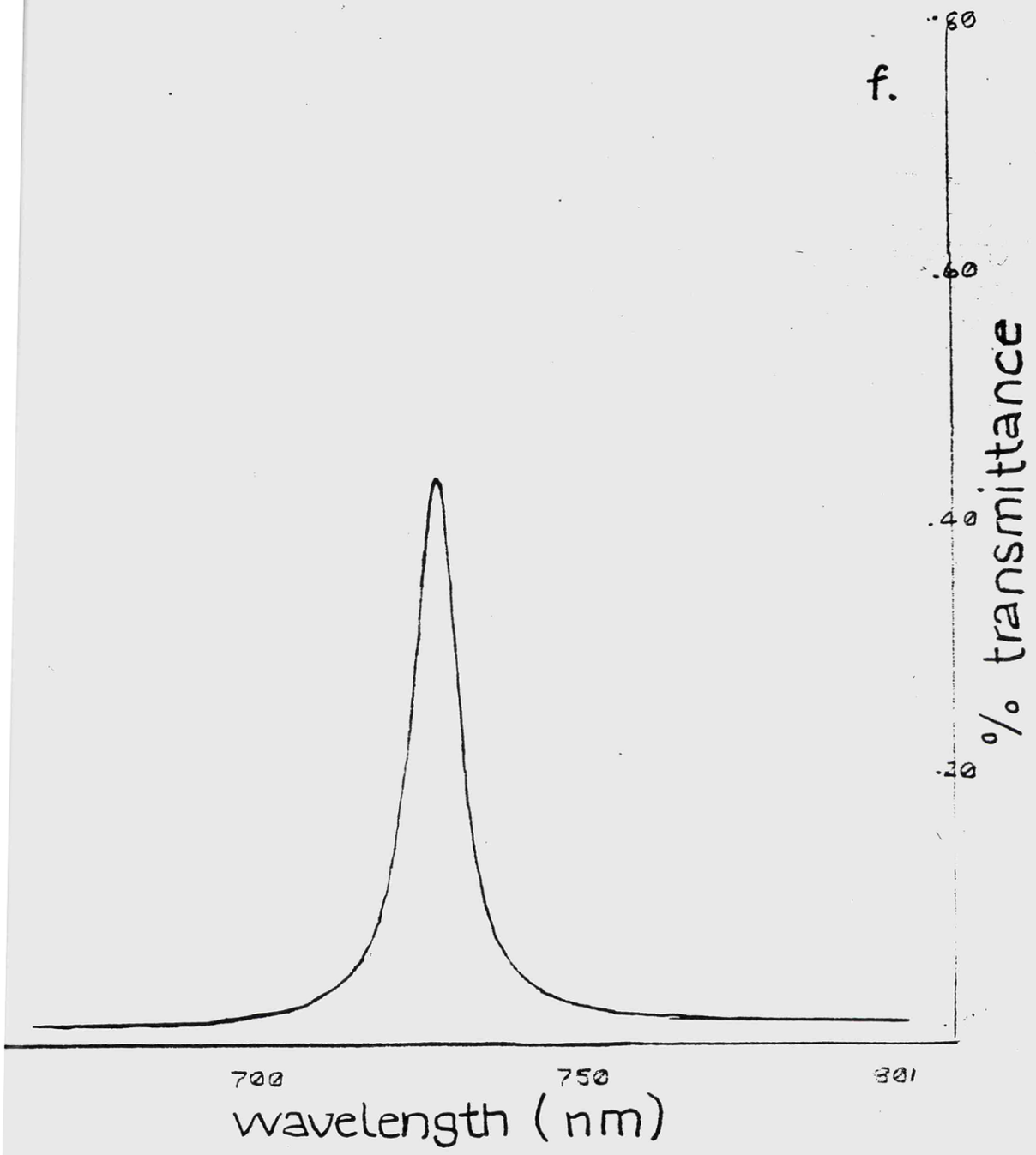
d.



e.



f.



media from stock solutions of their chlorides (150 mM). Aliquots were added at various times as specified to give the desired final concentration by dilution. When added to EGTA buffer calcium ions cause an acidification. In consequence the pH was readjusted to 6.5 with sodium hydroxide (1M) following addition. Lanthanum (III) ions were also added from a stock solution of the chloride in hydrochloric acid (1M). The pH was again readjusted to 6.5 following addition with NaOH (1M). Nitrate ions were added, at the times specified, from a stock solution of potassium nitrate (1M) to yield a final concentration of 5mM by dilution.

Unrolling Agonists.

Kinetin (Sigma Chemical Co., England) was prepared as a 25 mg/ml stock solution in sodium hydroxide (1.0M). To treat with kinetin, aliquots of stock solution were added to the experimental buffer stirred at 60 r.p.m. The pH was subsequently readjusted to 6.5 with hydrochloric acid (1.0M).

Gibberellic acid (Sigma Chemical Co., England) was prepared as a series of stock solutions by dilution. As with kinetin aliquots of stock solution were added to stirred buffer to yield the final desired concentration by dilution.

Calcium agonists.

Calcium ionophore A23187 was purchased as a Ca^{2+} salt [$Ca(A23187)_2$] (Sigma Chemical Co., England) and prepared as a stock solution (5 mg/ml) in dimethylsulphoxide (DMSO). To treat with A23187 aliquots were added to rapidly-stirred (180 r.p.m.) buffer solution to produce an opalescent colloidal solution. The tissue to be treated was immersed in this solution and gently agitated in an orbital shaker (60 r.p.m.). Equivalent amounts of DMSO were added in a similar fashion to all control treatments,

and where indicated Ca^{2+} (10 μM) also added.

5-Hydroxytryptamine (5-HT) treatments were supplied by addition of aliquots yielding the desired final concentration by dilution from a stock solution in deionized water. Pulsed treatments were performed by immersing the tissue in 5-HT/buffer for 30 minutes, draining onto filter paper in a funnel, treble washing in buffer and resuspending in fresh buffer. For comparison, ammonium treatments were performed in an identical manner using stock solutions of ammonium chloride.

1,2-diacylglycerol was obtained as 1,2-dioctanoyl-rac-glycerol (Sigma Chemical Co.) and prepared as a stock solution in absolute ethanol (25 mg/ml). Aliquots were added to rapidly-stirred (180 r.p.m.) buffer to yield a final concentration of 20 μM. Equivalent amounts of ethanol were added to non-treated samples in a similar fashion.

Complex Antagonists.

Gabaculin (3-amino-2,3-dihydrobenzoic acid) (Sigma Chemical Co., England) was directly dissolved in buffer for treatment.

W-compounds, derivatives of aminonaphthalene sulphonamide, W5, W7, W12 and W13 were obtained via the Sigma Chemical Company (England). Stock solutions were prepared in 1:1 DMSO/ethanol; W7 25 and 2.5 mg/ml, W5 10 mg/ml, W12 (10 mg/ml) and W13 (10 mg/ml). Aliquots of stock solution were added to rapidly-stirred buffer (180 r.p.m.) to yield the desired final concentration by dilution. Equivalent amounts of DMSO/ethanol were added to control treatments.

Other Chemical Treatments.

Valinomycin was purchased from Calbiochem (Cambridge Biosciences, Cambridge, England). A stock solution was prepared in 1:1 DMSO/ethanol (10 mg/ml) and aliquots added to rapidly-stirred buffer (180 r.p.m.) to

yield a final concentration of 20 μM.

Osmotic treatments were effected by preparing a range of mannitol concentrations in buffer.

Preparation of material.

Primary leaves (approx 90 mm ± 5 mm long) were harvested. The apical 10 mm was removed by cutting with a razor blade. Initially the 20 mm section adjacent to this was isolated for experimental purposes. This was subsequently reduced to 10 mm without alteration in the behaviour of the system. For phytochrome assays and protochlorophyillide assays 30 mm sections were isolated for ease of manipulation.

Incubations.

Except where otherwise stated, sections were floated on buffer (30 ml) in 9 cm petri dishes, and subjected to a pre-incubation (45 minutes at 20°C in darkness) prior to light treatment. The onset of light treatment was generally defined as T = o (t_o) and chemical treatments given at various times relative to this. Incubation at 20°C in darkness followed from t_o for 24h, subject to the intervention of subsequent light treatments, manipulating under dim green safelight as necessary and variations in total incubation time as directed by experimental protocol in measuring time courses, for example. Experiments involving chemcial treatments that utilised organic solvents to prepare stock solutions (ionophores, W-compounds, diacylglycerol) were incubated in an orbital shaker at 60 r.p.m. This was to retard aggregation of the ionophores in emulsified solution and was subsequently extended to the other organic chemical treatments both as a precautionary measure, and by attempts to combine and compare treatments. The incubation protocol leading up to protochlorophyllide assay differed considerably from that described

above. The incubation volume consisted of buffer (3 ml) in a small perspex trough, 30 sections being floated in this. The total incubation period was less than 4h. Excepting the assay periods, which necessitated light exposure, the samples were maintained in darkness at 20°C.

Assay systems.

1. Unrolling.

Following incubation, sections were removed from the buffer, blotted dry on filter paper and placed in a dry petri dish. The visible leaf width at the mid-point of the sections' long axis was measured using a dissecting microscope and eyepiece graticule at 20 x magnification. Upon calibration of the graticule measurements were converted to an accuracy of 1/22 mm. For practical use, this figure was rounded to the nearest 0.05 mm. Results were expressed as the mean width of the sections given a treatment with accompanying standard deviate or standard error. Where necessary statistical tests were performed based on 'Student's' t-distribution or an assumption of a Normal distribution of Values as appropriate to the sample size.

2. Nitrate reductase activity.

The assay used was based upon that described by Hageman and Reed (1980) and utilised spectrophotometric nitrite determination. Following treatments, approximately 0.3g of tissue per sample was blotted dry, weighed and chopped finely under dim green light. This was then suspended in 2 mls of extraction buffer [N-morpholino propanesulphonic acid (MOPS) (5 mM, pH 7.5) potassium nitrate (0.1M), propan-2-oL (0.15% v/v)] and subjected to three cycles of vacuum infiltration (1 min/cycle). The samples were then placed in a shaking water bath at 33°C and incubated for 30 min at 60 Hz in darkness. Enzymic nitrite production from nitrate was then stopped by the addition of 1 ml sulphanilamide (1% w/v) in hydro-

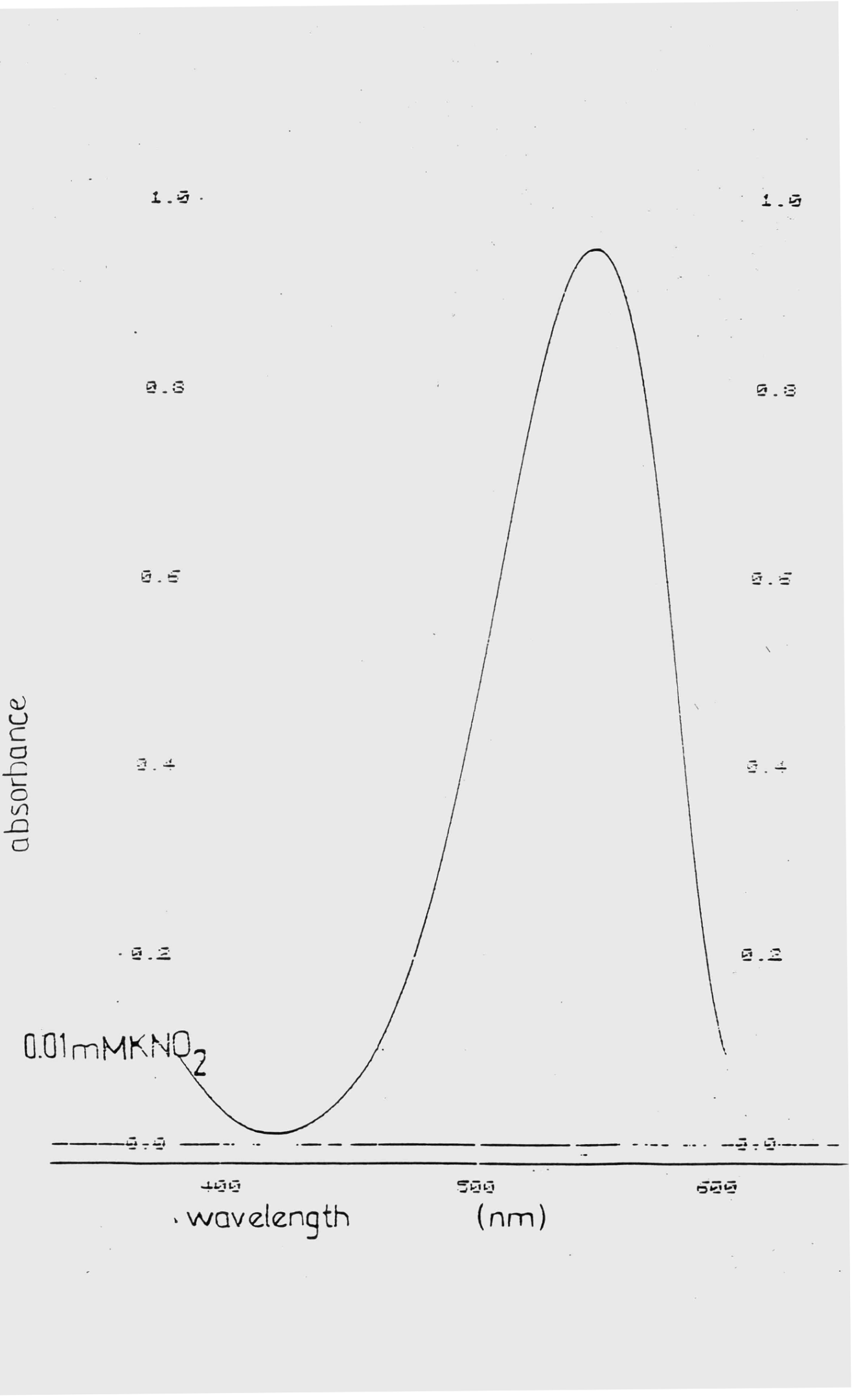
chloric acid (1 M). N-(1-naphthyl)-ethylenediamine dihydrochloride (N.E.D.) solution (0.02%), prepared and maintained in darkness, was then added (1 ml). The two reagents and the incubation medium were mixed thoroughly and the colour left to develop for 10 min.

Spectrophotometric scans of standard solutions of potassium nitrite containing the sulphanilamide /HCl/N.E.D. nitrite assay mixture indicated maximal sensitivity to nitrate around the 540-545 nm band of the visible spectrum following colorimetric development (Fig. 2.3). Using a series of standard potassium nitrite solutions and measuring absorbance at 545 nm, ten minutes after adding nitric assay reagents (sulphanilamide HCl/N.E.D.) a calibration curve was prepared to convert absorbance of the reagent mixture at 545 nm into nitrite concentration (Fig. 2.4). All absorbance measurements were performed relative to a reagent blank in distilled water using a Perkin-Elmer lambda-5 spectrophotometer. The nitrate reductase activity of samples was derived from the difference in absorbance at 545 nm of nitrite-assayed decanted solutions from samples taken before and after incubation with nitrate. Results were then expressed as μ mol$(NO_2^-)g(FW)^{-1}h^{-1}$.

3. Phytochrome assay.

Phytochrome destruction kinetics were measured using a Perkin-Elmer 156 dual beam spectrophotometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.). Measurements were performed in vivo on treated sections (30/1 ml cuvette, 5 mm path length) at various times following treatments. The $\Delta\Delta A$ value, reading at 730 nm and 800 nm at 20°C, was measured over three cycles of actinic FR (2 min) and R (2 min) with a 1 minute reading internal between light treatments. By comparison with the initial signal, Pfr/Ptot and Pr/Ptot were deduced. The assayed tissue was then blotted dry and weighed, the results being expressed in terms of $\Delta\Delta A\ g(FW)^{-1}$.

Fig. 2.3 Spectrophotometric scan showing absorbance of a 0.01 mM potassium nitrite standard solution (2 mls) following 10 min incubation with sulphanilamide/HCl (1 ml, 1% w/v in 1N acid) and N-(1-naphthyl)-ethylenediamine dihydrochloride (1 ml, 0.02% w/v).


1.0
0.8
0.6
0.4
0.2
absorbance
0.01mMKNO2
wavelength
(nm)

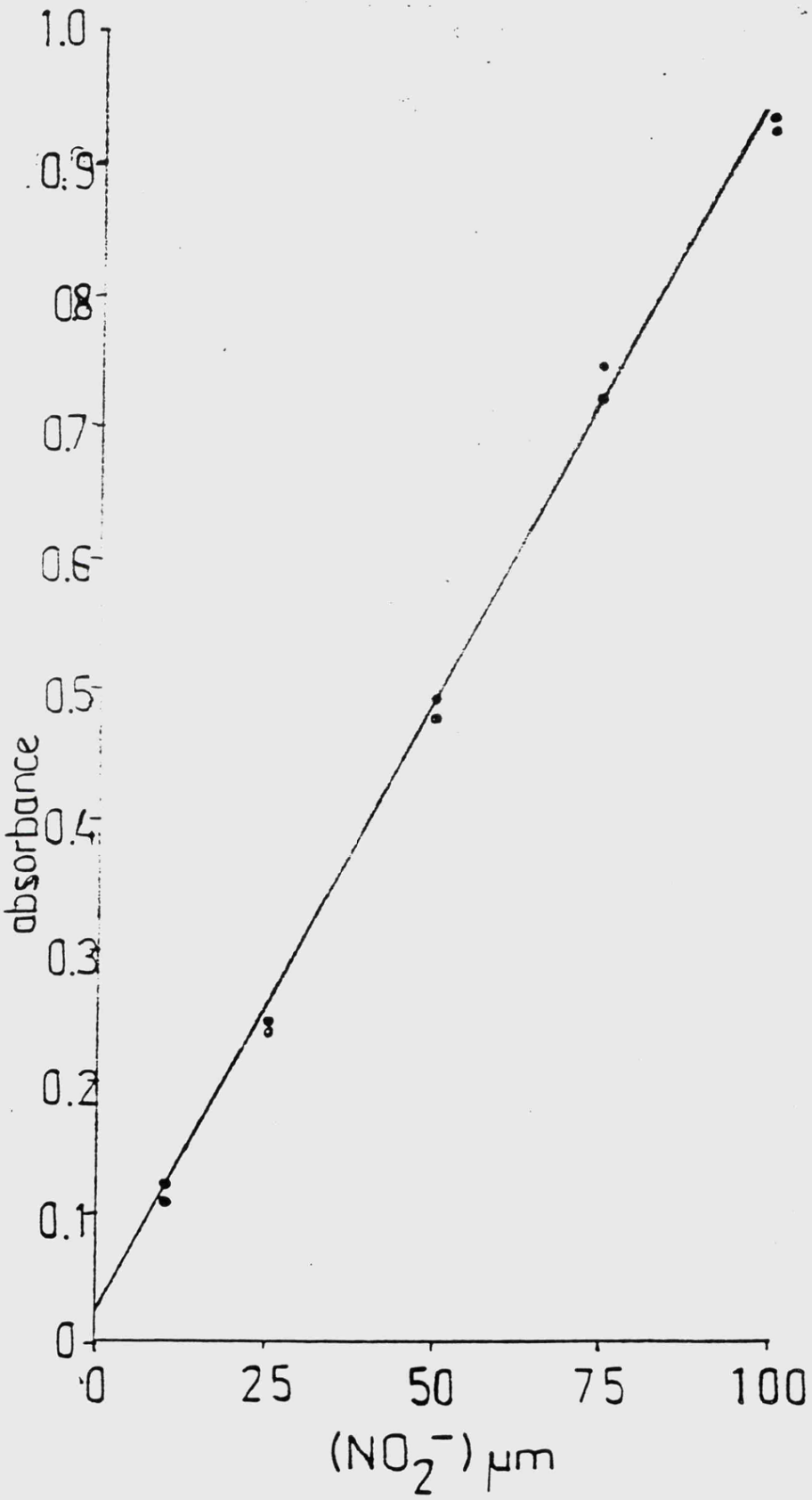

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
absorbance
0
25
50
75
100
$(NO_2^-)\ \mu m$

Fig. 2.4 Calibration curve: Absorbance of potassium nitrite standard solutions (2 ml) plus nitrite assay mixture (see text) after 10 minutes incubation, versus nitrite concentration. Absorbance measured at 545 nm.
Curve = line of best fit after linear regression (n = 2).
Equation: Absorbance = 0.094 (nitrite concentration) + 0.083
r = 0.999.

Photostationary state measurements were performed using an Aminco DW2A dual beam spectrophotometer (Aminco Instrument Corporation) at $0^{o}C$ reading at 660 and 730 nm for purified Avena phytochrome (a kind gift from Dr. G. Whitelam, University of Leicester, U.K.) and at $0^{o}C$ reading at 730 nm and 800 nm for in vivo phytochrome in leaf sections (30/1ml cuvette). Readings were obtained as before using cycles of actinic FR (2 min) and R (1 min) with a 30s reading internal. Results were expressed as percentage Pfr of Ptot. In calculating Pfr/Ptot the ratio of Pfr/Ptot at equilibrium was assumed to be 86% after Viersta and Quail (1983).

4. Protochlorophyllide assay.

The conversion of protochlorophyllide to chlorophyllide in vivo in etiolated tissue results in a quantitative absorbance change of a sample at 650 nm (Jabben and Mohr, 1975). This change was measured using the Aminco DW2A spectrophotometer in split beam mode with a semi-opaque reference cuvette. Protochlorophyllide conversion in 30 sections in a 1 ml cuvette was effected by the actinic red light source of the spectrophotometer (2 minutes). The ΔA_{650} before and after treatments was measured to quantify the amount of protochlorophyllide converted. Results were expressed as a cumulative total ΔA.

5. Calcium ion-selective electrode measurements.

Ca^{2+} activities were measured using an Orion Research model 92-20; electrode (Cambridge, Mass., U.S.A.) and double junction reference (model 92-02; Orion) linked to a pH meter (model 7050; Electric Instruments, Chertsey, Surrey, U.K.).

CHAPTER 3 : THE DEFINITION OF THE UNROLLING SYSTEM AND ITS RESPONSIVENESS TO EGTA

Throughout their history, studies of phytochrome-regulated leaf unrolling display a considerable diversity of approach. Barley, maize, oats, rice and wheat have all been used as experimental species, and although most protocols are based upon that of Virgin (1962), with sections being taken from etiolated primary leaves, treated and incubated in the dark for several hours prior to measurement, the duration and nature of the incubation, as well as the measurement technique, often differing between workers. Distilled water, tap water, phosphate buffer, citrate-phosphate buffer and sodium acetate buffer have all been used as incubation media over a pH range of 4.95 to 7.0. Methods of measuring the leaf width that have been used, range from direct observation with a dissecting microscope and eyepiece graticule, through measurement of projected images to computerized determination from photocopied images using a digitizer. With such a wide range of previous assaults on the problem, quantitative comparison between different workers' results becomes difficult, and the decision as to which specific techniques to use to extend this work somewhat arbitrary. Rather than adhere to any one system developed in the past, thereby implying judgement of the 'superiority' of one method over another, it was decided, for the purposes of this study, to incorporate elements from a variety of sources and build upon them as experimental need dictated change.

Some aspects of the experimental system are defined by practical

considerations, such as ease of manipulation. Thus, barley was chosen as the species to be studied for its rapid emergence of the primary leaf from the coleoptile when grown in the dark compared to oats or maize, and its ready availability compared to rice. The nature of the experimental treatments anticipated also restricts the options to be selected even at the very earliest stages. The expressed intention to study the role of calcium ions in leaf unrolling, made phosphate-containing buffers unsuitable as incubation media. This is because of the low solubility of calcium phosphate, which would severely limit the range of external calcium activities available in solution. There would also be a tendency towards variation in pH-buffering capacity with variations in Ca^{2+} concentration with calcium phosphate precipitating at millimolar calcium concentrations removing both Ca^{2+} and H^{+}-buffering phosphate from solution. Similarly the expected use of acidic chemical species, like EGTA, which could themselves alter the experimental pH, made some form of buffering desirable given the variation in unrolling with pH demonstrated by Kang (1971). Thus, water was deemed unsuitable on its own, as an incubation medium. It was decided to use one of the zwitterionic buffers described by Good et al (1972) to control the pH during the incubation stage of the experiments. There were several reasons for this, despite the fact that they had not previously been used in connection with cereal leaf-unrolling studies. Firstly, their high polarity renders biological membranes impermeable to their passage. Secondly, they are chemically inert. Thirdly, and most importantly, they are relatively unaffected by variations in temperature, concentration and ionic composition. These properties render them unlikely to interact with the system under study while retaining a relatively robust pH buffering capacity during experimental manipulation. On the basis of pK_a values at $20^{o}C$ and useful pH

ranges, MES was selected for the buffering medium (pK_a at 20^oC = 6.15, useful pH range = 5.6 to 6.8). With a Ca^{2+} buffer binding constant (Log K_m) of 0.7, it was of sufficiently low Ca^{2+} affinity for it not to interfere with the proposed work. It was decided to set the buffer to a working pH of 6.5, in line with that used by several previous workers (Loveys and Wareing, 1971b; Sundquist and Briggs, 1982).

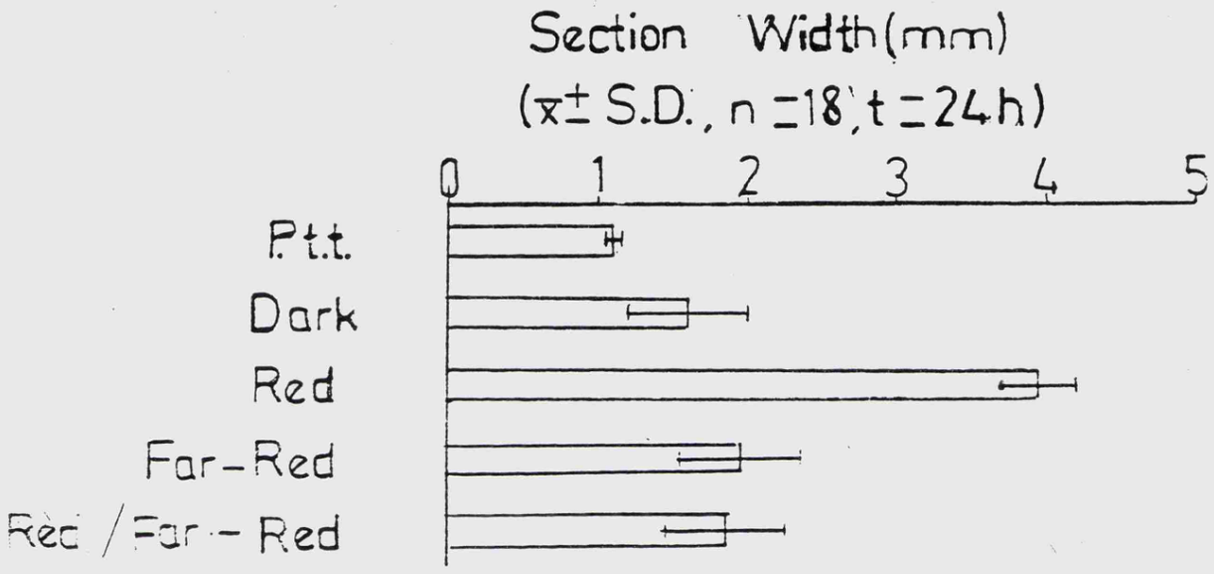
The use of an uncharacterised system like this necessitates some duplication of experiments performed prior to the main investigations, both to establish the presence of the phenomena to the studied (i.e. phytochrome-regulated leaf unrolling) and to provide a yard-stick for comparison with the qualitative themes and conclusions to be drawn from past studies.

Initial experiments were carried out to demonstrate the presence of phytochrome-regulated leaf unrolling in sections taken from the primary leaves of 6 day-old dark-grown barley (Hordeum vulgare L. var. 'Golden Promise') in MES-NaOH buffer (35 mM, pH 6.5), and a typical set of results is shown in Fig. 3.1.

It is seen that both sections kept in the dark throughout the 24h incubation period, and those subjected to five minutes R at the beginning of the incubation, unroll relative to sections measured at the beginning of the incubation (prior to treatment = ptt). The unrolling that occurs independently of light will be referred to as 'dark unrolling', to distinguish it from the considerably greater unrolling obtained when R(5 min) is supplied at the beginning of the dark incubation. The stimulatory effect of R can be blocked by immediate application of FR (10 min) at the beginning of the dark incubation.

This is indicative of the operation of a classical low-fluence phytochrome response, thus showing the presence of the desired phenomenon

Fig. 3.1 Unrolling of barley leaf sections in MES Buffer. R treatments and FR treatments were of 5 and 10 min duration respectively, and were given serially FR followed immediately after R. Treatment commenced at t_o following a 45 minute pre-incubation, and the sections measured at t_{24h} following a dark incubation at 20°C.


Section Width (mm)
(x̄ ± S.D., n = 18, t = 24h)
0
1
2
3
4
5
P.t.t.
Dark
Red
Far-Red
Red / Far-Red

in the chosen system. In making these measurements, it was noticed that the degree of unrolling was not evenly distributed along a section. Schönbohm (1985) demonstrated that the apical end of a section always unrolls more than the basal end. Because of this, it is necessary, when measuring visible leaf section widths as an assay of degree of unrolling, to define what is actually being measured. For this study the section width was defined as the shortest distance between the visible edges of the section at the mid-point between its cut ends. All subsequent unrolling data in this study are expressed as the mean value for this distance for the stated number of samples, less the mean width p.t.t.; the value thus obtained is described as the 'increase in leaf width'. Departures from this practice are clearly labelled.

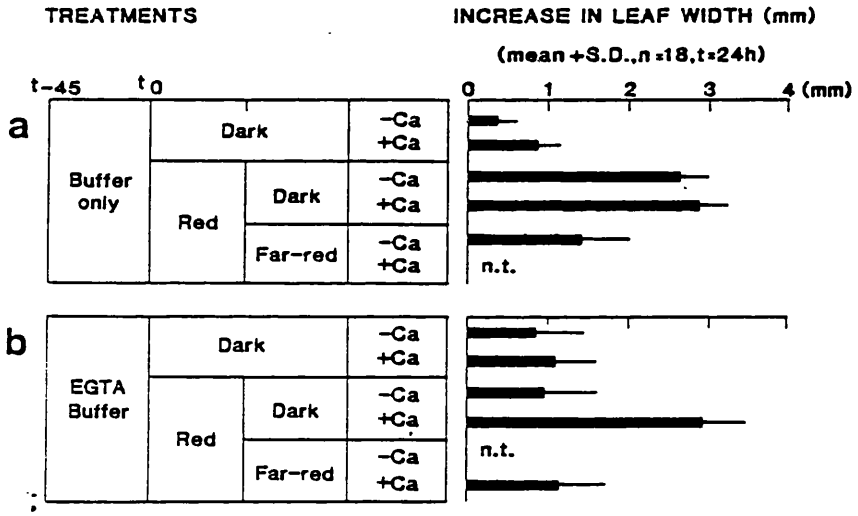
If a physiological process involves Ca^{2+}, it follows that a suitable change in the availability of Ca^{2+} in the system will affect the observed behaviour of that system in relation to the process measured. This premise provides a useful starting point in defining the role of Ca^{2+} in phytochrome-controlled leaf unrolling. An obvious way to alter the Ca^{2+} availability is to introduce a chelating agent to reduce the Ca^{2+} activity to very low levels. EGTA has been used extensively in this context in biological systems.

The EGTA molecule consists of an ethylene glycol bis aminoethylether backbone with a pair of carboxyl groups attached to both terminal nitrogen atoms. These orientate to 'cage' multivalent metal ions such as Ca^{2+}, effectively removing them from solution. It has been suggested by Tsien (1980), that the dimensions of the molecular backbone, and the small size of the Mg^{2+} ion, give rise to steric hindrance between the carboxyl groups accounting for the much lower affinity of the molecule for Mg^{2+} compared to Ca^{2+}, approximately 10000 fold at $20^{o}C$ with an ionic strength of 0.1

(Martell and Smith, 1974). It is this large difference in Ca^{2+}/Mg^{2+} affinity that has led to the preference for EGTA rather than EDTA, which shows a much smaller affinity difference. In spite of this there are some problems associated with the use of EGTA to characterise the role of Ca^{2+} in a system. Firstly, EGTA shows even higher affinity for some heavy metal ions than it does for Ca^{2+}. However, in a system such as leaf unrolling in buffer these should be present only in trace amounts compared to the Ca^{2+} levels. Secondly, the Ca^{2+} buffering capacity is strongly dependent on pH around 7, hence the need to pH buffer the system. Some acidification was measured on addition of Ca^{2+} to MES-EGTA buffer, due to changes in pKa values on Ca^{2+} chelation. Typically this was around 0.15 pH units and where appropriate, NaOH was added to correct this. Finally, the rate constants for Ca^{2+} binding are relatively low, making buffering of Ca^{2+} transients, e.g. those detected in muscle contraction, difficult. To increase the likelihood of abolition of any such effects by EGTA it was decided to introduce a pre-incubation stage, prior to other treatments, into all experiments involving the use of EGTA, there being evidence that chelating agents do deplete internal Ca^{2+} stores of plant cells, as well as removing extracellular Ca^{2+} (Miller and Kotenko, 1987).

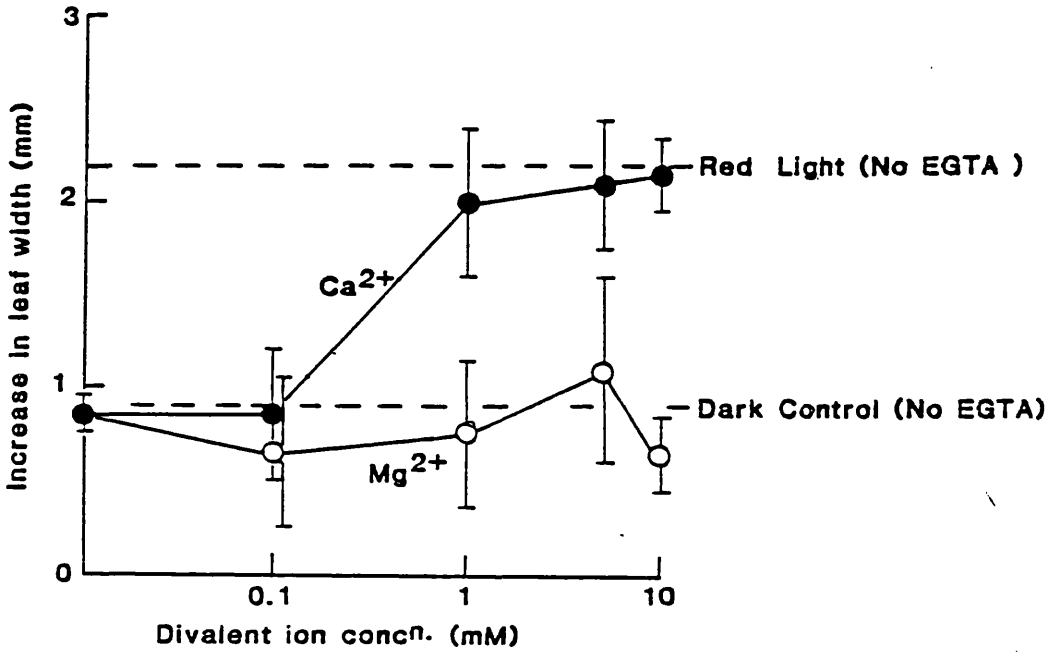
It can be seen from the above that the rationale behind the use of EGTA is to remove Ca^{2+} in a controlled manner, such that all processes with an absolute Ca^{2+} requirement will be inhibited, whilst leaving Mg^{2+} levels relatively unchanged. Fig. 3.2 compares unrolling in the presence and absence of added Ca^{2+} ion: (a) MES buffer only, and (b) MES/EGTA buffer, both with a 45 min. pre-incubation prior to light, and Ca^{2+} treatments. Fig. 3.2a duplicates much of Fig. 3.1, namely that R promotes an increase in unrolling and subsequent FR reverses this. Addition of Ca^{2+}

Fig. 3.2 Unrolling of barley leaf sections in a. MES buffer; b. MES-EGTA buffer. R treatments and FR treatments as for Fig. 3.1. Ca^{2+} (5 mM) was applied at $t_{5\ min}$ and the sections measured at t_{24h} following a dark incubation (n.t. = not tested).


