

UNIVERSITY OF LEICESTER

Phytochrome control of plastid gibberellin levels

A thesis submitted for the degree of

Doctor of Philosophy

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1980

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## Dedication

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For my parents.

## Abstract

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## Phytochrome control of plastid gibberellin levels.

J. R. Hilton

Previously published work has shown that phytochrome is present in association with etioplast-enriched organelle suspensions and that it mediates in vitro the levels of gibberellin-like substances (GA-LS) extractable from them into aqueous methanol. However, the technique used to isolate the organelle suspensions has recently been critically examined, both biochemically and with the use of the electron microscope and found to purify mitochondria to a similar extent to etioplasts. The validity of these results is therefore questionable.

This thesis presents results of experiments designed to study further the presence of phytochrome in association with etioplast-enriched suspensions and also re-examines the rôle of phytochrome in the mediation of GA-LS levels. When etioplasts are prepared free of mitochondrial contamination (as judged by biochemical marker enzymes) phytochrome remains in association with the etioplasts and also mediates the levels of GA-LS extractable from them. Suspensions enriched with mitochondria also contain spectrophotometrically-detectable phytochrome but exhibit no phytochrome mediation of extractable GA-LS levels.

It was previously hypothesized that photoconversion of etioplast-associated phytochrome to Pfr mediated a change in the permeability of the etioplast envelope membranes with respect to GA-LS thereby allowing an efflux of GA-LS out of the etioplasts into the surrounding medium. The results presented in this thesis show that a large proportion of the red light-mediated increase in GA-LS is retained within the etioplasts. The results suggest, therefore, that phytochrome photoconversion has an effect on the extractability of GA-LS from etioplasts possibly via conformational changes of the etioplast membranes.

Results of preliminary experiments with chloroplast-enriched organelle suspensions are also presented and suggest that phytochrome is detectable in association with chloroplasts (namely the envelope membranes) and that it mediates the levels of GA-LS extractable from them.

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## Abbreviations

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|                   |   |
|-------------------|---|
| MOPS              | N-morpholino-3-propanesulphonic acid.   |
| EDTA              | Ethylene diamine tetra acetic acid.   |
| HEPES             | 4-(2-dihydroxymethyl)-1-piperazine ethanesulphonic acid.                      |
| S.E.              | Standard error.   |
| FLB               | Prolamellar body.   |
| GA <sub>x</sub>   | Gibberellic acid <sub>x</sub> .   |
| w/v               | Weight by volume.   |
| v/v               | Volume by volume.   |
| A                 | Absorbance.   |
| $\lambda$         | Wavelength.   |
| Rf                | Relative band speed to front.   |
| Equiv.            | Equivalent.   |
| L                 | Litre.  |
| Pfr               | Far-red absorbing form of phytochrome.  |
| Pr                | Red absorbing form of phytochrome.  |
| M                 | Molar (concentration).  |
| TLC               | Thin-layer chromatography.  |
| $\Delta$          | Change.   |
| R/FR              | Red/far-red.  |
| log               | Logarithm.  |
| xKP               | x,000 g pellet.   |
| xKS               | x,000 g supernatant.  |
| [ <sup>3</sup> H] | Tritiated   |
| GA-LS             | Gibberellin-like substances.  |
| G-50 method       | Sephadex G-50 (coarse) gel filtration method of Wellburn and Wellburn (1971). |
| GC-MS             | Gas chromatography - mass spectroscopy  |
| GC-RC             | Gas chromatography - radio chromatography                                     |

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Dedication

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## Chapter 1

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### General Introduction

It was from the results of physiological experiments that the morphogenically-active plant chromoprotein phytochrome was discovered (Hendricks, 1974). Phytochrome is present ubiquitously throughout the whole of the eukaryotic green plant kingdom, and may enable plants to detect, and respond to, shading from other plants (Smith, 1975). Borthwick (1972) describes phytochrome as "a master control which keeps the plant in tune with its' surroundings through interplay with some of the various other regulatory systems". Phytochrome exists in two interconvertible forms; Pr absorbing maximally at 660 nm and Pfr absorbing maximally at 730 nm. Absorption of light by either form, results in conversion to the other form. In dark-grown plants phytochrome exists solely as Pr. Upon irradiation with red light Pr is photoconverted to Pfr, the so-called 'active' form of phytochrome. A large number of different phytochrome-mediated responses have been described (Smith, 1975). The time interval between the absorption of light by the pigment and the expression of the photomorphogenic response varies from a few seconds (Newman and Briggs, 1972) up to several hours (Mohr, 1966). Also, the rate of escape from photoreversibility varies from 1.5 min for 50% loss of photoreversibility (Fredericq, 1964) up to 9 hours (Pjon and Furuya, 1967). The signal-transducing mechanism is still unknown. There are three hypotheses on the mode of primary action of phytochrome, (a) phytochrome functioning through gene action (Mohr, 1966; Schopfer, 1977), (b) phytochrome activation of soluble enzymes (Tezuka and Yamamoto, 1974), and (c) phytochrome-induced changes in membrane properties (Hendricks and Borthwick, 1967). Currently, the most popular and simple hypothesis of the mode of action of phytochrome is an effect on membrane properties. Since its proposal in 1967 increasing support has been gained for this theory both from physiological and in vitro localization studies. To understand the mode of action of phytochrome in the cell it is very important to know where it is located. In very elegant studies Haupt and



co-workers, (Haupt et al., 1969; Haupt, 1970; Weisenseel and Haupt, 1974) studying the orientation of the chloroplasts in the green alga Mougeotia, have suggested that the dichroic photoreceptor changes its direction of maximal absorption by  $90^{\circ}$  upon photoconversion and conclude that in Mougeotia the phytochrome molecules are arranged in the plasma membrane.

Etzold (1965) also concludes that spatial organization of photoreceptor molecules responsible for germ tube growth in the fern Dryopteris filix mas is located in the plasma membrane. From work on the immunocytochemical localization of phytochrome Pratt and co-workers (Pratt and Coleman, 1971; Pratt et al., 1974; Coleman and Pratt, 1974 a and b; Pratt and Coleman, 1976) have made considerable progress towards localizing phytochrome within the cell. In cells which have been exposed to green safe-light only, phytochrome possesses no discernible unique association with a single organelle or membrane type. Stain for phytochrome as Pr is observed in association with the nuclear membrane, with mitochondria, with amyloplasts and with plastids, as well as being widely distributed throughout the cytoplasm. These observations are consistent with the possibility that phytochrome, as Pr, is present largely as a soluble protein in the cytoplasm of etiolated plant tissue. As they point out, one may still not conclude, however, that there is not a biologically significant association of a small, but active, pool of Pr with some unidentified sub-cellular component.

Mackenzie et al., (1975) have shown that Pfr, the so-called physiologically active form of phytochrome, has a different sub-cellular distribution. It appears that phytochrome migrates, after photoconversion to Pfr, from a relatively uniform distribution to discrete, as yet unidentified, areas. This movement is very rapid and can be detected clearly within two minutes at  $3^{\circ}\text{C}$ . The results obtained from immunocytology seem to rule out a unique association of phytochrome with one sub-cellular component. However, they

indicate that phytochrome, as a result of photoconversion, can change its' sub-cellular distribution. It would appear therefore, that there are two types of phytochrome present in the cell; one inherently associated with the membranes and another soluble in the cell, only becoming associated with particulate material (and thus becoming pelletable) following photoconversion to the Pfr form (Quail et al., 1973, Furuya and Manabe, 1976; Marmé et al., 1973; Quail and Gressel, 1976).

Another method used to localize phytochrome has been that of cell fractionation. From work with purified sub-cellular fractions phytochrome has been localized in association with mitochondria, (Gordon, 1961; Manabe and Furuya, 1974) plasma membrane, (Marmé, 1974; Quail and Hughes, 1977;) rough endoplasmic reticulum (Williamson et al., 1975) and etioplasts (Evans and Smith, 1976a; Cooke and Kendrick 1976; Kraack and Spruit, Pers. Comm.). There is, therefore, a large body of evidence to suggest that phytochrome is present in cells, and further, that it is present in association with purified organelle fractions.

The first observation of the presence of GA-LS in association with plastid fractions was that of Stoddart (1968). He showed significant levels of GA-like activity associated with crude chloroplast (1KP) fractions isolated from the leaves of light-grown Brassica oleracea L. and Hordeum vulgare L. When expressed on a total chlorophyll or a fresh-weight basis approximately 16% of the gibberellin-like activity found in the leaf could be accounted for in this fraction. Similar fractions have also been shown to be capable of GA biosynthesis in vitro (Stoddart, 1969). An association between chloroplasts and GA-LS has also been shown by Railton and Reid (1974). Chloroplasts isolated from the leaves of Solanum andigena L. exposed to short days contained smaller quantities of GA-LS than did chloroplasts isolated from leaves maintained under long days. Gibberellins have also

been reported in association with chloroplast fractions isolated from ivy leaves (Frydman and Wareing, 1973) and from the leaves of Pisum sativum L. (Railton and Reid, 1974). In more recent work, Cooke and Saunders (1975) and Cooke et al., (1975), using a lettuce hypocotyl bioassay (Frankland and Wareing, 1960), showed that GA-LS were associated with etioplast-enriched fractions isolated from the leaves of dark-grown wheat seedlings. They used a 1,000g plastid-enriched fraction isolated from the leaf homogenate, and also, a further purified etioplast-enriched fraction isolated by the Sephadex G-50 (coarse) gel filtration technique of Wellburn and Wellburn (1971). Results of Evans and Smith (1976a) also showed GA-like activity to be associated with etioplast-enriched suspensions isolated by the G-50 method. Evans and Smith (1976a) used etiolated barley leaves and assayed the GA-like activity using a modification of the barley aleurone layer bioassay described by Jones and Varner (1967).

Evans and Smith, (1976a) and Cooke and Kendrick, (1976) showed that the GA-like activity extractable into aqueous methanol was increased markedly if the etioplast-enriched suspensions were ultrasonicated prior to extraction. The ultrasonication treatment, as well as rupturing the etioplasts, would be expected to solubilize at least some of the material associated with lipo-protein or membranes. These results suggest therefore, that a large proportion of the plastid-associated GA-LS was strongly associated with the plastid membranes and that disruption of these membranes rendered the GA-LS more readily extractable into aqueous methanol. In 1977, Browning and Saunders used a non-ionic detergent, Triton X-100, to disperse membranes in chloroplast suspensions isolated from wheat leaves. The levels of GA-like activity subsequently extractable (as assessed by the lettuce hypocotyl bioassay) were markedly higher following this treatment than from chloroplast preparations which had been ultrasonicated. The quantities extracted

were far greater than the quantities extracted from the vegetative tissues of any other plant species. The results suggest that the bulk of the chloroplast-associated GA-LS are localized in the chloroplast membranes in a specific manner rather than simply partitioned into the membrane lipids. One possibility is that the triton-extractable gibberellins are intimately associated with non-polar proteins or other molecules located within, rather than on, the periphery of membranes. Since this report numerous workers have attempted to repeat these experiments and have been unsuccessful in obtaining the very high levels of GA-LS claimed to be extractable from chloroplasts by this technique (Pers. Comm.). However, Hall, (Pers. Comm.) has been successful in extracting small increases in the level of GA-LS with Triton X-100 over and above those extractable by solvent extraction alone. The authenticity of the results of Browning and Saunders (1977) remains questionable therefore.

When segments of etiolated barley or wheat leaves are irradiated with red light there is a rapid increase in the level of extractable GA-like activity (Reid et al., 1968; Beevers et al., 1970; Loveys and Wareing, 1971). Similarly, there is an increase in the level of subsequently extractable GA-LS in the homogenates of etiolated barley leaves following an irradiation with red light (Reid et al., 1972). This work led Evans and Smith, (1976a) and Cooke and Kendrick, (1976), to study the effects of red light treatment on the levels of GA-LS extractable from plastid-enriched fractions isolated from etiolated barley and wheat leaves respectively. Both groups of workers observed that the levels of GA-LS subsequently extractable into aqueous methanol were much higher in etioplasts given a brief irradiation with red light, than in those maintained in darkness. They also established that an irradiation with far-red light immediately subsequent to the red irradiation reversed the effect of the red irradiation on the levels of

extractable GA-LS. The induction of the response of increase in GA-LS by red light and its reversal by immediate subsequent far-red, indicated that the photoreceptor involved in the response was phytochrome.