TREATMENTS
INCREASE IN LEAF WIDTH (mm)
(mean +S.D., n =18, t=24h)
t-45
t0
0
1
2
3
4 (mm)
a
Buffer only
Dark
−Ca
+Ca
Red
Dark
−Ca
+Ca
Far-red
−Ca
+Ca
n.t.
b
EGTA Buffer
Dark
−Ca
+Ca
Red
Dark
−Ca
+Ca
Far-red
−Ca
+Ca
n.t.

(5mM) at the onset of treatment does not detectably alter this. However, it will be demonstrated in later chapters, using larger samples, that Ca^{2+} may have small, but detectable, effects on both dark and R-stimulated unrolling. Since these effects are small relative to the difference between dark-unrolling and R-stimulated unrolling, it is not necessary to discuss them here. Fig. 3.2b shows that dark unrolling is not substantially affected by EGTA; it is therefore Ca^{2+}-independent. R-stimulated unrolling is, however, completely abolished in the presence of EGTA. If Ca^{2+} is supplied at a concentration in excess of that required to saturate the EGTA (5mM Ca^{2+} vs 2mM EGTA) then R-sensitivity of unrolling is restored in a FR-reversible fashion. This indicates that the inhibiting effect of EGTA on R-stimulated unrolling is due to its Ca^{2+}-chelating properties and not an effect unrelated to chelation. From this it appears that phytochrome-controlled unrolling is a Ca^{2+}-dependent process. Similar results have been obtained with EGTA for several other phytochrome-regulated phenomena, including the Onoclea spore germination system (Wayne and Hapler, 1984). Avena mitochondrial ATPase activity (Serlin et al, 1984), nuclear protein phosphorylation in Pisum (Datta et al, 1985) and light-dependent graviperception in maize (Reddy et al, 1987). The Ca^{2+}-dependence of R-stimulated unrolling reveals nothing as to the actual role of Ca^{2+} in the response beyond establishing that such a role exists. To confirm this, the effectiveness of Mg^{2+} as a substitute for Ca^{2+} was evaluated over a range of added concentrations from 0.1 mM to 10 mM, into EGTA buffer. This was performed to allow the Ca^{2+}-dependence of unrolling to be assessed in terms of likely physiological relevance by the criteria suggested by Hepler and Wayne (1985). These are: that the phenomena under study should be 'markedly stimulated' at Ca^{2+} activities less than 100 μM, and that such phenomena 'will not be stimulated by Mg^{2+}

Fig. 3.3 The effect of increasing applied concentrations of Ca^{2+} (●——●) or Mg^{2+} (o——o), supplied at $t_{5\ min}$ (relative to the onset of R on R-stimulated unrolling of barley leaf sections in MES-EGTA buffer. R-treatment followed a 45 minute pre-incubation in buffer, with or without EGTA. Sections were measured at t_{24h} following a dark incubation at 20°C. Upper dashed line; unrolling of R-treated controls without EGTA. Lower dashed line; dark unrolling.


3
2
1
0
Increase in leaf width (mm)
Red Light (No EGTA)
Ca2+
Dark Control (No EGTA)
Mg2+
0.1
1
10
Divalent ion concn. (mM)

at 1 mM'. The results of this assessment are shown in Fig. 3.3. It is seen from this that Mg^{2+} additions from 0 through to 10 mM have no effect on the inhibition of R-stimulated unrolling by EGTA. It is likely, therefore, that the inhibition is due to Ca^{2+}-chelation and is not a non-specific effect due to lack of divalent cations. The added concentration of Ca^{2+} necessary to overcome this inhibition lies between 0.1 mM and 1 mM. It may appear from this that unrolling fails to meet the first of the above criteria; however, the free Ca^{2+} ionic activity at a concentration of 1 mM in EGTA (2 mM) is much less than 1 mM. Using a Ca^{2+} ion selective electrode, it proved possible to measure the free Ca^{2+} activity in the MES/EGTA buffer used for the experiment for given additions of Ca^{2+}. The values obtained are shown in table 3.1.

Due to limitations imposed by electrode sensitivity, it was not possible to calibrate the electrodes below 10 μM, hence the lack of a signal for additions of Ca^{2+} to cause a concentration change up to 0.5 mM. At a concentration of 1 mM, the Ca^{2+} activity rises to 40 μM. Taken in conjunction with Fig. 3.3, it follows that R-stimulated unrolling will proceed in EGTA buffer provided there is an extracellular Ca^{2+} activity of 40 μM. This value provides an upper limit for the threshold Ca^{2+} activity for unrolling to proceed, which may have a value considerably lower than this in practice. R-stimulated leaf unrolling therefore shows complete stimulation at Ca^{2+} activities well below 100 μM; thus both criteria of Hepler and Wayne (1985) are fulfilled, and the Ca^{2+}-dependence is of likely physiological relevance.

To approach the question of how R-stimulated unrolling depends on the presence of Ca^{2+}, it was decided to examine the temporal variation in Ca^{2+}-dependence relative to the R treatment, if any. Using this strategy, Wayne and Hepler (1984) and Mische et al (1987) have shown that the Ca^{2+}

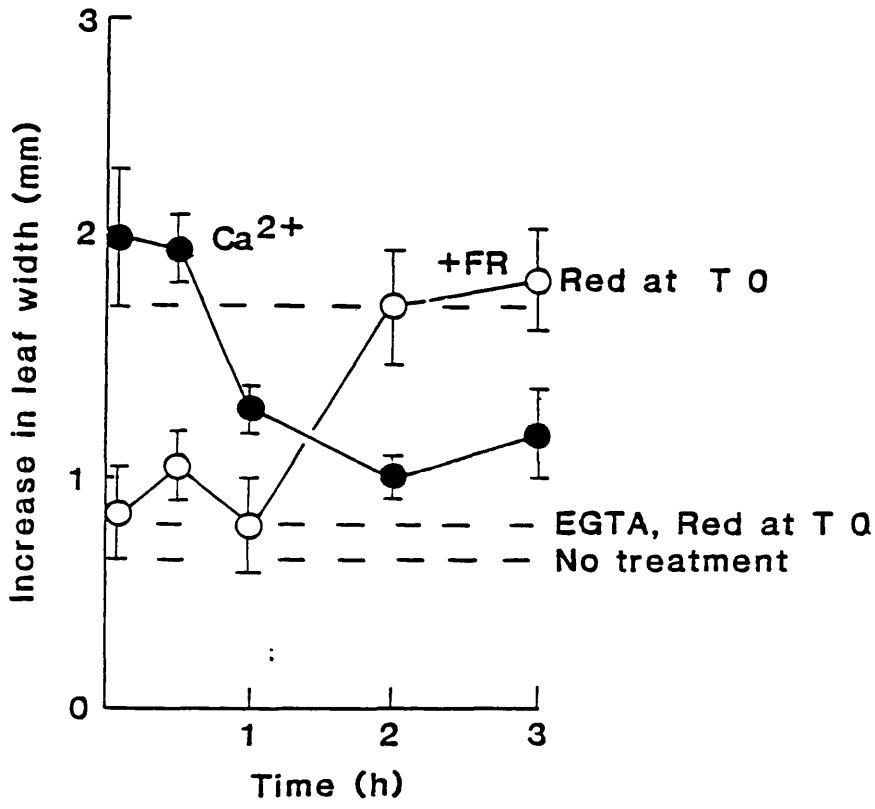
Table 3.1 Ca^{2+} activity in MES/NaOH buffer (35 mM pH 6.5) containing EGTA (2 mM) for addition of Ca^{2+}. Measurement performed in 30 ml buffer at $20^{o}C$.

Added Ca^{2+} Concentration (mM)	Ca^{2+} activity (μM)
0	> 10
0.1	> 10
0.5	> 10
1.0	40
5.0	3.16×10^{3}

requirement of fern spore germination can persist for many hours after light treatment in the absence of Ca^{2+}, germination being induced by added Ca^{2+} even after the response has become refractory to FR reversal. This indicates that the initial stages of the transduction chain from photoconversion of phytochrome to germination are Ca^{2+} independent, and that a relatively stable intermediate state can be arrived at without the participation of Ca^{2+} (although whether such a state could exist given 'normal' levels of Ca^{2+} is not proven). Such experiments can yield information regarding the path taken in a response and allow dissection of the response into components which may be studied in isolation. To attempt this in the leaf-unrolling system, sections were incubated in EGTA buffer, treated with R, and the effect of delaying the Ca^{2+} treatment for increasing periods assessed for changes induced in the subsequent degree of unrolling. At the same time, sections were incubated in MES buffer, treated with R and FR with increasing periods between R and FR. This was to establish the time taken for the system to become refractory to FR (the escape time). The results in Fig. 3.4 show that FR treatment (open circles) completely loses its reversing effect on R treatment if delayed by 2h, although it is fully capable, if given within 1h of R, of reversing the R effect and abolishing the response. Therefore, the escape time lies between 1h and 2h for this system. This is in close agreement with previous determinations of escape time for the leaf unrolling response in barley (Smith, 1975; Sundquist and Briggs, 1982) which put the escape time between 1½ and 3h.

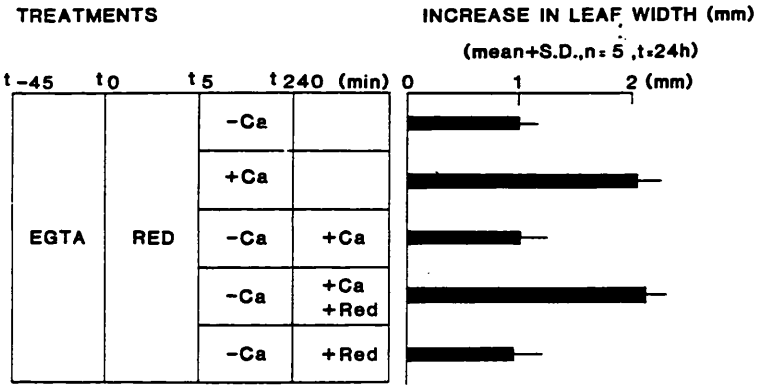
The effect of delaying Ca^{2+} supply, relative to R,. in EGTA-treated sections (closed circles), almost mirrors the FR escape curve. Up to 30 min after R, Ca^{2+} can overcome the EGTA inhibition. However, by 1h it is substantially less effective, and by 2h there is little or no effect of

Fig. 3.4 The effect of delayed Ca^{2+} (5 mM) treatment, until the times indicated, on unrolling in R-treated (5 min at t_o) barley leaf sections in MES/EGTA buffer following a 45 min pre-incubation (o——o). Also, the effect of delayed FR (10 min) following R (5 min at t_o) on barley leaf-section unrolling in MES buffer (●——●). Sections were measured at t_{24h} following a dark incubation at 20^oC.


3
2
1
0
Increase in leaf width (mm)
Ca2+
+FR
Red at T 0
EGTA, Red at T 0
No treatment
1
2
3
Time (h)

adding Ca^{2+} back to the system. This indicates that the EGTA-treated sections 'escape' from responsivity to Ca^{2+} between 30 min and 2h after R. This is very different to the situation described for fern spore germination where photoreversibility and the Ca^{2+} requirement could be temporally separated. It is impossible to do this for leaf unrolling. One explanation of this difference could be that Ca^{2+} deprivation in EGTA for long periods causes irreversible degenerative changes in the barley leaf tissue; for example, by de-stabilising the cell membrane. Under these circumstances, supply of Ca^{2+} will have no effect because the mechanism of unrolling is no longer functional. Recently Bossen et al (1988) demonstrated escape from responsivity to Ca^{2+} within 10 min for the phytochrome-mediated wheat protoplast swelling response during EGTA treatment. However, no attempt was made to assess whether or not this was an irreversible toxic effect of EGTA. To examine this possibility in the barley leaf unrolling system, sections in EGTA Buffer were subjected to a second R treatment after they had become refractory to Ca^{2+} following an initial R treatment. This was examined both in the presence and absence of simultaneous added Ca^{2+} and was performed 4h after the initial R treatment. The aim was to show whether or not the unrolling mechanism remained fully functional during extended Ca^{2+} deprivation in excess of the period used in the previous experiment. The results are shown in Fig. 3.5. It is seen that Ca^{2+} overcomes EGTA inhibition of R-stimulated unrolling if supplied immediately after the R treatment, but has no effect on its own if withheld until 4h after the onset of the R treatment. These results are in agreement with those of Fig. 3.4. A second R treatment 4h after the first, has no effect in the absence of Ca^{2+}. If both Ca^{2+} and R are given together, 4h after the first R treatment, then full unrolling is induced, even though neither the second R treatment nor Ca^{2+}

Fig. 3.5 Unrolling of barley leaf sections as measured 24h after R (5 min) in EGTA buffer: the effect of delayed Ca^{2+} (5 mM) treatment and a second 5 min supplementary R treatment both given at t_{4h}.

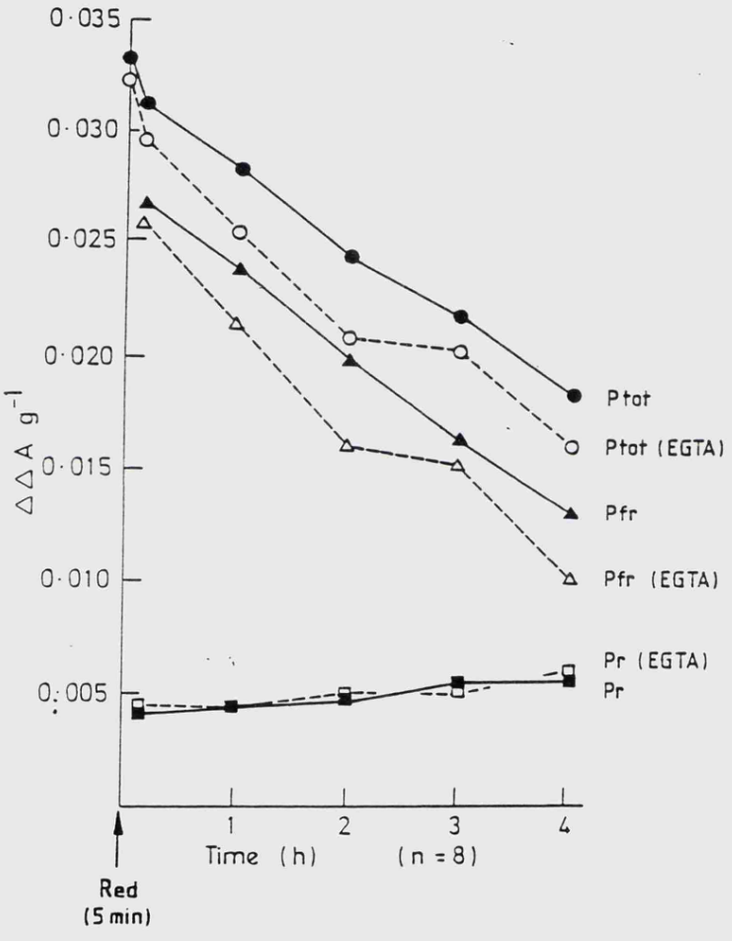

TREATMENTS
INCREASE IN LEAF WIDTH (mm)
(mean+S.D.,n= 5 ,t=24h)
t -45
t 0
t 5
t 240 (min)
0
1
2 (mm)
EGTA
RED
-Ca
+Ca
-Ca
+Ca
-Ca
+Ca
+Red
-Ca
+Red

have any effect if given in isolation at this time. This clearly demonstrates that the unrolling mechanism remains undamaged during EGTA treatments lasting up to 4h + ¾h pre-incubation. Therefore, damage to the system is an inadequate explanation of the 'escape' from responsivity to Ca^{2+} seen in Fig. 3.4. Instead, it would appear that R sets up an unstable state in the tissue that must interact with Ca^{2+} for unrolling to proceed. In the absence of Ca^{2+}, this state disappears within 2h of R treatment and can only be re-created by subsequent illumination. The fact that this period coincides with the time in which the response is photoreversible, indicates that a Ca^{2+}-dependent step must occur relatively early in transduction of the light signal. This is very different to the situation described for fern spore germination (Wayne and Hepler, 1984; Mische et al 1987). However, the two responses are very different, spore germination being an 'all or nothing' event, whereas unrolling is a continuous process. In an 'all or nothing' event, a point is reached where the system is committed, apparently independently of Ca^{2+} in the case of the fern spore. This is not seen in a continuously variable process such as leaf unrolling.

An obvious component of the system that appears following R and interacts with Ca^{2+} is Pfr itself. Since the system decays, during EGTA treatment within the period of escape from photoreversibility, the relationship of phytochrome to other components of the system must be dynamic. The possibility exists, therefore, that EGTA treatment exerts its effect through altering phytochrome/Pfr levels so that functional loss of Pfr occurs more rapidly and the system decays through this accelerated loss. To test this possibility, the loss of total phytochrome and Pfr following saturating R treatment was measured in sections incubated in darkness in MES/NaOH buffer or MES/NaOH/EGTA buffer, and the results

compared. Phytochrome and photoequillibrium measurements were performed using dual beam spectrophotometry reading at 730 nm and 800 nm. These wavelengths are especially suitable for measuring phytochrome in vivo as neither Pr nor Pfr absorb at 800 nm, whereas only Pfr absorbs at 730 nm, allowing absorbance changes specifically due to the conversion of Pr to Pfr to be measured. Neither protochlorophyllide nor chlorophyllide absorb at either wavelength and thus changes in absorbance due to the photoreduction of chlorophyllide are avoided during measurement. Short, rapid alterations in the measuring beams induce absorbance changes in the sample dependent on the ratio Pfr/Pr in the sample. Measurement of the initial value of this and the extreme values following subsequent saturating FR and R treatments allows calculation of total phytochrome content and the relative proportions of Pfr in terms of the unit difference of the changes in absorbance after R and FR ($\Delta\Delta$ A, see Smith, 1975 for a full treatment and theoretical background). Measurements were performed at 20°C, to minimise equilibration times and signal drift, over a 4h period following R and a 45 min pre-incubation prior to this. Pfr and Pr levels have been calculated on the basis of a photoequilibrium value of 86% Pfr following saturating R, after Vierstra and Quail (1983), and the results presented in Fig. 3.6. The total phytochrome (P_{tot}) declines to nearly half its original level, following R, over the 4h dark incubation. This loss is matched by, and so presumably consists of, an almost identical loss in Pfr, which undergoes destruction with zero order kinetics ($k \simeq 10^{-5} \Delta\Delta A\ s^{-1}$). Vierstra and Quail (1986) assert that Pfr destruction exhibits first order kinetics. However, the rate of Pfr destruction in etiolated barley leaf tissue has not been determined previously, and Pratt and Briggs (1965) also found zero order Pfr destruction in Zea coleoptiles. This indicates considerable variation in

Fig. 3.6 Loss of total phytochrome (P_{tot}) and Pfr from barley leaf sections in MES Buffer and MES-EGTA buffer following R (5 min).


0·035
0·030
0·025
0·020
0·015
0·010
0·005
ΔΔA g⁻¹
Ptot
Ptot (EGTA)
Pfr
Pfr (EGTA)
Pr (EGTA)
Pr
1
2
3
4
Time (h)
(n = 8)
Red
(5 min)

destruction kinetics between species, and given the variation in extent of autoregulation of Pr synthesis (Lagarias, 1987; Quail, 1987) may imply that rates of synthesis and destruction of phytochrome are not central to the function it performs in etiolated tissue.

Pr does not change significantly over the 4h of the experiment, although there appears to be a slow accumulation (less than 10% increase over 4h) which is presumably due to Pr synthesis. This, and the correlation betwen Pfr loss and loss of P_{tot} indicates that dark reversion of Pfr to Pr does not occur in barley leaves.

Incubation in EGTA buffer does not substantially affect any of the parameters discussed above. The values of P_{tot} and Pfr may be slightly lower at any given time in EGTA-treated tissue, compared with controls, but the rate of P_{tot} loss arising from Pfr destruction appears the same, and Pr levels are indistinguishable from control tissue throughout. This is in agreement with Furuya et al (1965). Using EDTA (2 mM) as a chelating agent, no effect on the in vivo destruction of phytochrome could be detected at pH 6 in etiolated Avena coleoptiles. At pH 8, the onset of destruction of Pfr could be delayed for up to 2h by EDTA. Between these pH values, the inhibition of destruction during the first hour after R varied in a more or less linear fashion, there only being a small effect at pH 6.5. These observations have never been adequately explained, sodium azide (0.5 mM) showing a similar impermanent inhibiting effect on destruction. Whether or not chelating agents can temporarily protect Pfr in barley leaf tissue has not been established in the present study, it would however appear that EGTA does not affect Pfr levels at pH 6.5 and another explanation for the escape from responsivity to Ca^{2+} during EGTA treatment must be sought. Further questions concerning this observation are raised by the apparent dose/response relationship of R-stimulated

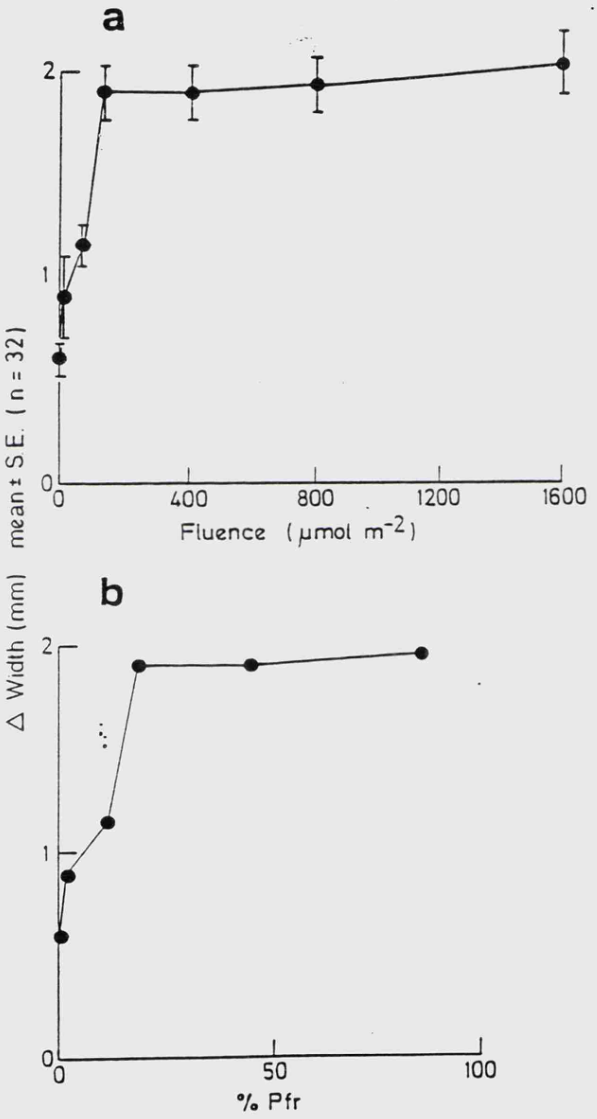
unrolling. This was obtained by varying the duration of the R and measuring the subsequent unrolling in MES buffer. The fluence rate was measured at the sample surface (13.6 μMol m^{-2} s^{-1}) and the fluence for each treatment calculated. At the same time samples incubated in a similar manner were assayed for Pfr/P_{tot} using dual beam spectrophotometry as before, the results being expressed as percentage Pfr. Fig. 3.7a shows the magnitude of the response in terms of fluence. There is an initial rapid increase in unrolling saturating around 136 μmol m^{-2}. This is broadly in line with previous determinations of saturating fluence for leaf unrolling in barley (Smith, 1975; Sundquist and Briggs, 1982) any variations probably being due to different light sources and seed stocks. Higher fluences do not appear to affect unrolling.

Fig. 3.7b shows the response in terms of the amount of Pfr generated. 11% Pfr gives less than half the maximum response, which is attained with 19% Pfr. Photoequilibrium at 86% Pfr requires a fluence of approximately 800 μmol m^{-2}. Therefore there is a large excess of phytochrome present in the sections relative to the requirement for unrolling.

Taken together, Figs. 3.4 - 3.7 contain a paradox based on the following observations:

1. EGTA appears to inhibit unrolling through depletion of Ca^{2+};
2. Unrolling requires approximately 19% of the initial P_{tot} to be converted to Pfr for full induction;
3. 4h after the initial R treatment approximately 33% of the initial P_{tot} is present as Pfr in EGTA treated sections;
4. The level of Pfr described in '3' plus added Ca^{2+}, fail to induce unrolling 4h after R in EGTA buffer, despite the presence of a fully functional unrolling mechanism;
5. A second R treatment given with Ca^{2+} to EGTA treated sections

Fig. 3.7a, b Relationship between the degree of unrolling measured 24h after R and a) The R fluence applied; b) The percentage of total phytochrome (P_{tot}) generated as Pfr (100% = 0.033 $\Delta\Delta$ A $(g\ FW)^{-1}$) of barley leaf sections.


a
2
1
0
0
400
800
1200
1600
Fluence (μmol m-2)
Δ Width (mm) mean ± S.E. (n = 32)
b
2
1
0
0
50
100
% Pfr

induces full unrolling despite the pre-existence of saturating levels of Pfr.

The paradox resides in the absence of a correlation between spectrophotometrically detectable Pfr and its apparent physiological activity (or lack of it). The situation differs from the well-known Pisum and Zea 'paradoxes' (Chon and Briggs, 1966; Hillman, 1966; Hillman, 1967) where physiological effects are detected indicating the presence of active amounts of Pfr, where none can be detected spectrophotometrically. Instead the converse appears to be true, large amounts of Pfr are detectable accompanied by an apparent lack of physiological activity. This situation bears a resemblance to that encountered during some cases of escape from FR reversibility. For example, Hopkins and Hillman (1966) described the following measurements in etiolated Avena coleoptile sections which exhibit an elongation response to R. The promotion can be partially reversed 5h after R by light sources generating less than 48% Pfr in a photostationary state. Reversibility is lost 8h after the initial R treatment, even though 22% Pfr is still present at this stage. Superficially this also shows appreciable amounts of Pfr that appear inactive. However, the situation described above for EGTA-treated etiolated barley leaf tissue is far more extreme, there being an excess of 'inactive' Pfr detectable above what would be expected, in physiological terms, to induce the response, when supplied with Ca^{2+}, 4h after R. Yet there must be some potentially physiologically active phytochrome present for the leaf sections to be able to respond to Ca^{2+} and R when supplied. Whether or not any of the spectrophotometrically detectable Pfr contributes to this remains to be established.

Conclusions.

1. Initial experiments show that R-stimulated leaf unrolling in barley

is a Ca^{2+}-dependent process. The threshold external Ca^{2+} activity for the response to proceed lies below 40 μM.

2. Over 2-4h in EGTA/R-treated tissue, an escape from responsivity to Ca^{2+} occurs, by which measurable Pfr becomes decoupled from the degree of response observed. This paradoxical situation is resolved by subsequent R treatment. In this sense it resembles an extreme form of the classical-type escape from FR-reversibility.

These two groups of observations comprise the dual lines of investigation to be developed in the remainder of this study:

i. The role of Ca^{2+} in unrolling and

ii. The reality and possible significance behind the apparent change in properties in Pfr seen during EGTA treatment.

CHAPTER 4 : Ca^{2+} AND THE PHOTOCONTROL OF NITRATE REDUCTASE ACTIVITY IN ETIOLATED BARLEY

In the last chapter, an uncoupling of measurable Pfr from the degree of the unrolling response was described during extended EGTA treatment and subsequent supply of Ca^{2+}. The explanation of such observations could reside with a property of, or process associated with, the Pfr molecule. Alternatively they could be due to the behaviour of some early part of the transduction path of unrolling under the control of, but not including Pfr. To ascertain whether or not the 'uncoupling' is specific to unrolling in etiolated barley leaf tissue and not a more general property of phytochrome-regulated processes, it was decided to examine the effect of similar experimental treatments to those described in the previous chapter on another phytochrome-regulated response. The response chosen was the R-stimulated increase in nitrate reductase activity (NRA) in etiolated tissue. This was chosen on the basis of previous determinations of the lag before onset of the response on transfer to white light of magnitude 2 - 4h (Nasrulhaq-Boyce and Jones, 1977), and the duration of the response (24h) (Travis et al, 1970). These parameters are similar to the corresponding properties of the unrolling response, which have previously been determined for barley. The lag time for unrolling has been measured as approximately 4h (Poulson and Beevers, 1970), although a dark pre-incubation can eliminate this, suggesting it is a response to segment isolation. The relatively short pre-incubations used in the present study mean, however, that a significant lag phase will probably exist.

The time course for unrolling to become complete will depend on many variables, including the temperature, buffer, and age of tissue. Sundquist and Briggs (1982) measured the time course of R-stimulated unrolling in etiolated barley sections as taking 20 - 24h to complete, and although there will be some variation around this in the present study, empirical observation confirms it to be of a similar magnitude.

Nitrate reductase (NR) catalyses the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) in plants and fungi, comprising the first step in NO_3^- assimilation to ammonia and thence incorporation into amino acids. The enzyme is a 100-110 kD homodimer with flavin adenine dinucleotide, cytochrome b557 and molybdenum as ubiquitous prosthetic groups (Cambell and Smarelli, 1986). The reaction utilizes NADH or, less frequently,NADPH, to furnish the reducing hydrogens to yield water. Genetically distinct forms of the enzyme exist to accommodate the two different reducing coenzymes (Sorger et al, 1986). Unless specifically stated, the discussion here will be limited to the NADH-dependent NR.

NR occupies the first step of a major biosynthetic pathway, and is thus of considerable potential regulatory importance. Three factors are proposed to combine to induce NR in plants. These are: NO_3^-, Light and an as yet undefined 'plastidic signal'.

There is a considerable body of work concerning the substate-induction of NR by NO_3^- in barley. It has been shown that two forms of NR exist in monocots, including barley: a major NADH specific, NO_3^- inducible NR and a minor constitutive NADPH-specific NR (Campbell and Smarelli, 1986). Somers et al (1986) demonstrated that the induction of barley NR by NO_3^- is due to synthesis of the enzyme rather than activation of pre-existing molecules. Cheng et al (1986) further demonstrated that NO_3^- induces an increase in translatable mRNA for NR. It would thus

appear that NO_3^- operates at the gene level to induce NR although there is also evidence that it may stabilize mRNA in maize (Yamazaki et al, 1986).

The influence of light on NRA is harder to define. Although NO_3^- can induce NRA in dark-grown tissue in some species, light massively increases this (Duke and Duke, 1978; 1979). The coupling of NR to light is a necessary relationship, as without light-generated reduced ferrodoxin to drive the reduction of NO_2^-, toxic NO_2^- concentrations can be generated by NR if fully active (Duke and Duke, 1984).

The initial light-induced rise in NRA in etiolated nitrate-grown barley tissue appears to be due to de novo synthesis of enzyme (Somers et al, 1983). This is supported by the observed lag of about 2h before NRA begins to increase (Nasrulhaq-Boyce and Jones, 1977). However, Johnson (1976) was unable to detect a lag in mustard.

Both red light, through phytochrome, and blue light, through both phytochrome and the blue light receptor are able to induce NRA (Rajasekhar and Oelmuller, 1987). Photosynthesis does not contribute to the process beyond providing a supply of organic carbon as a source of energy or structural units. In barley, however, normal plastid development is necessary for light-induced NRA (Deane-Drummond and Johnson, 1981), but this is not a photosynthetic effect. The identity of the 'plastidic signal' is obscure, but it also appears a necessary prerequisite for NO_3^- induction of NR (Rajasekhar and Oelmuller, 1987). Indeed, the pre-competent phase of NR induction in seed germination may be due to immature plastid states. Certainly the plastidic signal only appears to operate once plastid development has started (Mohr, 1984; Oelmuller et al, 1986).

The heirarchy of interaction between NO_3^-, light and the 'plastidic signal' is not clear. However, given NO_3^- and normal plastid development, light is clearly the limiting factor in the induction of NRA (Duke

and Duke, 1984).

Phytochrome control of NRA was first demonstrated by Jones and Sheard (1972) in Pisum as a low fluence response (LFR). Nasrulhaq-Boyce and Jones (1977) failed to find a similar response in dark-grown barley tissue although this is misleading, as the response has been clearly demonstrated in maize (Rao et al, 1980), wheat (Ramaswamy et al, 1983) and barley (Whitelam, personal communication). Like leaf unrolling, the R-stimulated increase in NRA is a classical LFR responding to short illuminations (< 30 min) and reversed by FR given immediately after R. There is also a high irradiance response (HIR) type induction of NRA (Duke and Duke, 1979). As with most phytochrome-regulated phenomena, little is known regarding the transduction pathways of the LFR and HIR induction of NRA although, perhaps significantly in the context of the present study, it has recently been demonstrated that NR from Amaranthus is Ca^{2+}/calmodulin-activated in vitro (Sane et al, 1987).

Blue light has much the same induction effect as the LFR, although the receptor is different, as the response is not FR-reversible (Rao et al, 1982). This suggests some common elements in transduction. However, there is also the possibility of a direct effect of blue light on the flavin moeity of the enzyme. 'Reactivation' of the CN^--treated enzyme occurs in response to blue light in vitro (Maurino et al, 1983) and there is some evidence for similar processes in vivo (Aryan et al, 1983). Despite these observations, it must be stressed that the in vivo Blue-induction, like the LFR results from de novo enzyme synthesis (Duke and Duke, 1984), and shows a similar time course of induction.

NRA can be measured in several different ways, principally divisible into in vivo and in vitro techniques. The latter undoubtedly give higher enzyme activities following extraction and allow measurements to be made

in non-limiting conditions. However, the enzyme is highly unstable once extracted and protectants such as bovine serum albumen, and polyvinyl-pyrrolidine must be included in the extraction medium (Hageman and Reed, 1980). Activity is assayed either in terms of the NO_2^- generated or depletion of NADH in the assay medium. NO_2^- is determined colorimetrically after a reaction with acidified sulphanilamide reagent and N-(1-Naphthyl)ethylene-diamine dihidrochloride (NED). Colour development *in vitro* can be interfered with by residual NADH and other unknown factors. This necessitates post-assay oxidation and precipitation steps in precise determinations.

Although yielding lower values, enzyme activities obtained *in vivo* do correlate with *in vitro* rates (Hageman and Reed, 1980). *In vivo* determinations will always tend to underestimate NRA, based as they are on an NO_2^- assay. Some NO_2^- loss does occur under dark anaerobic conditions (Radin, 1973). The same comments in relation to the colorimetric NO_2^- assay mentioned above also apply to *in vivo* determinations.

It was decided to assay NRA by an *in vivo* technique as the quickest and simplest available. As well as the drawbacks already discussed, *in vivo* measurements do not exclude the influence of light-affected factors other than the induction of NR which are not present or limiting in the *in vitro* techniques. Examples of these factors include; NO_3^- (Jones and Sheard, 1979), NADH and ATP:ADP ratios (Reed and Canvin, 1982). These factors may limit the usefulness of *in vivo* measurements in the study of NR. However, in physiological terms, NRA is a meaningful quantity in itself, and any R-induced change in its value will serve the purpose of this study if it can be shown to be otherwise independent of unrolling. It must also be noted that light-induced changes *in vivo* involve the induction of NR synthesis so changes in the enzyme level will

be a component of the observed response in vivo.

The first step in examining the induction of NRA by R is to observe changes in NRA with time (if any) in order to establish the profile of the response. In determining this, the protocols were kept as similar as possible to that used in the previous chapter, with NRA being assayed instead of leaf unrolling. The material used and incubation procedures were essentially the same except the tissue was grown using NO_3^- (20 mM) containing water and KNO_3 (5 mM) was added to the buffer. Leaf tissue samples were removed at various times relative to R (5 min) and assayed to generate a time course. NRA was derived from the NO_2^- produced during a 30 min dark incubation in MOPS/Propan-2-ol/KNO_3 at pH 7.5, 33°C. NO_2^- was assayed colormetrically following the sulphanilamide/NED reaction. The results are expressed in $mol(NO_2^-)g(FW)^{-1}h^{-1}$ and are shown graphically in Figs. 4.1 and 4.2.

Fig. 4.1 follows changes in NRA in the short term, over the first hour following R. There is little or no difference in NRA at the beginning of R and 60 min later. However, between these time points there is a gradual increase in NRA peaking around 20 min and thereafter decreasing again. This promotive effect is small, only reaching about 150% of dark control values, but consistent. To the author's knowledge, this is the first examination of changes in NRA to response to R over such a short time scale in barley. Nasrulhaq-Boyce and Jones (1977) did not commence measurements until 1h after the onset of light. The most obvious comparison is with Johnson (1976). Here, no lag was detected in the photoinduction of NRA in mustard. This response appeared independent of protein synthesis, suggesting it was a different response to the longer term, slower changes in NRA observed elsewhere. This is also supported by the absence of a lag before its appearance. It is possible that such

Fig. 4.1 Nitrate reductase activity of etiolated barley leaf sections in MES/NO_3^- buffer at 20°C between t_0 and t_{1h} following R (5 min) at t_0 following a 45 min dark pre-incubation. Results given as mean ± S.D. (n = 3).

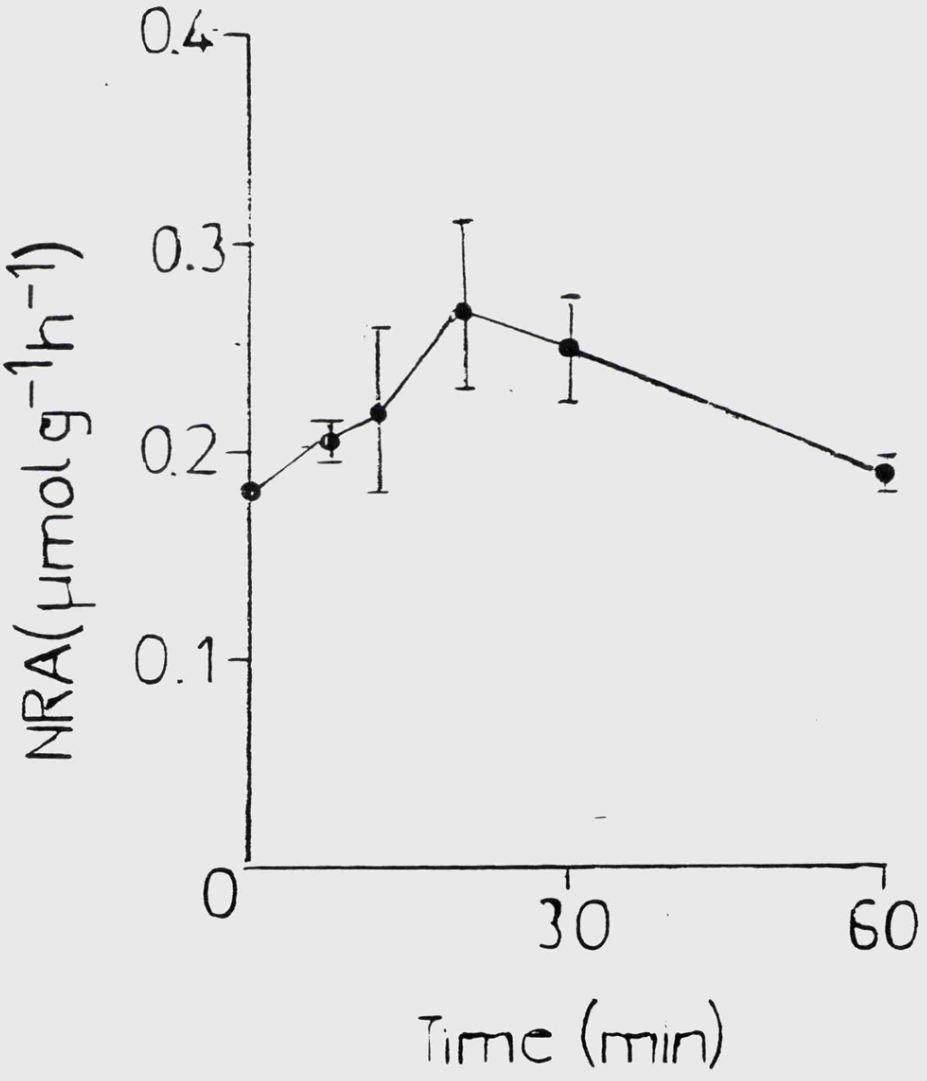
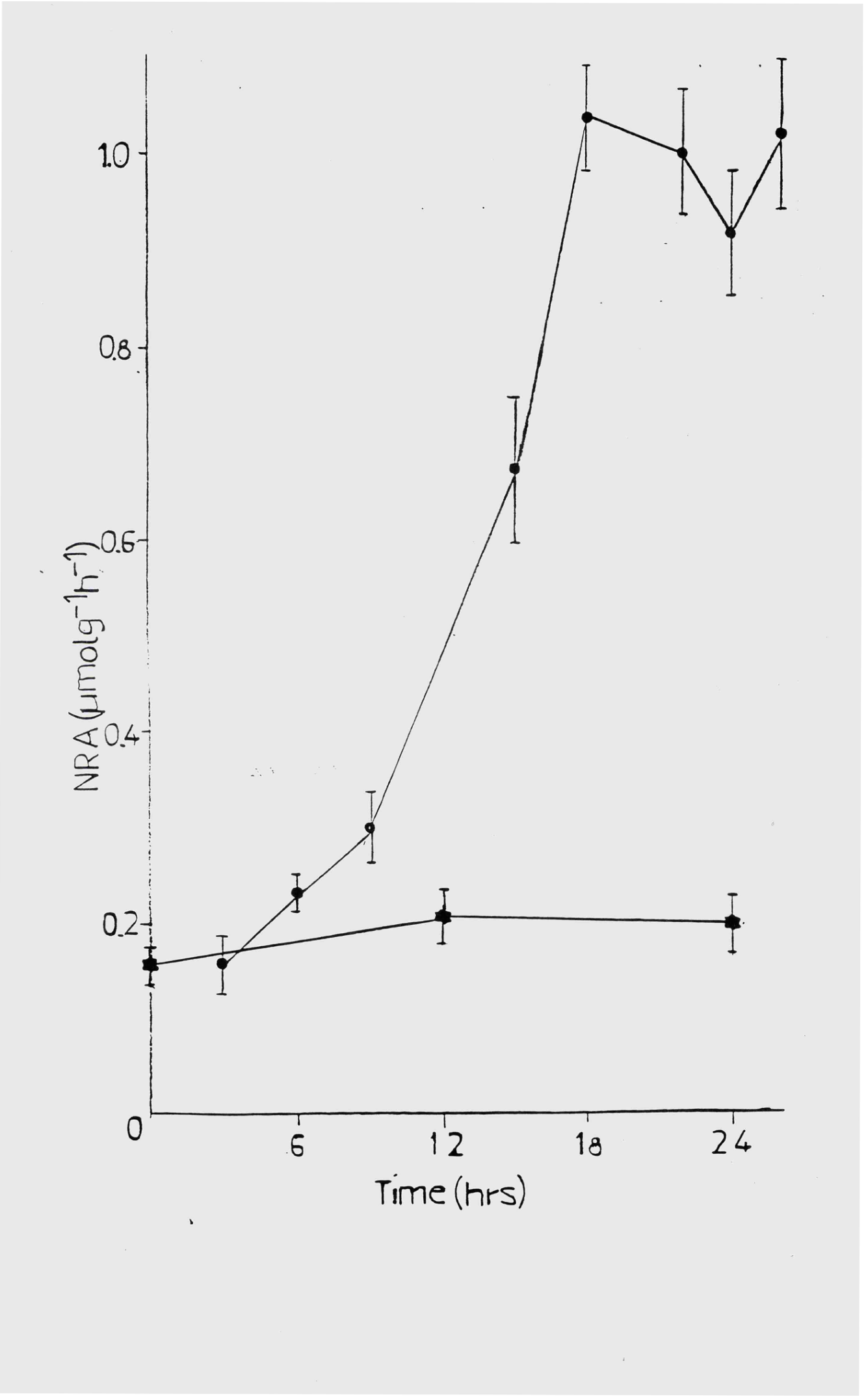

0.4
0.3
0.2
0.1
0
NRA(μmol g⁻¹h⁻¹)
30
60
Time (min)

Fig. 4.2 NRA in etiolated barley leaf sections during dark incubation in MES/NO_3^- buffer at 20°C between t_o and t_{26h}. Upper trace; R (5 min) at t_o: Lower trace; Dark control. Incubation started at $t_{-45\ min}$. Results given as mean ± S.D. (n = 3).


1.0
0.8
0.6
0.4
0.2
0
NRA (μmolg⁻¹h⁻¹)
6
12
18
24
Time (hrs)

rapid responses are the product of using *in vivo* techniques rather than *in vitro*, although the importance of *in vivo* measurements is emphasised in these circumstances.

Fig. 4.2 shows changes in NRA in the longer term, over 26h, following R. The lower curve shows dark control values taken at 0, 12h and 24h through the experiment. No change is detected over this period. In contrast, the NRA in R-treated tissue begins to increase around 6h and continues to rise until 18h after R when a plateau is reached around 500% of the dark control value through to 26h. This confirms the earlier statement that R massively increases the level of NRA in etiolated NO_3^--grown tissue, and, in contrast to Nasrulhaq-Boyce and Jones (1977) shows that barley NRA does respond to short R treatments.

These induction kinetics are somewhat slower than those measured by Rao *et al*, (1980) for maize. There, NRA peaked 4h after R (5 min) and declined to dark control levels at 18h. However the time course of appearance is similar to that obtained by Travis et al, (1970) for induction in dark/NO_3^--grown barley leaves on transfer into continuous white light, in which NRA increased, following a lag of about 6h, to reach a plateau at 24h. On return to darkness a slow decrease over the next 24h was observed in this study, but this was not clearly detected until 12h after the return to darkness, that is, 36h after the onset of illumination. It would therefore appear that both the induction of NRA by light and the decay in its activity are somewhat slower in barley than in maize.

For the purposes of this study, and given the time courses involved, it was decided not to pursue the rapid phase of NRA increase and to concentrate on the much larger, slower second phase, both for its similarities to unrolling in duration and its relatively higher signal to noise ratio.

When assaying NO_2^- to determine NRA, it is necessary to eliminate the possibility that what is being measured is a change in tissue permeability to NO_2^- and not due to enzymic activity changing. This can easily be tested by boiling the tissue prior to the addition of sulphanilamide and NED, and cooling. Boiling disrupts the cells and releases all sequestered NO_2^-. The results of this are shown in Table 4.1.

Although boiling increases the extraction of NO_2^- in both D- and R-treated tissue, the rise in NRA induced by R persists. The increase in NRA caused by R is therefore due to a change in NRA.

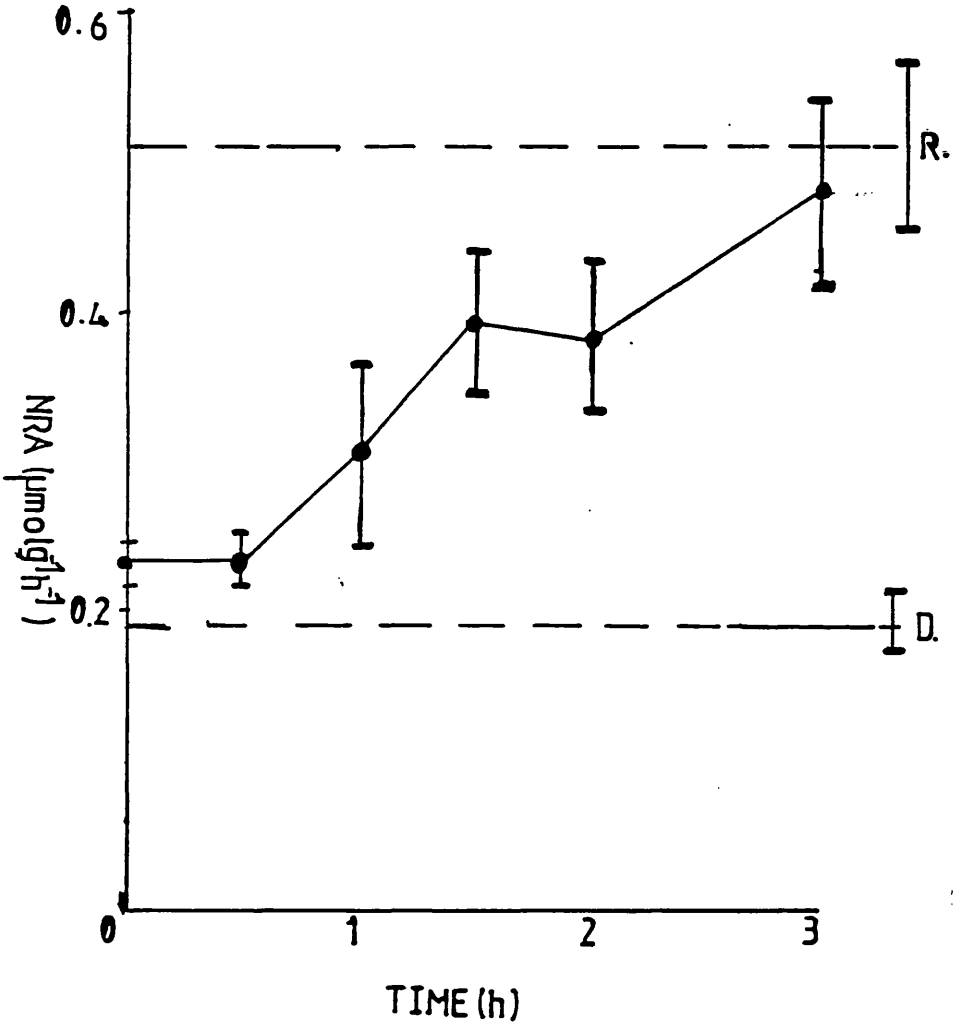
To demonstrate that what was being measured was a classical LFR the escape time from FR-reversibility was determined for the response. Experiments were performed on R-treated tissue in MES/NaOH buffer plus NO_3^- (5 mM) with increasing lags between the R and FR (10 min) treatments from 0 to 3h. The tissue was assayed for NRA as before 20h after the R treatment, during which time the tissue was incubated in the dark in buffer. The results are shown in Fig. 4.3. The magnitude of the R-induced response is seen to be smaller than in the previous figure. This is because a 20 min incubation time MOPS/isopropanol/NO_3^- was used to generate NO_2^- for assay rather than 30 min as used previously. This lowers the apparent NRA because of a lag of 5 - 10 min before NO_2^- starts to accumulate (Hageman and Reed, 1980), which will be proportionately greater for a 20 min incubation combined to a 30 min incubation. The cause of the lag is not clear, but may indicate time taken for the substate, NO_3^- , to penetrate sections, the isopropanol to permeablize the tissue, or simply for sufficient NO_2^- to accumulate under anaerobic conditions to diffuse out of the tissue.

FR given up to 30 min after R treatment almost completely removed the effect of R on NRA. The response therefore shows classic LFR photo-

Table 4.1 Effect of boiling on NRA in dark and R-treated etiolated, NO_3^--grown barley leaf tissue measured 20h after R (5 min).

	NRA ($\mu molg^{-1}h^{-1}$)	NRA (boiled)($\mu molg^{-1}h^{-1}$)
Dark	0.180 ± 0.0 72	0.231 ± 0.094
R	0.872 ± 0.016	1.248 ± 0.251

Fig. 4.3 The effect of delayed FR (until the times indicated) following R (5 min at t_0) on NRA measured at t_{20h} following a dark incubation in etiolated barley leaf sections in MES/NO_3^- buffer (mean ± S.D; n = 3).


0.6
0.4
0.2
0
NRA (μmol g⁻¹ h⁻¹)
R.
D.
1
2
3
TIME (h)

reversibility. If delayed beyond this time, FR is progressively less effective in removing the R effect until, 2 - 3h after R, the system becomes refractive to FR. The FR-escape time is thus between 2h and 3h.

Previous estimates of FR-escape time for NRA have shown considerable variation between species. In general, dicotyledonous species exhibit rapid escape, as little as 5 min for cauliflower florets (Whitelam et al, 1979) upto 15 min for mustard cotyledons (Starr et al, 1980). Monocotyledonous species would appear to show much slower escape kinetics on the basis of this study for barley leaves, and an escape time of approximately 2h for maize (Sharma and Sopory, 1984). The escape from FR-reversibility and onset of response in both barley (this study) and maize Rao et al, 1980) very nearly coincides. This argues against the development of a phytochrome-independent stable intermediate state in the transduction of the response, contrary to the inference of Sharma and Sopory (1984) and Rajasekhar and Oelmuller (1987).

The similar time-courses to the onset of a response and escape times for NRA and unrolling following R poses the question of how closely the two are related, if at all, beyond the initial requirement of Pfr generation. If one were wholly or partially dependent upon the other the objectives outlined at the beginning of this chapter could not be achieved. Three observations have a bearing upon this. The first is the fact that R-induction of NRA does not appear to be restricted to any specific plant organ (Duke and Duke, 1984), occurring in both floral (Whitelam et al, 1979) and root (Augsten and Michel, 1981) tissue, as well as leaves. It would therefore appear that NRA does not depend on leaf unrolling <u>per se</u>. Similarly, when barley seedlings are grown in the dark without added NO_3^-, that is, under non-inductive conditions as in Chapter 3, leaf unrolling still occurs in the presence of much reduced NRA

induction. Therefore leaf unrolling does not appear to correlate with the amount of NRA induced. The two processes are not completely independent, since both require Pfr, and so must share some common elements. To examine this further, dose/response determinations were performed for NRA and the results expressed in terms of the amount of Pfr generated by the light dose, measured as in Fig.3.6 and shown in Fig. 4.4.

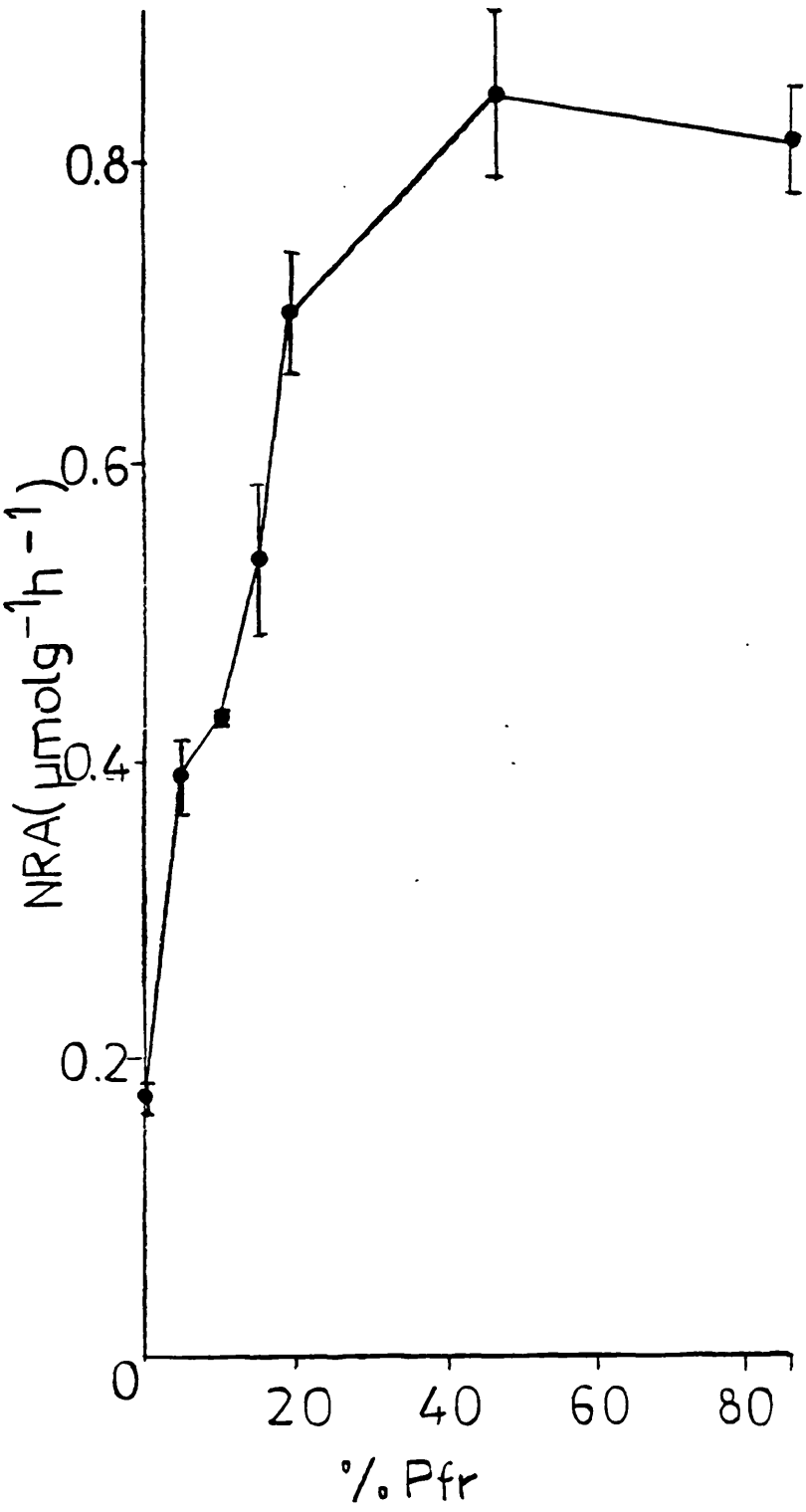
A linear positive relationship between Pfr and NRA, measured 20h after R, appears to operate upto approximately 20% Pfr. Whitelam et al (1979) found a similar relationship in cauliflower floret tissue. This indicates that, under inductive conditions, NRA is limited by Pfr levels in etiolated tissue. This ceases to apply above 20% Pfr, and the response requirement saturates between 20 and 40% Pfr. NRA induction by R is therefore rather less sensitive to Pfr levels than leaf unrolling, which clearly saturates below 20% Pfr (Fig. 3.6). Why this should be is not clear, although it provides further evidence for the essentially separate nature of the NRA and unrolling responses to R.

Using the above observations as a basis for assuming that the two responses are unrelated, it is now possible to examine the effect of EGTA and Ca^{2+} treatments on R-induced NRA. By comparison with the results of Chapter 3, the discrimination of standard phytochrome-specific effects from unique properties of each response in the context of Ca^{2+}/EGTA treatment is possible.

The theoretical approaches and rationale of the experiments to investigate this were essentially the same as the parallel experiments in Chapter 3, as were the treatment protocols. As such only brief descriptions of experimental procedures are necessary.

Tissue was incubated in EGTA buffer, as in Chapter 3, with the addition of 5 mM KNO_3. After 45 min, R (5 min) was given and Ca^{2+} (5 mM)

Fig. 4.4 Relationship between NRA measured at t_{20h} following R at t_o, and the percentage total phytochrome present as Pfr [100% = 0.033 $\Delta\Delta$ A.(g FW)$^{-1}$] measured in response to increasing R doses at t_o in etiolated barley leaf sections (mean $\pm$ S.D; n = 3).


0.8
0.6
0.4
0.2
0
NRA(μmolg⁻¹h⁻¹)
20
40
60
80
% Pfr

supplied at various times relative to this in a similar manner to the experiment shown in Fig. 3.4. NRA was assayed 20h after R. The results are shown in Fig. 4.5, and bear a clear resemblance to those in Fig. 3.4. EGTA treatment inhibits the appearance of NRA, and Ca^{2+} supplied within 40 min of R can substantially overcome this. However, by 1h after R the effectiveness is much reduced and, by 2h, is completely lost. The appearance of NRA is therefore Ca^{2+}-dependent, and undergoes 'escape' from responsivity to Ca^{2+} during EGTA treatment within 2h of R.

This work was extended, as before, to test whether or not the observed phenomenon is a result of EGTA toxicity and the results are similar to those obtained for unrolling in Chapter 3. Fig. 4.6 shows the effect of a second R treatment, given 2h after the first, during EGTA treatment, with, and without the concomitant addition of Ca^{2+} on NRA 20h after the first R. This is a similar protocol to that shown for Fig. 3.5. The second R treatment is ineffective in inducing NRA unless Ca^{2+} is supplied, neither Ca^{2+} or R alone given 2h after the first R being effective. Therefore, the results shown in Fig. 4.5 are not due to toxic EGTA effects.

Given that Fig. 3.6 shows loss of Pfr in similar circumstances to, and tissue samples as those described here; and by applying the same arguments used as in Chapter 3 to the data in Figs. 4.5 and 4.6, the same paradox as described for unrolling is arrived at for the NRA response. This is that measurable Pfr ceases to correlate with the observed response, indicating a decoupling that can only be resolved by subsequent R treatment. Indeed the paradox is more acute in the case of the NRA response in that the second R is given after a shorter period when reasonable Pfr levels are correspondingly higher. This presumably also applies to unrolling although it has not been tested for the effectiveness of a

Fig. 4.5 The effect of delayed Ca^{2+} (5 mM) treatment, until the times indicated, on NRA measured at t_{20h} in R-treated (5 min at t_0) barley leaf sections in MES/EGTA/NO_3^- buffer following a 45 min pre-incubation. Upper dashed line; NRA in R-treated controls in MES/NO_3^- buffer. Lower dashed line; NRA in MES/NO_3^- buffer in the absence of R-tratment (mean ± S.D., n = 3).

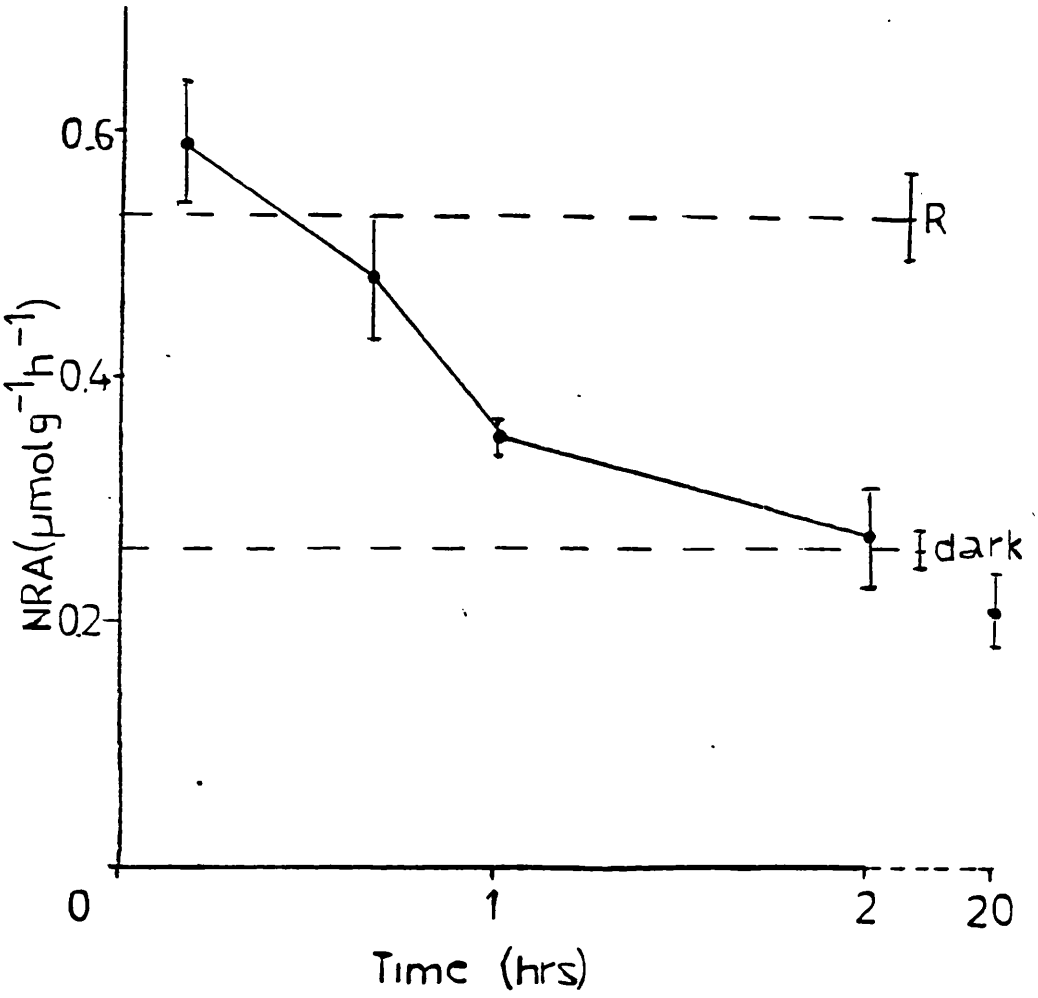
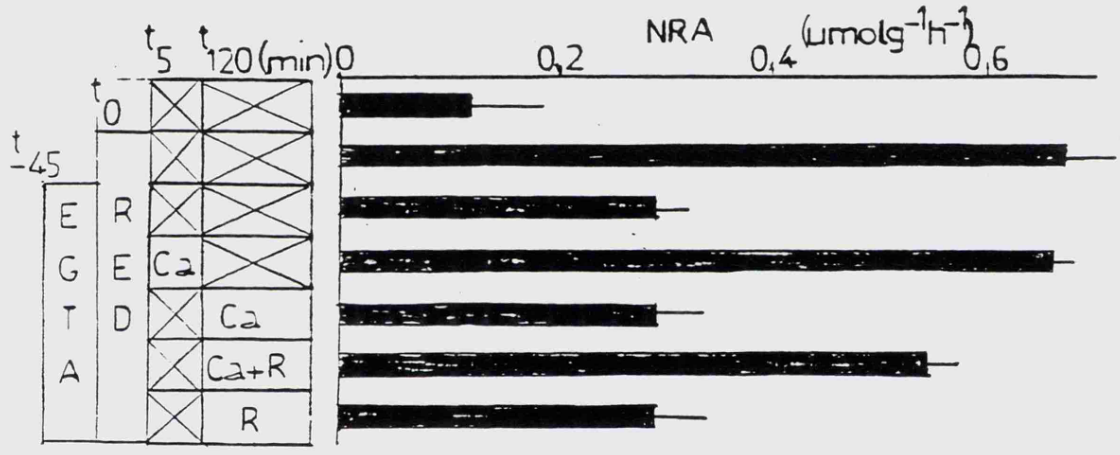

0.6
0.4
0.2
0
NRA(μmol g⁻¹ h⁻¹)
R
dark
1
2
20
Time (hrs)

Fig. 4.6 NRA measured at t_{20h} after R (5 min at t_o) in MES/EGTA/NO_3^- buffer: the effect of delayed Ca^{2+} (5 mM) and a second 5 min supplementary R-treatment, both given at t_{2h} (mean ± S.D., n = 3).


NRA
(µmolg⁻¹h⁻¹)
0
0,2
0,4
0,6
t5
t120 (min)
t0
t-45
EGTA
RED
Ca
Ca
Ca+R
R

shorter delay before the second R treatment with Ca^{2+}.

The previous grounds noted for assuming the independence of unrolling and NRA induction, and the close similarity between Figs. 3.4 and 4.5, and Figs. 3.5 and 4.6 respectively, indicate that the basic cause of the paradox lies with a property of, or in close relation to, the phytochrome molecule itself. This is reinforced by the relationship between escape from FR reversibility and escape from responsiveness to Ca^{2+} during EGTA treatments. For both NRA induction and unrolling the 'Ca^{2+} escape time' appears less than the 'FR escape time'. Apart from this similarity, both NRA and Ca^{2+}-based 'paradoxes' involve uncoupling of measurable Pfr from degree of response, both are overcome by subsequent R and both occur over broadly similar time scales. It is therefore possible that both are manifestations of the same process, a putative Pfr signal attenuation mechanism. The slight mismatch of time scales involved for the two processes may be due to the fact that escape from Ca^{2+} responsivity is only detectable in the presence of EGTA, and the attenuation process itself may be accelerated in the absence of Ca^{2+}. There are certainly logical objections to envisaging a process that appears to involve deactivation of the phytochrome signal within the FR escape time, since FR reversibility would seem to demand 'active' Pfr to be present as a prerequisite. If it is indeed the case that the two phenomena represent the same process, it should be possible to duplicate these effects with any treatment which blocks the response in question, and which can be removed as required. Although unrolling is not immediately amenable to such experiments, NRA induction provides an excellent system to test this hypothesis via the manipulation of NO_3^- availability.

It has already been stated that NRA is dependent on the presence of NO_3^- for full induction by R. By omitting the addition of NO_3^- (20 mM)

during the growth of material, the required block on the response is provided which can be removed by the addition of NO_3^- (5 mM) at suitable times to MES/NaOH buffer lacking added NO_3^-. The experimental protocols were essentially the same as used in Figs. 4.5 and 4.6 except that material was grown and incubation buffer prepared with the omission of NO_3^- as described above, and NRA was assayed 24h after R. The results are shown in Figs. 4.7 and 4.8.

Fig. 4.7 shows the effect of supplying NO_3^- (5 mM) at various times relative to R (5 min) on NRA 24h after R. The magnitude of the response is considerably reduced (0.4 $\mu molg^{-1}h^{-1}$ maximum) compared to that observed with tissue pretreated with NO_3^-, indicating that NO_3^- is, to some extent, required prior to light treatment for full induction. In the absence of added NO_3^- no induction occurs, therefore R-stimulated NRA has an absolute NO_3^- requirement. This is in agreement with Deane-Drummond and Johnson (1980) who showed that the constitutive NRA of barley does not undergo photoinduction, in contrast to the situation in soy bean (Duke and Duke, 1984). Witholding NO_3^- for increasing periods beyond the R treatment leads to a progressive decline in NRA induced until, between 1h and 2h escape from responsivity to NO_3^- occurs.

That the effect shown in Fig. 4.7 is phytochrome related is shown in Fig. 4.8. A second R treatment, ineffective by itself, induces NRA when NO_3^- is supplied. Escape from FR reversibility, responsivity to Ca^{2+} and responsivity to NO_3^- would therefore appear to share several common properties. It is suggested here that they are all manifestations of the same process.

Fig. 4.7 apparently contradicts the study of Sharma and Sopory (1984), which demonstrated responsivity to added NO_3^- in maize, after FR-escape, upto 8h after R. From this observation a heirarchy of inducing

Fig. 4.7 The effect of delayed NO_3^- (5 mM) treatment, until the times indicated on NRA measured at t_{20h} in R treated (5 min at t_0) barley leaf sections grown without added NO_3^-, incubated in MES buffer (mean ± S.D., n = 3).