The first evidence for phytochrome action in isolated etioplasts was reported by Wellburn and Wellburn (1973) who showed that plastid development at the ultrastructural level was enhanced by red light and that this enhancement could be reversed by subsequent far-red light. As previously stated, using immunocytochemical techniques Coleman and Pratt (1974a and b) observed phytochrome to be present in association with the plastids of dark-grown plants. Evans and Smith (1976a); Cooke et al., (1975) and Kraack and Spruit, (Pers. Comm.) measured phytochrome spectrophotometrically in etioplast-enriched suspensions and further, Evans and Smith (1976b) and Cooke and Kendrick, (1976) localized phytochrome in exclusive association with the envelope membranes when etioplasts were further sub-fractionated. No phytochrome was detectable in association with either the stroma or the prolamellar body-enriched fractions. However, only about 4% of the phytochrome extractable from the leaves was present in association with the envelope fraction and this led Pratt, (1978) and other workers (Pers. Comm.) to suggest that the phytochrome was present solely as a soluble contaminant. Quail et al., (1976) attempted to show a correlation between phytochrome and plastids. On a sucrose density gradient the structures bearing the bulk phytochrome showed a different sedimentation behaviour and a different isopycnic buoyant density from the etioplast membranes which were identified using carotenoids as a plastid marker. However, in contrast to Evans and Smith, (1976b) and Cooke and Kendrick, (1976), Quail et al., (1976) used a crude pellet following centrifugation of an homogenate and not a further purified plastid fraction. There was therefore mounting evidence that phytochrome was present in association with etioplasts and that it was in

exclusive association with the envelope membranes. The photoconversion of etioplast-associated phytochrome to Pfr resulted in increased levels of methanol-extractable GA-LS (Evans and Smith 1976a; Cooke and Kendrick, 1976). When red light-treated etioplast suspensions were subsequently centrifuged at 6,000g and GA-LS extracted from both the 6KS and 6KP fractions, the majority (approximately 80%) of the red light-mediated increase in GA levels was present in the 6KS fractions (Evans and Smith 1976a; Cooke and Kendrick, 1976). These results led Evans and Smith, and Cooke and Kendrick to postulate that phytochrome was having an effect on the permeability of the etioplast envelope membranes with respect to GA-LS. Evans and Smith (1976a) proposed that the photoconversion of Pr to Pfr by red light results in an efflux of gibberellins out of the plastids resulting in internal depletion which in turn stimulates biosynthesis via release from a feed-back inhibition mechanism. The alternative explanation, initially advanced by Cooke and Saunders, (1975), was that the increased biological activity reflects release from a 'bound' form (attachment to either proteins or membranes) within the plastids and in this case the effects upon envelope permeability would be a secondary correlated process facilitating efflux of the released gibberellins. Both Evans and Smith, (1976a) and Cooke and Kendrick, (1976) performed sonication treatments on isolated etioplasts and compared the levels of GA-LS subsequently extractable with those extractable from red light-treated suspensions. Evans and Smith (1976a) reported that the levels of GA-LS subsequently extractable were similar but Cooke and Kendrick (1976) reported that significantly higher levels of GA-LS could be extracted from red light-treated etioplasts. Cooke and Kendrick (1976) suggested, therefore, that red light was having more than one effect on the etioplast preparations and that there was the possibility of an effect of red light on the metabolism of GA's within the etioplasts. They investigated this hypothesis by comparing the levels of

acidic and non-acidic ethyl-acetate-soluble GA-LS present in etioplast sub-fractions. Non-acidic ethyl-acetate-soluble GA-LS present in dark-maintained extracts were converted by red light to acidic ethyl-acetate-soluble GA-LS and this response was exclusively associated with the envelope membrane-enriched fractions. The far-red reversal of this response was not demonstrated and therefore the involvement of phytochrome can still be questioned. However, it seemed likely that both the phytochrome-mediated response and the phytochrome itself were localized in association with the envelope membranes of the etioplasts.

Since 1967, when Hendricks and Borthwick first suggested the involvement of membranes in the primary action of phytochrome, many experiments have, directly or indirectly, demonstrated phytochrome-induced changes in membrane properties. The etioplast system described was therefore another example of such a mechanism.

In 1977, Quail critically reviewed both biochemically, and with the use of electron microscopy, the Wellburn and Wellburn (1971) Sephadex G-50 (coarse) gel filtration technique for the isolation of etioplasts. He concluded that there was a high contamination of the etioplasts by mitochondria and that the phytochrome present in association with the column eluate was present solely as a soluble contaminant running in the void volume of the column. As previous experiments on the phytochrome control of GA-LS in etioplasts relied upon the isolation of etioplasts by this method, the whole authenticity of this response was now severely questioned.

The interest in the effects of light on the GA content of plants dates back to the findings of Lockhart (1956, 1958 a and b) and Lockhart and Deal (1960). They showed that GA treatment prevented the inhibition of stem growth by light in seedlings of a number of species including pea, bean, cucumber and pumpkin.

In pea seedlings the question of light inhibition was linked to dwarfism since Sale and Vince (1960) and Gorter (1961) found that dwarfism in this species is determined by light; in the dark all varieties attain a similar height whereas in the light the dwarf varieties are more inhibited than the tall ones. Previously, Brian and Hemming (1955) and Brian (1957) had reported that GA could counteract dwarfism in light-grown pea seedlings. Lockhart (1959, 1961, 1964) concluded that stem elongation, in species where GA reverses light inhibition, is regulated by light through some effect on GA metabolism probably involving decreased biosynthesis or increased destruction. Later work by Roesel and Haber, 1964; Vince 1967; Russell and Galston, 1968) showed that the reversal of light inhibition of stem elongation by GA is not complete. There are, therefore, very complex relationships between GA effects and light inhibition of growth and, it is not surprising that a simple, consistent relationship between light and GA content has not yet been reported.

Kende and Lang, (1964) and Jones and Lang, (1968) were unable to find differences in GA-like activity extractable from light- or dark-grown tall and dwarf pea varieties. They also found that light-grown dwarf pea seedlings did not respond to their own endogenous GA's. In contrast to these results, Köhler (1965, 1970, 1971) found that light-grown pea seedlings contained substantially more GA than dark-grown ones and that dwarf seedlings contained more than tall seedlings. Köhler (1971) also found evidence to suggest more rapid GA metabolism in the faster-growing seedlings. Other workers have also found evidence of faster metabolism of GA's in dark-grown than light-grown seedlings (Musgrave and Kende, 1970; Railton, 1974 et al., 1974; Stoddart et al., 1974). In contrast to the situation in pea seedlings much less GA-like activity was extractable from seedlings of Phaseolus coccineus grown in a 16 hour photoperiod than from the corresponding dark-grown controls (Brown



et al., 1967; Crozier and Audus, 1968). Also, the metabolism of GA is different in P. coicineus; [ $^3\text{H}$ ]GA $_4$  is metabolized faster in light-grown than in dark-grown plants (Brown et al., 1967). Interactions between light and GA's giving more unified results are the effects of short-term irradiations with red light. Brief exposure of lettuce seeds (Köhler, 1966), apple seed embryos (Smoleńska and Lewak, 1971) etiolated barley leaf sections (Reid et al., 1968), etiolated wheat leaf sections (Beevers et al., 1970) and etiolated pea seedlings (Nešković and Konjević, 1974) to red light causes a transient increase in the extractable GA-like activity. The red light mediated increase in GA-like activity in leaves has been suggested to be due to a phytochrome-mediated release of GA from the etioplasts (Cooke et al., 1975; Evans and Smith, 1976).

This thesis presents detailed experiments designed to study further the rôle of phytochrome in the mediation of etioplast-associated gibberellin levels. Information is also presented from experiments designed to study the rôle of phytochrome in the mediation of GA levels in plastid fractions isolated from light-grown plants.

Experimental details

(a) Preparation of plant material

(1) Dark-grown

Seedlings of Hordeum vulgare L. cv. Julia, 1976 (Stevens, Shardlow, Derbyshire, England and Rothwell Plant Breeders, Lincoln, England) were grown in moist vermiculite at 24°C in total darkness for 6 days (except where stated).

(2) Light-grown

Seedlings of Hordeum vulgare L. (as above) were grown at the University of York in moist vermiculite for 6 days (except where stated) in a 16h photoperiod at 20°C. Phillips warmwhite colour 29 fluorescent tubes provided a photon fluence rate of 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

Seedlings of Triticum aestivum L. cv. Maris Dove, 1978 (Dickson, Brown and Tait, Altringham, England) were grown for 7 days in Levington Universal compost in a 16h photoperiod at 20°C with a photon fluence rate of 75  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The spectral energy distribution of the artificial light source is shown in Fig. 1, page 25.

Seedlings of Spinacia oleracea L. Hybrid 102 (Yates and Co., Australia) were grown at the University of York in moist vermiculite for 2 weeks in a 12h photoperiod at 25°C (20°C night temperature) with a photon fluence rate of 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Phillips warmwhite colour 29 fluorescent tubes. The seedlings were transferred to an aerated nutrient solution containing 6mM  $\text{KNO}_3$ ; 1mM  $\text{KH}_2\text{PO}_4$ ; 4mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ; 46 $\mu\text{M}$   $\text{H}_3\text{BO}_3$ ; 0.32 $\mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 1.2 $\mu\text{M}$   $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ; 4mM  $\text{Ca}(\text{NO}_3)_2$ ; 2mM  $\text{MgSO}_4$ ; 9 $\mu\text{M}$   $\text{MnCl}_2$ ; 0.76 $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 24 $\mu\text{M}$  NaFeEDTA for a further two weeks. During the subsequent 3 weeks the seedlings were maintained in liquid culture in a greenhouse at 21-23°C with an 8.5h photoperiod. Plants were harvested 7 weeks after sowing.

(b) Light sources

The red light source consisted of four 'warm white' 15W fluorescent tubes, filtered through one layer of No. 14 Ruby Cinemoid and one layer No. 1 Yellow Cinemoid (Rank Strand Electric, Kingsway, London, England). The resulting photon fluence rate was  $2.3 \mu\text{mol m}^{-2}\text{s}^{-1}$  at the sample surface. The source of far-red light consisted of four 250W single coil tungsten bulbs filtered through 10cm of running water, one layer of No. 5A Deep Orange Cinemoid and one layer of No. 20 Deep Blue Primary Cinemoid. The photon fluence rate at the sample surface was  $4.6 \mu\text{mol m}^{-2}\text{s}^{-1}$ . Safe-lights consisted of either one 40W incandescent bulb or one 20W fluorescent tube filtered through two layers of No. 39 Primary Green Cinemoid. Spectral energy distributions of the light sources and safe lights are shown in Figs. 2(a), (b) and (c), page 26.

(c) Light treatments

Except where stated, organelle suspensions were irradiated with red light for 5 min. During the subsequent 5 min they were either returned to darkness or irradiated with far-red light. Control suspensions were maintained in darkness for the entire treatment period. All treatments were performed at  $24^{\circ}\text{C}$ .

(d) Isolation of organelle fractions

Harvesting and all subsequent procedures were performed at  $2 - 4^{\circ}\text{C}$  under a dim green safe-light except where stated.

(1) Dark-grown plant material

(1.1) Preparation of the crude plastid pellet

80g fresh weight laminae segments of Hordeum vulgare L. were finely chopped with a razor blade and gently homogenized in a pestle and mortar in 25mM MOPS containing 3mM EDTA (di-sodium salt), 14mM 2-mercaptoethanol and 250mM

sucrose adjusted to a final pH of 7.5. A tissue to buffer ratio of 1:2 (w/v) was used. The homogenate was gently exuded through twelve layers of muslin. The resultant filtrate had a pH of 7.1 which was maintained throughout the isolation procedure. The filtrate was centrifuged at 6,000g for 1 min. in a Sorvall model RCB-2 Superspeed centrifuge and the 6KP fractions were re-suspended in 5 cm<sup>3</sup> initial extraction buffer.

(1.2) Sephadex G-50 (coarse) gel filtration [G-50 method]

A modification of the Wellburn and Wellburn (1971) gel filtration technique, described by Evans (1975), was used to isolate etioplasts. In brief, a crude plastid pellet, approximately 5 cm<sup>3</sup> in volume, was loaded on to a loosely-packed Sephadex G-50 (coarse) gel filtration column 50 cm long and 1.1 cm in diameter. The Sephadex G-50 (coarse) had been previously expanded overnight at 3-4°C in isolation buffer. Fractions 0.5 cm<sup>3</sup> in volume were eluted from the column with extraction buffer. Before light-treatment the organelle-containing fractions eluting from the column were pooled. The resultant organelle suspension was approximately 9 cm<sup>3</sup> in volume.