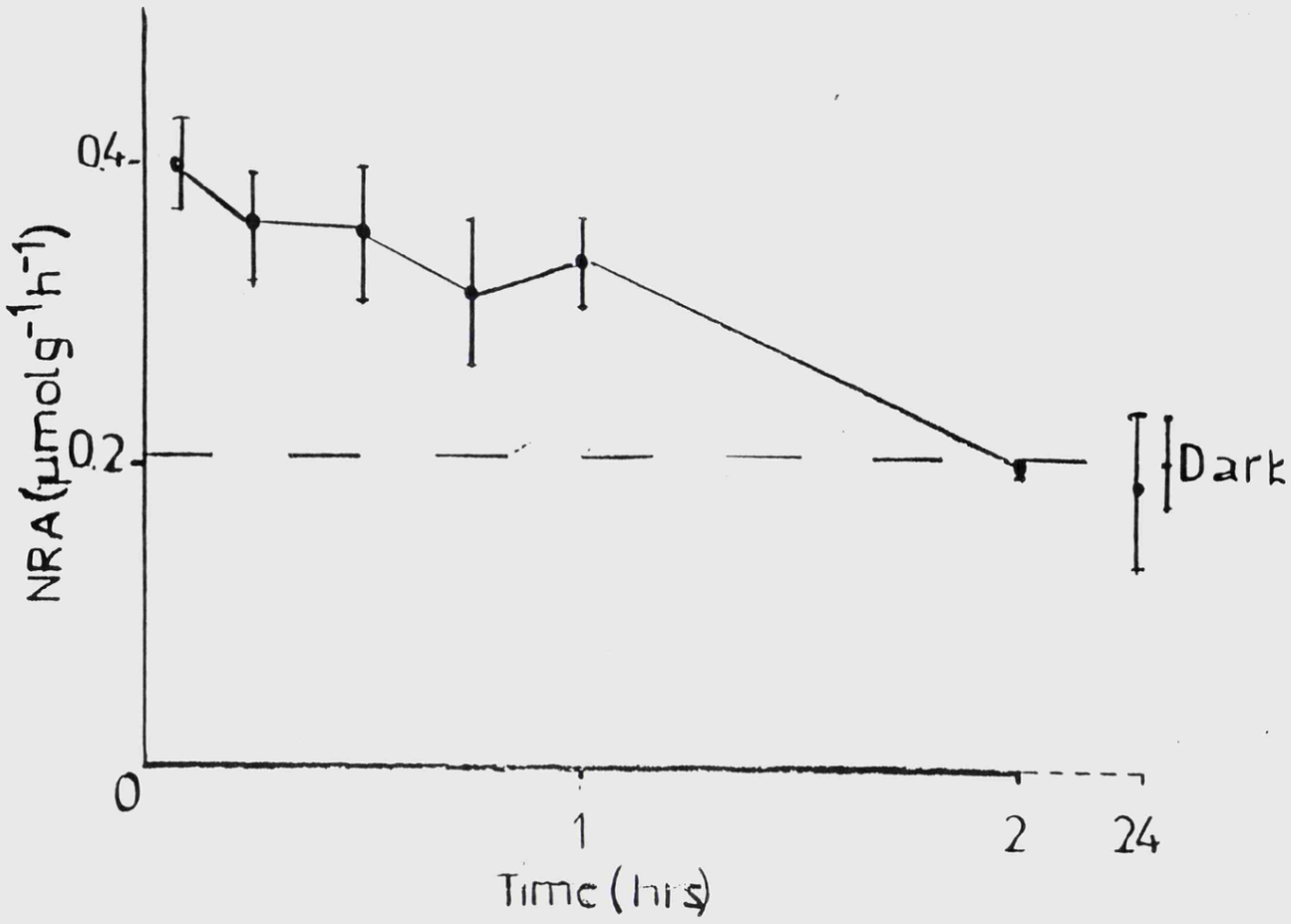
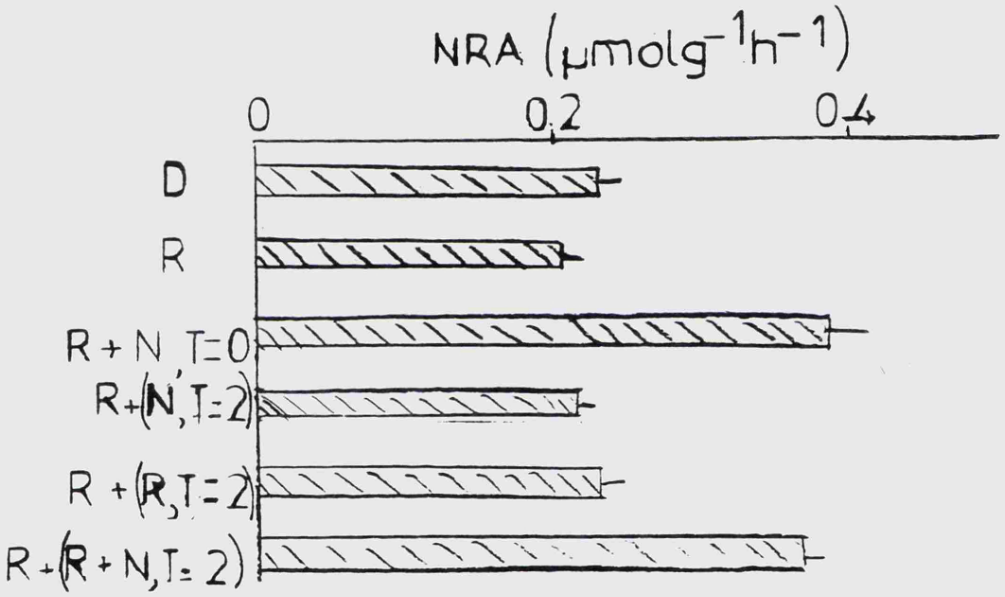

0.4
0.2
0
NRA (μmol g⁻¹ h⁻¹)
Dark
1
2
24
Time (hrs)

Fig. 4.8 NRA measured at t_{20h} after R (5 min at t_0) in non-NO_3^--grown etiolated barley leaf sections, incubated in MES buffer: the effect of delayed NO_3^- (N) and a second R treatment, both given at t_{2h} (mean ± S.D., n = 3).


NRA (μmolg⁻¹h⁻¹)
0
0,2
0,4
D
R
R + N, T=0
R + (N, T=2)
R + (R, T=2)
R + (R + N, T=2)

factors for NRA in which NO_3^- dominates over the light requirement was proposed. The loss of effectiveness of NO_3^- in the present study indicates a different situation to that in maize, although need for NO_3^- prior to R for full induction does indicate a heirarchical relationship between inducting factors.

Conclusions.

1. Unrolling and NRA induction are essentially distinct and independent phytochrome responses.
2. A process operates during an arrested phytochrome response, at least for unrolling and induction of NRA, that attenuates the Pfr signal, which can only be restored by subsequent R.
3. In many respects the process resembles that which causes FR escape and may be identical with it.
4. The induction of NRA by R in barley is Ca^{2+}-dependent, as well as requiring the presence of NO_3^- and, according to previous workers, the 'plastidic factor' (Deane-Drummond and Johnson, 1980; Rajasekhar and Oelmuller, 1987). This Ca^{2+} requirement *in vivo* is a new finding although the enzyme is activated by Ca^{2+} *in vitro* (Sane et al, 1987).
5. NRA induction in etiolated barley in response to R is a two phase process, the second phase at least requiring some prior exposure to NO_3^-.

Like unrolling, the NRA induction response has generated two areas of interest, one centred around the attenuation of the Pfr signal and the other around the role of Ca^{2+} in the transduction of the signal. Possible mechanisms of attenuation are examined in the next chapter, but the role of Ca^{2+} in NRA induction lies outside the scope of this study and time and space do not allow its pursuit here. It is, however, of some

relevance to general patterns of the role of Ca^{2+} in phytochrome responses. To this end, some relevant data are presented in Chapter 6.

CHAPTER 5 : THE MECHANISM OF UNCOUPLING AND RECOUPLING OF MEASURABLE Pfr AND RESPONSE

The roots of all the so-called 'phytochrome paradoxes' lie in the divorce between spectrophotometric measurements of Pfr and measurable responses. Most workers, from Hillman (1967) through Kronenberg and Kendrick (1986) have invoked some sort of compartmentation to explain these discrepancies, and suggested 'bulk' and 'active' pools of phytochrome. Although the specific details vary, it is generally suggested that undetectably small pools of 'active' phytochrome exist which, during *in vivo* measurements, are swamped by the signal from the 'bulk' of the phytochrome. Such explanations work well for the very low fluence responses (VLFRs) such as the *Zea* paradox and can easily be adapted to the escape from responsivity to Ca^{2+} discussed in this study. In support of such notions, the existence of unstable dark and stable 'green' phytochrome as separate species is often cited, as is the fact that 124 kD undegraded *Avena* phytochrome can be induced to dark revert by the binding of a monochonal antibody *in vitro* (Vierstra and Quail, 1986). This indicates that the properties of phytochrome can be modified by its molecular environment and hence its cellular location. However, there are problems with this concept in that there is a danger of explaining away observations in terms of the undetectable or unmeasurable. It can be argued that this is unscientific, placing speculation about the mode of action of phytochrome in a realm not amenable to experimental manipulation and yielding few testable predictions.

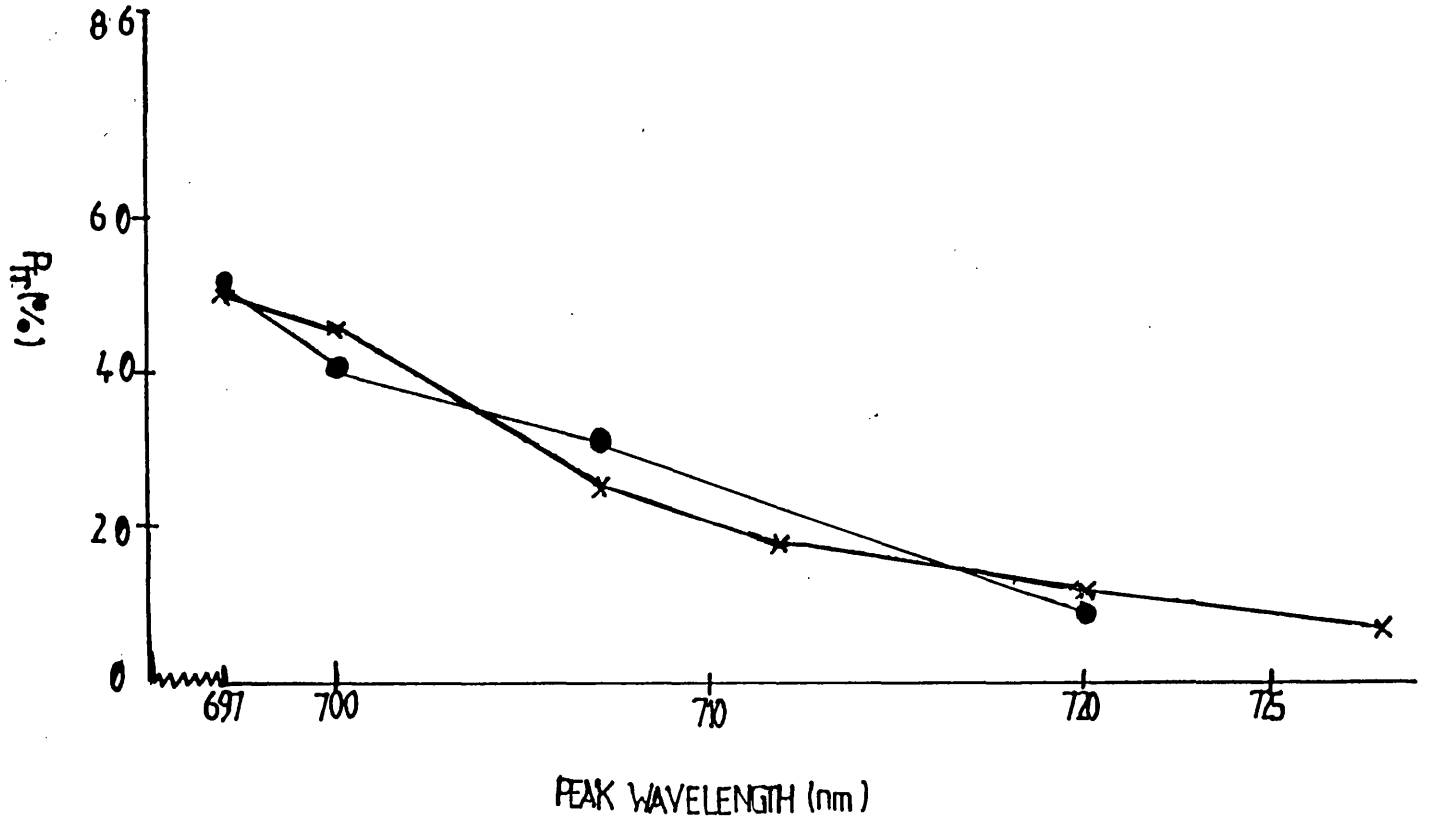
Kronenberg and Kendrick (1986) question the necessary corollary of the 'active' phytochrome concept, that the majority of phytochrome in etiolated plants is non-functional, as being contrary to all previous experience.

In seeking to explain the paradox residing in the escape from responsivity to Ca^{2+} during leaf unrolling, or the induction of NRA in etiolated barley leaves, it was decided to avoid the theoretical difficulties of the type described above by using the radical differences between the Ca^{2+}/phytochrome paradox and the other phytochrome paradoxes to experimental advantage. Unlike the Zea, Pharbitis or Pisum paradoxes, the escape from Ca^{2+} responsivity involves an unmodified low fluence response occuring in the presence of detectable Pfr throughout. As such it is well characterised and more open to experimental manipulation. The divorce that occurs in the uncoupling of responsivity from measured Pfr level, must presumably have followed a 'marriage' of some kind. Without stretching the analagy too far, it is possible to design experiments to see if this is indeed the case. Using a range of narrow band interference filters and a projector, Pfr/P_{tot} was measured for the photostationary states generated after 15 min illumination for peak wavelengths over the range 697 nm to 728 nm. Both Pfr and Pr absorb light in this range and, given time to equilibrate, a photostationary state where interconversions between Pfr and Pr are equal is attained. If the relative efficiences of interconversion between Pr and Pfr, or the immediate stability of Pfr and/or Pr are affected by their molecular environment, this should show up as a deviation in the photostationary state obtained when this environment changes. Thus by examining the behaviour of phytochrome in two very different environments, purified in vitro and measured in vivo any such deviation should be apparent.

Purified 124 kD Avena phytochrome was compared to in vivo $\Delta\Delta$ A measurements on etiolated barley leaf tissue. Measurements were performed at 0°C reading at 600 and 730 nm for Avena phytochrome, and 730 nm and 800 nm for barley leaf tissue and are expressed as percentage Pfr at photostationary state in Fig. 5.1. This shows that over the range of peak illumination wavelengths from 697 nm to 720 nm there is a steady decline in the percentage Pfr at photostationary state for both purified Avena phytochrome and barley phytochrome in vivo. The decline is from approximately 50% Pfr to 9 - 12% Pfr. The decline continues for barley phytochrome in vivo to 7% at 728 nm. This was not measured for Avena phytochrome. Throughout the range the results for barley in vivo and purified Avena phytochrome follow each other closely even though their molecular environments are markedly different. They are also broadly similar to the determinations of Lagarias et al (1987), also for purified 124 kD Avena phytochrome. The slightly higher values obtained here are probably the result of using different filters to generate photostationary states. Therefore, within the limits of this comparison, it appears that the Pfr/P_{tot} set up by a given light treatment is an intrinsic property of the molecule itself and not dependent on the environment of the molecule. It follows from this that the initial dose/response relationship enshrines the 'marriage' of measurable Pfr and response. Divorce is a subsequent process.

If measurable Pfr and the unrolling response for example are initially coupled and subsequently uncoupled during Ca^{2+}-deprivation (or NO_3^- deprivation in the case of NRA induction), the question arises as to whether or not recoupling occurs when the second light treatment is supplied with Ca^{2+} (or NO_3^- for NRA induction). Until now saturating light doses have been used and the presence of large amounts of Pfr before

Fig. 5.1 Phytochrome photostationary state measurements: effect of increasing peak transmitted wavelength from actinic source via interference filters. (o——o) 124kD *Avena* phytochrome; (x——x) *in vitro* measurements on etiolated barley leaf tissue. Measurements derived from $\Delta\Delta A$ signal between 660 nm and 730 nm (*Avena* phytochrome) and 730 m and 800 nm (barley leaf tissue) at $0^{o}C$.

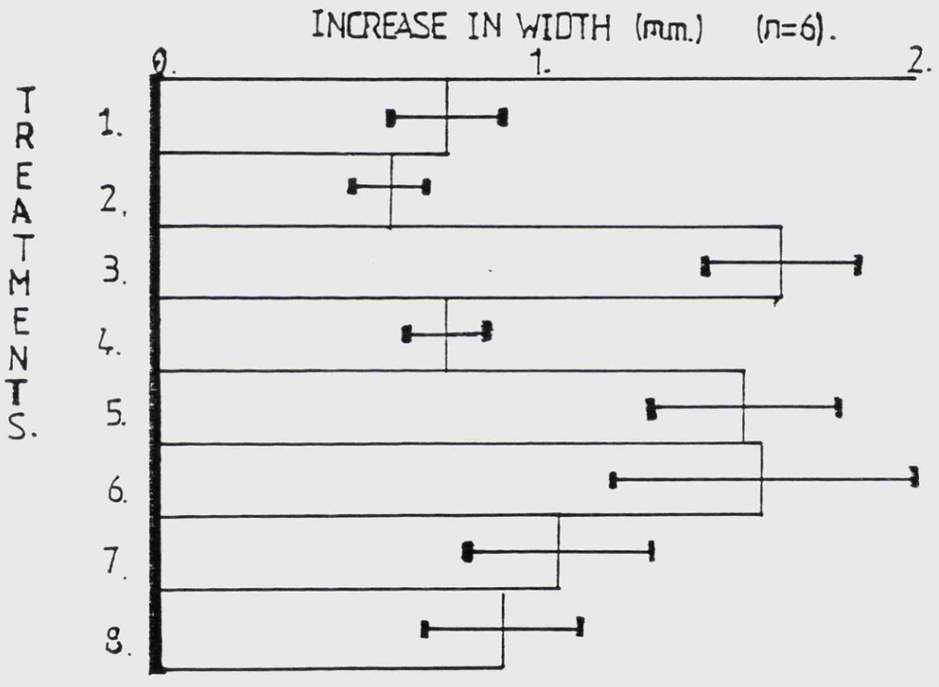

80
60
40
20
0
P_{FF}(%)
697
700
710
720
725
PEAK WAVELENGTH (nm)

the second illumination precludes the use of subsaturating doses to obtain a dose/response relationship in terms of Pfr. However, using the filters as described above to set up photostationary states, it is possible to reduce the measurable Pfr level when giving the second light treatment to a range of values from 7% Pfr through to 50%, thus 're-setting' the Pfr levels throughout the tissue. If true recoupling is occuring then it may be predicted that the dose/response relationship will be the same as for the first light treatment, as shown in Fig. 3.7b for leaf unrolling. The results of this determination are shown for the unrolling response in Fig. 5.2. Leaf sections in EGTA buffer were R-treated, Ca^{2+} withheld until 4h later and then illuminated for 15 min using projector and filter combinations giving 25%, 18%, 12% and 7% Pfr (as determined in Fig. 5.1). As seen before Ca^{2+} withheld until 4h after R had lost its effectiveness in confering responsivity to R. Light sources generating a photostationary state greater than 18% gave a full response when Ca^{2+} was supplied 4h after R. Sources generating 12% and 7% were ineffective in inducing the response. This agrees closely with Fig. 3.7b, where 19% Pfr gave the maximal response and 11% Pfr very little promotion. Therefore it is possible to recouple the uncoupled response in such a way that an identical dose/response relationship is obtained. This indicates that the effect of the second light treatment is essentially the same as the first and that the first need not make any contribution to the response if the necessary 'coupling factor' (Ca^{2+} in this case) is absent long enough for uncoupling to occur. The mechanism of the paradox would therefore appear to operate as follows: Red light sets up sufficient Pfr to induce the response; but, due to the absence of a coupling factor, transduction does not proceed to any stable intermediate state. As time after R increases, and the coupling factor is withheld, an unspecified change

Fig. 5.2 Photostationary state (Pfr) dose/response determination for second supplementary light treatment at t_{4h} following incubation in MES/EGTA buffer, R (5 min) at t_o and Ca^{2+} (5mM) at t_{4h}. Unrolling measured at t_{24h} following dark incubation. Photostationary state treatments = 15 min duration, Pfr levels derived from Fig. 5.1. (Mean ± S.D., n = 6).

Treatments:

1. None (= Dark control), MES buffer
2. R at t_o, MES/EGTA buffer
3. R at t_o, Ca^{2+} at $t_{5\ min}$, MES/EGTA buffer
4. R at t_o, Ca^{2+} at t_{4h}, MES/EGTA buffer
5. As treatment '4' + source generating 25% Pfr at t_{4h}
6. As treatment '4' + source generating 18% Pfr at t_{4h}
7. As treatment '4' + source generating 12% Pfr at t_{4h}
8. As treatment '4' + source generating 7% Pfr at t_{4h}



(For Treatments 1-8 see Legend.)

occurs such that the activity of phytochrome is progressively lost, and when the coupling factor is supplied, there is no inducing signal even though Pfr remains. The plant tissue has 'forgotten' its exposure to light. Only by creating new Pfr can the response be induced, and this induction operates exactly as if the tissue has never been illuminated, utilizing the same transduction path to completion.

These data are of relevance to the proposal of Smith (1983), that Pfr is not the sole active form of phytochrome, but that activity may result from antagonistic actions of Pfr and Pr, such that the ratio Pfr/Pr or Pfr/P_{tot} may represent the active component. The question as to what the active species of phytochrome is remains unanswered to date, although if phytochrome operates in a ratio-based mechanism then responses would be predicted to be independent of phytochrome concentration within reasonable limits. This is clearly demonstrated by Figs. 3.6, 3.7 and 5.2 in combination. The fact that the proportion of phytochrome as Pfr for a given degree of unrolling remains constant while the measurable P_{tot} declines by nearly 50% is strong evidence for the hypothesis. This being the case, uncoupling could be viewed as a time-dependent inhibition of phytochrome activity and recoupling, the light-driven reactivation requiring phytochrome photoconversion. The main weakness of this scenario is its dependence on in vivo spectrophotometric measurements of phytochrome in bulk. An alternative explanation of the data in terms of 'active' Pfr would propose that the loss of phytochrome seen in Fig. 3.6 is not relevant to the response and that a stable sub-population of phytochrome is functionally active in Pfr. This explanation requires; i) that: the 'bulk' phytochrome be functionally irrelevant; ii) that this bulk is specifically degraded following photoconversion to Pfr, and iii) that a specific separate deactivation process operates on the

'active' Pfr pool. Furthermore a source of 'new' Pfr is required to allow recoupling.

As previously stated, the concept of bulk and active phytochrome proposed by Hillman (1967) has been attacked by Kendrick and Kronenberg (1986) as illogical on the grounds that the reaccumulation of bulk phytochrome to high levels seen on return of a plant to darkness, and its autoregulation both indicate functional importance. They further suggest that the destruction/accumulation process is analogous to the accommodation mechanism of vertebrate vision. This is more compatible with the ratio-based hypothesis of action. A more complete evaluation of the two hypotheses in the context of the unrolling response is clearly warranted. It has already been noted that the ratio hypothesis can adequately explain all the data, but that the Pfr-active hypothesis requires that certain conditions be met prior to this. Although not disproven, the criterion of 'bulk-irrelevance' has been shown to be untestable by virtue of the impossibility of defining an 'active' pool of phytochrome within present techniques.

The second condition, that a specific deactivation process occurs whereby Pfr loses its functional capacity with time is not a new idea. Haupt and Reif (1979) demonstrated that Mesotaenium cells 'forgot' a brief R treatment that sensitized cells to subsequent continuous R or blue treatments if a lag greater than 10 min was introduced between treatments. This observation is consistent with either hypothesis of action and serves only to reinforce the case for some time-dependent deactivation of phytochrome molecules that is separate from destruction.

The third condition does allow experimental approach. If recoupling is to occur in a stable pool of phytochrome after deactivation, then a source of responding phytochrome molecules in the recoupled response may

be defined and tested for. These are: pre-existing Pr (old Pr), Pr synthesized since the first light treatment (new Pr) and Pfr left over from the first light treatment (old Pfr). This last category may be divided into deactivated Pfr and old Pr that is still active but present at sub-threshold levels for induction. Any of these categories could individually, or in combination, be responsible for the response following recoupling.

The maximum photoconversion of Pr to Pfr is estimated at approximately 86% (Vierstra and Quail, 1983). Thus 14% of the initial Pr remains as old Pr after the first light treatment. Assuming that Pfr is the active form of phytochrome it can be shown that old Pr alone is not enough to induce a response after recoupling and conversion to Pfr. Even under saturating conditions in the absence of any remaining Pfr in the responding compartment, the new Pfr generated from old Pr would only constitute 86% x 14% (initial P_{tot}) = 12.04%. Since it has already been shown that measurable phytochrome is coupled to the response at the level of dose/response determination, this is seen to constitute insufficient Pfr for induction according to Figs. 3.7 and 5.2. Therefore old Pr cannot totally fulfil the requirement.

It is possible that this shortfall is met from <u>de novo</u> synthesis of Pr. New Pr alone would appear to be equally incapable of supplying the requirement given the very low rate of accumulation seen in Fig. 3.6 (approximately 0.00035 $\Delta\Delta$ A $g^{-1}h^{-1}$ or 1% of initial P_{tot}). However, accelerated synthesis in the 'active' compartment is a possibility that could be masked against a general background signal. This possibility was examined by the use of a specific inhibitor of tetrapyrrole biosynthesis, gabaculin. The inhibition of synthesis of the phytochrome tetrapyrrole chromophore by gabaculin was first described by Gardner and

Gorton (1985). Tetrapyrrole structures including chlorophyll as well as the phytochrome chromophore are built up from 5-aminolevulinic acid (ALA) which undergoes a dimerization and dehydration step to form porphobilinogen, the basic structural unit of all pyrrole compounds. The subsequent reactions to form cyclic tetrapyrroles and the opening of the ring structure to form linear tetrapyrroles are well characterised and have been detailed by Krogman (1973).

Gabaculin (3-amino-2,3-dihydrobenzoic acid) has previously been used to inhibit phytochrome synthesis in peas, oats and maize with varying degrees of success (Gardner and Gorton, 1985; Elich and Lagarias, 1987), but never before in barley.

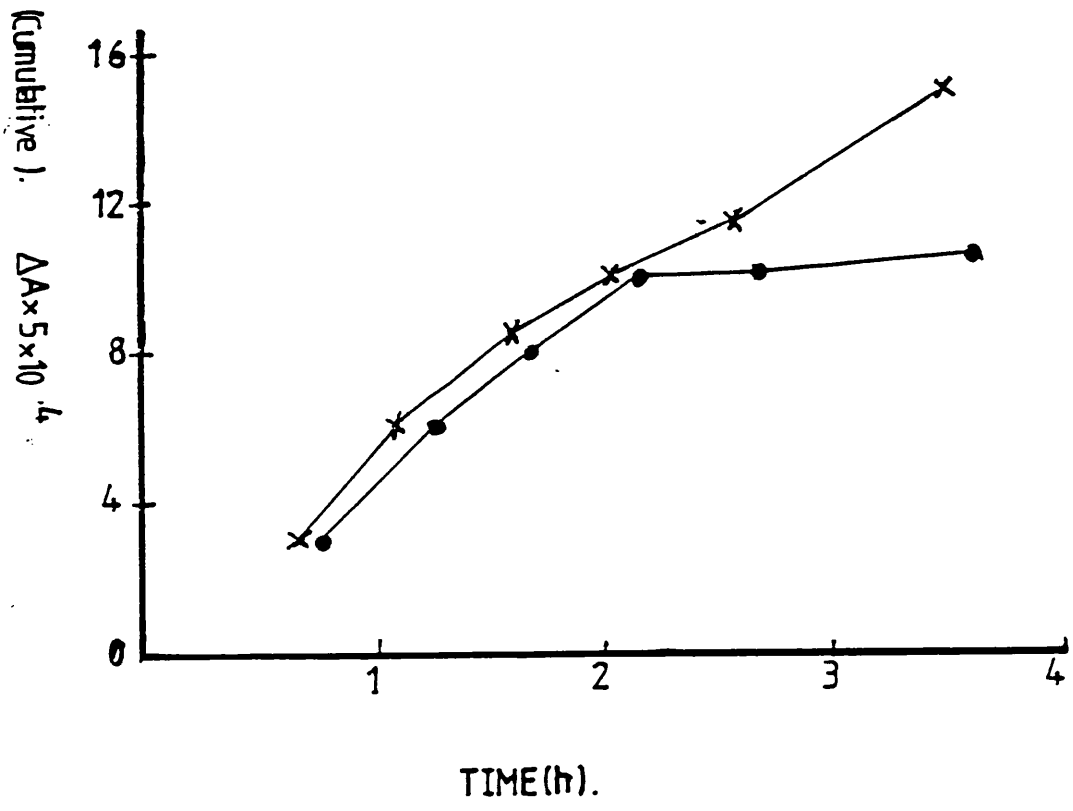
Gabaculin is an inhibitor of mammalian pyridoxalphosphate-linked Y-aminobutyrate (GABA) aminotransferase first isolated from Streptomyces toyocaensis (Koboyishi et al, 1976; Rando and Bangerter, 1976). It is unusual as an enzyme inhibitor in that although a portion of the molecule resembles the substance GABA it is not a competitive inhibitor. Rather it becomes activated by the enzyme and forms a stable molecule with the cofactor pyridoxal phosphate (Rando and Bangerter, 1977). This compound, carboxyphenylpyridoxamine phosphate, because of its stability and size, remains associated with the active site of the enzyme, preventing further enzymic activity (Rando, 1977). As such it is a highly potent, irriversible, and specific inhibitor. In plants, gabaculin inhibits the synthesis of ALA (Gardner and Gorton, 1985). The target enzyme is not known, but inhibition appears to involve the 5-carbon path of ALA synthesis from glutamate or α-ketoglutarate rather than the condensation of succinyl COA and glycine seen in mammals. The former path is almost the exclusive source of ALA for chlorophyll biosynthesis and is based in the plastids. Haem-containing cytochromes etc. appear to be derived from

the latter source of ALA based in the mitochondria. The effectiveness of gabaculin in inhibiting both chlorophyll synthesis (Corriveau and Beale, 1985) and phytochrome synthesis (Gardner and Gorton, 1985) is good evidence that they share the 5-carbon path as a source of ALA. This is further suggested by the observation that both *in vivo* and *in vitro* succinyl-CoA/glycine-based ALA synthesis is unaffected by gabaculin (Corriveau and Beale, 1986).

The rapidity and effectiveness of gabaculin in blocking porphyrin biosynthesis by the 5-carbon route was demonstrated in etiolated barley leaf sections by monitoring the regeneration of protochlorophyllide in control and gabaculin (3 mM) treated tissue after R treatments (2 min) at 30 min intervals. R treatment empties the protochlorophyll(ide) pool by a photochemical reduction mechanism to yield chlorophyll(ide) with a corresponding change in absorbance properties. This initiates re-synthesis starting from ALA (Castelfranco and Chereskin, 1982). Thus, the inhibition of ALA synthesis will manifest itself as an absence of photoreducible protochlorophyllide in subsequent R treatments given dark incubation between. Protochlorophyllide regeneration can be monitored by measuring the change in absorbance of a sample at 650 nm resulting from each R treatment (Jabben and Mohr, 1975). Using a dual beam spectro-photometer in split beam mode with a semi-opaque reference cuvette, the very small changes in absorbance produced by this transition *in vivo* can readily be monitored. The results are shown in Fig. 5.3.

In etiolated barley leaf sections incubated in MES buffer only, protochlorophyllide regeneration proceeds at a fairly constant rate, throughout the 3½h of the experiment. The protochlorophyllide pool is apparently being emptied by R treatment before feedback inhibition of the synthetic pathway begins to operate. There is thus a continuous flow of

Fig. 5.3 Regeneration of photochlorophyllide during serial R treatments (2 min), at the times indicated in untreated (upper trace) and gabaculin (3 mM)-treated (lower trace) etiolated barley leaf sections, with interim dark incubation at 20°C. Photochlorophyllide conversion measured as change in absorbance at 650 nm before and after R.


(Cumulative). ΔA×5×10⁻⁴
16
12
8
4
0
1
2
3
4
TIME(h).

material through the system. When identical leaf material is incubated in MES buffer and gabaculin (3 mM) from the onset of the first light treatment, protochlorophyllide regeneration is unaffected for the first 2h, closely following the control levels. After this, regeneration is effectively blocked and accumulation ceases. Therefore, at some point around 2h after the initial R treatment, gabaculin has penetrated the tissue, blocked the synthetic path, and all intermediate pools have been depleted. This is a measure of the time taken for gabaculin to take effect in the leaf section/MES buffer system, as well as a clear demonstration of its effectiveness.

In a separate experiment barley seedlings were dark grown in gabaculin (0.2 mM) and subjected to in vivo dual beam spectrophotometry to measure phytochrome levels. The results are shown in Table 5.1. It is seen that in gabaculin treated tissue, 75% depression of the level of spectrally detectable phytochrome is seen. Therefore gabaculin does inhibit phytochrome synthesis in barley. Similar results have been reported for peas (Gardner and Gorton, 1985) and oats (Elich and Lagarias, 1987).

The above results indicate that gabaculin totally blocks the synthetic path to the tetrapyrroles in the short term, although some long term compensation may operate in the case of the phytochrome chromophore (Note: gabaculin concentrations in germination experiments were more than 10-fold lower than in Figs. 5.3 and 5.4). These observations were used to evaluate the effect of blocking chromophore synthesis on the escape from Ca^{2+} responsivity and recoupling process. By extending the pre-incubation time to 2h 40 min and incorporating gabaculin into the buffer medium, from Fig. 5.3, tetrapyrrole synthesis was blocked from the onset of the first R treatment and throughout the remainder of the experiment.

Table 5.1 The effect of gabaculin (0.2 mM) on phytochrome synthesis in etiolated barley seedlings.

Treatment	Δ A g^{-1} ± S.D.(n = 3)
Untreated	0.016 ± 0.001
Gabaculin	0.004 ± 0.001

Gabaculin treated seedlings were grown for 6d in gabaculin solution (0.2 mM) in darkness at 20°C. Phytochrome measurements were performed at 20°C reading between 660 nm and 730 nm, following saturating R and FR treatments.

The effect of gabaculin on unrolling per se as well as the recoupling process was assessed by comparison of gabaculin treated and non-treated leaf sections. The results are shown in Fig. 5.4. The inclusion of gabaculin has a small but consistent effect on the unrolling process itself. It had previously been noted that gabaculin grown seedlings exhibited growth-retardation indicating some long-term toxicity. The reduction in unrolling may be a manifestation of this.

Blockage of chromophore synthesis by gabaculin has no detectable effect on the recoupling phenomenon. This indicates that de novo chromophore synthesis, and hence new Pfr, need not be part of the recoupling process, although an alternative explanation is that the chromophore and/or intermediate compounds, are present at sufficient levels for Pr synthesis to be unaffected during the period of uncoupling. Against this explanation is the fact that the levels of key intermediates upto and including Protoporphyrin IX will be depleted by protochlorophyllide re-synthesis following R in the presence of gabaculin. Not only is this a rapid process, as shown in Fig. 5.3, it also accounts for much of the flow of material through the pathway.

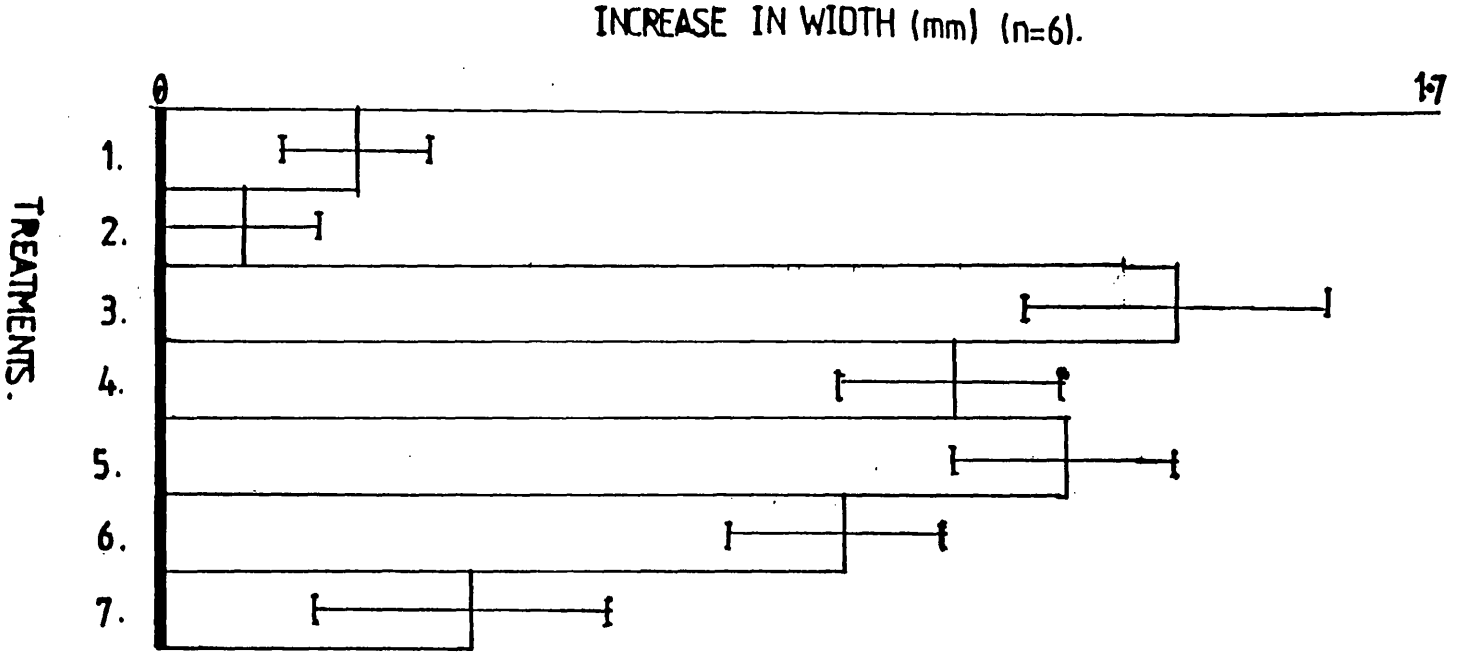
Fig. 5.5 clarifies the possible importance of old Pfr to the response, showing dose/response determinations for the second R treatment after the supply of Ca^{2+} a. 2½h after the first R treatment, b. 4h after the first R treatment. The results are presented in terms of duration of light treatment, as the Pfr levels detectable, generated in the primary R treatment (60 - 75% P_{tot}) would obscure the nature of the changes induced by low fluence secondary R pulses 4h later.

Both curves in Fig. 5.5 for the second R treatment show complete saturation of the response at a fluence of 68 μmol m^{-2}. In comparison, the dose/response for the primary R treatment in the presence of Ca^{2+} in

Fig. 5.4 Effect of gabaculin treatment (3 mM) on the unrolling and the recoupling processes. Gabaculin treatment (and pre-incubation) commenced at $t_{-2h\ 40\ min}$. Unrolling measured at t_{24h} following a dark incubation. (Mean ± S.D., n = 6).

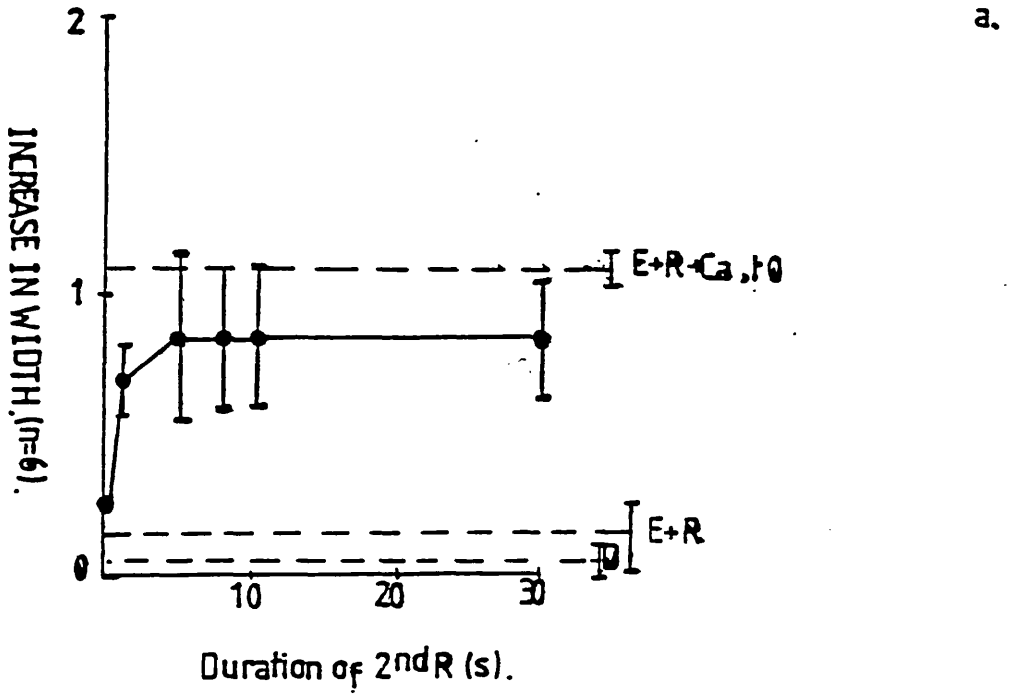
Treatments:

1. None (Dark control), MES buffer
2. R at t_o, MES/EGTA buffer
3. R at t_o, Ca^{2+} at $t_{5\ min}$, MES/EGTA buffer
4. As treatment '3' with inclusion of gabaculin in buffer
5. R at t_o, Ca^{2+} at t_{4h}, R at t_{4h}, MES/EGTA buffer
6. As treatment '5' with inclusion of gabaculin in buffer
7. R at t_o, Ca^{2+} at t_{4h}, MES/EGTA buffer



(For Treatments 1-7 see Legend.)

Fig. 5.5 Non-photostationary (subsaturating) phytochrome (Pfr) dose/response determination for second supplementary light treatment at: a. $t_{2h\ 30min}$; b. t_{4h} following incubation in MES/EGTA buffer, R (5 min) at t_0 and Ca^{2+} (5 mM) coincident with the onset of the second R treatment. (Mean ± S.D., n = 6). Fluence rate = 13.6 μ-mol $m^{-2}s^{-1}$ at sample surface.


a.
2
1
0
INCREASE IN WIDTH (n=6).
E+R+Ca, t0
E+R
10
10
20
30
Duration of 2nd R (s).

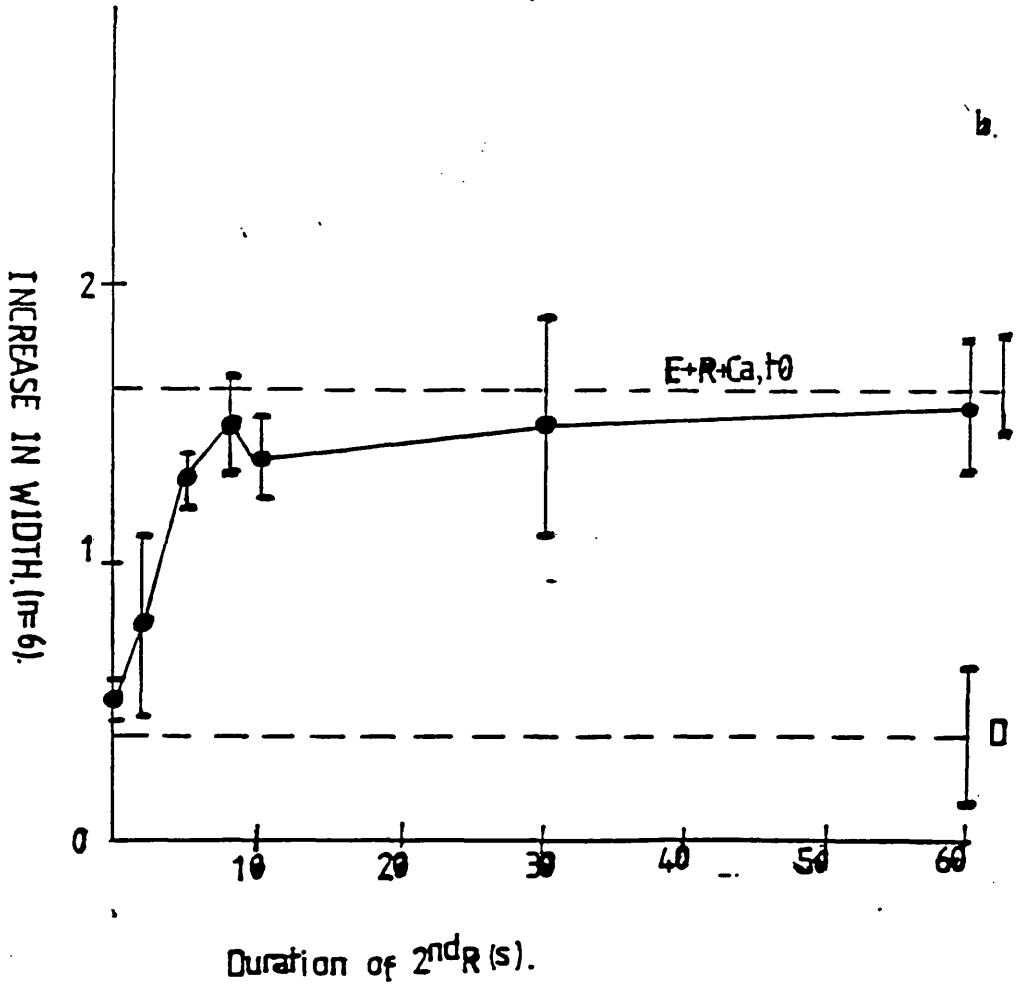

b.
2
1
0
INCREASE IN WIDTH (n=6).
E+R+Ca, t0
0
10
20
30
40
50
60
Duration of 2nd R (s).

Fig. 3.7a only shows 40% of the full response at this dosage. This indicates that the reactivation process is more sensitive than the primary response to R. However, Fig. 5.2 contradicts this, indicating an identical dose/response. The only explanation of this is that some of the original phytochrome remains 'coupled' at sub-optimal levels at the time of secondary illumination. In terms of the 'ratio' hypothesis, this means that less phytochrome needs to be photoconverted in the secondary response for induction by reactivation (possibly reassociation) or; in terms of the 'active' Pfr hypothesis, that some 'old' Pfr remains active reducing the amount of new Pfr required in the active compartment. Both explanations are supported by comparison of Figs. 5.5 'a' and b. The response to 14 μmol m^{-2} R at 2½h appears to be greater than at 4h. Therefore as the delay between primary and secondary R treatments increases, sensitivity to the secondary R decreases, further suggesting some carry over of a declining activity from the primary treatment at sub-optimal levels.

It is now possible to define the upper limits for the levels of Pr and Pfr from various sources within the active compartment according to the active Pfr hypothesis from the foregoing experiments. Taking the 4h timepoint of secondary R treatment, the light dose required for saturation in the secondary response corresponds to a photoconversion of 10 - 11%. From Fig. 3.7 it may thus be deduced that the residual active 'old' Pfr is around 8 - 9% of the initial P_{tot} in the active compartment. The remainder of the phytochrome present in this compartment must consist of old Pr (14% of initial P_{tot}), new Pr, and an unknown quantity of old 'inactive' Pfr. Given that the P_{tot} in the active pool must remain constant according to the 'active Pfr' hypothesis, either the tissue re-synthesizes 75 - 80% of its functional phytochrome in 4h or deactivated

old Pfr can be reactivated by a previously unknown process. Unless the functionally active pool is very small such a high rate of resynthesis is likely to be wasteful both energetically and in terms of chromophore and protein, although this does not rule it out as a possibility.

In conclusion, although it is not possible to eliminate either hypothesis as an explanation of the mode of action at phytochrome in the response, the ratio-based models offer a simpler, more comprehensive explanation of the data presented, the only condition being that some reversible, time-dependent phytochrome deactivation process occurs in the decoupled state. This is also a feature of the 'active' Pfr hypothesis, although the reversibility of such a process in this case is not absolutely necessary. A further condition of this 'active-pool' hypothesis is that the bulk of the phytochrome in etiolated plants be functionless in the LFR, an unlikely if untestable proposition given its behaviour following light treatment.

Whatever the mechanism of induction, the phytochrome deactivation process emerges as a potentially significant phenomenon.

CHAPTER 6 : TOWARDS A MECHANISM FOR LEAF UNROLLING

Having investigated the changes that occur in the immediate properties of phytochrome following the induction of unrolling (and NRA), some elements of the transduction path to unrolling will now be considered. Previous investigations of the phenomenon are, as has already been stated, somewhat fragmentary in the information they provide, and, as such, offer no clear starting point. It was decided to proceed from the basic observations of Chapter 3; namely that unrolling arising from R treatment is a Ca^{2+}-dependent process and that no stable intermediate state is produced if Ca^{2+} is withheld for at least 2h. In this way a coherent picture of the system may emerge that will hopefully offer points of reference to the data of other workers. The majority of the data to be presented here deals with aspects of unrolling directly involving Ca^{2+}, and the existence and implications of forming a steady state in transduction will only be dealt with briefly due to temporal constraints.

It was demonstrated in Chapter 3 that R-stimulated unrolling is Ca^{2+}-dependent in itself beyond the absolute non-specific Ca^{2+} requirement demonstrated by most living systems. At this level of Ca^{2+}-dependence, it is possible to envisage three general ways in which Ca^{2+} could participate in the response. These are:

1. Ca^{2+} is required to be present for the response to proceed i.e. as a co-factor. This is Ca^{2+}-dependence in its narrowest sense.
2. Ca^{2+} is a participating part of the transduction chain through which a signal to respond is transmitted. This will be referred to as a

'Ca^{2+}-mediation'.

3. The response may be induced by an independent path involving Ca^{2+} as a participating agent, although the stimulus under observation does not utilize Ca^{2+} beyond showing 'type 1' Ca^{2+}-dependence as described above. This will be referred to as 'independent Ca^{2+}-activation'.

Roux et al (1986) have proposed Ca^{2+}-mediation as a vehicle for the transduction of a variety of phytochrome-regulated phenomena. Some of the evidence pertaining to this has already been described in Chapter 1. In seeking to identify Ca^{2+}-mediation when it occurs much use has been made, often retrospectively, of the 'rules' set down by Jaffe (1980) and stated specifically in connection with plant phenomena by Hepler and Wayne (1985). These state that:

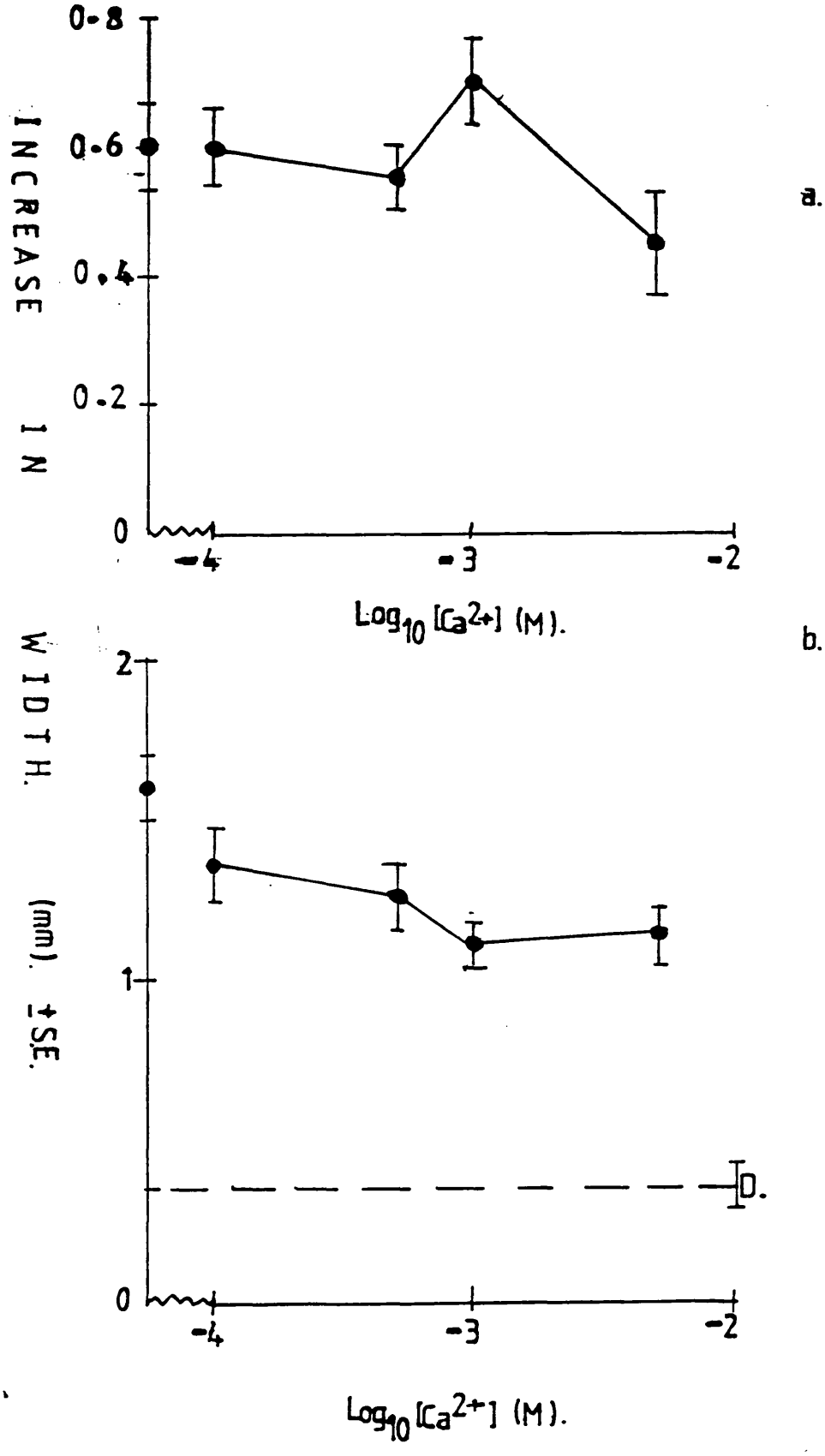
1. The responses should be preceded or accompanied by a rise in intracellular $[Ca^{2+}]$.
2. Blockage of the natural increase in Ca^{2+} should inhibit the response.
3. Experimental generation of an intracellular rise in Ca^{2+} should stimulate the response.

These 'rules' involve a number of unjustified assumptions, as will be demonstrated in later chapters, and over-zealous application may generate misleading conclusions. In practice they are very difficult to implement in an unequivocal fashion. For a variety of technical reasons, it is difficult to measure cytoplasmic Ca^{2+} activity ($[Ca^{2+}]_{cyt}$) in all but a very small number of plant systems (Thomas, 1986). This makes the application of rule '1' something which must be deferred pending the emergence of robust, accurate techniques of Ca^{2+} measurement in plant cells. Rule '2' is readily applied through the agency of EGTA, and this

was performed for unrolling in Chapter 3. This leaves rule '3' as an approach point to examine the idea of Ca^{2+} in unrolling. In isolation it allows the differention of Ca^{2+}-mediation or independent Ca^{2+}-activation from type 1 Ca^{2+}-dependency. Arguably, it is as difficult to test as rule '1', as $[Ca^{2+}]_{cyt}$ should be detectably elevated, and this requires measurement. However, it does suggest approaches whereby treatments with predicted effects on $[Ca^{2+}]_{cyt}$ could be examined for their effects on R-stimulated unrolling.

The basic aim should be to disturb the Ca^{2+} homoeostasis of the cell. Intracellular Ca^{2+} appears very tightly regulated in all systems studied to date (Hepler and Wayne, 1985), and, as such, may prove difficult to disturb. However, a necessary preliminary experiment, is to examine the effect of variations in external Ca^{2+} ($[Ca^{2+}]_{ext}$), over a wide range, on both dark and R-stimulated unrolling. To maximise sensitivity and facilitate statistical analysis, sample sizes were increased sufficiently to allow the assumption of normal distribution of results within a treatment. Samples were isolated and incubated as before, for 24h following R-treatment, or maintained in darkness throughout. The MES/NaOH buffer used contained a range of added Ca^{2+} concentrations from 0 through to 5 mM. The buffer was prepared in double-deionized water (conductivity $< 0.1\ \mu S\ cm^{-1}$), and plastic ware washed thoroughly in the same was used throughout. This 'low Ca^{2+} buffer' allowed accurate knowledge of the initial $[Ca^{2+}]_{ext}$ during experiments, and reduced the risk of uncontrolled variation in Ca^{2+} levels between experiments arising from the source of water used and contaminated apparatus. The results are shown in Figs. 6.1a and 6.1b. Fig. 6.1a shows the response of dark unrolling to variation in $[Ca^{2+}]_{ext}$. It can be seen that there is little or no variation in unrolling in the dark over a wide range of concentrations of

Fig. 6.1 Effect of variation in external calcium concentration on unrolling of etiolated barley leaf sections in MES buffer: a. in the absence of light treatment; b. following R, 5 min at t_0. Measurements were performed at t_{24h} following a dark incubation at 20°C. (Mean ± S.E., $n \geq 37$).


0·8
0·6
0·4
0·2
0
INCREASE IN
-4
-3
-2
Log10 [Ca2+] (M).
a.
2
1
0
WIDTH. (mm). ±SE.
D.
-4
-3
-2
Log10 [Ca2+] (M).
b.

the Ca^{2+} added to the low Ca^{2+} buffer. This is consistent with the notion that internal Ca^{2+} levels are tightly regulated and maintained independently of external levels. It is also consistent with the implication, from Chapter 3, that 'dark unrolling' is not necessarily Ca^{2+}-dependent, or that the Ca^{2+} requirement, if any, is satisfied at all levels. There is an indication that at Ca^{2+} levels > 5 mM unrolling may be inhibited, although this is not statistically significant. Possible underlying causes of this could include loss of cell wall plasticity or cytotoxicity.

These effects are seen more clearly in Fig. 6.1b. This shows R-stimulated unrolling across a range of added Ca^{2+} concentrations. Addition of Ca^{2+} to the low Ca^{2+} buffer at any level from 0.5 mM upwards significantly reduces unrolling relative to that obtained in low Ca^{2+} buffer only ($0.01<p<0.02$). This is contrary to the hypothesis that Ca^{2+} has an active role in unrolling. However it is consistent with the proposed role of Ca^{2+} in wall extensibility. At very low external Ca^{2+} levels, Ca^{2+} would be expected to be leached from the cell walls where it is thought to be important in cross-linking wall components, thus increasing extensibility by reducing these links (Baydoun and Brett, 1988). Like all forms of growth, unrolling must involve cell expansion, and hence, wall extension. The role of Ca^{2+} in reducing unrolling becomes clear. This hypothesis is favoured over notions of cytoxicity because; firstly, the magnitude of the effect is relatively small over a wide range of added Ca^{2+} concentrations, and secondly, the reduction in response is manifested at $[Ca^{2+}]_{ext}$ of similar magnitude to the expected Ca^{2+} concentrations in cell walls.

The measured $[Ca^{2+}]_{ext}$ in low Ca^{2+} buffer is less than 10 μM (limit of detection), bringing the data point for R-stimulated unrolling for Fig.

6.1b into the range of $[Ca^{2+}]_{ext}$ where EGTA was found to be inhibitory. This emphasises the Ca^{2+} homoeostasis of plant cells under normal circumstances and indicates that the Ca^{2+} activity in EGTA buffer may be considerably lower than the 1 - 10 μM range. At such levels and in non-standard conditions Ca^{2+} activities are difficult to assess or calculate accurately. At present it is only necessary to suggest that the apparent contradiction has more basis in technical limitations than anything else.

Overall, both parts of Fig. 6.1 show a relative insensitivity of the unrolling mechanism to $[Ca^{2+}]_{ext}$, for all but extreme values. To overcome this could be to find a way to bypass the normal Ca^{2+} regulatory mechanism of the cell and, by variations in $[Ca^{2+}]_{ext}$, assess the role of changes in intracellular Ca^{2+} levels in unrolling. Ionophores are chemcials that greatly increase the permeability of biological membranes to ions in a semispecific manner. By using the appropriate ionophore it is possible to 'short circuit' the normal regulatory mechanisms for Ca^{2+} in the cell and forge a more direct link between $[Ca^{2+}]_{ext}$ and $[Ca^{2+}]_{cyt}$. However, there are many problems associated with their use and care is required in interpretation of experiments. The specific nature of these problems will become apparent as this chapter progresses.

The ionophore chosen was the antibiotic A23187. This is a weakly acidic molecule that forms complexes with Ca^{2+} with the formula $Ca(A23187)_2$. These complexes are readily soluble in organic solvents and almost insoluble in water. In a living system, A23187 becomes concentrated in biological membranes. Where a Ca^{2+}-concentration gradient exists across the membrane, and Ca^{2+} exchange to and from the A23187 complex is possible, A23187 will carry Ca^{2+} down the concentration gradient providing an unregulated Ca^{2+} 'channel'. In this way changes in $[Ca^{2+}]_{cyt}$ may be driven by changes in $[Ca^{2+}]_{ext}$, although, without

measuring both and knowing the relevant ion pump and channel kinetics, the efficiency of the linkage cannot be determined. As such experiments can only, at best, be partially quantitative.

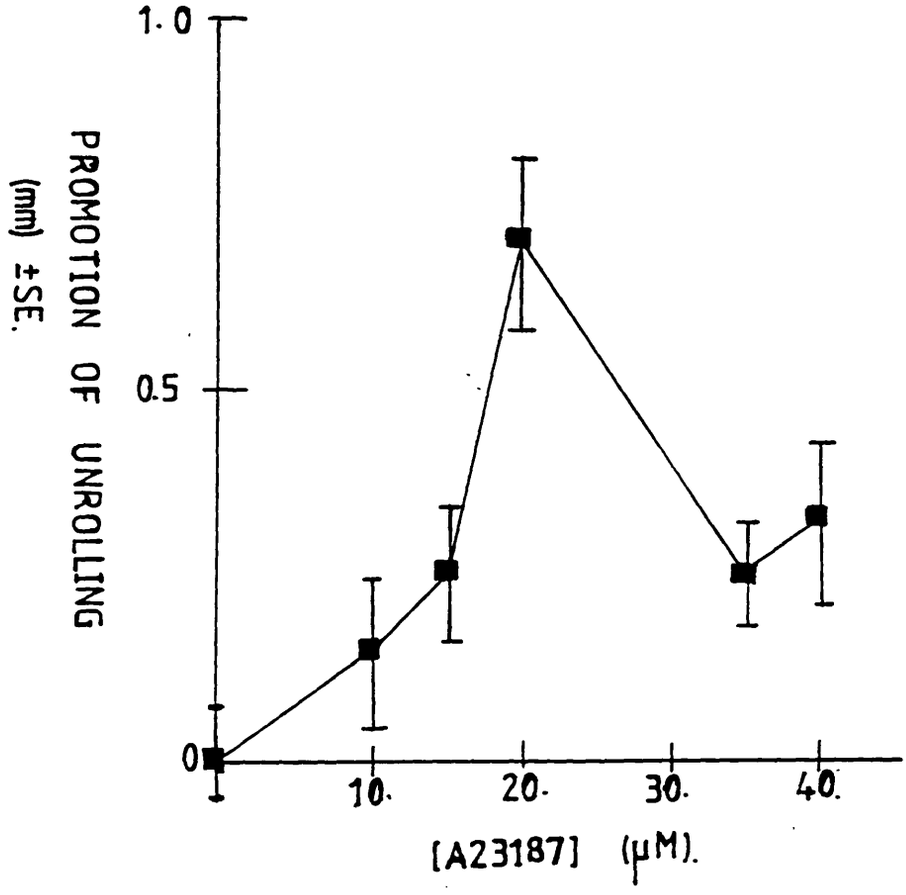
Another problem in using A23187 is that of limitations on ion selectivity. Many divalent ions will bind to A23187, and Ca^{2+} and Mg^{2+} exhibit similar affinites. However, by excluding Mg^{2+} and assuming cations such as Mn^{2+} and other heavy metals to be present only as traces, the significance of this can be reduced.

The other main area of difficulty associated with the use of ionophores is often overlooked. Because they disolve in membranes, they may disrupt them in various ways and this disruption may itself induce the response rather than the passasge of ions. There is some evidence that this may be the case with A23187. Sha'afi et al (1986) demonstrated that A23187 caused stimulated association of actin with the cytoskeleton of rabbit neutrophils independently of the presence or absence of Ca^{2+} in the incubation medium. While this does not exclude the possibility of A23187 acting on internal reservoirs of Ca^{2+}, the same study showed, using fMet-Leu-Phe to induce the same response, that the rise in $[Ca^{2+}]_{cyt}$ measurable during the response could be inhibited with quinacrine without affecting the response. Taken together this indicates that a rise in $[Ca^{2+}]_{cyt}$ may not be necessary, and that A23187 may have agonistic effects independent of those due to ion movement. By careful manipulation of $[Ca^{2+}]_{ext}$ it is possible to separate Ca^{2+}-dependent and independent A23187 effects.

To examine the effect of A23187 on unrolling, the effect of a variety of concentrations of A23187 on dark unrolling were tested. Although very insoluble in aqueous media, colloidal solutions of A23187 can be prepared from stock solutions in dimethylsulphoxide (DMSO) by

adding microliter amounts to rapidly stirred buffer. The Ca^{2+} salt of A23187 was used in these experiments, and concentrations of A23187 are calculated on the basis of an empirical formula weight of 543 for [A23187 $Ca_{\frac{1}{2}}$]. The experimental protocol used was identical to that in Chapter 3 except that throughout the dark incubation the sections were kept on an orbital shaker operating at 60 rev/min. This was to retard ionophore precipitation/aggregation. No light treatments were given. The results are shown in Fig. 6.2 and are expressed as the promotion in unrolling above that of the untreated controls. It is seen that all concentrations tried were promotive relative to untreated sections. However, a concentration of 20 μM A23187 is markedly more effective in increasing unrolling than the others tried. This contrasts with the finding of Wayne and Hepler (1984) that concentration variations over the range 5 μM – 96.5 μM A23187 showed little difference in effectiveness in the Onoclea spore germination response. Two variables other than A23187 could explain the relative ineffectiveness of higher concentrations of A23187 to stimulate unrolling relative to the 20 μM datum. Both Ca^{2+} and DMSO concentrations will vary with A23187 (since the calcium salt of the ionophore in DMSO is used) and either could be responsible. Ca^{2+} is highly cytotoxic, and, if A23187 were very efficient in linking external and internal components, the micromolar $[Ca^{2+}]_{ext}$ may lead to progressive inhibition of unrolling. Efficiency would be expected to increase with high ionophore concentrations. Equally feasible is inhibition by DMSO. In preliminary experiments, DMSO was markedly, although not totally, inhibitory of R-stimulated unrolling at 1% (v/v) concentration. No effect was seen at 0.1%. The concentrations included here are intermediate and an optimization between promotion of unrolling by A23187 and inhibition by DMSO can be envisaged. Whatever the mechanism of

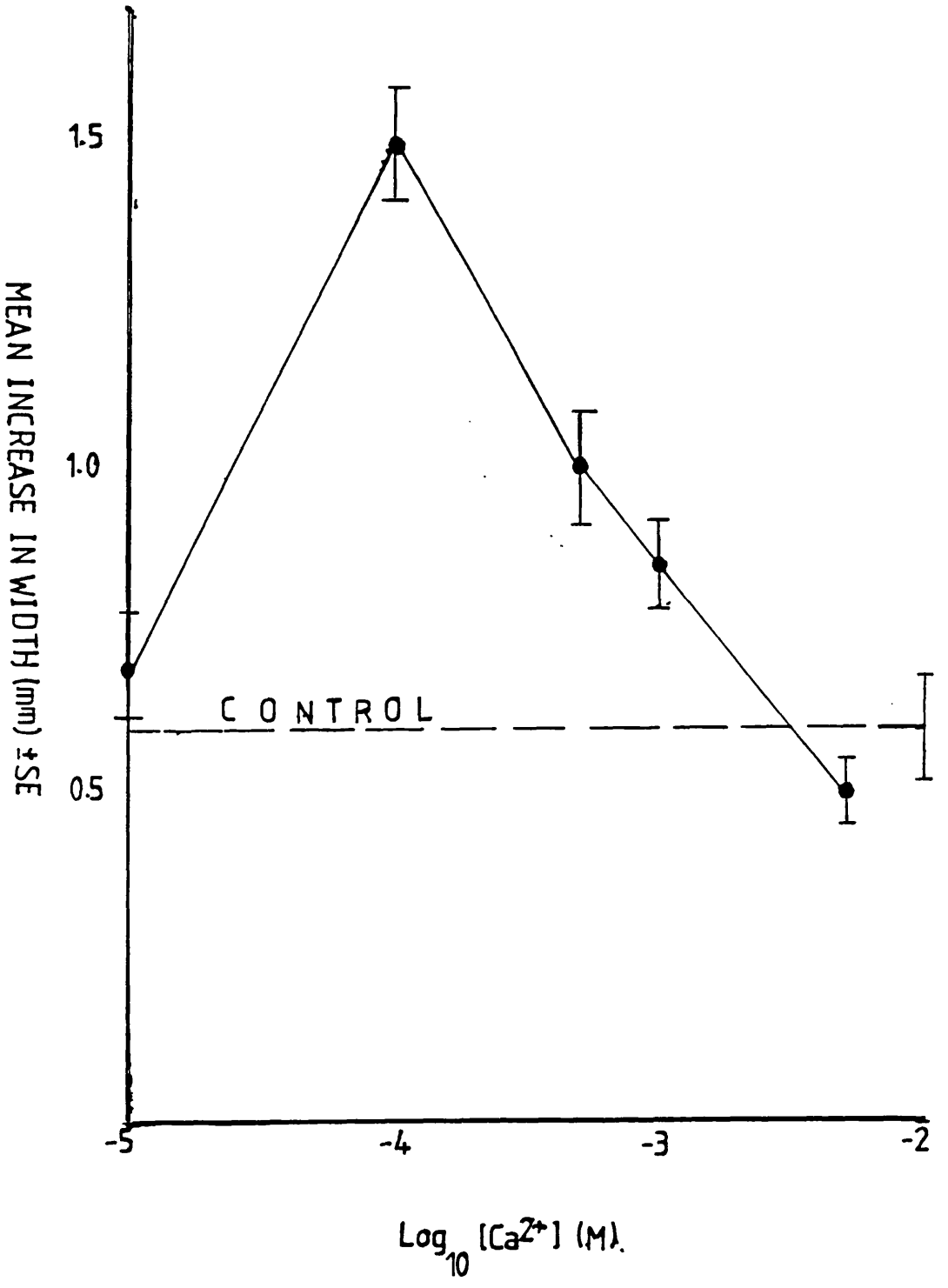
Fig. 6.2 Promotion of unrolling of etiolated barley leaf sections in MES buffer by A23187: effect of ionophore concentration. Unrolling measured at t_{24h} following a dark incubation at 20°C. (Mean ± S.E., $n \geq 31$).


1.0
0.5
0
PROMOTION OF UNROLLING
(mm) ±SE.
10.
20.
30.
40.
[A23187] (μM).

optimisation, the promotive action of A23187 is clearly demonstrated.

To pursue this further the A23187 concentration was fixed at 20 μM for subsequent work thus fixing the DMSO level and contribution of Ca^{2+} from the Ca^{2+}/A23187 salt. Appropriate additions of DMSO and Ca^{2+} were then made to non-A23187 treated tissue within experiments, such that levels were constant between treatments, except where variables such as $[Ca]_{ext}$ were under study. This is the case in Fig. 6.3 which shows the magnitude of the A23187 effect at 20 μM, over a range of added Ca^{2+} concentrations to low Ca^{2+} buffer. The aim of this experiment is to demonstrate the Ca^{2+}-dependent aspects of the phenomenon. It can be seen that without any Ca^{2+} addition very little promotion of dark unrolling is obtained with A23187. The $[Ca^{2+}]_{ext}$ in these circumstances may be as high as 10 μM if the empirical formula of $Ca_{\frac{1}{2}}$ A23187 is used. However, since this is essentially insoluble and it is not clear how much Ca^{2+} enters into true solution, it is only given as an upper limit. The error this introduces into the other additions of Ca^{2+} is never greater than 10% and not relevant within the limitations of this experiment. Addition of 100 μM Ca^{2+} gives a large promotion of dark unrolling. This demonstrates that A23187 and Ca^{2+} interact to promote unrolling since neither supplied externally in isolation is sufficient to induce the response. The optimum $[Ca^{2+}]_{ext}$ for maximal unrolling under these conditions is probably less than 100 μM, as the response with A23187 decreasing with increasing $[Ca^{2+}]_{ext}$ above this, is an approximate semilogarithmic relationship, such that no promotion at all is attained at 5 mM Ca^{2+}. Compared to Fig. 6.1a, A23187 is seen to sensitize the unrolling process in the dark to $[Ca^{2+}]_{ext}$. This sensitization indicates that the ionophoric conduction of Ca^{2+} must be able to determine unrolling above dark control levels in the absence of light treatment. This is consistent with the

Fig. 6.3 Effect of Ca^{2+} concentration on A23187-promoted unrolling of etiolated barley leaf tissue in MES buffer following 24h dark incubation at 20°C. Lower dashed line shows unrolling in untreated controls. (Mean ± S.E., n ≥ 46). A23187 concentration = 20 μM.



A23187 = 20 μM.

third of Jaffe's rules that experimentally generated increases in $[Ca^{2+}]_{cyt}$ should be able to substitute for light treatment in the response, although in this case, there is a clear optimum elevation, above which the response diminishes. This was not demonstrated by Bossen et al (1988) for the wheat leaf protoplast swelling response, where an increase in $[Ca^{2+}]_{ext}$ from 0.1 mM to 1 mM had no effect on the response induction with A23187. No attempt was made to see if the swelling response occurred in the absence of Ca^{2+} with A23187. This might have been due to the stabilizing nature of Ca^{2+} on protoplast membranes. In other studies, Wayne and Hepler (1984) omitted to examine the role of $[Ca^{2+}]$ in A23187-induced spore germination in Onoclea. Only Serlin and Roux (1984) have previously demonstrated that the effect of A23187 in mimicing phytochrome responses is dependent on the supply of Ca^{2+}. This was for the chloroplast rotation response in Mougeotia, but no dose/response determinations for $[Ca^{2+}]_{ext}$ were performed. The present study therefore provides the first clear evidence for a phytochrome-regulated phenomenon that the ionophoric movement of Ca^{2+}, as determined by the magnitude of the concentration gradient, positively affects the response. Thus, unrolling appears to be Ca^{2+}-activated, rather than Ca^{2+}-dependent, in the type 1 sense described earlier.