(1.3) Post G-50 sucrose centrifugation

1 cm<sup>3</sup> aliquots of the pooled G-50 organelle suspensions were layered on to an equal volume of 20% (w/v) sucrose and centrifuged in an MSE bench centrifuge at approximately 500 g for 1 or 1.5 min. Following a 1 min. centrifugation, the 0.5KP fractions derived from separate aliquots were re-suspended in approximately 9 cm<sup>3</sup> original extraction buffer and used as an etioplast-enriched organelle suspension. The 0.5KS fractions remaining after a 1.5 min. centrifugation were further centrifuged at 27,000g for 10 min. in a Sorval model RCB-2 Superspeed centrifuge. The 27KP fractions were re-suspended in approximately 9 cm<sup>3</sup> original extraction buffer and used as a mitochondrial-enriched organelle suspension. Sucrose solutions were prepared in original extraction buffer minus sucrose.

(1.4) Discontinuous sucrose density gradient centrifugation

A crude plastid pellet, approximately 5 cm<sup>3</sup> in volume, was layered on to a discontinuous sucrose density gradient of 7.5 cm<sup>3</sup> 25% sucrose (w/v), 12.0 cm<sup>3</sup> 40% sucrose (w/v) and 7.5 cm<sup>3</sup> 25% sucrose (w/v) and centrifuged in an SW27 swing-out rotor in a Beckman L2-65B ultracentrifuge for 20 min. at approximately 9,350g. Two fractions, namely those banding at the upper i.e. the 40%/25% sucrose interface and the lower i.e. the 40%/55% sucrose interface were fractionated from the gradient, diluted with original extraction buffer, and subsequently used as the organelle-containing fractions. Each fraction was approximately 9 cm<sup>3</sup> in volume. Sucrose solutions were prepared in original extraction buffer minus sucrose.

A general procedure for the extraction of organelle fractions (1.2, 1.3 and 1.4) is shown in Fig. 3, page 27.

(1.5) The isolation of radioactively-labelled organelle suspensions

Etioplast suspensions were isolated from the first leaves of Hordeum vulgare L. seedlings following incubation in [<sup>3</sup>H] gibberellin A<sub>9</sub> by a modification of the method described by Leech et al., (Pers. Comm.) (see Experimental Details (d) (2.1) page 14). Table 1, page 28, shows the reduction in the activity of cytochrome c oxidase, a mitochondrial membrane marker enzyme, from the homogenate to the re-suspended etioplast-enriched pellet. The results suggest that the level of contamination of the etioplast suspension by mitochondrial membranes was very low (8.4%).

(2) Light-grown plant material

(2.1) The isolation of chloroplast suspensions from small amounts of leaf material

Chloroplast suspensions were isolated from the first leaves of Hordeum vulgare L. and Triticum aestivum L. seedlings by a modification of the method described by Leech et al., (Pers. Comm.) (except where stated) and finely

chopped with a razor blade for 5 min. in a solution containing 50mM HEPES, 400mM sorbitol, and 0.75 mM  $MgCl_2$ . The pH of the final solution was adjusted to 7.6. The homogenate was gently exuded through eight layers of muslin and centrifuged at approximately 500g in an MSE bench centrifuge for 5 min. through a layer of isolation buffer, the density of which had been increased by replacing the sorbitol with 400mM sucrose. The 0.5KP fraction was re-suspended in approximately 4 cm<sup>3</sup> original extraction buffer. Table 2, page 29, shows the reduction in the activity of cytochrome c oxidase, a mitochondrial membrane marker enzyme, from the total homogenate to a re-suspended chloroplast pellet and suggests that contamination of the chloroplast suspension by mitochondrial membranes was only 5.8%.

(2.2) The isolation of chloroplast suspensions from spinach leaves

Chloroplast suspensions were isolated from the leaves of Spinacia oleracea L. by a modification of the method described by Cockburn, Walker and Baldry (1968), using a tissue to buffer ratio of 1:2 (w/v). Laminae, without midribs, were homogenized for 2 x 1 sec bursts in an Atomix blender in a semi-frozen solution containing 10mM  $Na_4P_2O_7 \cdot 10H_2O$ , 330mM sorbitol and 5mM  $MgCl_2$ , adjusted to a final pH of 7.5. Sodium isoascorbate was added after pH adjustment to a final concentration of 2mM. The homogenate, after passing through six layers of muslin and two layers of 25  $\mu$ m nylon cloth, was immediately centrifuged from rest to 3,000g and then to rest in an MSE 18 centrifuge fitted with a fast brake. Unless stated otherwise, the re-suspended 3KP fractions were washed by centrifuging at 500g for 15 min. through a layer of isolation buffer, the density of which had been increased by replacing the sorbitol with 330 mM sucrose.

(2.3) The isolation of chloroplast suspensions from barley seedlings grown under varying phytochrome photoequilibria

Laminae segments of Hordeum vulgare L. 3 cm in length 1 cm from the apex were homogenized using a tissue to buffer ratio of 1:2 (w/v) with a Polytron

homogenizer model PT 10 20 3500 in a semi-frozen solution containing 25mM HEPES, 2mM EDTA (di-sodium salt) and 300 mM sucrose, adjusted to a final pH of 7.5. Sodium isoascorbate was added, immediately prior to homogenization, to a final concentration of 2mM. The homogenate, after passing through four layers of muslin and eight layers of muslin, was centrifuged at 3,000g for 1 min. in an MSE high-speed centrifuge, model Mistral 6L. The 3KP fraction was washed in isolation buffer and re-suspended in approximately 5 cm<sup>3</sup> of a solution containing 25mM HEPES, 330mM sucrose, 2mM EDTA (di-sodium salt), 1mM MgCl<sub>2</sub> and 1mM MnCl<sub>2</sub> adjusted to a final pH of 7.6.

(e) The isolation of chloroplast envelope membrane preparations

Envelope membrane-enriched preparations were isolated from Spinacia oleracea L. chloroplasts by the method described by Mackender and Leech (1970). The general procedure is outlined in Fig. 4, page 30. Chloroplast pellets prepared as described on page 15, d(2.2), were re-suspended in 30 cm<sup>3</sup> isolation buffer (from which sorbitol had been omitted) for 15 min. at 0°C. The resultant suspension of lysed chloroplasts was homogenized in a Ten-Bröeck ground glass homogenizer by raising and lowering the plunger rapidly three times. After dilution with 60 cm<sup>3</sup> incubation buffer, the suspension was centrifuged at 3,000g for 10 min. in an MSE 18 high speed centrifuge and floating material removed. The 3KS fraction was decanted and re-centrifuged at 20,000g for 30 min. in a Beckman L2-65B ultra-centrifuge. The 20KP fraction was re-suspended in approximately 4 cm<sup>3</sup> isolation buffer and used as the chloroplast envelope membrane-enriched preparation. Samples for chlorophyll assay were immediately removed and the remainder of the envelope membrane-enriched preparation was frozen and stored under liquid nitrogen for approximately 24h.



(f) Preparation of 'soluble' phytochrome (for general procedure, see Fig. 5, page 31)

The 6KS fraction obtained following the first 6,000g centrifugation as outlined on page 12, d(1.1) was centrifuged at 100,000g in a Type 75 Ti rotor in a Beckman L5-65B ultracentrifuge for 60 min. Aliquots of the 100KS fraction (total volume approximately 100 cm<sup>3</sup>) were used as 'soluble' phytochrome.

(g) Estimation of phytochrome pelletability

Preparations of 'soluble' phytochrome and G-50 organelle-enriched suspensions were brought to 24°C in a water bath, and, if appropriate, mixed by pipetting particulate suspensions via a wide-bore pipette into 'soluble' phytochrome preparations. Usually, 5 cm<sup>3</sup> of the particulate suspension and 10 cm<sup>3</sup> of the 'soluble' phytochrome were used. Pre-mixed samples were irradiated for the stated periods and immediately centrifuged at 6,000g for 1 min. in a Sorvall model RCB-2 Superspeed centrifuge. When samples were irradiated separately prior to mixing, the mixture was incubated at 24°C for 5 min. before centrifugation. Pellets were re-suspended in 2.5 cm<sup>3</sup> 25mM MOPS buffer, pH 7.5, for phytochrome and protein assays.

(h) Estimation of the movement of soluble substances into a discontinuous sucrose density gradient

100 µl [<sup>3</sup>H] gibberellin A<sub>1</sub> (1,2 [<sup>3</sup>H] GA<sub>1</sub>) (6 x 10<sup>5</sup> dpm µl<sup>-1</sup>, 48 Ci m mol<sup>-1</sup>) were layered on to a discontinuous sucrose density gradient [see Experimental Details, page 14, d(1.4)] in a solution of 25 mM MOPS buffer to a final volume of 5 cm<sup>3</sup> and centrifuged at approximately 9,350g for 20 min. in an SW27 swing-out rotor in a Beckman L2-65B ultracentrifuge. Radioactivity recovered in samples fractionated from the gradients following centrifugation was estimated using the tritium channel of a Packard Tri Carb liquid scintillation spectrometer AAA Model 544 using Brays scintillation

fluid (Bray, 1960). The counting efficiency varied between 35% and 55%. Fig. 6 (a), page 32, shows the distribution of radioactivity (following TLC) associated with the [ $^3\text{H}$ ] gibberellin  $\text{A}_1$  applied to the sucrose density gradient, and (b) the distribution of radioactivity in fractions isolated from the sucrose density gradient following centrifugation.

The movement of the soluble proteins haemoglobin and catalase into a discontinuous sucrose density gradient was estimated in a similar manner.  $1.5 \text{ cm}^3$  catalase ( $1 \text{ mg cm}^{-3}$ ) and  $0.5 \text{ cm}^3$  haemoglobin (total absorbance at 410nm approximately 2.0 A units) were layered on to a discontinuous sucrose density gradient in 25 ml MOPS buffer to a final volume of  $5 \text{ cm}^3$ . After centrifugation, haemoglobin was estimated by measurement of absorbance at 410nm and catalase activity was estimated by the method described by Lück (1965) in the samples fractionated from the gradient. Fig. 7, page 33, shows the results obtained from these measurements.

(j) Estimation of the uptake of [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  into detached leaves of *Hordeum vulgare* L. and into plastid-enriched suspensions subsequently isolated

(1) Light-grown plants

Whole shoots of 5 day old (except where stated) *Hordeum vulgare* L. seedlings were excised and incubated in a solution containing 50mM HEPES, 400mM sucrose, 0.75mM  $\text{MgCl}_2$  pH 7.6 and [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  ( $2,3[^3\text{H}]. \text{GA}_9$ ) (approx.  $43 \text{ Ci m mol}^{-1}$ ,  $19 \times 10^4 \text{ dpm } \mu\text{l}^{-1}$ ) to a final concentration of  $2.4 \times 10^{-11} \text{ M}$ . at  $24^\circ\text{C}$  for 24h in constant light ( $58 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) (for spectral energy distribution see Fig. 8, page 34). At the end of the incubation period, plastids were isolated from the leaves by a modification of the method described by Leech et al., (Pers. Comm.) as described on page 14, d(2.1). The resultant 0.5KP fraction was re-suspended in approximately  $4 \text{ cm}^3$  original extraction buffer.

(2) Dark-grown plants

Whole shoots of 6-day old (except where stated) Hordeum vulgare L. seedlings were excised and incubated in a solution containing 25mM MOPS, 3mM EDTA (di-sodium salt), 14mM 2-mercaptoethanol and [ $^3\text{H}$ ] gibberellin A<sub>9</sub> (2,3 [ $^3\text{H}$ ]GA<sub>9</sub>) to a final concentration of  $2.4 \times 10^{-11}\text{M}$  at 24°C for 24h in complete darkness. Plastids were isolated from the shoots by a modification of the method described by Leech et al., (Pers. Comm.) as described on page 14, d(2.1). The isolation medium was 25mM MOPS and its' density was increased by increasing the concentration of sucrose from 250mM to 500mM. The resultant 0.5KP fraction was re-suspended in approximately 4cm<sup>3</sup> original extraction buffer.

After light treatment, organelle suspensions, approximately 2cm<sup>3</sup> in volume, were layered on to a discontinuous sucrose density gradient of 2.5cm<sup>3</sup> 25% (w/v) sucrose and 2.5cm<sup>3</sup> 55% (w/v) sucrose, and centrifuged at approximately 9,350g in an SW27 swing-out rotor for 20 min. in a Beckman L2-65B ultra-centrifuge to separate the radioactivity associated with the organelle and the soluble fractions. After centrifugation, 1cm<sup>3</sup> aliquots fractionated from the gradient were dissolved in 9cm<sup>3</sup> Brays scintillation fluid (Bray, 1960).