This conclusion offers little insight into how Ca^{2+} acts in unrolling. It shows that a treatment, that increases the movement of Ca^{2+} into cells, induces unrolling. It is possible that A23187 operates by disturbing the electrochemical gradient (via ion movement) and this causes induction of a response. Serlin and Roux (1984) examined this possibility using Valinomycin. This is an antibiotic isolated from Streptomyces fulvissimus (Brockman and Schmidt-Kastner, 1955) which acts as a potassium (K^+) ionophore. In the Mougeotia chloroplast rotation

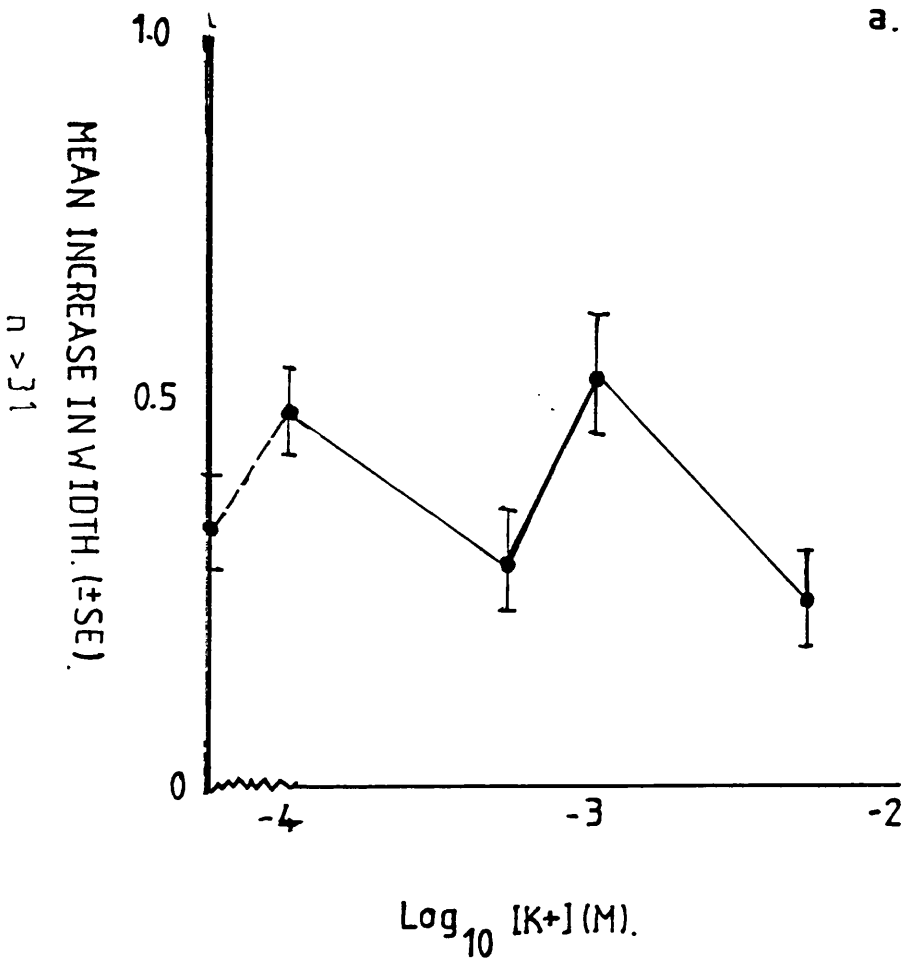
system, substitution of Valinomycin for A23187 failed to induce the response. However, this is not an adequate test as the external $[K^+]$ was not varied to provide a range of polarization effects. It is possible that superoptimal or suboptimal concentrations of K^+ were provided.

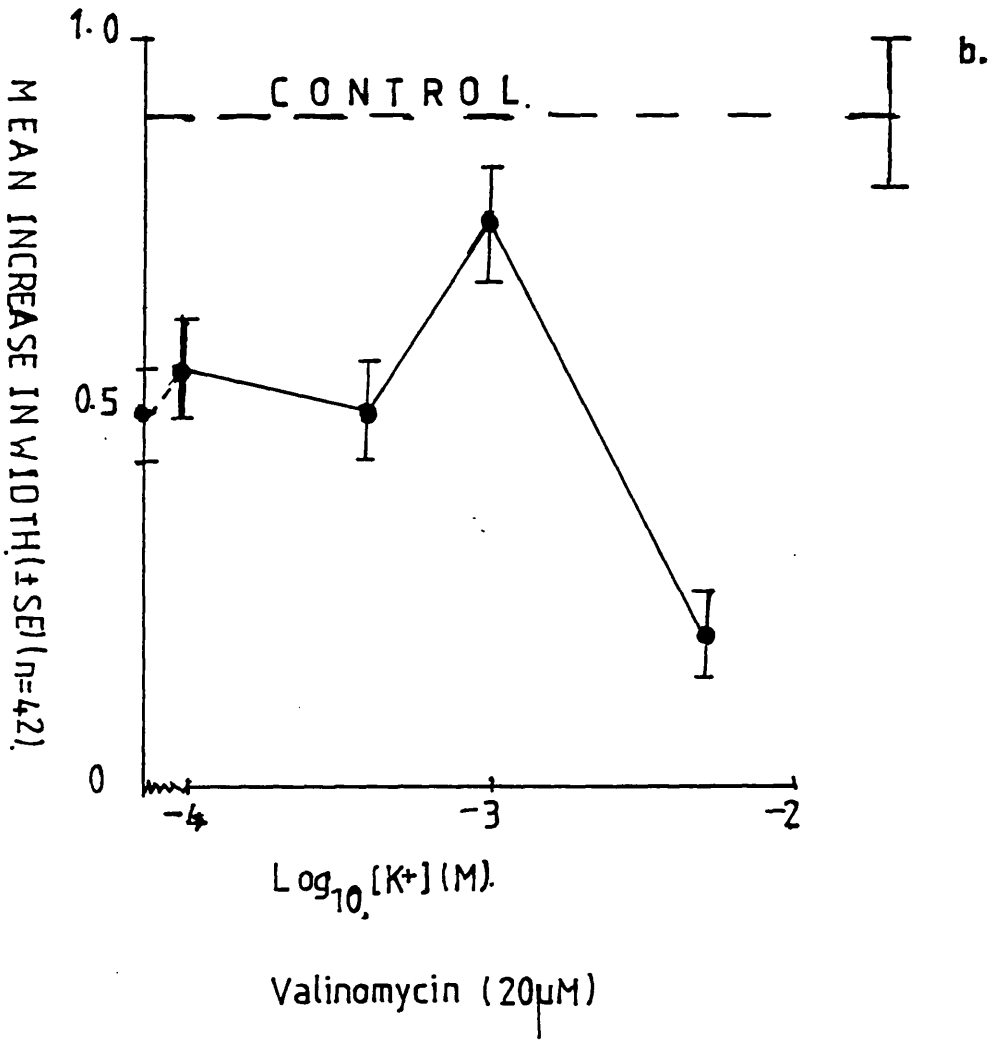
In using Valinomycin to substitute for A23187 in the unrolling response, a wide range of $[K^+]_{ext}$ values were used and compared to the effect of the same values in the absence of ionophore. Valinomycin was dissolved in ethanol (20 mgs/ml) and added to rapidly-stirred buffer to give a final concentration of 20 μM. Leaf sections were incubated on an orbital shaker using the same protocol as used with A23187. The results are shown in Figs. 6.4 'a' and 'b'. Equivalent amounts of ethanol were added to control experiments.

Fig. 6.4a shows the effect of a range of $[K^+]_{ext}$ values from 0.1 mM to 5 mM compared to dark unrolling in the absence of added K^+. It can be seen that variation in $[K^+]$ has little or no effect on dark unrolling.

Fig. 6.4b shows the effect of same range of $[K^+]_{ext}$ values in the presence of Valinomycin (20 μM). All data lie below the control values (no ionophore of K^+ added) and, with the exception of the 1 mM K^+ datum, show highly significant inhibition ($p < 0.01$). This shows that K^+ movement cannot substitute for Ca^{2+} movement. It also demonstrates an apparently negative effect of electrophysiological perturbation on unrolling. At lower concentrations of K^+, the basis of this inhibition is very probably due to loss of cellular K^+. Around 1 mM $[K^+]_{ext}$, the relatively high external value will reduce this loss such that something approaching the normal situation may persist. Increasing $[K^+]_{ext}$ to 5 mM profoundly inhibits dark unrolling. The most likely explanation for this is an ionophore-induced influx of K^+, since the 1 mM datum most closely resembles the control value. If this is the case then Fig. 6.4b shows

Fig. 6.4 Effect of variation in external K^+ concentration on unrolling in etiolated barley leaf sections in MES buffer: a. in the absence of, and b. in the presence of Valinomycin (20 μM) during dark incubation. Measurements performed at t_{24h} following dark incubation at 20°C.


a.
1.0
0.5
0
MEAN INCREASE IN WIDTH (±SE).
n > 31
-4
-3
-2
Log10 [K+] (M).


b.
1·0
CONTROL
0·5
0
MEAN INCREASE IN WIDTH (±SEM) (n=42).
−4
−3
−2
Log10 [K+] (M).
Valinomycin (20μM)

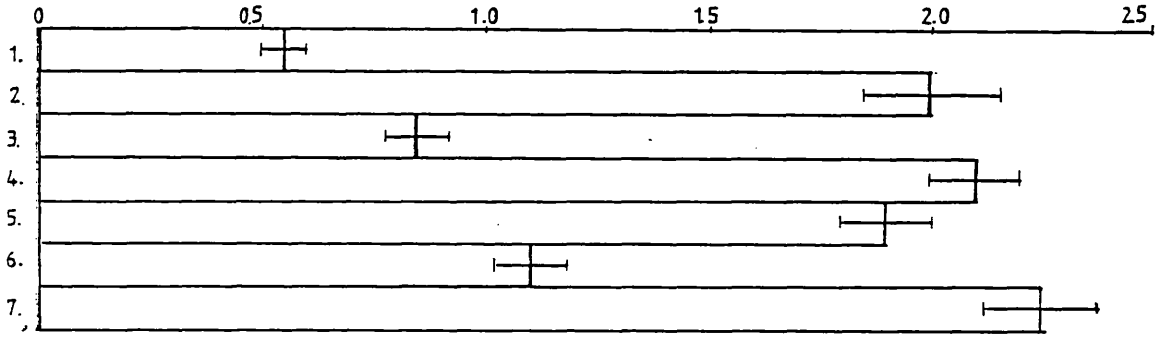
the effects of both hyperpolarizing K^+ loss through to hypopolarizing K^+ gain to the cell, both extremes of which are inhibitory. It is therefore unlikely that the effect of A23187 with Ca^{2+} is a purely electrochemical phenomenon.

If a rise in $[Ca^{2+}]_{cyt}$ does provide a chemical signal to promote unrolling, and photoconversion of phytochrome exerts its effect via such a rise as suggested by Roux et al (1986), then, as well as being able to substitute for R, the Ca^{2+}/A23187 effect should disappear in the presence of R if both R, and the rise in $[Ca^{2+}]_{cyt}$ operate as part of the same mechanism. This can be tested by introducing an R treatment at the beginning of the incubation and comparing A23187 treated and untreated sections. The results are shown in Fig. 6.5. Low Ca^{2+} buffer was used as an incubation medium and the presence or absence of Ca^{2+} (100 μM) was also assessed. Both R and A23187 appear to be promotive. R much more so than A23187, and given together in the absence of added Ca^{2+} little or no difference from R given in isolation is detectable. The effect of incorporating Ca^{2+} into the incubation medium differs for R and A23187 treatments. As seen in Fig. 6.1b there is a small reduction in R-stimulated unrolling with Ca^{2+}. However, the addition of Ca^{2+} increases the magnitude of the A23187 effect as seen in Fig. 6.3. This difference in response to added Ca^{2+} is not inconsistent with common paths of action, the A23187 depending on a source of external Ca^{2+} to generate the effect. However, A23187 is seen to produce an additive effect above the R effect on unrolling in the presence of Ca^{2+}. This effect is significant ($p < 0.04$) when the effect of Ca^{2+} on R-stimulated unrolling is compared to R-stimulated unrolling in the presence of Ca^{2+} and A23187. This indicates that R and A23187 may operate differently to induce unrolling. The only previous simultaneous comparison of R and A23187

Fig. 6.5 The effect of R (5 min at t_0) on A23187 induced unrolling of etiolated barley leaf sections in the presence and absence of Ca^{2+} (100 μM). Unrolling measured at t_{24h} following a dark incubation at 20°C.

Treatments:
1. No treatment (Control)
2. R (5 min at t_0)
3. A23187 (20 μM)
4. As treatment '3' + R (5 min at t_0)
5. As treatment '2' + Ca^{2+} (0.1 mM)
6. As treatment '3' + Ca^{2+} (0.1 mM)
7. As treatment '4' + Ca^{2+} (0.1 mM)

MEAN INCREASE IN WIDTH (mm ± SE, n≥44)



For Treatments see legend.

effects is in spore germination on Onoclea (Wayne and Hepler, 1984). There, no additive effects were detected, although it should be noted that:

1. The leaf unrolling system has already been shown to have very different properties to the Onoclea system with regard to its regulation by phytochrome (see Fig. 3.4 and comments).
2. The magnitude of the A23187 effect on spore germination is relatively small compared to that demonstrated here. It is thus possible that any effect in Onoclea is too small to be detected.

Further evidence that A23187 operates in a different manner to R treatment is furnished by the effect of A23187 on NRA induction. Fig. 6.6 shows NRA, measured by the same protocol as in Chapter 4, for NO_3^--grown leaf sections incubated in Ca^{2+} buffer plus 100 μM Ca^{2+} and KNO_3 (5 mM). It can be seen that A23187 is almost as effective as R in inducing NRA in the presence of Ca^{2+}. Furthermore the effects of R treatment and A23187 appear to be additive when given together in the presence of Ca^{2+} (100 μM) in much the same way as leaf unrolling, except that the difference is more clearly marked. The manifestation of additive effects in unrelated responses indicates that the difference in action of phytochrome and A23187 must be quite fundamental.

One explanation of these observations is that the phytochrome system, although saturated by light, does not itself saturate the response mechanism. A23187, by virtue of its continuous presence, provides an extra stimulus giving an additive effect. There are two arguments against this:

1. In isolation A23187 is at best only as effective as R in inducing the response despite its continuous presence.
2. If the response mechanism was not saturated by phytochrome-

Fig. 6.6 Promotion of nitrate reductase activity in etiolated barley leaf sections by A23187 in the presence and absence of R treatment (5 min at t_0). Nitrate reductase activity measured <u>in vivo</u> by nitrite assay at t_{20h} following a dark incubation in MES Buffer + Ca^{2+} (0.1 mM) at 20°C (mean ± S.D., n = 3).

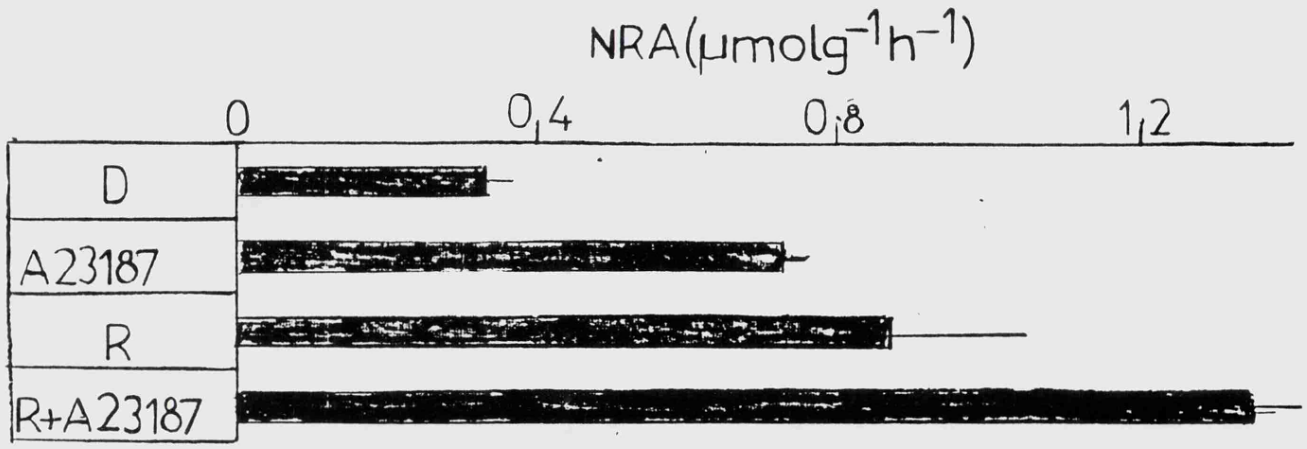

NRA(μmolg⁻¹h⁻¹)
0
0,4
0,8
1,2
D
A23187
R
R+A23187

Table 6.1 Comparison of single R (5 min at t_0) treatment and serial R (5 x 5 min at 2½h intervals) treatments on induction of leaf unrolling in etiolated barley leaf sections. Sections measured at t_{24h} following a dark interim incubation at 20°C.

Treatment	Mean increase in leaf width (mm) mean ± SE n ≥ 47
None (Dark control)	1.3 ± 0.139
Single R	2.3 ± 0.13
Serial R	2.1 ± 0.16

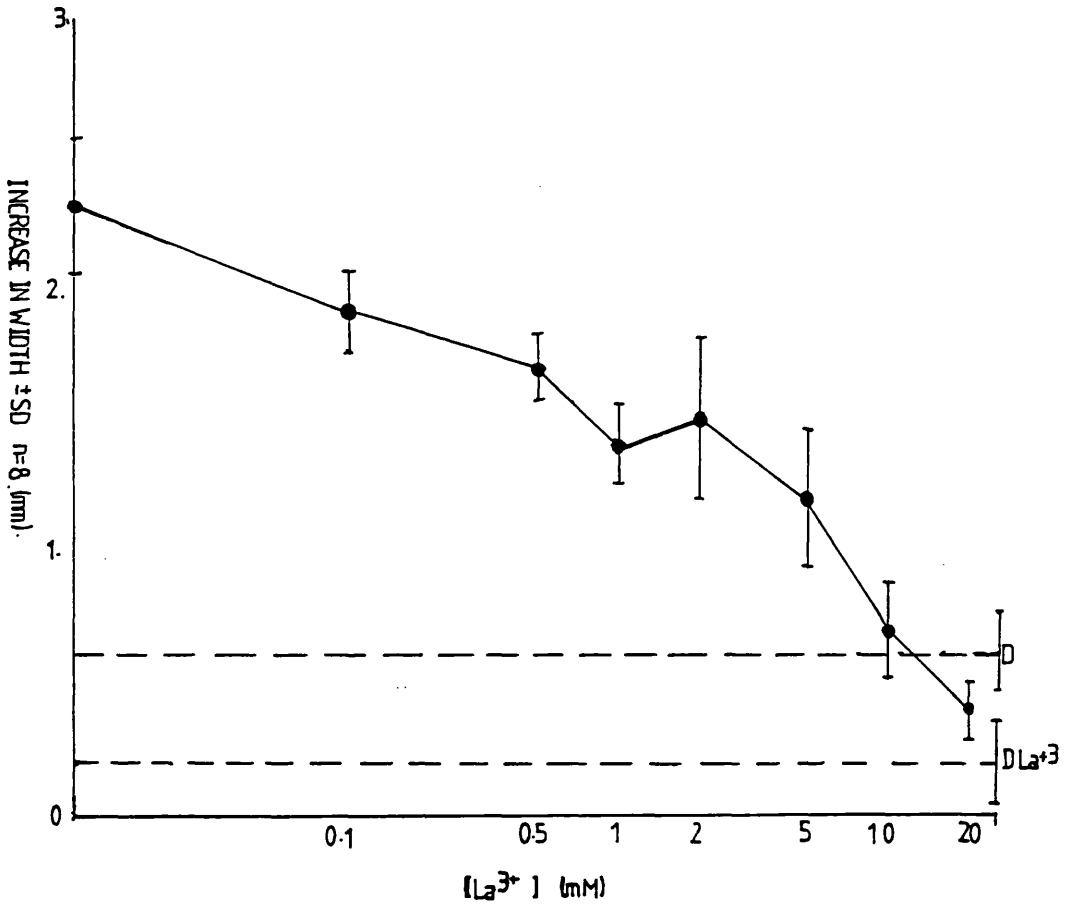
saturating R, then serial R-treatments would be predicted to give a greater effect than a single one.

As shown in Table 6.1, this is not the case. This supports the conclusion that A23187 and phytochrome exert at least partially distinct influences on the unrolling mechanism as providing the most plausible explanation of the data. Further evidence in support of this conclusion will be presented in later chapters.

If A23187 cannot be relied on to accurately mimic the effect of R on unrolling, other approaches are necessary to explore the basis of the Ca^{2+}-dependence of the process. These include the use of various inhibitors with known or supposed effects on aspects of Ca^{2+} metabolism in the cell. One of these is the Lanthanum ion (La^{3+}), which has been described at various times as; an agent to displace Ca^{2+} from its binding sites or membranes, a Ca^{2+} transport blocker and a 'Ca^{2+} antagonist' (Wayne and Hepler, 1984; Rincon and Hanson, 1986; Blumwald and Poole, 1986). It is probable that the first properties combine to create antagonism, although the relative significance of displacement and transport blocking to the inhibition of a response is difficult to determine. In intact tissue, La^{3+} must operate at the outer surface of the plasma membrane since it does not penetrate plant cells (Thomson et al, 1973). Overall, its effects, although disruptive to Ca^{2+} metabolism, are hard to define and its true mode of inhibition (if any) may be quite complex.

The effect of La^{3+} on R-stimulated leaf unrolling was examined using an adaptation of the standard protocol developed in Chapter 3. A range of concentrations of Lanthanum III chloride [pre-dissolved in HCL (1 M)] in MES/NaOH buffer were prepared and the tissue pre-incubated for 2h prior to R and full dark incubation. The results are shown in Fig. 6.7. It is seen that La^{3+} does inhibit unrolling. The concentration/response

Fig. 6.7 The effect of variation in La^{3+} concentration in MES buffer on unrolling of etiolated barley leaf sections following R (5 min at t_o). Unrolling measured at t_{24h} following a dark incubation at 20°C. La^{3+} supplied at t_{-2h}.


INCREASE IN WIDTH ± SD n=8 (mm)
3.
2.
1.
0
D
DLa+3
0·1
0·5
1
2
5
10
20
[La3+] (mM)

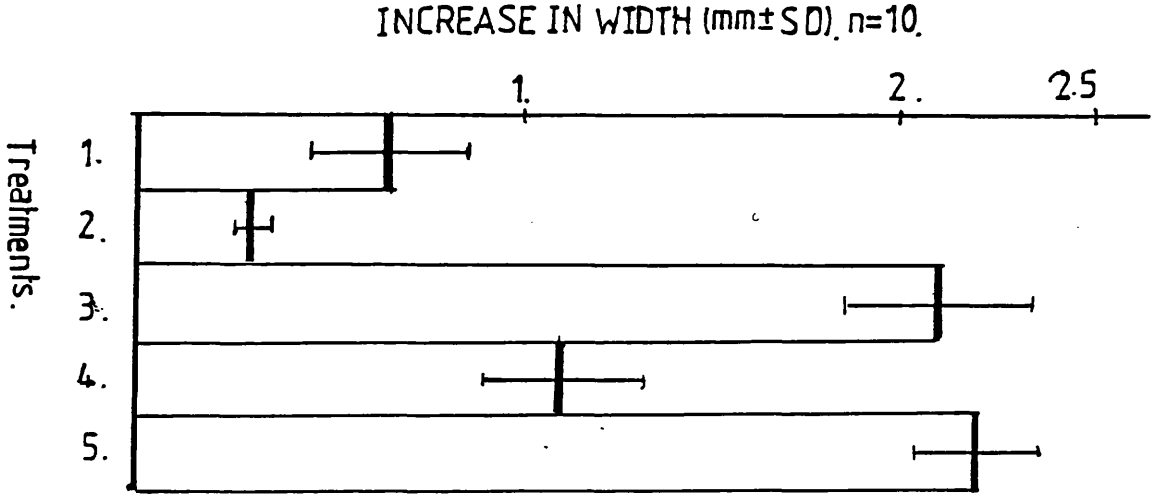
curve can be divided into two approximately semi-log/linear phases, which intersect at the region of 1 - 2 mM La^{3+} addition. This may represent two La^{3+} sensitive phenomena. La^{3+} is cytotoxic; because of this, and the likelihood of the higher concentrations generating more side effects, it was decided that the first region of the dose/response curve (0 — 2 mM) was more likely to be relevant to perturbed Ca^{2+} metabolism. Accordingly 2 mM was chosen as the concentration giving maximal inhibition of this aspect of the response. The effect of delaying La^{3+} supply until $t_{1h\ 30\ min}$ relative to R treatment, following a 2h pre-incubation was then examined. The results are shown in Fig. 6.8. This shows that, given 2h before R, La^{3+} partially inhibits R-stimulated unrolling, as was seen in Fig. 6.7. However, given 1½h after R La^{3+} has no effect at this concentration (2 mM). This is all the more remarkable given the finding that dark unrolling appears to be inhibited by La^{3+}.

The transient effect of La^{3+} on unrolling is similar to that seen in the Onoclea system by Wayne and Hepler (1984) and reaffirms the notion that Ca^{2+}-dependent effects are important within the first 2h of unrolling induction. If the La^{3+} effect is due to perturbed Ca^{2+} metabolism, the induction of unrolling may involve Ca^{2+} at the plasma membrane.

A more specific technique to elucidate aspects of Ca^{2+} metabolism in a response, utilizes a series of aminohexyl-naphthalenesulphonamide derivatives developed by Hidaka and Tanaka (1982). These are known collectively as W-compounds, and are inhibitors of the activity of the calcium receptor protein Calmodulin (CM). This protein is ubiquitous to eukaryotes and functions as a Ca^{2+} receptor at cellular Ca^{2+} levels (Means and Dedman, 1980). Alteration of Ca^{2+} binding to CM can lead to the alteration in activity of a variety of proteins interacting with CM in vitro. These include nitrate reductase (Sane et al, 1988), a variety

Fig. 6.8 Effect of delayed La^{3+} supply on unrolling of R-treated (5 min at t_0) etiolated barley leaf sections in MES buffer, with 2h pre-incubation. Measurements performed at t_{24h} following a dark incubation at 20°C.

Treatments:
1. Dark control
2. La^{3+} (2 mM) at t_{-2h}
3. R 5 min at t_0
4. Treatments '2' and '3' combined
5. As treatment '3' + La^{3+} (2 mM) at $t_{1h\ 30min}$



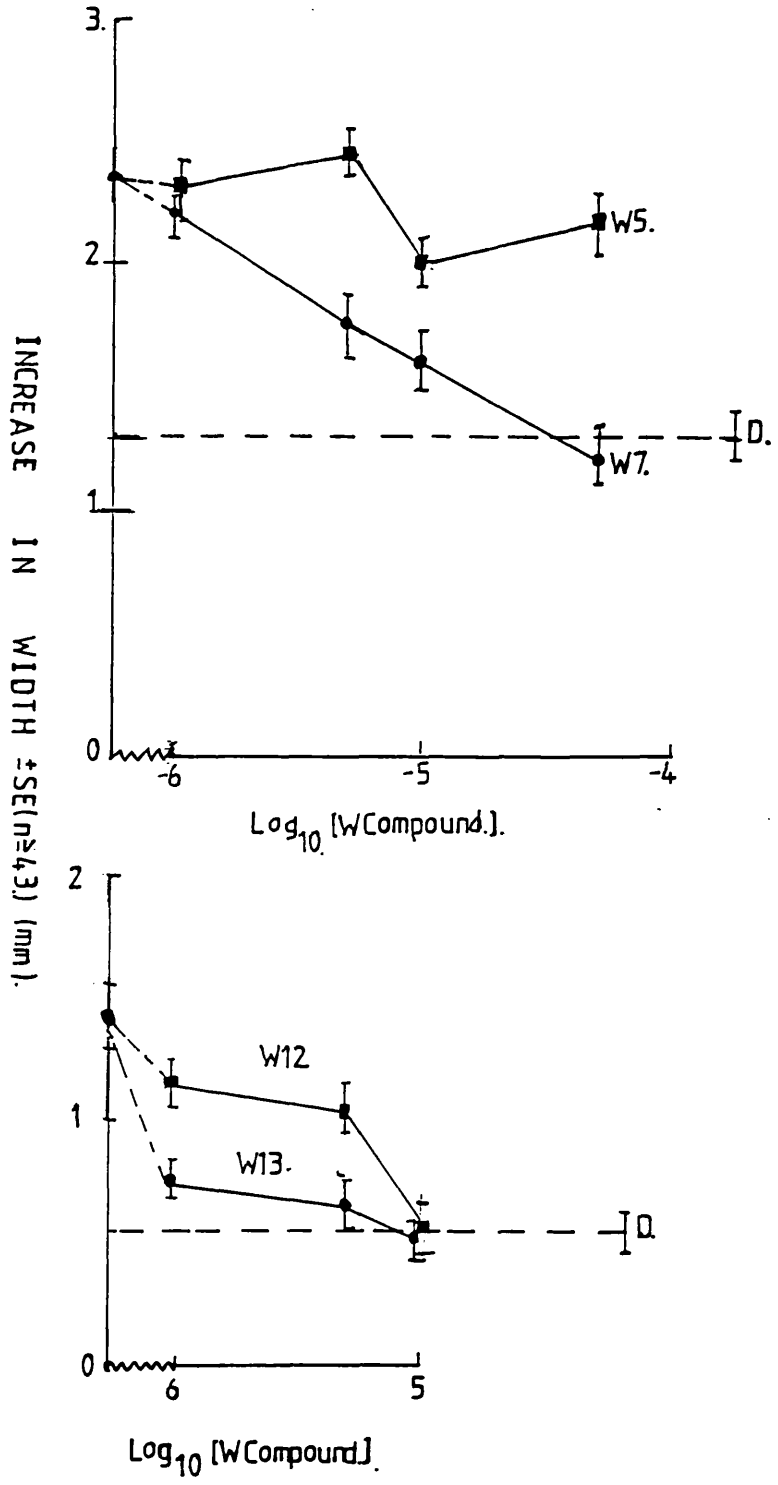
(See legend for treatments.)

of protein kinases (Polya et al, 1986), NAD kinase (Dieter, 1986), and phosphatidic acid phosphatase (Paliyath and Thompson, 1987). Also, a variety of cellular activities including Ca^{2+} transport (Zocchi and Hansen, 1983), phosphatidyl inositol turnover (Sandelius and Morré, 1986) are stimulated by addition of exogeneous CM. These findings demonstrate that CM may be influential in response co-ordination and integration.

The W-compounds are best used in pairs with matched hydrophobicity indicies but differing anti-calmodulin activity within a pair as described by Serlin and Roux (1984). This is achieved by substituting chlorine atoms into the molecule and making use of isomeric variations. In this way a pair can be used to assess at least some of the CM-independent effects of treatment separately from effects due to CM inhibition. This overcomes many of the limitations of other frequently used CM antagonists such as Trifluoroperazine which, because of their hydrophobicity, generate side effects. Most W-compounds are relatively hydrophilic in comparison.

Two pairs of W-compounds were chosen to investigate unrolling. These were W_5 - W_7 and W_{12} - W_{13}. In each pair the latter member has a higher CM affinity than the former. Any increase in inhibitory activity with the latter member that is consistent across a range of concentrations, between pairs is likely to be due to CM binding. This strategy was successfully used by Serlin and Roux (1984) to implicate CM in chloroplast rotation. The results of their use to inhibit unrolling are shown if Figs. 6.9 'a' and 'b'. All W-compounds were dissolved in 1:1 DMSO/Ethanol and added to rapidly stirred low Ca^{2+} buffer incorporating 100 μM Ca^{2+}. Samples were maintained on an orbital shaker as with the A23187 treatments described earlier. Light treatments were given after an arbitrary 4h pre-incubation period for tissue penetration. Control treatments contained DMSO/Ethanol levels equivalent to that

Fig. 6.9 The effect of W-Compounds on unrolling of R-treated (5 min at t_0) etiolated barley leaf sections. Measurements performed at t_{24h} following a dark incubation at 20°C. a) Comparative effect of W5 (upper curve) and W7 (lower curve);
b) Comparative effect of W12 (upper curve) and W13 (lower curve).
Dashed line = Dark control value.


INCREASE IN WIDTH ±SE(n≥43) (mm).
3.
2
1
0
-6
-5
-4
W5.
W7.
D.
Log10 [WCompound.].
a.
2
1
0
6
5
W12
W13.
D.
Log10 [WCompound].
b.

present at the highest concentration of the heaviest W-compound (by molecular weight) being tested.

Fig. 6.9a shows the effect of W5 and W7 on R-stimulated unrolling. W5 has little effect on the response throughout the concentration range whereas W7 shows concentration dependent inhibition in a semi-log/linear fashion down to dark control levels at a concentration of 50 μM. The appearance of significant inhibition ($p < 0.02$) at the 5 μM level for W7 relative to both control and W5 (5 μM) shows the high degree of response sensitivity. In fact, this concentration gives greater than 50% inhibition. For comparison, 50 μM W7 is required to give half maximal inhibition of Ca^{2+}/CM activation of myosin light chain kinase in vitro (Kanamori et al, 1981) and 100 μM W7 is required for 50% inhibition of chloroplast rotation (Wagner et al, 1984). Fig. 6.9b shows that the response is even more sensitive to W12 - W13 inhibition. All concentrations of both W12 and W13 were significantly inhibitory ($p < 0.04$ for 1 μM W12) indicating major CM-dependent effects. However, significantly greater inhibition by W13 over W12 was obtained for both 1 μM and 5 μM concentrations ($p < 0.01$). Serlin and Roux (1984) have also found that W12 - W13 was a more inhibitory combination than W5 - W7, but failed to show significant inhibition below 5 μM W12. The minimum W13 concentration tested in their study was 2.5 μM, giving significant inhibition. From this it is not possible to ascertain whether unrolling is more sensitive to inhibition, although the response to W12 suggests that it may be in a non-specific sense.

Although consistent differences in inhibition within pairs of W compounds should not be interpreted as absolute proof of the involvement of CM (Roberts et al, 1986), the presence of significant differences in inhibition at such low concentrations in both pairs tested is evidence

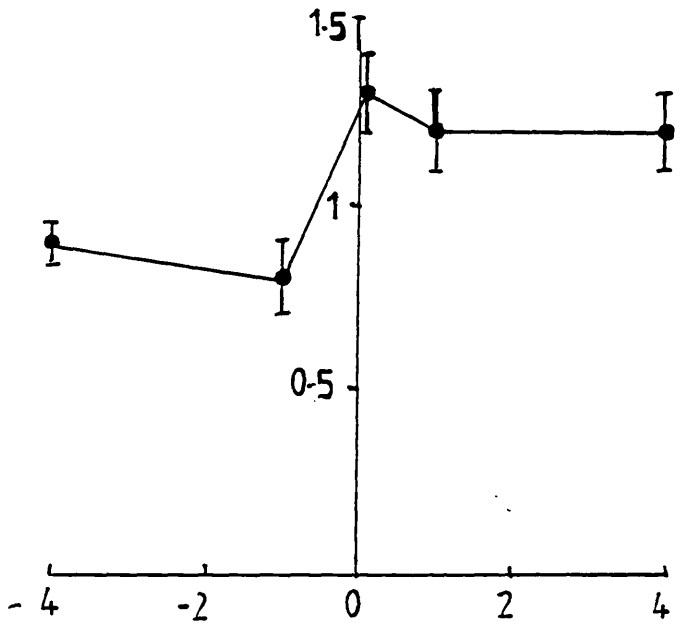
that CM is involved in the R-stimulated unrolling response.