GA-LS were extracted as described in Fig. 9, page 35, and following TLC were absorbed directly into Tritium absorber D (Fisons, Loughborough, England) to a final volume of 10cm<sup>3</sup>.

Whole-tissue samples were combusted in an Intertechnique Oxymat model JA 101 and the products from the tritium chamber were absorbed directly into Tritium absorber D to a final volume of 10cm<sup>3</sup>.

Radioactivity was determined using the tritium channel of a Beckman liquid scintillation counter model LS 3133P. The counting efficiency varied between 35% and 50%.

(k) Sub-fractionation of radioactively-labelled plastid suspensions

Radioactively-labelled plastid-enriched suspensions were sub-fractionated by a modification of the method described by Mackender and Leech, 1970 (see experimental details, page 16(e), and general procedure Fig. 4, page 30). The 3KP fraction was used as the thylakoid/prolamellar body-enriched fraction, the 20KP fraction as the envelope membrane-enriched fraction and the 20KS fraction as the soluble, stroma-enriched fraction. 1cm<sup>3</sup> fractions were re-dissolved in 9cm<sup>3</sup> Brays scintillation fluid (Bray, 1960) and the radioactivity was estimated using the tritium channel of a Beckman liquid scintillation counter model LS 3133P. The counting efficiency varied between 35% and 50%.

(l) Phytochrome measurement

Total phytochrome was measured at 24°C, using a Perkin Elmer 156 dual wavelength spectrophotometer. Calcium carbonate was used to increase effective path length over a 1cm path length plastic cuvette unless otherwise stated. Actinic and measuring beams were set at 660nm and 730nm. All samples were pre-irradiated with sufficient red light to effect maximum conversion of protochlorophyllide to chlorophyllide prior to measurement. Actinic irradiations were of 60 sec. duration.

Fractions isolated from sucrose density gradients were centrifuged at 27,000g for 10 min. in a Sorvall model RCB-2 Superspeed centrifuge. The 27KP fractions were re-suspended in the original volume of extraction buffer in order to obtain fractions of similar sucrose concentrations prior to phytochrome measurement.

The difference spectrum of chloroplast envelope membrane phytochrome was constructed at 25°C using a Shimadzu recording spectrophotometer model UV 300.

(m) Chlorophyll assay

Chlorophyll was extracted into acetone to a final concentration of 80% (v/v) and estimated by the method described by Mackinney (1941).

(n) Carotenoid assay

Total carotenoids were extracted into acetone to a final concentration of 80% (v/v) and estimated as outlined by Davies, (1976).

(o) Catalase assay

Catalase was assayed by the method described by Lück (1965).

(p) Cytochrome c oxidase assay

Cytochrome c oxidase was assayed by the method described by Smith (1955). All fractions to be assayed passed through at least one cycle of freezing and thawing to effect disruption of the mitochondrial membranes.

(q) Succinate dehydrogenase assay

Succinate dehydrogenase was assayed by the method described by Slatter and Bonner (1952).

(r) Protein assay

All samples were precipitated in 10% (v/v) trichloroacetic acid at 0°C for 30 min. and centrifuged in an MSE bench centrifuge at approximately 500g for 5 min. The 0.5KP fractions were washed and re-dissolved in the original volume of 2mM NaOH, and the protein content determined by the method described by Lowry et al., (1951) using bovine serum albumin as a standard.

(s) Extraction and fractionation of GA-like substances (for general procedure see Fig. 9, page 35)

After light treatment, aliquots of organelle fractions were immediately

homogenized in ice-cold methanol to a final concentration of 80% (v/v). After extraction, the homogenates were centrifuged at 2,000g for 5 min. in an MSE bench centrifuge and the 2KS fraction decanted and reduced to the aqueous phase in vacuo at 35°C. The aqueous phases were re-dissolved in a similar volume of 0.5M phosphate buffer pH 8.5 and partitioned three times against equal volumes of re-distilled ethyl acetate after which the pH was lowered to 2.5 with 0.8M HCl.

The pooled organic phases, following similar partition against re-distilled ethyl acetate at low pH, were reduced to dryness in vacuo at 35°C and re-dissolved in a small volume of re-distilled ethanol:ethyl acetate 1:1 (v/v). TLC was performed on 0.25mm layers of silica gel (Macherey-Nagel and Co., Duren, Germany) using chloroform:ethyl acetate:acetic acid, 60:40:5 (v/v/v) as the developing solvent which was allowed to run 10cm from the point of sample application. After being thoroughly air dried at room temperature, 1cm zones, scraped from the TLC plates, were eluted three times in 0.5cm<sup>3</sup> re-distilled ethanol:ethyl acetate, 1:1 (v/v). The pooled eluates were reduced to dryness under a stream of nitrogen and re-dissolved in 1cm<sup>3</sup> sterile water prior to bioassay.

(t) Estimation of GA-like activity

GA-like activity was estimated using a modification of the Jones and Varner (1967) barley aleurone layer bioassay. Half-seeds of Hordeum vulgare L.cv. Himalaya, 1974 (Professor J D Maguire, Washington State University, USA) were used. Embryo-less halves of the barley seeds were sterilized for 20 min in 1 % (v/v) sodium hypochlorite. At the end of the sterilization period the half-seeds were washed at least three times with sterile water and transferred to sterile filter paper in sterile petri dishes so that the cut ends of the half-seeds were not in contact with the paper, nor were they in contact with each other. The half-seeds were imbibed for 48h at room

temperature, ensuring that the filter paper was moist, but not flooded, with sterile water. At the end of the imbibition period ten sterile half-seeds were transferred to sterile conical flasks containing  $0.5\text{cm}^3$  sterile  $0.01\text{M}$  succinate buffer pH 4.8,  $0.5\text{cm}^3$  sterile  $0.1\text{ M}$  calcium chloride, 1 drop chloramphenicol ( $0.5\text{ mg cm}^{-3}$ ) and  $1\text{ cm}^3$  test solution. Conical flasks were incubated on an orbital shaker at  $25^\circ\text{C}$  for 24h at approximately 50 oscillations  $\text{min}^{-1}$ . After incubation, the liquid was decanted, the half-seeds rinsed with  $2\text{ cm}^3$  distilled water and the combined solutions were centrifuged at  $2,000g$  for 5 min. in an MSE bench centrifuge. The 2KS fraction was used as the enzyme solution and the  $\alpha$ -amylase activity induced was estimated using starch as the substrate, and iodine/hydrochloric acid as the terminator of the reaction.  $\alpha$ -amylase activity, induced in the half-seeds by the test solutions, was calculated from the absorbance of the resulting solutions at  $620\text{nm}$ . Fig. 10, (a) and (b), page 36, shows the results obtained in the above bioassay procedure when standard solutions of  $\text{GA}_3$  were used as the test solutions.

(u) Estimation of the recovery of GA-LS following TLC

$5\text{ }\mu\text{l}$  [ $^3\text{H}$ ] gibberellin  $\text{A}_1$  ( $1,2[^3\text{H}].\text{GA}_1$ ) ( $6 \times 10^5\text{cpm }\mu\text{l}^{-1}$ ,  $48\text{ Ci m mol}^{-1}$ ) were applied to a TLC plate and chromatographed [for Experimental Details see page 21 (s)]. Following reduction to dryness under a stream of nitrogen, the ethanol:ethyl acetate eluates were re-dissolved in  $10\text{ cm}^3$  Brays scintillation fluid (Bray, 1960) and the radioactivity estimated using the tritium channel of a Packard Tri-carb liquid scintillation spectrometer AAA model 544. The counting efficiency varied between 35% and 50%. The recovery of radioactivity, following TLC, was 77% of that originally applied to the thin-layer plate.

(v) Estimation of the relative mobilities of authentic GA's following TLC

Aliquots of the standard GA solutions, containing either  $\text{GA}_3$ , a mixture of

GA<sub>4</sub> and GA<sub>7</sub>, or GA<sub>9</sub>, were applied to TLC plates and chromatographed as previously described [see Experimental Details, page 21 (s)]. After being thoroughly air-dried at approximately 24°C, the plates were developed using a solution of 5% (v/v) sulphuric acid in ethanol and dried in an oven at 100°C for 5 min. The positions of the authentic GA's were assessed, both visually in daylight and under ultra-violet light. The relative mobilities of these compounds were calculated using the following equation:

$$\text{Relative band speed to front (Rf)} = \frac{\text{Distance moved by solvent front}}{\text{Distance moved by X}}$$

where X is the test compound. Estimations of the Rf values of authentic GA's were performed each time a group of TLC plates was developed.



Spectral energy distribution of the artificial light source under which seedlings of Hordeum vulgare L. were grown.

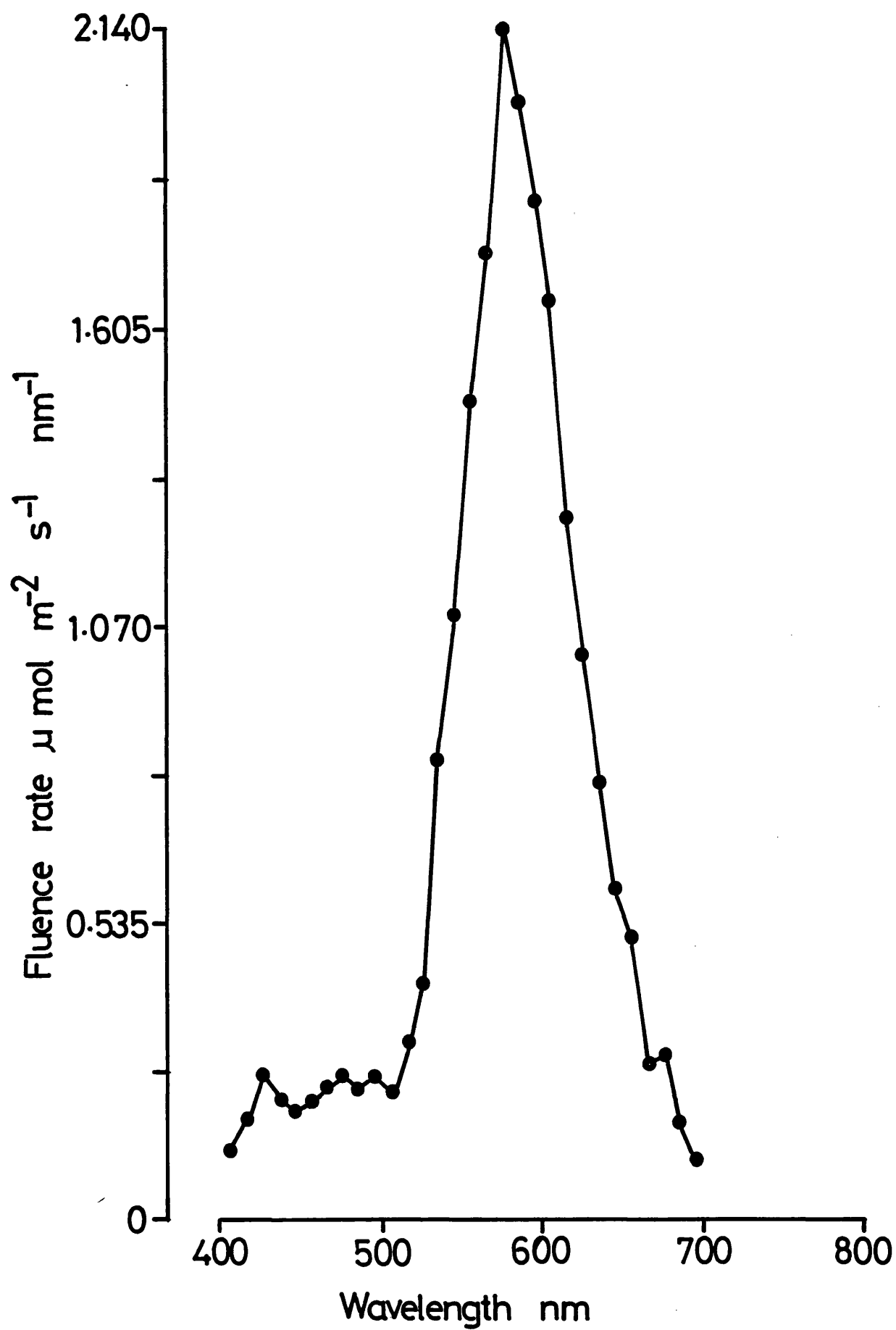


Fig. 2 (a), (b) and (c)

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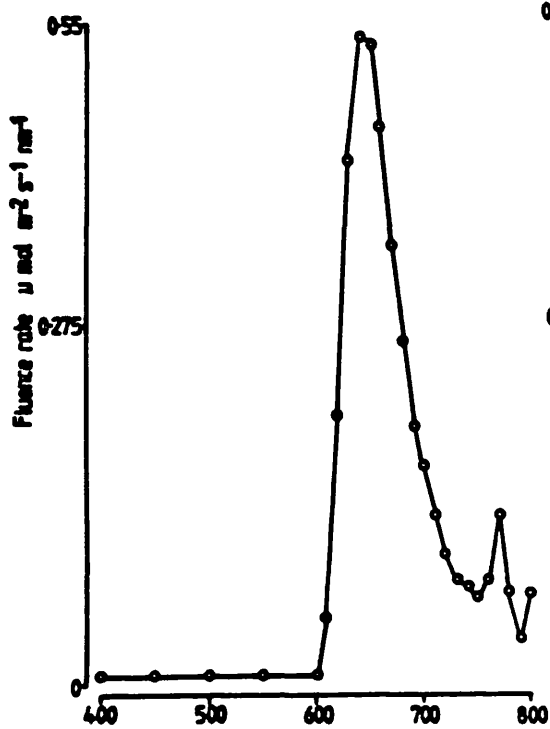
Spectral energy distributions of the light sources used for the irradiation of organelle fractions, and of a representative green safe-light used throughout the course of dark-room manipulations.