As with La^{3+}, the effect of delaying application of W7 was investigated. Fig. 6.10 shows the results of this experiment. All samples received a 4h pre-incubation and W7 was added at various times relative to R. It is seen that W7 (20 μM) supplied upto 1h before R, gives maximal inhibition of the response. This inhibition is significantly reduced ($p < 0.01$) if supply of W7 is withheld until after R. The data indicate that the shift in effectiveness in W7 must result from the inhibition of a process that is complete within 1h of R at most (assuming W7 takes all of the 1h prior to illumination to penetrate the tissue). The similarity to the La^{3+} data in Fig. 6.8 is obvious. In this case though, W7 continues to inhibit the response significantly after the time-dependent shift has occurred (R control not shown). Since the W5 dose response (Fig. 6.9) shows little indication of side effects due to hydrophobicity/detergent properties, it is possible that this persistant inhibition results from a more general disruption of cell function due to CM inhibition. It could also represent a secondary slower function of CM in unrolling potentiated by the transitory effect indicated within the early stages of unrolling.

Another chemical that has been used to probe the putative role of Ca^{2+} in phytochrome responses is 5-Hydroxytryptamine (5-HT). This is a mammalian neurotransmitter that hydrolyses membrane polyphosphorinositides to yield diacylglycerol (DG) and inositol-1,4,5,-trisphosphate (IP3). IP_3 subsequently mobilises Ca^{2+} (Berridge and Irvine, 1984). There is no evidence for an endogeneous role for 5-HT in plants. Despite this it has been tested for activity in plant systems, seemingly on the basis of the very incomplete evidence that a functional signal transducing Phosphoinositide (PI) cycle exists in plants. This ignores the fact that, in

Fig. 6.10 Effect of delayed W7 addition on unrolling of R-treated (5 min at t_0) etiolated barley leaf sections in MES buffer. Measurements performed at t_{24h} following a dark incubation at 20°C.

MEAN INCREASE IN WIDTH (mm) ± SE n≥ 36.

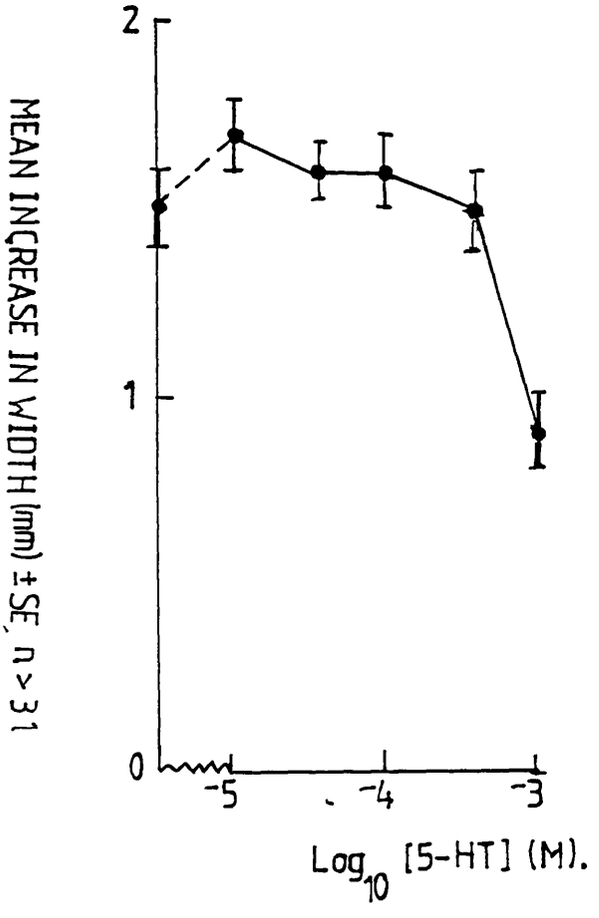


TIME OF ADDITION (h).

mammalian systems, many receptors couple to the PI cycle, and, to reiterate, there are no grounds for supposing a 5-HT recepter to exist in plants. The use of 5-HT to initiate PI-driven Ca^{2+} release in plant cells is thus rather questionable and based on many assumptions. Nevertheless, 5-HT has been shown to be active in mimicing some phytochrome responses. Das and Sopory (1985) showed that 5-HT could substitute for R in a non-additive way in stimulating Ca^{2+} uptake in maize protoplasts, it would therefore seem that 5-HT can cause redistribution of Ca^{2+} at the cellular level. Subsequently 5-HT was shown to substitute for red light in light-dependent gravitropism in maize roots, another phytochrome mediated effect (Reddy et al, 1987). The same report contains some very incomplete data to show that both R and 5-HT increase the level of IP_3 measurable. Unfortunately the data are expressed as percentages derived from three and two replicates respectively and so are not amenable to statistical analysis.

To investigate the properties of 5-HT in relation to leaf unrolling the effect of a range of concentrations on dark unrolling in low Ca^{2+} buffer incorporating Ca^{2+} (100 μM) was ascertained. The results are shown in Fig. 6.11. No significant effects were observed except at 1 mM where a substantial inhibition was seen. It was noted, however, that all concentrations greater that 10 μM gave rise to browning of the leaf tissue. This indicated that 5-HT was toxic which could mask any promotive effect. To reduce the risk of this the duration of the treatment with 5-HT was limited to 30 min. After this period the sections were drained, washed in buffer and resuspended in buffer without 5-HT for the remainder of the incubation. Non-5-HT treated controls were also washed and resuspended at this time as washing was noted to have a small promotive effect on dark unrolling. The effect of these 'pulsed' 5-HT

Fig. 6.11 The effect of 5-Hydroxytryptamine (5-HT) on dark unrolling of etiolated barley leaf sections in MES buffer: the effect of concentration. Measurements performed at t_{24h} following a dark incubation at 20°C.


MEAN INCREASE IN WIDTH (mm) ± SE, n > 31
2
1
0
-5
-4
-3
Log10 [5-HT] (M).

treatments on unrolling in the dark and following R treatment is shown in Fig. 6.12. Both concentrations of 5-HT used gave highly significant promotion of dark unrolling ($p < 0.001$). No additive effect is seen with R. A pulse of 5-HT (50 μM) can thus partially mimic the effect of R in inducing unrolling. It should also be noted that the concentration of 5-HT effective in this is an order of magnitude lower than any previously published figure shown to be active in plants.

Although one explanation of these data is that 5-HT generates a Ca^{2+} transient in the cells of the leaf section and that this induces unrolling, in a similar way to R, there is an alternative. Plant cell membranes possess a relatively unspecific amine transporter (Sanders D., Personal communication). 5-HT possesses an amino group, and is charged in solution. Transport of 5-HT by this route into cells could initiate cell depolarization and thus induce a response. To test this possibility ammonium chloride (NH_4Cl) was substituted for 5-HT as a charged species containing the amino moiety. The results are shown in Table 6.2. No effect is seen with continuous NH_4^+ treatment. There is a suggestion of a promotive effect with pulsed treatment but this is not significant ($0.29 < p < 0.3$). It is therefore unlikely that amino transport can mimic the 5-HT effect, which indicates that a separate mechanism is responsible.

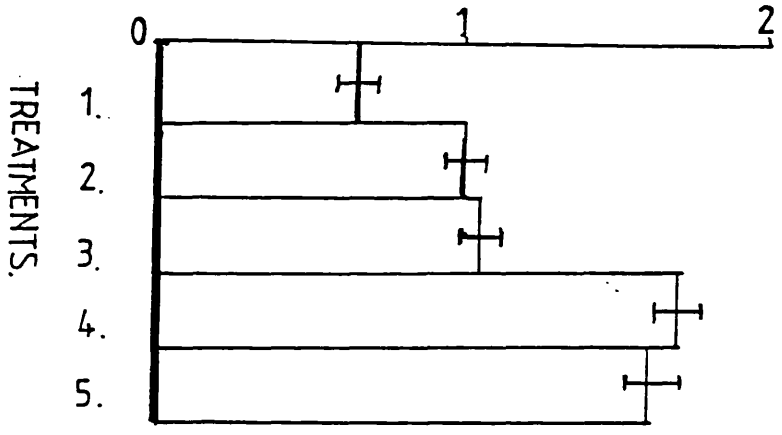
This completes the work relating to Ca^{2+} for this chapter. There follow some data that, although unrelated to the role of Ca^{2+} in unrolling, have some bearing on the range of activities Ca^{2+} may mediate in unrolling.

Virgin (1962) was the first worker to look at inhibition of unrolling by the external osmoticum during incubation, and, since leaf unrolling results from cell expansion, the end process of transduction

Fig. 6.12 The effect of 5-Hydroxytryptamine pulse (30 min at t_o) on the unrolling of R-treated (5 min at t_o) and untreated etiolated barley leaf sections in MES buffer. Measurements performed at t_{24h} following a dark incubation at 20^oC.

Treatments:
1. Non-treatment (Dark control)
2. 5-HT pulse (30 min at 50 μM) at t_o
3. 5-HT pulse (30 min at 100 μM) at t_o
4. R (5 min at t_o)
5. Combination of treatments '4' and '2'

MEAN INCREASE IN WIDTH (mm) ± SE n ≥ 35.



(For treatments see legend.)

Table 6.2 The effect of continuous and pulsed NH_4^+ treatments on dark unrolling.

Treatment	Mean increase in leaf width (mm) ± SE (n ≥ 35)
Dark	0.90 ± 0.092
NH_4^+ continuous (50 μM)	1.10 ± 0.08
Dark (washed at 30 min)	1.05 ± 0.139
NH_4^+ pulse 30 min at 50 μM)	1.25 ± 0.13

must involve osmotic uptake of water. Fig. 6.13 shows the effect of decreasing external osmotic potential (Π_0) on leaf unrolling in both dark and R-treated tissue. Tissue was incubated in a range of concentrations of mannitol in MES buffer to effect this. It is seen that reductions in Π_0 as small as 0.12 MPa reduce both dark and R-stimulated unrolling. The R-stimulated response is reduced to dark control levels by a reduction in Π_0 of 0.24MPa. This is a much smaller figure than the previous estimate of Kang (1971) in which a reduction of 0.85 MPa was required. Reductions of this magnitude (0.24MPa) are reported to affect cell growth, wall synthesis and protein synthesis in plants (Hsaio et al, 1976) and either of these latter two factors could affect unrolling, which is a form of cell growth, in addition to the simple limitation of osmotic uptake of water by Π_0. That factors involved in wall plasticity operate in R-stimulated unrolling is shown by Table 6.3 which shows the effect of incubating unrolled R-treated sections in mannitol concentrations sufficient to severely limit unrolling.

Mannitol (0.7M) is seen to inhibit R-stimulated unrolling well below dark control levels. However, when R-treated, unrolled tissue is incubated in mannitol the unrolling is not reversed. In fact expansion apparently continues. Therefore irreversible changes occur during unrolling, whose inhibition by moderate osmotic stress limit unrolling rather than simple inhibition of water uptake by Π_0. Once these changes have occurred unrolling seems relatively insensitive to osmotic inhibition. The fact that expansion continues once commenced even when placed in mannitol (0.7M) strongly suggests that it is the induction of unrolling rather than the process itself that is initially inhibited. That growth continues at such a high value for Π_0 (- 1.7 MPa) is remarkable, as it must be close to the point of incipient plasmolysis (zero

Fig. 6.13 The effect of decreasing osmotic potential on the unrolling of R-treated (upper curve) and dark control (lower curve) sections from etiolated barley leaves in MES buffer/mannitol. Measurements performed at t_{24h} following a dark incubation at 20°C. Light treatment = 5 min at t_0. (Mean ± S.E., n ≥ 31)

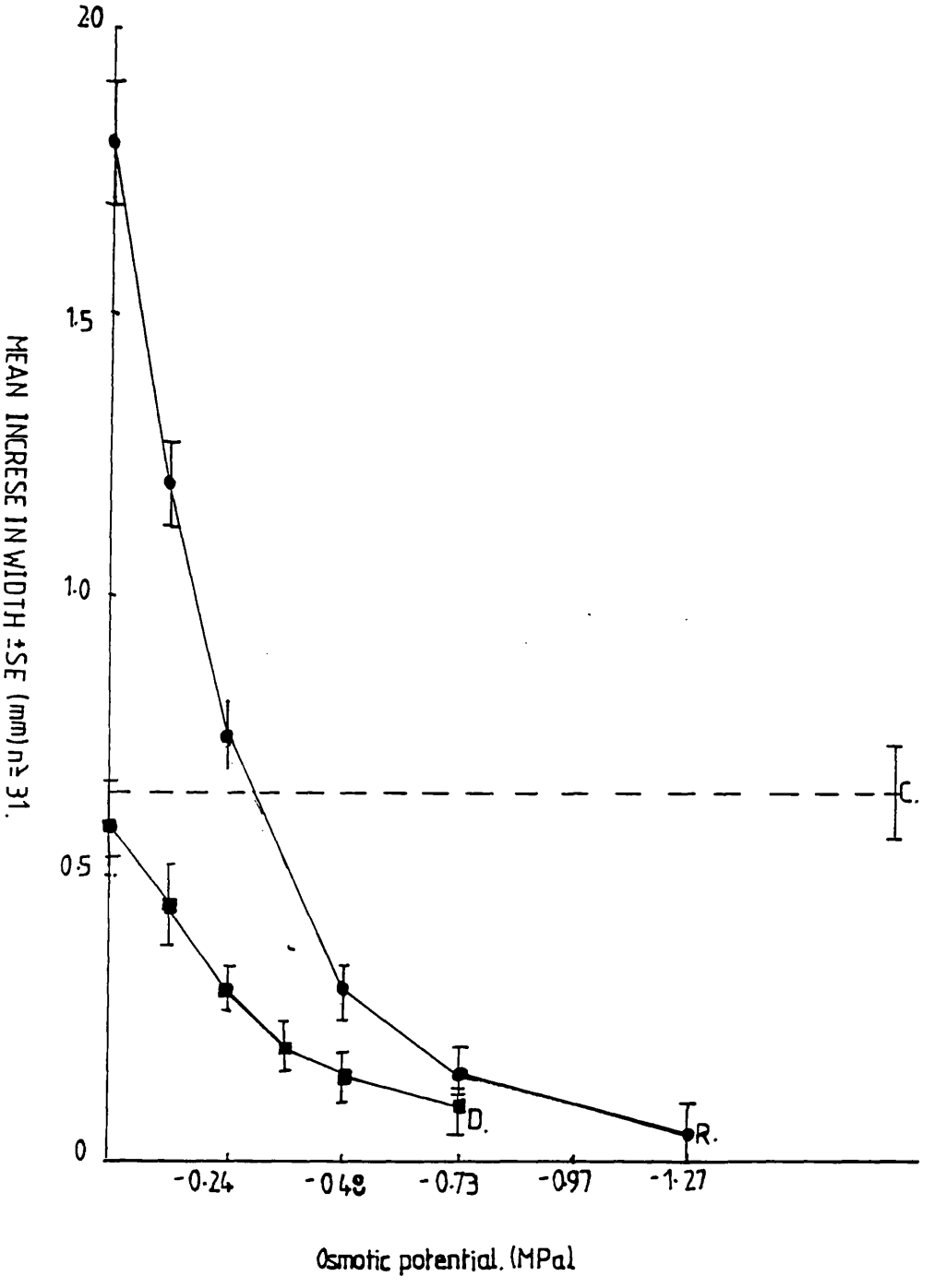

2.0
1.5
1.0
0.5
0
MEAN INCRESE IN WIDTH ±SE (mm) n≥ 31.
C.
D.
R.
-0.24
-0.48
-0.73
-0.97
-1.27
Osmotic potential. (MPa)

Table 6.3 The Effect of Mannitol (0.7 M) on R-stimulated unrolling and on unrolled tissue.

Treatments		Mean increase in width (mm) ± SE (n ⩾ 30)
Dark control	Incubated 24h	0.55 ± 0.07
R at t_o	Incubated 24h	2.0 ± 0.11
Buffer/0.7 M Mannitol, R at t_o	Incubated 24h	0.15 ± 0.03
R treated, incubated (24h) then placed in Mannitol (0.7M)/buffer (24h)		2.5 ± 0.13

turgor). The fact that turgor would be expected to be low under these circumstances suggests that wall plasticity may be modulated in unrolling. Other workers have suggested this before (Virgin, 1962; Rajagopal and Masden, 1981).

Conclusions.

1. In common with some other phytochrome responses A23187 can induce unrolling in the absence of light. This appears to result from the ionophoric conduction of Ca^{2+}. However, the A23187 and R effects do not appear to operate in the same way, thus a simple rise in $[Ca^{2+}]_{cyt}$ does not appear to present an adequate model of phytochrome action. Therefore unrolling may be Ca^{2+} activated but is not necessarily Ca^{2+}-mediated.
2. Studies with La^{3+} and the W-compounds indicate the likelihood that Ca^{2+} receptors especially CM are central to the induction of unrolling. Temporal shifts in inhibition indicate a role within the first hour after R. Sites and/or channels at the plasma membrane are indicated.
3. The neurotransmitter 5-HT stimulates unrolling in the absence of light. The lack of additive effects with R indicates it shares the same response path. One effect of 5-HT in biological systems is to activate the PI cycle, which mobilizes Ca^{2+} from internal stores.
4. There are osmotically-sensitive irreversible changes, probably involving cell wall properties that accompany the induction of unrolling by R. By deduction from Chapter 3 these must occur after the first Ca^{2+}-dependent step in unrolling.

CHAPTER 7 : Ca^{2+} AND PLANT GROWTH SUBSTANCE-STIMULATED LEAF UNROLLING

R-treatment is not the only natural factor that induces leaf unrolling in dark tissue. A variety of chemicals may be isolated from plants that induce unrolling with varying degrees of activity, in complete darkness, when supplied exogenously (Reid et al 1968; Beevers et al, 1970; Sundquist and Briggs, 1982). Of principal interest from a physiological standpoint are the effects of the gibberellins and cytokinins, both recognized as important plant growth substances. Like A23187 in this study, both exhibit additive effects with R (Kang, 1971; Sundquist and Briggs, 1982). It has been suggested that the cytokinin effect is mediated through endogenous gibberellins (Loveys and Wareing, 1971b). If this is so, and if, as seems likely, R-stimulated unrolling does not operate entirely via changes in endogenous gibberellins, then the prospect that at least two mechanisms of unrolling etiolated tissue are operational presents itself. That this situation resembles that proposed with regard to A23187 and light in the previous chapter, raises the possibility that A23187 and the plant growth substances share a mechanism. This chapter attempts to analyse this suggestion further.

As a starting point, it was decided to define the dose/response relationship for unrolling in response to gibberellins and cytokinins within the system developed for this study. Previous studies show variation in the responsiveness of tissues to added growth substances which presumably arises from the assay system. This makes such experi-

ments a necessary precaution. The growth substances selected for the investigation were gibberellic acid (GA_3) and kinetin). Dissolved to form the appropriate stock solution, a range of concentrations of growth substance in buffer were prepared and the dark-unrolling induced by these treatments measured following incubation of dark grown leaf sections therein.

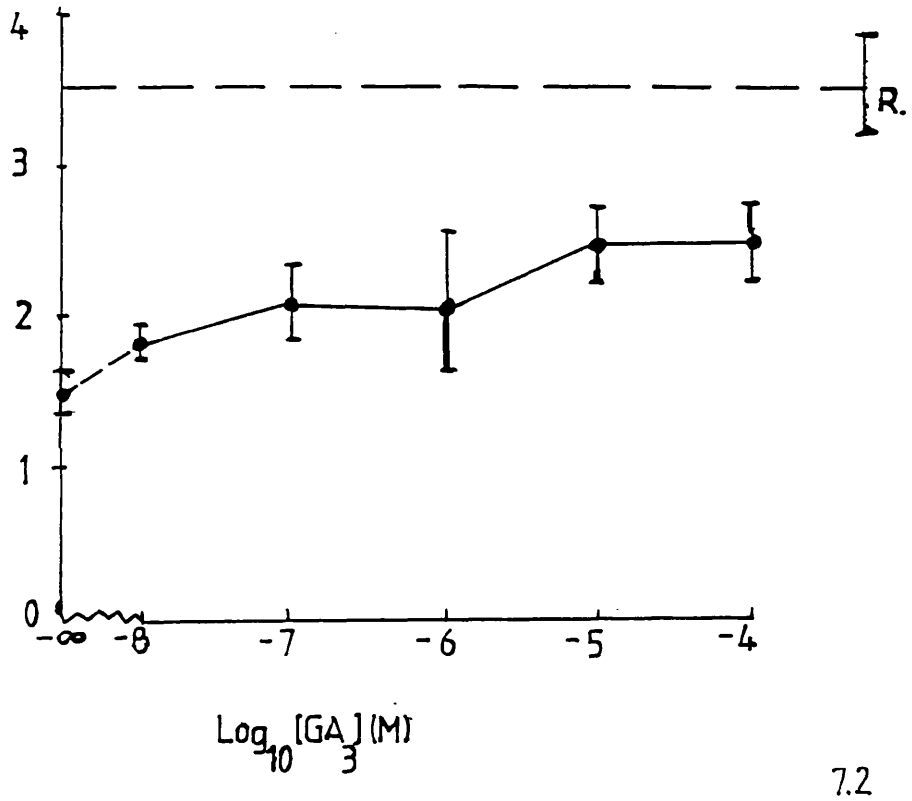
The concentration/response curve of dark unrolling in response to GA_3 is shown in Figure 7.1. Unrolling appears very sensitive to GA_3. As little as 10 nM gives some promotion. However, the overall magnitude of the response is small, and the increase in response with increasing GA_3 limited. Previous determinations in barley have yielded conflicting results. Kang (1971) failed to detect any response to GA_3 in dark-unrolling. However, the data of Poulson and Beevers (1970) and Sundquist and Briggs (1982) suggest an optimum promotion around 1 - 20 μM GA_3. The absence of an optimum concentration here bears closer resemblance to the results obtained using wheat leaves (Beevers et al, 1970; Loveys and Wareing, 1971b). These also show a poor response to increasing GA3 concentration and a small overall response. In the absence of an optimal concentration, a concentration of 100 M was chosen for further experiments.

The concentration/response curve for kinetin and dark-unrolling is shown in Figure 7.2. Promotion of dark unrolling is seen at 100 nM and the response increases with increasing kinetin concentration. There is no optimum point below 100 μM. The increase in response with increasing concentration is much greater than that seen with GA_3, as is the magnitude of the response obtained. This appears to be the case in previous studies (Kang, 1971). The influence of kinetin on unrolling has not been so widely studied as that of GA_3. However, a previous dose/response

Fig. 7.1 The effect of Gibberellic acid concentration on dark unrolling of etiolated barley leaf sections in MES buffer: measurements performed at t_{24h} following a dark incubation at 20°C. (Mean ± S.D., n = 5)

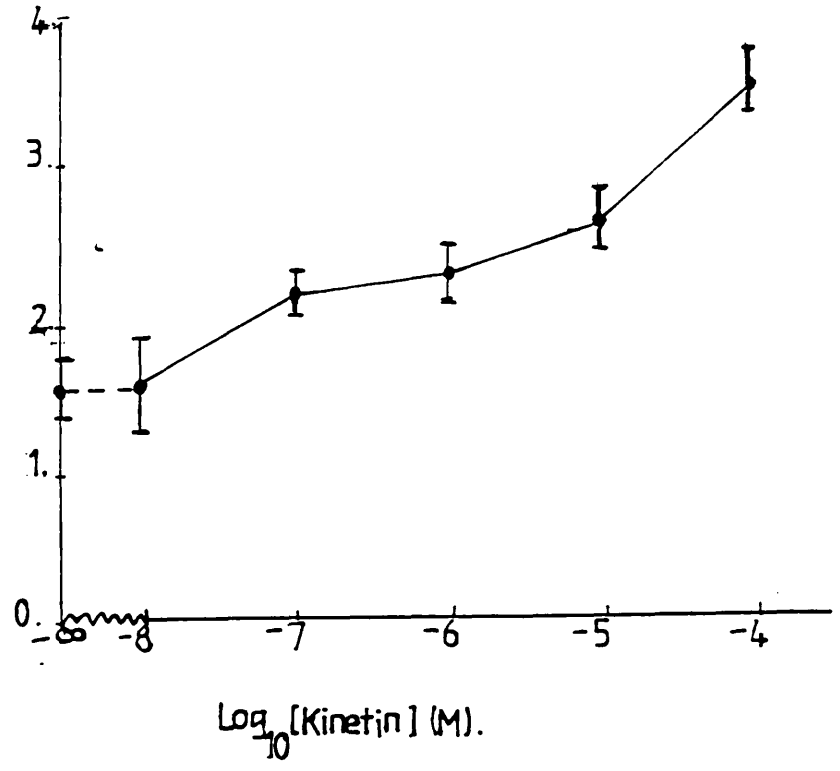
Fig. 7.2 The effect of Kinetin concentration on dark unrolling of etiolated barley leaf sections in MES buffer: measurements performed at t_{24h} following a dark incubation at 20°C. (Mean ± S.D., n = 5)

7.1



MEAN INCRESE IN WIDTH ± SD n = 5.

7.2



determination in barley indicates an optimum concentration for induction of 1 - 10 μM (Kang, 1971). Why this is not seen in the present system is not clear. On the basis of the dose/response relationship, a kinetin concentration of 100 μM was chosen for further experiments. As well as giving a large response, the equivalence of dosage would allow comparison to similar experiments including GA_3.

Having selected appropriate experimental concentrations, the Ca^{2+} dependence of GA_3 and kinetin induced unrolling was investigated. This was effected using EGTA and Ca^{2+} as in Chapter 3, substituting GA_3 or kinetin treatment for R at the end of the 45 minute pre-incubation.

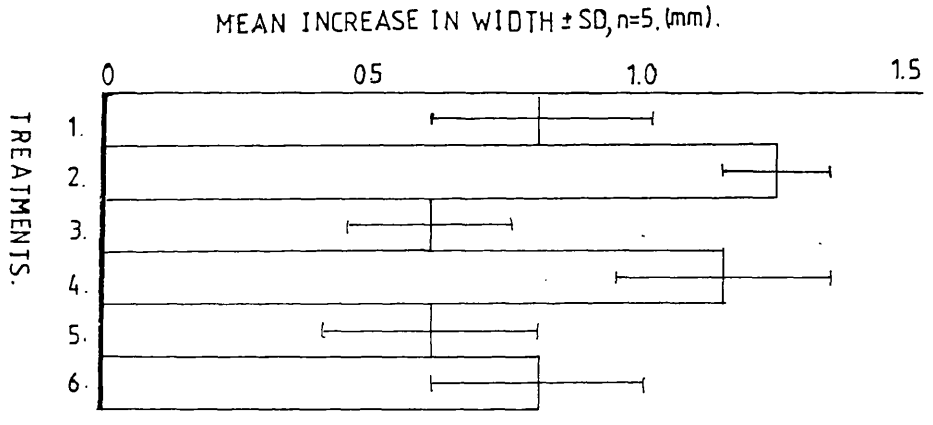
Figure 7.3 shows the effect of EGTA, Ca^{2+} and Mg^{2+} on GA_3-induced unrolling. Because of the small sample sizes and the relatively small response obtained with GA_3 compared to that seen with R or kinetin, interpretation of this figure is difficult, but the following trends are indicated. As seen in Figure 7.1, GA_3 increases unrolling in the dark. EGTA inhibits this promotion, but does not affect dark unrolling substantially. Ca^{2+} (5 mM) may inhibit the GA_3 effect in isolation. However, with EGTA the inhibitory effect of both EGTA and Ca^{2+} disappears, restoring GA_3 promotion. Mg^{2+} (5 mM) probably does not substitute for Ca^{2+} in removing the EGTA effect.

Superficially GA_3-stimulated unrolling appears, like R-stimulated unrolling, to be Ca^{2+}-dependent. However, what is more important is the possible loss of effectiveness of GA_3 in the presence of Ca^{2+} (5 mM) alone. It is not clear whether this represents the reduction in response seen with both dark and R-stimulated unrolling with increasing $[Ca^{2+}]_{ext}$ (see Fig. 6.1), or a separate interaction of Ca^{2+} and GA_3 indicative of a different response mechanism. It may be noted in this context that the A23187 effect was completely abolished at 5 mM $[Ca^{2+}]_{ext}$

Fig. 7.3 The effect of EGTA, Ca^{2+} and Mg^{2+} on the Gibberelic acid-induced unrolling of etiolated barley leaf sections during dark incubation. Measurements performed at t_{24h}.

Treatments:

1. None (control)
2. Gibberellic acid (0.1 mM)
3. Gibberellic acid + EGTA buffer
4. As treatment '3' + Ca^{2+} (5 mM) at t_o
5. As treatment '2' + Ca^{2+} (5 mM) at t_o
6. As treatment '3' + Mg^{2+} at t_o



(For treatments see legend.)

(Fig. 6.3).

Fig. 7.4 shows the results of a similar experiment to that described for Figure 7.3, using kinetin instead of GA_3. Caution is again required in interpretation of this figure because of the small sample sizes. However, similar conclusions may be drawn from this figure as the previous one. These are that kinetin promotes the degree of unrolling in the dark considerably; that this promotion is Ca^{2+}-dependent and inhibited by EGTA; that Ca^{2+} (5 mM) alone appears to inhibit much of the promotive effect of kinetin, but can remove the inhibitory effect of EGTA; and that Mg^{2+} (5 mM) cannot substitute for Ca^{2+}.

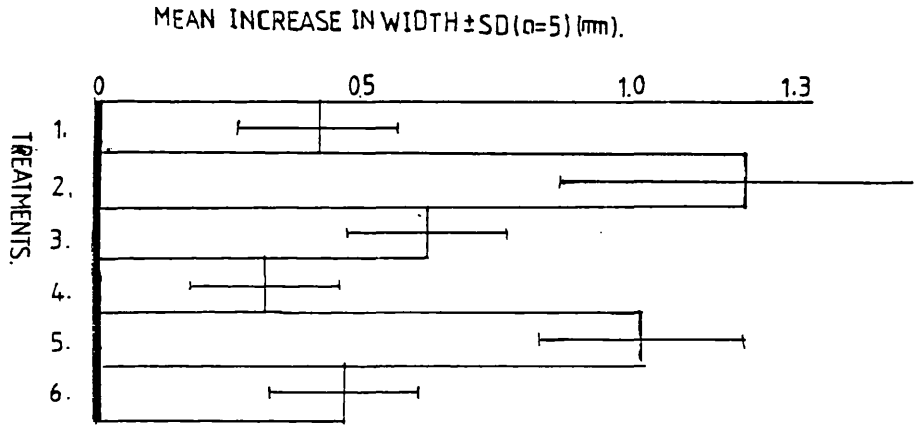
If these growth regulating substances do operate in a similar way to A23187 to induce unrolling then, unlike R treatment, there should be no additive effect with the ionophore. This was investigated using low Ca^{2+} buffer incorporating 100 μM Ca^{2+}, the effects of A23187 and the growth substances being compared. Preparation of A23187 solutions, incubation techniques and addition of equivalent amounts of DMSO to non-ionophore treated samples were as described in Chapter 6.

Figure 7.5a shows the effect of kinetin and A23187 on dark unrolling. Both A23187 and kinetin induce a large promotion in dark unrolling relative to the controls. However, there is no additive effect when the two treatments are combined. Although the treatments in combination appear to give a smaller promotion than kinetin alone, this effect is not significant ($0.17 < p < 0.18$).

Figure 7.5b shows the effect of GA_3 and A23187 on dark unrolling. Again both A23187 and GA_3 individually promote dark unrolling well above control values. As with kinetin there is no additive effect between GA_3 and A23187. However, this result is difficult to interpret as there appears to be a negative interaction between A23187 and GA_3 that is just

Fig. 7.4 The effect of EGTA, Ca^{2+} and Mg^{2+} on the kinetin-induced unrolling of etiolated barley leaf sections in MES buffer during dark incubation. Measurements performed at t_{24h}. (Mean $\pm$ S.D., n = 5)

Treatments:
1. None (control)
2. Kinetin (0.1 mM)
3. As treatment '2' + Ca^{2+} (5 mM) at t_o
4. As treatment '2' in EGTA buffer
5. As treatment '4' + Ca^{2+} (5 mM) at t_o
6. As treatment '4' + Mg^{2+} (5 mM) at t_o



(For treatments see legend)

Fig. 7.5 Interactions between growth regulators and A23187 (20 μM) in the induction of unrolling etiolated barley leaf sections in MES buffer during dark incubation. Measurements performed at t_{24h}.

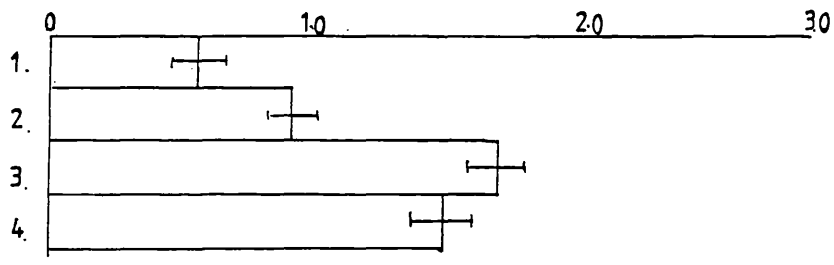
a. The interaction of kinetin (0.1 mM, from t_0) with A23187 treatment
Treatments:
1. None (control)
2. Kinetin
3. A23187
4. Kinetin + A23187

b. The interaction of GA_3 (0.1 mM from t_0) with A23187 treatment, together with the effect of R on GA_3 treatment (5 min at t_0)
Treatments:
1. None (control)
2. A23187
3. GA_3
4. A23187 + GA_3
5. R
6. R + GA_3

All treatments performed in the presence of Ca^{2+} (0.1 mM).

a.

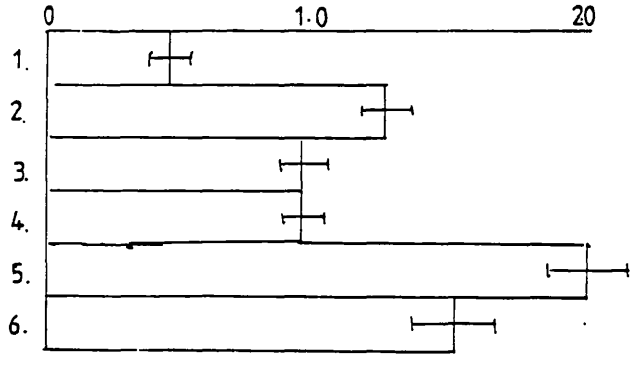
MEAN INCREASE IN WIDTH ± SE n ≥ 43.



TREATMENTS

MEAN INCREASE IN WIDTH ± SE n ≥ 43.

b.



(For treatments see legend).

significant ($P = 0.05$). There are several possible explanations for this, the most likely being that the high concentration of GA_3 used reduces the magnitude of the induced response. This is indicated by a comparison of the effect of R treatment and R and GA_3 treatment in combination. The effect of R is significantly reduced in the presence of GA_3 ($p < 0.05$). In passing it may be mentioned that the concentration of GA_3 used was considerably higher at 0.1 mM, than that expected from in vivo measurements in other systems. Alternative explanations of the data include invoking the existence of enhanced Ca^{2+}-transport via GA_3 leading to superoptimal $[Ca^{2+}]_{cyt}$ in the presence of A23187, or GA3-induced Ca^{2+}-sensitivity changes in the response profile. However, the fact that additive effects between GA_3 and R (and not negative interaction) have been demonstrated by other workers in unrolling systems (Sundquist and Briggs, 1982) using much lower concentrations of GA_3, indicates that the toxicity of high GA_3 concentrations is a more plausible explanation.

From the above it may be stated that GA_3, kinetin and A23187-induced unrolling share certain properties. All are inhibited at 5 mM $[Ca^{2+}]_{ext}$, and neither growth substance shows additive effects with A23187, although in the case of GA_3 this may be a toxic effect. In addition to this, if it is accepted that the negative interaction between R and GA_3 demonstrated here is in fact due to the high concentrations used, then the results of Sundquist and Briggs (1982) and Kang (1971) indicate that in certain circumstances GA_3 and kinetin, in common with A23187 (see Chapter 6), exhibit additive effects with R. This implies that there are at least two mechanisms by which unrolling of etiolated leaf sections may be induced.

Conclusions.

1. Unrolling in response to both GA_3 and kinetin is a Ca^{2+}-dependent process.
2. The induction of unrolling by R differs qualitatively from that induced by A23187 and growth substances in terms of its response to external $[Ca^{2+}]$ indicating that separate processes may be operating.