Fig. 2 (a) red light source

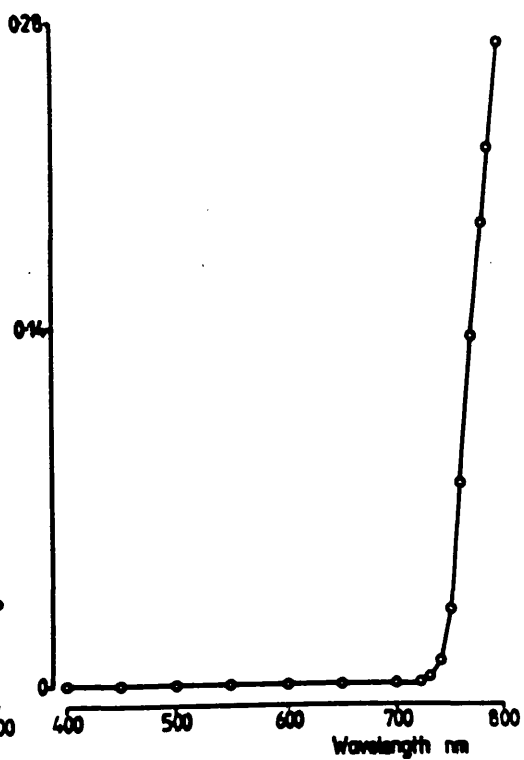
Fig. 2 (b) far-red light source

Fig. 2 (c) green safe-light

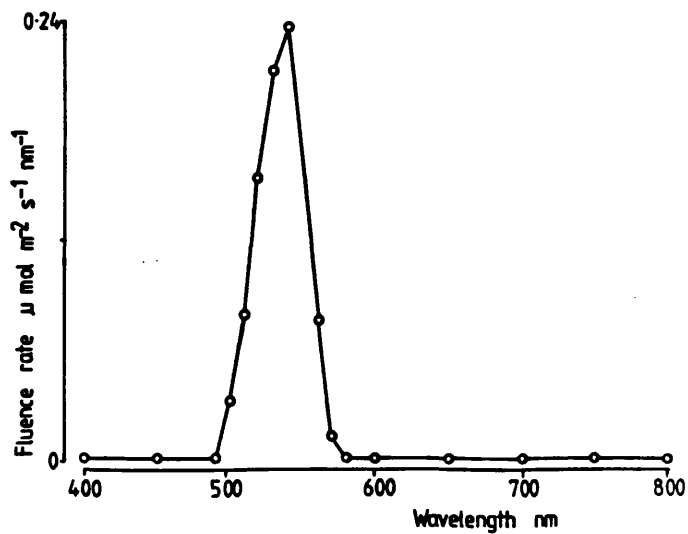
(a)



(b)



(c)



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General procedure for the extraction of organelle fractions from  
etiolated leaves of Hordeum vulgare L.

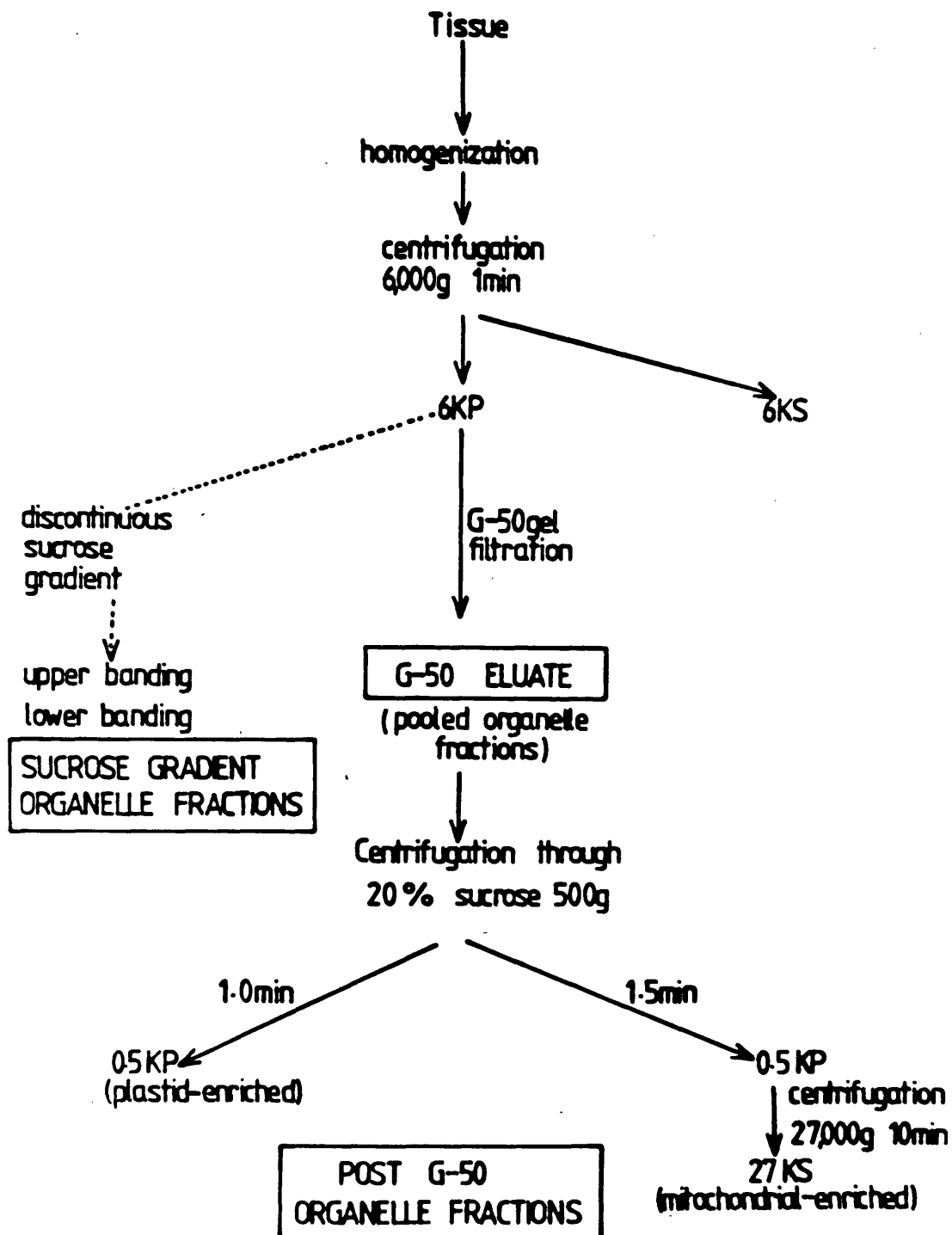


Table 1

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Measurement of cytochrome c oxidase activity in a total leaf homogenate and a re-suspended etioplast-enriched pellet (0.5KP) as an indication of the level of contamination by mitochondrial membranes.

Total volumes of fractions:

|                               |                    |
|-------------------------------|--------------------|
| Crude homogenate              | 10 cm <sup>3</sup> |
| Etioplast-enriched suspension | 2 cm <sup>3</sup>  |

---

| Fraction | Total cytochrome c oxidase activity<br>n kat |
|----------|--|
|----------|--|

---

|                  |     |
|------------------|-----|
| Crude homogenate | 2.4 |
|------------------|-----|

|                                  |     |
|----------------------------------|-----|
| Etioplast-enriched<br>suspension | 0.2 |
|----------------------------------|-----|

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Table 2

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Measurement of cytochrome c oxidase activity in a total homogenate and a re-suspended chloroplast-enriched pellet (0.5KP) as an indication of the level of contamination by mitochondrial membranes.

Total volumes of fractions:

Crude homogenate                      10 cm<sup>3</sup>

Chloroplast-enriched pellet      2 cm<sup>3</sup>

---

| Fraction | Total cytochrome c oxidase activity<br>n kat |
|----------|--|
|----------|--|

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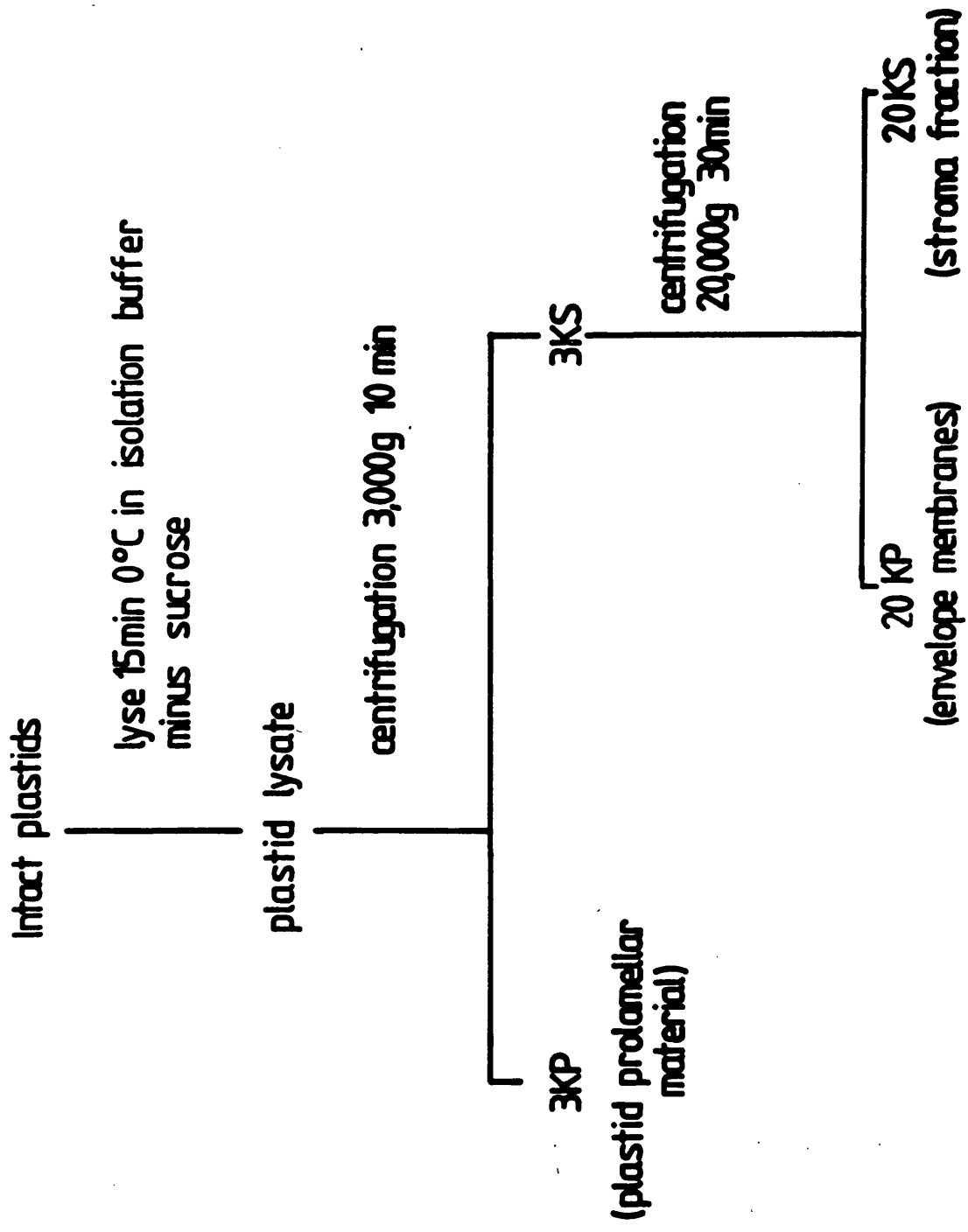
|                  |     |
|------------------|-----|
| Crude homogenate | 4.8 |
|------------------|-----|

|                                    |     |
|------------------------------------|-----|
| Chloroplast-enriched<br>suspension | 0.3 |
|------------------------------------|-----|

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General procedure for the isolation of envelope membrane-enriched fractions from plastids by the method described by Mackender and Leech, (1970).