CHAPTER 8 : Ca^{2+}-SENSITIVITY MODULATION AND UNROLLING

The implication of the previous chapters, that A23187-induced unrolling differs from phytochrome-induced unrolling, leaves the question of how phytochrome induces unrolling unanswered. The experiments involving A23187 indicate that unrolling may be Ca^{2+}-activated, but if this is the case then the phytochrome effect cannot be Ca^{2+}-mediated. Against this must be set the evidence for the involvement of CM in the phytochrome-induced response, and the responsivity to 5-HT, both of which are indicative of Ca^{2+}-mediative mechanisms of the sort proposed by workers from Haupt and Weisenseel (1976) through to Roux et al (1986) as general models of phytochrome action. The conflict between these two lines of evidence is clear, and implies a missing factor that may resolve the apparent contradictions of the data. The aim of this chapter is to attempt this resolution and lay the foundations for the hypothesis of phytochrome action in unrolling to be developed in the next (final) chapter.

The majority of work on Ca^{2+}-signalling in plants has concentrated on the prospect of information being conveyed via changes in Ca^{2+} activity (amplitude modulation). This ignores the possible contribution to signal transduction from controlled variation of receptor affinity for Ca^{2+}, allowing response induction in the absence of a change in $[Ca^{2+}]$ (sensitivity modulation). The possible importance of sensitivity modulation in plant cell signalling at the primary messenger level was first proposed by Trewavas (1981). Hepler and Wayne (1985) have

discussed the possibility of sensitivity modulation at the second messenger level in specific relation to Ca^{2+}-sensitivity, but there is only limited evidence for such a scenario in plant systems. In animal systems there is evidence that the sensitivity of Protein kinase C to Ca^{2+} activation is increased by the second messenger 1,2-Diaclyglycerol (1,2-DG). Attempts have been made to isolate Protein kinase C activity in plants. The activity of protein kinase C is a function of a variety of co-factors including some phospholipids, Ca^{2+}, 1,2-DG, and free fatty acids. These properties have been used to screen kinase activity isolated from various plant membrane preparations. Several enzyme activities have been demonstrated that show activation by one or more of the above factors either singly or in combination (Schafer et al, 1985; Muto and Shimogarawa, 1985; Morré, 1986; Olah and Kiss, 1986; Elliot and Skinner, 1986; Klucis and Polya, 1987). The modulation of Ca^{2+} sensitivity has not been demonstrated in response to 1,2-DG, although Klucis and Polya (1987) have shown effective reduction in the Ca^{2+} requirement for activation by free fatty acids in a preparation from silver beet leaves. Also lacking is a functional demonstration of a physiological process in plants modulated by these protein kinase C-type enzymes, apart from protein phosphorylation itself. In animal systems the enzyme is implicated in the regulation of cell proliferation, exocytosis and DNA synthesis (Berridge, 1984; Gomperts, 1986).

An obvious approach in the search for plant processes regulated by protein kinase C is to look for effects produced by the co-factors/activators at low concentrations. With the exception of Ca^{2+}, and its attendant ambiguity due to sensitivity <u>vs</u> amplitude changes, this has not been investigated. Of the other co-factors, phospholipids and free fatty acids may be present at saturating levels in the resting state (Klucis and

Polya, 1987). However, 1,2-DG is not a recognized stable component of membranes, like phospholipids, or generated in the general metabolism of the cell, like free fatty acids may be. 1,2-DGs will only be generated by the action of phospholipase C on phospholipids, a step which is central to signal transduction in animal cells. As such, 1,2-DGs are ideal factors to manipulate protein kinase C activity *in vivo*, since they are not generally abundant in the cell and have no other known biological effects. It was decided to use 1,2-DG to evaluate the possible significance of sensitivity modulation in leaf unrolling, and thus provide a model system of an *in vivo* protein kinase C driven process in plants. This study took advantage of the availability of short chain, highly pure 1,2-DGs, shown by Lapetina et al (1985) to be potent *in vivo* and *in vitro* activation of protein kinase C. Their relatively high solubility in aqueous media makes them especially suitable for *in vivo* work. The DG selected for this work was 1,2-dioctanoyl-*rac*-glycerol (DiC8:0), which was prepared in a stock solution of ethanol and added to rapidly stirred 'low Ca^{2+}' buffer incorporating 0.1 mM Ca^{2+} to give a final concentration of 20 μM. Equivalent amounts of ethanol were added to non-DiC8:0 treated samples. Leaf sections were added and dark incubated on an orbital shaker at 60 revs/min. The effect of DiC8:0 on unrolling in the dark is shown in Fig. 8.1. The inclusion of 1,2-DG massively increases dark unrolling relative to control sections. This provides the first demonstration of the biological activity of 1,2-DG in plants *in vivo*, and, since the only known biological effect of 1,2-DG is to activate protein kinase C, provides circumstantial evidence for the existence of non-Ca^{2+}-mediated processes involving phosphorylation in the induction of unrolling.

The effect of 1,2-DG on unrolling was compared to that of R (5 min)

Fig. 8.1 (Upper histogram)

The effect of DiC8:0 on unrolling of etiolated barley leaf sections in MES buffer in the presence of Ca^{2+} (0.1 mM). Sections were measured at t_{24h} following a dark incubation at 20°C. DiC8:0 was added at t_o. Sections were incubated on an orbital shaker at 60 revs/min.

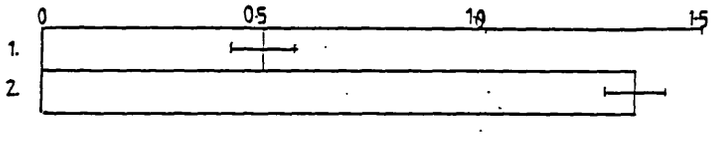
Treatments:
1. No treatment
2. DiC8:0

Fig. 8.2 (Lower histogram)

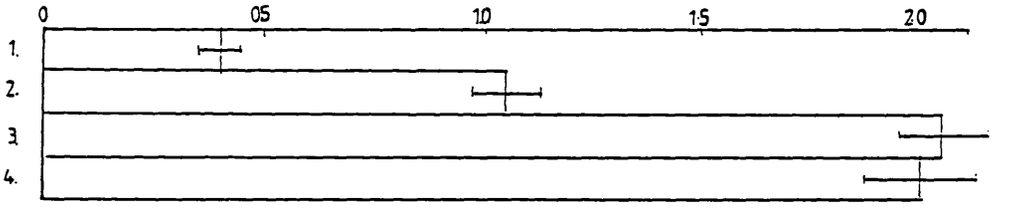
See legend Fig. 8.1 for conditions. Effect of DiC8:0 and R treatment (5 min at t_o) in combination.

Treatments:
1. No treatment
2. DiC8:0
3. R
4. R + DiC8:0

MEAN INCREASE IN WIDTH ± SE n≥41 (mm).



MEAN INCREASE IN WIDTH ± SE n≥50 (mm).



TREATMENTS.

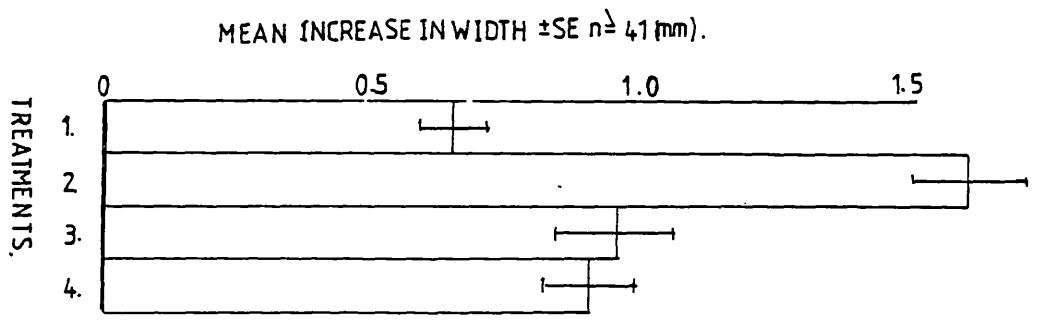
(for Treatments see legend.)

in a similar experiment to that described above, with the introduction of a R treatment immediately after the addition of DiC8:0. The results of this are shown in Fig. 8.2. The orignal observation of 1,2-DG promoted unrolling in darkness, is reproduced. The magnitude of this promotion is seen to be smaller than the effect of R, an observation that may be due to non-optimization of reagent concentrations. It is also apparent that the effects of R and 1,2-DG are not additive. It follows from this that 1,2-DG operates differently in unrolling to both A23187 and the plant growth substances GA_3 and kinetin. The complete disappearance of the promotive effect of 1,2-DG in the presence of R could indicate a shared response path, or it could represent the inhibition of the 1,2-DG response path by R. Similar comments may be applied to the absence of additive effects between 5-HT and R in Chapter 6, and it should be noted in this context that the predicted mode of action of 5-HT from animal studies involves protein kinase C activation via generation of 1,2-DG.

If R and 1,2-DG operate via exactly the same mechanism, or if 1,2-DG comprises part of the transduction chain of phytochrome in the response, then the interaction between 1,2-DG and A23187 would be predicted to be the same as that between R and A23187, that is, additive effects will be seen between A23187 and 1,2-DG. Testing this necessitated the combination of A23187 and 1,2-DG treatments. Since A23187 is prepared in a DMSO stock solution and DiC8:0 in absolute ethanol appropriate additions of DMSO and/or ethanol were made to rapidly stirred experimental solutions (low Ca^{2+} MES incorporating 100 μM Ca^{2+}) to bring all solutions within an experiment to the same concentration of DMSO/ethanol (0.2% DMSO and 0.06% ethanol v/v). Apart from this treatment protocols and incubation details were as described previously for A23187 and DiC8:0 treatments. The results are shown in Fig. 8.3. It was found that both A23187 and DiC8:0-

Fig. 8.3 The effect of DiC8:0 on A23187-induced unrolling of etiolated barley leaf sections in MES buffer containing Ca^{2+} (0.1 mM). Measurements performed at t_{24h} following a dark incubation at 20°C on an orbital shaker (60 revs/min). Ethanol and DMSO concentrations were equalized between treatments.

Treatments:
1. No treatment
2. A23187 (20 µM at t_o)
3. DiC8:0 (20 µM at t_o)
4. Treatment '2' + treatment '3'



(for Treatments see legend.)

mediated promotion of dark unrolling were clearly demonstrable in the modified medium to standardize DMSO/ethanol concentrations. However, unlike the interaction between R and A23187 (see Chapter 6, Fig. 6.5), a negative interaction was found betwen A23187 and DiC8:0. In the presence of DiC8:0, the stimulatory effect of A23187 disappeared completely. The absence of a negative interaction with R (Fig. 8.2) indicates that unlike GA_3, toxicity is not the cause of this. Rather it implies antagonism between the treatments, and furthermore, that Phytochrome and DiC8:0 cannot operate in exactly the same manner to induce unrolling, even though there are no additive effects between them. Equally, the comparison reinforces the earlier conclusion that R and A23187 also differ in their mode of action.

The basis of the DiC8:0-mediated inhibition of the A23187 effect while remaining an unrolling agonist itself, is not clear. However, certain parallels exist with neutrophil cells in animals, where both protein kinase C activation and elevated cytosolic Ca^{2+} can induce lysosomal secretion independently, but kinase activation inhibits the Ca^{2+}-activated route (Gomperts, 1986). In suggesting such a scenario, it is necessary to assume the existence of protein kinase C or a protein kinase C-like enzyme in etiolated barley leaves, and that DiC8:0 acts via such an enzyme. There is much circumstantial evidence to indicate that this notion may be true for at least some plant cells. However proof is lacking. It is possible to predict some of the properties and consequences of this proposed interaction and look for them in the unrolling system. An expected property of 1,2-DGs and protein kinase C is a shift in Ca^{2+} sensitivity; the sensitivity modulation discussed earlier. The unrolling system is highly amenable to this sort of determination, and experiments were performed to assess the effect of

DiC8:0 on the Ca^{2+}-sensitivity of unrolling. Fig. 8.4 shows the effect of $[Ca^{2+}]_{ext}$ on DiC8:0-induced unrolling in low Ca^{2+} MES buffer incorporating a range of Ca^{2+} concentrations. At all levels of Ca^{2+} up to 0.5 mM even in the absence of added Ca^{2+}, DiC8:0 induced significant promotion of dark unrolling ($p < 0.01$). There was also a clear optimum $[Ca^{2+}]_{ext}$ for DiC8:0 promotion around 50 μM Ca^{2+}. This demonstrates that DiC8:0-promoted unrolling is sensitive to variations in $[Ca^{2+}]_{ext}$. A23187 also sensitized unrolling to $[Ca^{2+}]_{ext}$ (Fig. 6.3). However, DiC8:0 would not be expected to exhibit Ca^{2+}-ionophoric properties from its chemical structure and a change in the cell sensitivity to Ca^{2+} is thus more likely.

The relationship between A23187-induced Ca^{2+}-sensitization, DiC8:0 and $[Ca^{2+}]_{ext}$ is clarified in Fig. 8.5, which examines the interaction between A23187 and DiC8:0 in the presence and absence of Ca^{2+} (100 μM) in the buffer medium. As shown previously, the promotive effects of A23187 and DiC8:0 in isolation are significantly increased in the presence of Ca^{2+} (100 μM). In combination in the presence of Ca^{2+} (100 μM), there is a negative interaction, as seen in Fig. 8.3, which is not seen in the absence of added Ca^{2+} (Ca^{2+} standardized to 10 μM in the absence of Ca^{2+} addition). This indicates that the negative interaction is a product of superoptimal $[Ca^{2+}]_{ext}$. However, since Ca^{2+} (100 μM) is actually promotive for A23187 in isolation (and hence 10 μM Ca^{2+} in low-Ca^{2+} buffer is suboptimal) a Ca^{2+}-sensitivity change in the optimum $[Ca^{2+}]$ for unrolling appears to have occurred. The effect of DiC8:0 is thus to lower the optimum $[Ca^{2+}]_{ext}$ for A23187 promoted unrolling. If, as indicated by Chapter 6, A23187 operates through changing $[Ca^{2+}]_{cyt}$, then DiC8 operates by shifting the response curve to lower levels in a Ca^{2+} sensitivity modulation. This is as predicted if the site of action of

Fig. 8.4 The effect of Ca^{2+} concentration on DiC8:0-induced unrolling of etiolated barley leaf sections in MES buffer. Measurements performed at t_{24h} following dark incubation at $20^{o}C$ on an orbital shaker (60 revs/min). DiC8:0 and Ca^{2+} supplied at t_{o}.

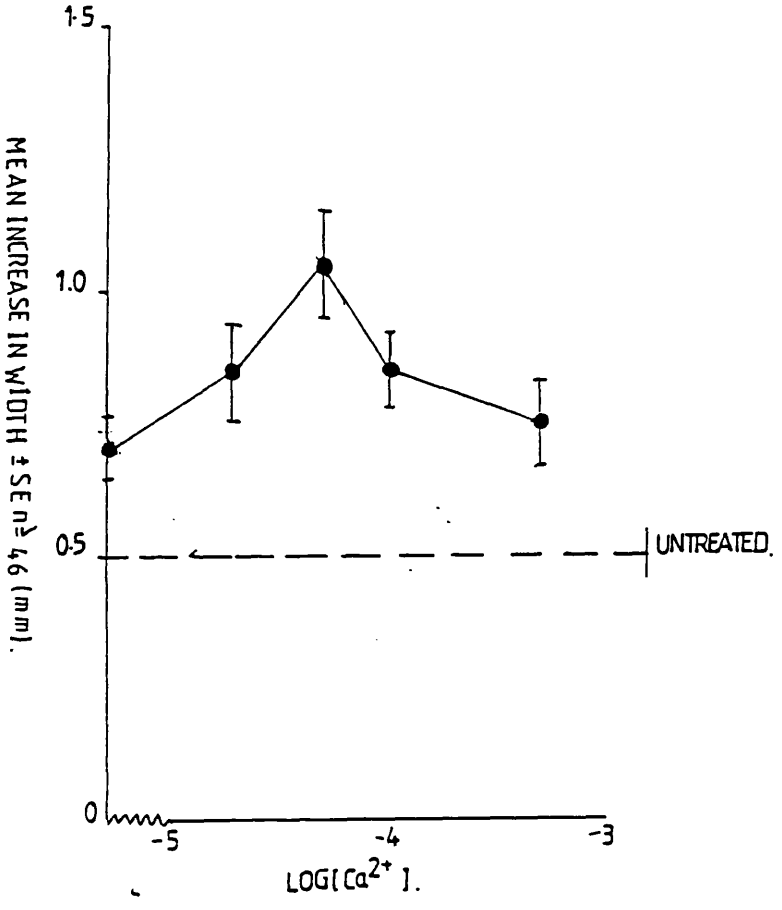
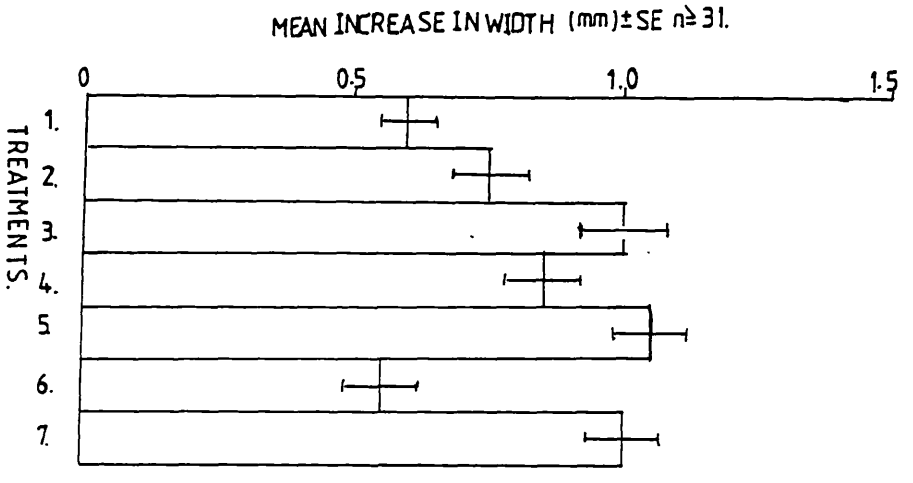

1·5
1.0
0·5
0
-5
-4
-3
UNTREATED.
LOG[Ca2+].
MEAN INCREASE IN WIDTH ± SE n 4 6 (mm)

Fig. 8.5 The effects of Ca^{2+} availability on the interaction between A23187 and DiC8:0 in the induction of unrolling of etiolated barley leaf tissue. Conditions as per legend Fig. 8.3 except for Ca^{2+} concentration.

Treatments:

1. No treatment
2. DiC8:0 (20 μM at t_o)
3. As treatment '2' + Ca^{2+} (0.1 mM)
4. A23187 (20 μM at t_o)
5. As treatment '4' + Ca^{2+} (0.1 mM)
6. As treatment '5' + DiC8:0 (20 μM at t_o)
7. As treatment '4' + DiC8:0 (20 μM at t_o)



(For treatments see legend.).

DiC8:0 is a protein kinase C-type molecule and would represent the first *in vivo* demonstration of such a property in plants.

The aim of this chapter has been to reconcile the circumstantial evidence that phytochrome-induced unrolling involves Ca^{2+}-signalling with the finding that an amplitude modulated Ca^{2+} signal, while capable of inducing unrolling in itself does not accurately mimic phytochrome-stimulated unrolling. It was proposed that Ca^{2+}-sensitivity modulation could explain these discrepancies. While Ca^{2+}-sensitivity modulation may underlie the effect of DiC8:0, comparison of Figs. 6.5 and 8.5 clearly demonstrates that this does not account for the role of phytochrome in leaf unrolling.

In conclusion, the mode of action of phytochrome in the induction of unrolling remains unknown. However, the foregoing data allow the elimination of both Ca^{2+}-mediative hypotheses and those based on sensitivity modulation as currently formulated. An alternative proposal of a testable hypothesis of phytochrome action in unrolling will now be presented. This hypothesis will be discussed in the next chapter.

CHAPTER 9 : OVERVIEW

The work in this study divides into two broad subject areas, one concerning the behaviour of phytochrome in etiolated plant systems, the other evaluating the feasibility of Ca^{2+}-mediative hypotheses of phytochrome action and alternatives to this in the induction of unrolling. As such, they are best treated separately, although the relevant common elements will be incorporated as necessary.

Time-dependent changes in the behaviour of phytochrome in etiolated barley (see Fig., 9.1)

Following R-treatment and photoconversion, a progressive loss of phytochrome activity occurs, that is undetectable spectrophotometrically. The manifestation of this loss in responses as diverse as leaf unrolling and NRA suggests it is fundamental, in barley at least. Since this has not been clearly described prior to the present study, such a process is not represented in the many models of phytochrome action that have previously been described (see Kronenberg and Kendrick, 1986, for review). However, no theoretical difficulties are presented by its incorporation into a range of models, whether ratio-based or Pfr-based hypotheses are applied. Most models postulate the association of phytochrome with a reaction partner 'X', an association stimulated in some way by the appearance of Pfr. It is only necessary to propose that X and phytochrome undergo a relatively slow dissociation process leading to loss of activity. This can be extended to suggest that FR accelerates the dissociation process and explain the similarity between FR-escape time and

Fig. 9.1 Proposed hypothesis of Phytochrome action to account for Pfr deactivation. After Schäfer (1975) and Eilfeld et al (1987). (X = proposed phytochrome reaction partner)

SYNTHESIS
Pr
hv
LUMI R
28000S-1
X?
META Rα
X
370 S-1
META Rε (X) (nacentPfr?)
HIR
20S-1
Pfr (X')
LIR
X
hv
'OLD' Pfr
DESTRUCTION

deactivation time. A readily testable prediction of this model is that the deactivation process should set an upper limit on the range of escape times demonstrated for a range of phytochrome responses in a given system. It is also likely that several responses will cluster around this upper value. Processes with shorter escape times are proposed to be complete or at least progress to a steady committed state within this time limit, the Onoclea germination system providing an example of this type.

Two questions are raised by the existence of the deactivation process. These are: 'i' What is the physiological significance of such a process?; and 'ii' How might such a process be detectable at the cell level?

The physiological significance of the process is, at present, unknown. However, the attenuation of the phytochrome signal has parallels in the regulation of many other cell signals, for example the conversion of cyclic AMP to AMP by AMP-phosphodiesterase, or 1,2 DG to Phosphatidic acid by Diacylglycerol kinase. The removal of the activating signal is necessary for the control of a response over longer periods and to allow flexibility of response. In the two examples given above, the attenuation process is not reversible, the activating signal (AMP or 1,2-DG) only being regenerated in an indirect path. It is not clear whether or not this is the case for phytochrome, as discussed in Chapter 5, although there are grounds for supposing it to be the case. Pfr can be reconverted to Pr at any time before or after deactivation by light and R is capable of effecting both Pr → Pfr and Pfr → Pr transitions, albeit with different efficiencies. The possibility therefore exists for cyclical conversion of 'old' deactivated Pfr to 'new' Pfr in a uni-directional manner by secondary R treatment, as seen in the recoupling of the response in Chapters 3 - 5. It is then required that only the

'new' Pfr be able to couple with X. In support of this, in vitro determinations of the photoconversion Pr ⇌ Pfr in 124 kD Avena phytochrome indicate that conversion intrinsically proceeds via a unique sequential pathway (Eilfeld et al, 1987). A consequence of this is that any of the intermediates in this pathway could constitute a 'nascent' Pfr state which couples with X and then undergoes 'dark relaxation' to Pfr proper. The last steps of this process have rate constants measured at 380 s^{-1} and 20 s^{-1}, two to three orders of magnitude slower than the initial steps following photoconversion (Eilfeld et al, 1987), indicating that the intermediates immediately prior to Pfr may be stable long enough to interact with the hypothetical X. Fig. 9.1, which is based upon Schafer's model of phytochrome action (Schafer, 1987) summarizes the above speculations and shows how the concepts of phytochrome deactivation and 'new' or nascent Pfr could fit into such models. The roles assigned to the intermediate stages are pure supposition and merely serve to illustrate the principles involved. Similar schemata can be developed for phytochrome ratio-based hypotheses.

The development of a cellular model of the deactivation process is a necessary step in the further investigation of its mechanism. An obvious candidate for this is the phenomenon of sequestering, the redistribution of phytochrome in the cell into discrete areas following brief R-irradiation. Although this has been linked to phytochrome destruction (Shanklin et al, 1988) there is evidence that sequestering is a heterogeneous process involving a rapid phase (1 - 2s) and a subsequent slow aggregation (McCurdy and Pratt, 1986). In soybean, at least, sequestation appears to be a slow process continuing up to 1h after R (Cope and Pratt, 1988). There is ample scope within these observations for the formation of active complexes and subsequent deactivation as well as

destruction.

The behaviour of sequestered complexes in the presence of low levels of Ca^{2+}, and during serial light regimes, may provide an experimental guage of the functional importance of sequestration, even allow the testing of models of the type shown in Fig. 9.1. If not, then much will remain speculative until a clearer notion of the primary biochemical events following photoconversion emerges.

The mechanisms of control of leaf unrolling.

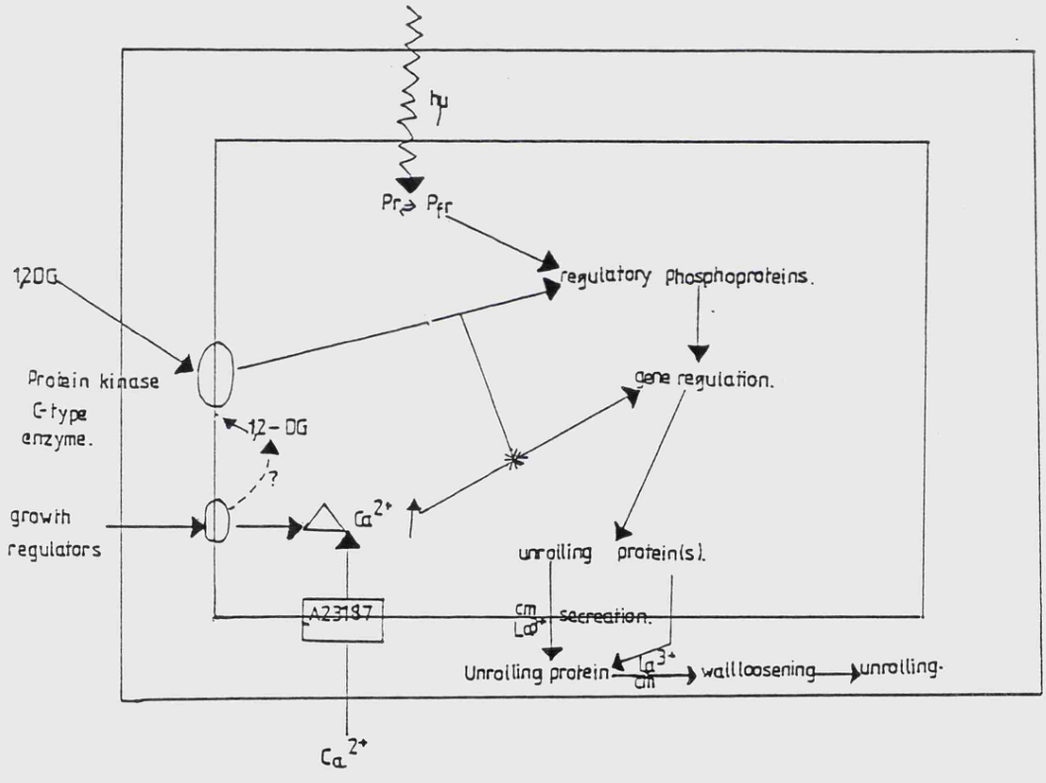
The finding that R and A23187 operate in different ways to induce unrolling is of considerable importance, and highlights the danger of uncritically applying Jaffe's rules, as adapted by Hepler and Wayne (1985), when proposing a role for Ca^{2+}-activation in plant phenomena . This study highlights two important weaknesses of this approach; first, that it tends to under-emphasize the possible importance of Ca^{2+}-sensitivity modulation and second, that, without specific extensions, it fails to differentiate sufficiently between Ca^{2+}-activated processes and Ca^{2+}-mediated processes when both show Ca^{2+}-dependence. In the light of this, the importance of clearly defining the relationship between R, A23187 and Ca^{2+}, is essential in analysing the interaction between phytochrome and Ca^{2+} in any response. With the possible exception of chloroplast rotation in Mougeotia, this does not appear to have been adequately performed to date for any of the many phytochrome-controlled phenomena that may involve Ca^{2+}. This is important because, taken in isolation, much of the data of this study could be used to support a Ca^{2+}-mediative hypothesis of phytochrome action, even though this cannot be the case. The warning implicit in this is obvious.

The finding that phytochrome-induced leaf unrolling is not Ca^{2+}-mediated leaves open the question of how mediation actually does occur.

This study has characterised three main classes of unrolling induction; that used by R via phytochrome, those involving a Ca^{2+}-amplitude modulated signal, and possibly those involving changes in cell sensitivity to Ca^{2+}. Although all three may share elements of transduction, and response, each must have some unique feature setting it apart. It is possible that the latter two classes represent individual aspects of a bifurcating cell signal path similar to the PI based pathway of animal cells. In support of this, cytokinins do appear to induce similar changes in soybean protoplast PI synthesis to those seen in animal cell transduction (Connett and Hanke, 1987). The apparent similarity of action of A23187 and the plant growth substances GA_3 and kinetin in unrolling also supports this, although the negative interaction between A23187 and 1,2-DG does not. However, A23187 may induce a different response profile of Ca^{2+} levels compared to those produced in a receptor-coordinated pattern of Ca^{2+} amplitude and sensitivity changes. The lack of data regarding the interaction of growth substances and A23187 across a range of $[Ca^{2+}]_{ext}$ values, and between 1,2-DG and growth substances precludes further discussion on this point.

Although no direct evidence as to the nature of the transducing elements in the phytochrome-controlled response is given in the data, current developments regarding rapid phytochrome effects and intrinsic properties of the molecule may provide a useful hypothesis of transduction. This hypothesis is described in Fig. 9.2, and has for a starting point, the observation that 1,2-DG and R do not show additive effects, even though they operate differently with regard to their interaction with A23187. Assuming a protein kinase C-type enzyme to be the target for 1,2-DG, a consequence of treatment will, in addition to altered Ca^{2+} sensitivity, be protein phosphorylation. Recent work indicates that

Fig. 9.2 Hypothetical mechanisms for the cellular induction of the unrolling process. For explanation see text.


hν
Pr ⇄ Pfr
1,2DG
regulatory Phosphoproteins.
Protein kinase
C-type
enzyme.
1,2-DG
gene regulation.
?
growth
regulators
Ca2+
unrolling protein(s).
A23187
cm
Ca2+
secreation.
Unrolling protein
Ca2+
cm
wallloosening
unrolling.
Ca2+

the phytochrome molecule itself may be a protein kinase (Wong et al, 1987; 1988) and rapid protein phosphorylation follows stimulation of the phytochrome system in corn (McFadden and Pooviah, 1988). The possibility exists therefore, that phytochrome and the protein kinase C-type target of 1,2-DG could phosphorylate the same population of protein or proteins (possibly among others), that then induce unrolling. The difference between 1,2-DG effect and phytochrome would then reside exclusively in changes in Ca^{2+}-sensitivity induced by 1,2-DG but not by phytochrome. Given the time-scale of induction and manifestation of unrolling (hours) it is likely that the phosphorylated proteins will act at the level of gene regulation, a notion that presupposes the existence of 'unrolling' proteins. The modulation of production of these is proposed to control the unrolling mechanism itself. The existence of such a protein or proteins has been inferred several times before (Carr and Reid, 1966; Kang, 1971; Rajagopal and Masden, 1981). The Ca^{2+}-mediated/activated unrolling path, stimulated by A23187 and growth regulators, is also proposed to regulate the activity of these unrolling proteins by a separate path that can be inhibited by 1,2-DG via the protein kinase C-type enzyme.

At the same time A23187 is proposed to inhibit the DiC8:0 path by admitting excessive amounts of Ca^{2+} to tissue sensitized to Ca^{2+} by 1,2-DG (via the protein kinase C-type enzyme). This would account for the negative interaction seen in the presence of external Ca^{2+} between 1,2-DG and A23187 and suggests an extreme Ca^{2+} sensitization to inhibitory levels, although direct inhibition of a secretory process as part of the mechanism as described by Gomperts (1986) is equally possible. The mode of action of the Ca^{2+} signal may involve Ca^{2+}/CM regulation of protein phosphorylation and gene switching.

The irreversible nature of the unrolling process once in progress indicates that changes in the wall properties may be more important to the mechanism than changes in osmotic parameters. Rajagopal and Masden (1981) first suggested that proline rich cell wall glycoproteins, like extensin, may be important in unrolling. If the unrolling protein or proteins are extensin-like molecules then the next stage in unrolling following synthesis would be expected to be translocation, followed by secretion and action on the cell wall leading to unrolling. Processes such as translocation and secretion are very likely to be sensitive to calmodulin antagonists, and it is proposed that this is the likely site of action of the W compounds in R stimulated unrolling, given the evidence against the operation of a Ca^{2+} signal in the primary transduction chain. Against this it may be pointed out that withdrawal of Ca^{2+} appears to inhibit unrolling without generating a steady state, that is, before the gene regulation stage. However, all this shows is that some part of this path is Ca^{2+}-dependent. Datta et al (1985) demonstrated the Ca^{2+}-dependence of protein phosphorylation in pea nuclei in response to red light, illustrating the Ca^{2+} sensitivity of such processes. A further site of W compound action and also La^{3+} action may be on the end process of wall loosening itself. La^{3+} can only act extracellularly. Cell walls contain significant amounts of CM (Biro et al, 1984), the function of which is by no means clear. If this could participate in wall loosening it would provide a likely site of antagonism by La^{3+} and the W compounds.

Many elements of this hypothesis are already demonstrated in various systems and it is feasible to perform similar demonstrations in barley. An essential starting point is the examination of the phosphorylations (if any) induced by R and 1,2-DG treatments. Common target proteins would be

of obvious regulatory interest. Further work could attempt to characterize the proposed unrolling proteins, using molecular biological techniques not available to Kang in 1971 when increased synthesis of a stable protein was found to correlate with unrolling in barley.

In conclusion, this study serves to focus attention on several areas of interest; namely, the phytochrome deactivation process, the inadequacy of existing Ca^{2+}-based hypotheses to describe unrolling, and the importance of Ca^{2+} sensitivity modulation in plants. It is hoped that these, and the data pertaining to them contained in this study may contribute to a better understanding of the mode of action of phytochrome and the control of leaf unrolling in etiolated barley.

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ABSTRACT

The role of Calcium ions (Ca^{2+}) in the phytochrome-controlled unrolling of 6-d-old etiolated barley leaf sections was evaluated through the manipulation of light treatments, Ca^{2+} levels and antagonists and agonists of Ca^{2+} metabolism.

The Ca^{2+}-chelator ethyleneglycol-bis-(βaminoethylether) - N, N, N', N', - tetraacetic acid (EGTA) inhibited the response. Ca^{2+} (activity 40 μM) restored responsivity when supplied within 1h of light treatment. Subsequent escape from responsivity to Ca^{2+} involved uncoupling of measurable phytochrome from response level. A second Red-light treatment (R) given, with Ca^{2+}, to EGTA-treated sections following Ca^{2+} escape from the initial R effect induced full unrolling. Similar results were obtained with the relatively independent phytochrome-controlled nitrate reductase activity induction in the sections. It is proposed that Ca^{2+} escape arises from phytochrome signal attenuation.

Measurable Pfr and response recoupled following Ca^{2+}-escape for the second R, given with Ca^{2+}. This relationship was identical to that observed with a single light treatment without EGTA, despite a 50% reduction in phytochrome levels at the time of the second R. This indicated that either the ratio of Pfr/P_{tot} governs response induction, or that the majority of detectable phytochrome is functionally irrelevant. It is suggested that phytochrome deactivation is reversible via phytochrome cycling.

A23187-induced movement of Ca^{2+} also induced unrolling. This, and the R effect appeared to be separate, thus Ca^{2+} does not mediate the phytochrome signal via simple amplitude modulation. Treatment with 1,2-Diacylglycerol (1,2,-DG) induced unrolling. The interaction of 1,2-DG and A23187 demonstrated Ca^{2+} sensitivity modulation, indicating involvement of Protein Kinase C. The interaction of A23187 and phytochrome did not show this. It is suggested that the induction of unrolling centres around protein phosphorylation via separate 1,2,-DG, Ca^{2+}-sensitive and phytochrome regulated kinases.


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