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The preparation of 'soluble' phytochrome and G-50 organelle-enriched fractions from a crude homogenate of the first leaves of 6 day old Hordeum vulgare L. seedlings.

Aliquots marked \* were used in subsequent experiments.

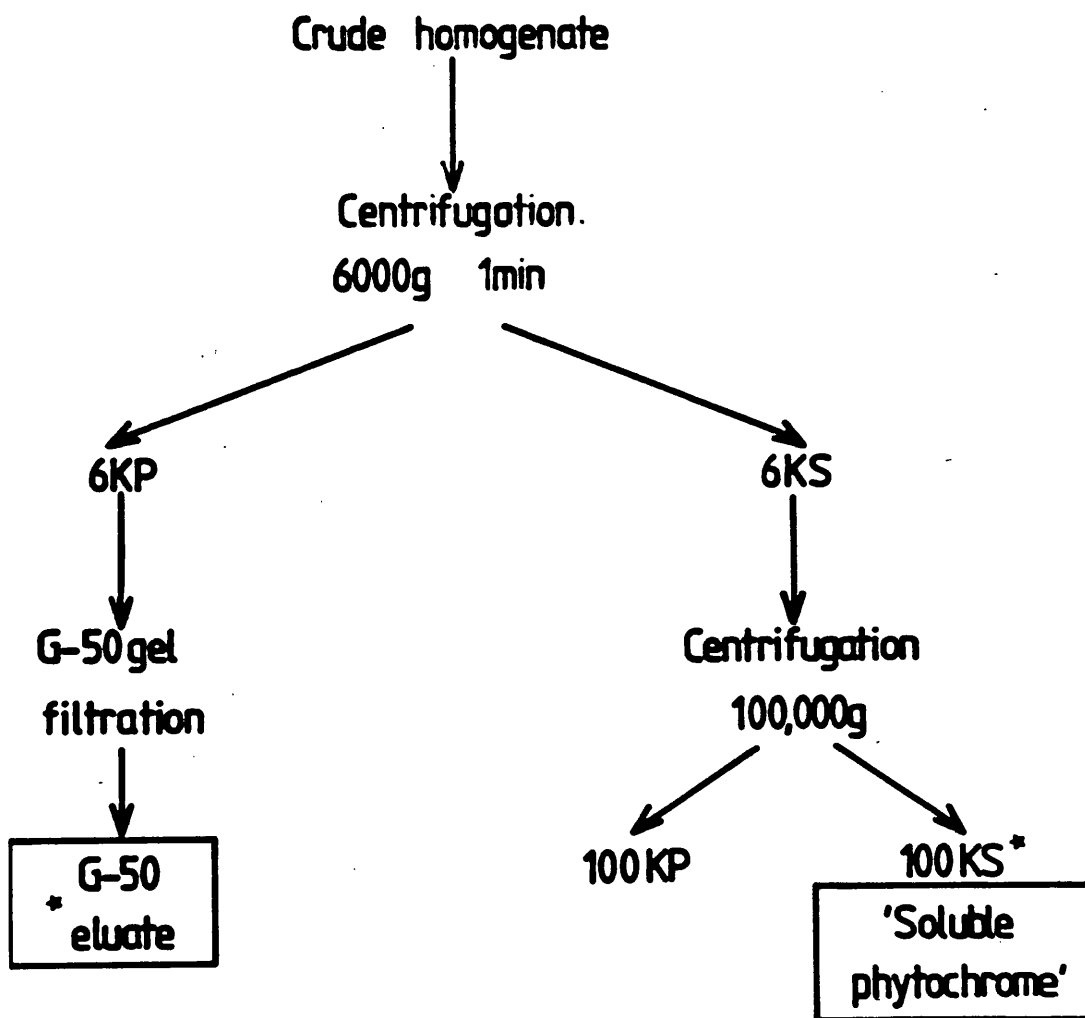
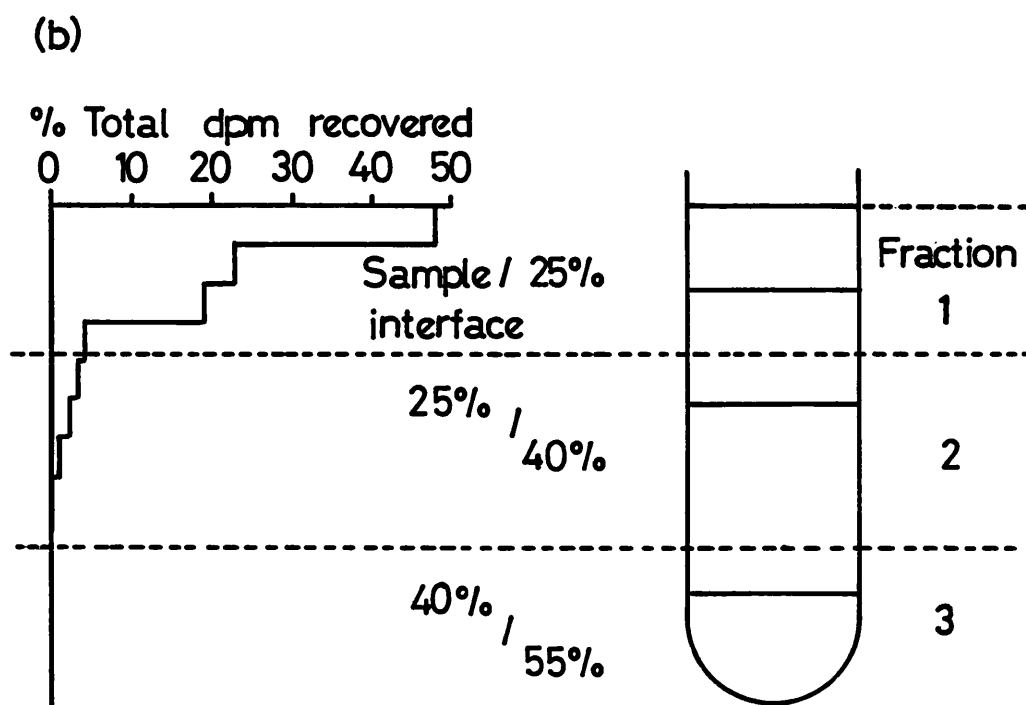
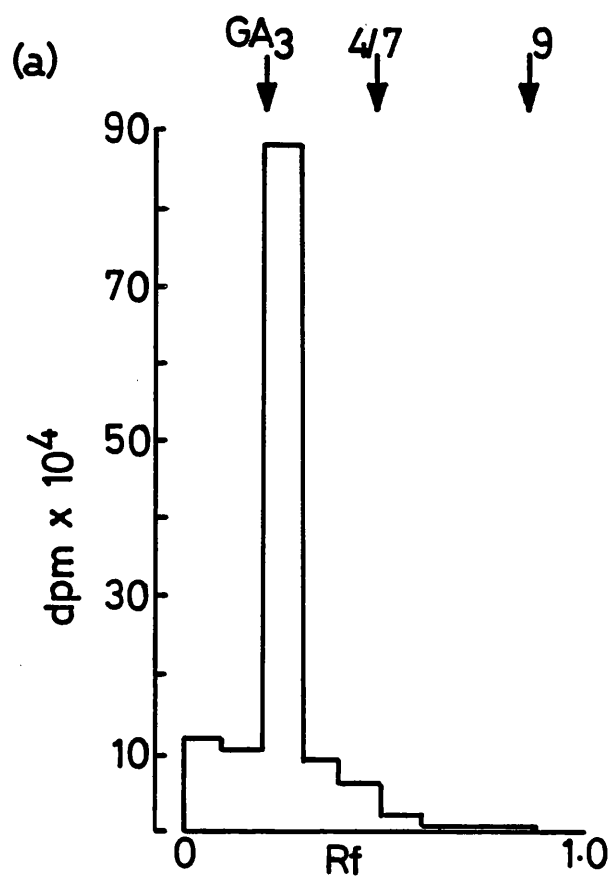


Fig. 6 (a) and (b)

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- (a) The distribution of radioactivity (following TLC) associated with the [ $^3\text{H}$ ] gibberellin  $A_1$  applied to a discontinuous sucrose density gradient.
- (b) The movement of [ $^3\text{H}$ ] gibberellin  $A_1$  into a discontinuous sucrose density gradient during centrifugation at 9,350g for 20 min.



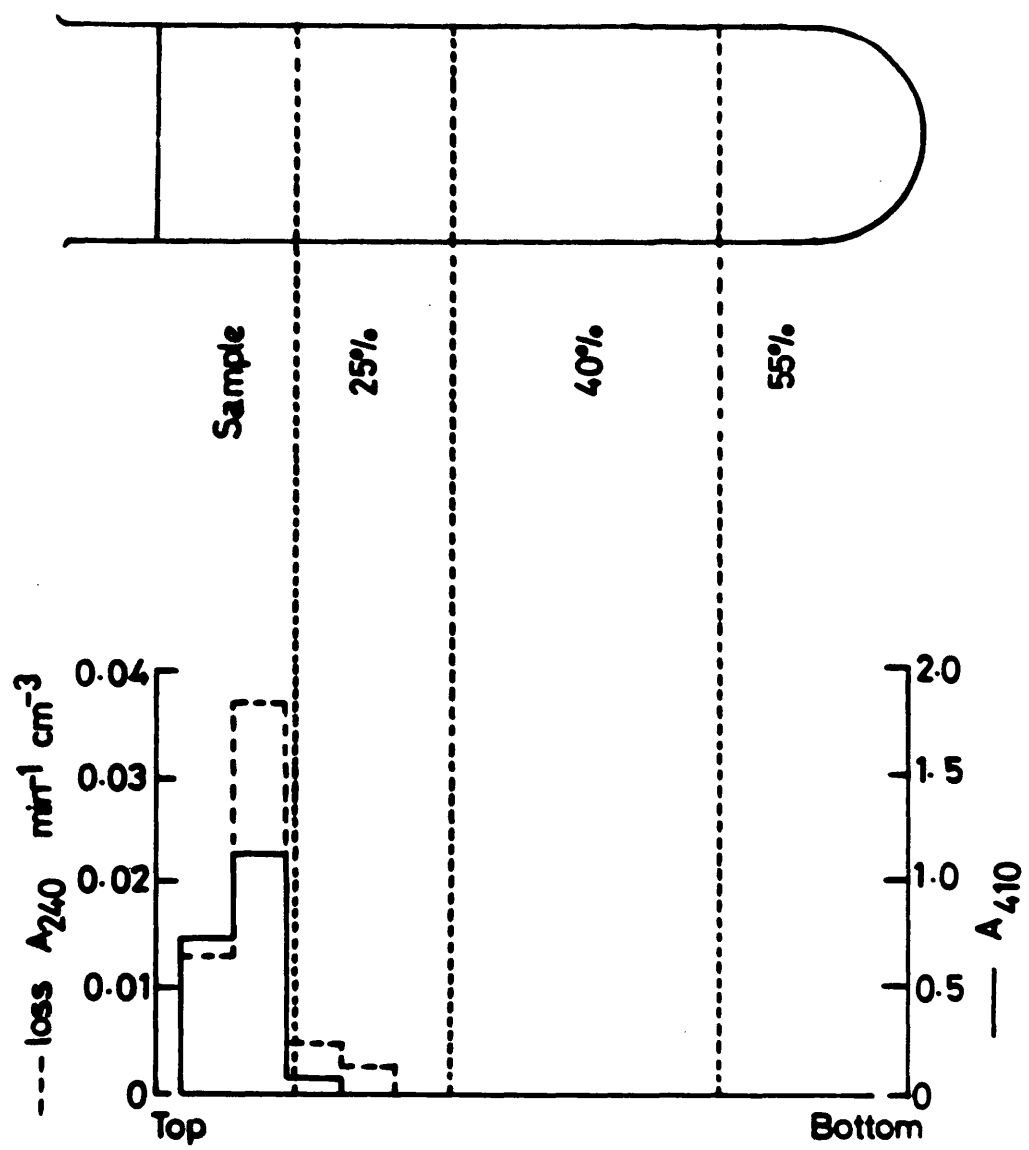


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Movement of the proteins catalase and haemoglobin into a discontinuous sucrose density gradient during centrifugation at 9,350g for 20 min.

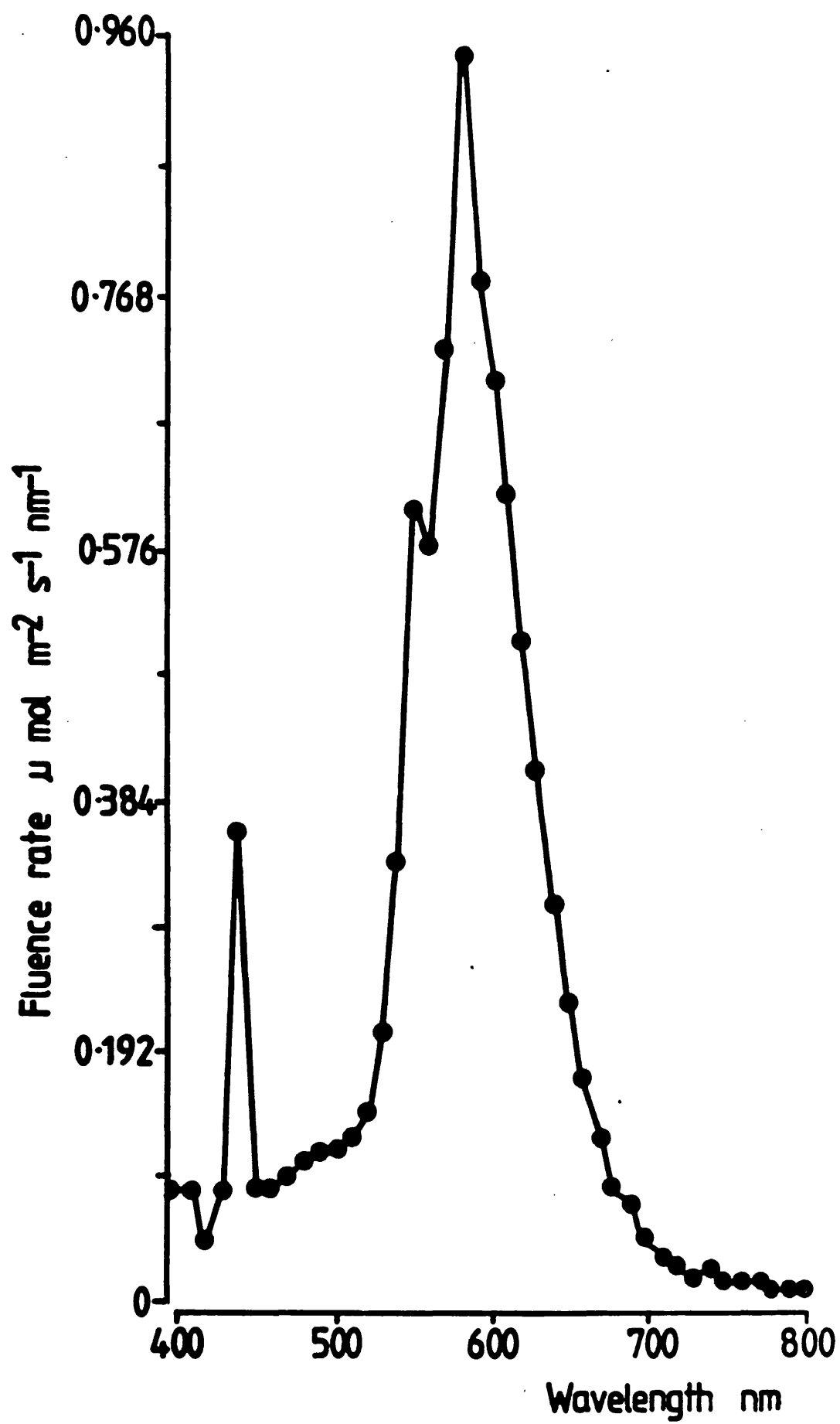
- - - - catalase

———— haemoglobin

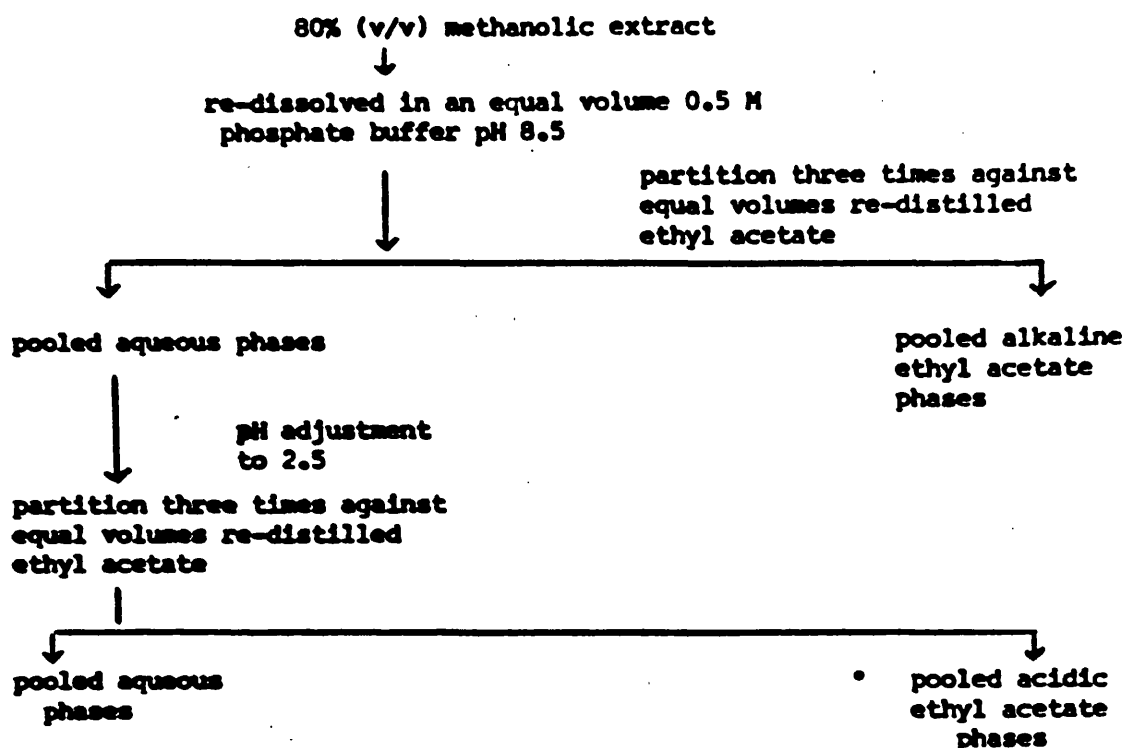


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Spectral energy distribution of the artificial light source under which whole shoots of Hordeum vulgare L. were incubated in a buffered solution containing [<sup>3</sup>H] gibberellin A<sub>9</sub>



General procedure for the extraction, fractionation and estimation of GA-LS.



- Reduced to dryness and re-dissolved in re-distilled ethanol : ethyl acetate 1 : 1 (v/v).

Thin-layer chromatography using chloroform : ethyl acetate : acetic acid, 60 : 40 : 5 (v/v/v) as the developing solvent. Solvent allowed to run 10 cm from the point of sample application.

1 cm zones eluted from the TLC plates with re-distilled ethanol : ethyl acetate, 1 : 1 (v/v) and reduced to dryness under a stream of nitrogen.

Samples re-dissolved in 1 cm<sup>3</sup> sterile water and assayed using a modification of the Jones & Varner (1967) barley aleurone layer bioassay.

Fig. 10 (a) and (b)

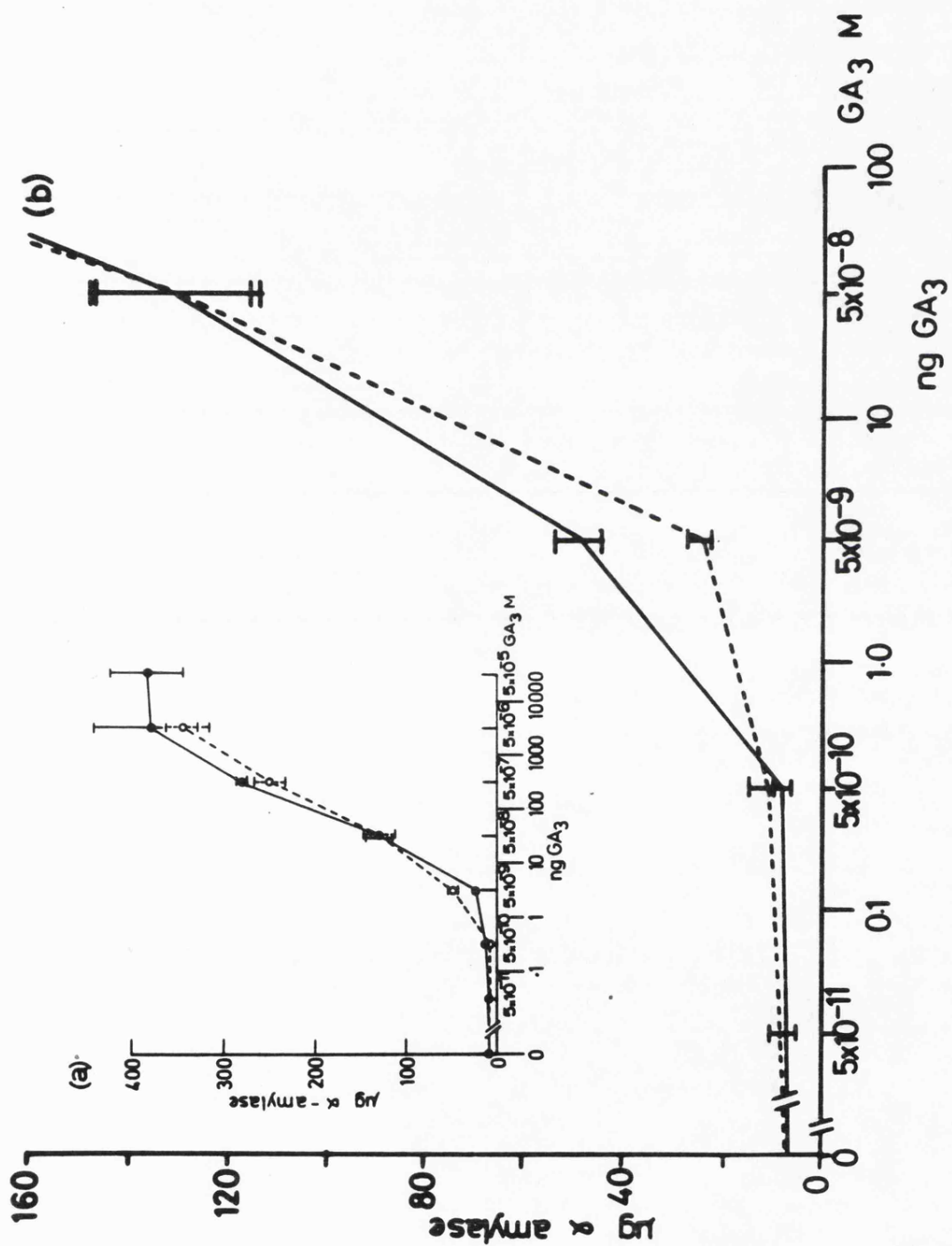
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$\alpha$ -amylase activity induced in a barley aleurone layer bioassay (Jones and Varner, 1967) when standard solutions of  $GA_3$  were used as the test solutions.

(The two lines represent results obtained with the two different batches of seed used in the experiments)

(a) Complete dose-response curve

(b) Expanded detail of the working range of  $\alpha$ -amylase activity.





Phytochrome and GA-like substances in plastid fractions isolated from dark-grown plants.

(a) The characterization of organelle fractions

As stated in the General Introduction all the previous work on the phytochrome control of the levels of methanol-extractable GA-like substances (GA-LS) in isolated etioplast suspensions have been performed using etioplast suspensions prepared by the Sephadex G-50 (coarse) gel filtration technique of Wellburn and Wellburn, (1971). In view of the comments of Quail, (1977) and other workers (Pers. Comm.) suggesting that the etioplasts prepared by this method are contaminated by mitochondria and, also, that the phytochrome present in association with these preparations is present solely as a soluble contaminant, it was considered necessary to re-investigate the characteristics of the G-50 column and to try to prepare etioplast-enriched suspensions by methods other than the G-50 column. In the absence of adequate electron microscope facilities it was found necessary to characterize the organelle fractions in terms of biochemical marker enzymes. As stated by Quail, (1979), when considering the positive aspects of using marker enzymes as opposed to using electron microscope data; (a) the amount of material represented by even 10 to 100 electron micrographs is of the order of  $10^3$  less than that used for a biochemical assay of a sub-cellular fraction and (b) great care is needed in the preparation and processing of samples for electron microscopy to ensure that unbiased, statistically valid electron micrographs of the fractions are obtained. When considering etioplast fractions it is necessary, as in other cases, to use a positive marker i.e. to locate the fractions enriched in the particular sub-cellular component for which the activity is a marker, and also, to use a negative marker, i.e. to establish the absence or low level of a particular sub-cellular component in fractions enriched for other organelles. Therefore, as mitochondria have been suggested to be the main contaminant of etioplast fractions, total extractable carotenoids were used as a positive marker for the identification of etioplast membranes and cytochrome c oxidase/

succinate dehydrogenase activities were used as negative marker enzymes to assess the contamination of the etioplast-enriched fractions by mitochondrial membranes. Carotenoids have been used as markers for etioplast membranes by several workers (Quail et al., 1976; Jesaitis et al., 1977; Ray, 1977) as indeed have cytochrome c oxidase (Cooper and Beevers, 1969; Batt and Venis, 1976; Hendricks, 1977; Benveniste et al., 1978) and succinate dehydrogenase (Lee, 1977; Sparace and Moore, 1979) been used for marker enzymes for mitochondria. These enzymes were therefore used routinely in the subsequent characterization of etioplast-enriched fractions.

- (1) Sephadex G-50 (coarse) gel filtration. [For Experimental Details see page 13, (d) 1.2]

The G-50 elution profile of Wellburn and Wellburn (1971) shows three peaks of  $A_{257}$  (Fig. 11, page 44). The first was claimed to correspond to intact plastids, the second to intact plastids and mitochondria, and a third to broken plastids. In contrast, the G-50 method profiles obtained by Evans and Smith, (1976a) and in this work, (Figs. 12 and 13, pages 45 and 46) show only single elution peaks. Fig. 14, page 47, shows the profiles of extractable carotenoids and cytochrome c oxidase/succinate dehydrogenase activities measured through a G-50 column eluate. The carotenoids and the mitochondrial marker enzymes have similar elution characteristics. The percentage recovery of these markers in a pooled G-50 eluate compared to that of the initial crude plastid pellet is shown in Table 3, page 48. The data suggest that etioplasts are purified to a similar extent following passage of a crude plastid pellet through a G-50 column and closely agree with those of Quail, (1977). They support his conclusion that etioplast suspensions prepared by the G-50 method have a significant level of mitochondrial contamination. Both Evans and Smith, (1976a) and Quail, (1977) have shown the elution profile of spectrophotometrically-detectable phytochrome to be closely similar to that of  $A_{260}$  measured through a G-50

column eluate. Similar results, showing a single elution peak, have been obtained in this work (Fig. 15, page 49). However, Quail, (1977) was unable to measure phytochrome spectrophotometrically in a re-suspended organelle pellet he obtained after further low speed centrifugation of pooled G-50 eluate fractions. He suggested that the phytochrome was present in the G-50 eluate solely as a soluble contaminant running in the void volume of the Sephadex G-50 column and that no pelletable phytochrome was present. In contrast, throughout this work, small amounts of phytochrome have been detectable in both the 6KS [ $2 \times 10 \Delta(\Delta A)$ ] and the 6KP, organelle-containing fraction [ $1.5 \times 10 \Delta(\Delta A)$ ] following centrifugation of the pooled eluate at 6,000g for 1 min. These results suggest that at least part of the total G-50 eluate phytochrome is truly organelle-associated (43%), despite a large proportion (57%) being non organelle-associated or soluble as suggested by Quail, (1977). Kraack and Spruit, (Pers. Comm.) have also been able consistently to measure pelletable or organelle-associated phytochrome in G-50 fractions isolated from etiolated maize leaves, both in complete darkness and under dim green safe-light. Their data support the observations that pelletable phytochrome is present in association with organelle suspensions isolated by the G-50 method.

The discrepancy between the results obtained by Kraack and Spruit (Pers. Comm.), Quail (1977) and those presented here may be due to the errors involved in the spectrophotometric measurement of phytochrome. There are reports of considerable variation between phytochrome measurements when  $Mg^{2+}$  and/or  $Ca^{2+}$  are present in the sample. Also, solutions of varying turbidity show very marked differences in  $\Delta(\Delta A)$  readings (Watson, Pers. Comm.). As  $CaCO_3$  was used to increase effective pathlengths in the cuvettes and the solutions were of varying turbidity (presumably due to varying numbers of plastids per sample) either, or a combination, of these factors may have produced the differences between readings. Throughout this work it has

proved virtually impossible to produce balance sheets of phytochrome measurements when comparing the phytochrome contents of crude fractions, purified organelle pellets and the corresponding supernatant fractions. In consequence, dilutions were performed for each sample and the maximum  $\Delta(\Delta A)$  reading obtained was taken as the phytochrome content of a particular fraction. An example of phytochrome measurements through G-50 etioplast purification is shown in Table 4, page 50. It is therefore extremely difficult to compare phytochrome measurements between different samples. However, the results presented here and those of Evans throughout her work are in close agreement. This is probably due to the very close similarity of the experimental techniques and measuring conditions. In order to reduce the error in phytochrome measurements due to varying numbers of plastids per sample,  $\Delta(\Delta A)$  readings were normally expressed on a protein basis. In retrospect, measurements could have been expressed in terms of total extractable carotenoids per sample.

(2) Post-G-50 sucrose centrifugation [see Experimental Details page 13 (d) (1.3)]

The 0.5KP and 27KS fractions obtained following centrifugation of equal volumes of pooled G-50 eluates through a buffered solution of 20% sucrose exhibited very distinct characteristics in terms of marker enzymes and of spectrophotometrically-detectable phytochrome when compared with the activities of the pooled G-50 eluate. The etioplast-enriched fraction contained 45% of the total extractable carotenoids, 12.5% of the total measureable phytochrome [ $0.5 \times 10^{-3} \Delta(\Delta A)$ ] and no detectable cytochrome c oxidase activity. The mitochondrial-enriched fraction contained 14% of the total extractable carotenoids, 44% of the total measureable phytochrome [ $1.75 \times 10^{-3} \Delta(\Delta A)$ ] and 97% of the cytochrome c oxidase activity (Table 5, page 51). It was therefore possible, by this method, to obtain an etioplast-enriched suspension containing measureable amounts of phytochrome

but no detectable cytochrome c oxidase activity (indicating the absence of mitochondrial contamination), and a separate fraction enriched with mitochondria.

(3) Discontinuous sucrose density gradient centrifugation  
[ For Experimental Details see page 14, (d) 1.4 ]

The elution profile obtained after the centrifugation of a crude plastid pellet through a discontinuous sucrose density gradient shows two peaks of extractable carotenoids (Fig. 16, page 52), one at the 25%/40% sucrose interface with high associated cytochrome c oxidase activity and another at the 40%/55% sucrose interface with no associated cytochrome c oxidase activity. Fig. 17, page 53, shows the distribution of phytochrome expressed in absolute units (a), and also the specific activity (b), through a sucrose density gradient. A large proportion, (approximately 40%) of the total phytochrome recovered from the gradient is not organelle-associated (i.e. is soluble), and remains on top of the gradient. A large proportion of the phytochrome passing into the gradient is associated with the upper banding fraction but a small amount is associated with the lower banding fraction.

(4) Discussion

Phytochrome has been shown to be present in association with etioplast-enriched fractions by several workers, (Cooke et al., 1975; Evans and Smith, 1976a and b; Kraack and Spruit, Pers. Comm.; Quail, 1977). All of these workers used the G-50 method to isolate the etioplast-enriched fractions. Cooke et al., (1975) and Evans (1975) showed electron micrographs of the G-50 organelle fractions they isolated and concluded that the etioplasts prepared by this method were intact, and not contaminated to any great extent with any other cellular organelles or debris. However, in a very detailed biochemical and electron microscope study of G-50 eluate

fractions isolated from etiolated oat shoots Quail (1977) concluded that mitochondria as well as etioplasts were purified to a similar extent by the G-50 method. He also proposed that the phytochrome he was able to detect spectrophotometrically in association with the G-50 organelle fractions was, in fact, merely a soluble contaminant running in the void volume of the G-50 column. He was unable to detect phytochrome when the G-50 organelles were pelleted by centrifugation and re-suspended. The data presented in this thesis is in close agreement with those of Quail in respect of mitochondrial contamination of the etioplast-enriched G-50 fractions but, in contrast to him, it was possible to measure small but consistent amounts of phytochrome associated with the G-50 organelles following centrifugation and re-suspension. Kraack and Spruit (Pers. Comm.) have been able also consistently to measure phytochrome spectrophotometrically in G-50 organelle fractions following centrifugation. Despite the mitochondrial contamination of the G-50 organelle fractions there is very little contamination by ribonucleoprotein material (Evans, 1975; Quail, 1977) and a high percentage of the etioplasts are intact (Kraack, Pers. Comm.). The volume of the organelle-containing G-50 eluate is an extremely convenient volume of organelle suspension to experiment with and it also has very distinct advantages in that it does not suffer from the defects inherent in organelle pellet re-suspension, which is very rarely accurate.

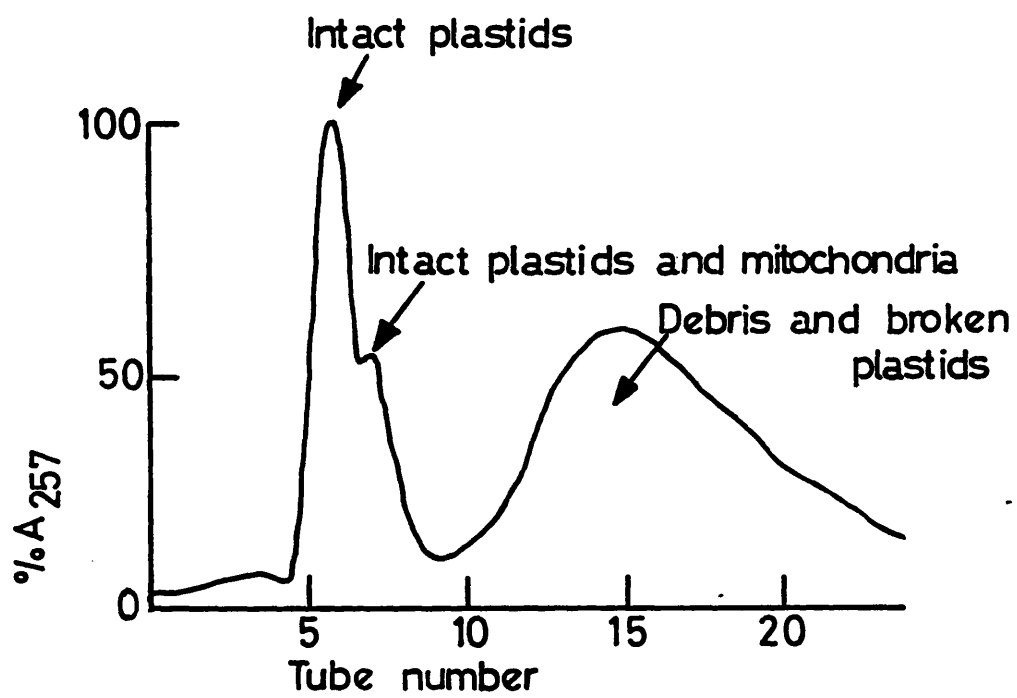
It was possible to obtain two distinct fractions, one enriched with plastids and another enriched with mitochondria (as judged by mitochondrial membrane marker enzymes) from the G-50 eluate by a single centrifugation of the eluate through a cushion of sucrose. Similar fractions were also obtained by the centrifugation of a crude plastid pellet through a discontinuous sucrose density gradient. These two latter methods of plastid isolation have problems inherent in using quite high concentrations of sucrose,

pellet re-suspension and dilution of the samples to a suitable volume for subsequent experimentation. Phytochrome was detectable in association with all the organelle fractions isolated. As sucrose has been shown to interfere with the spectrophotometric detection of phytochrome (Hopkins, Pers. Comm.) it was necessary to pellet, and subsequently to re-suspend the organelle fractions obtained from sucrose gradients in isolation buffer in order to obtain fractions of similar sucrose concentration prior to phytochrome measurement. More recently, however, Billet (Pers. Comm.) has shown that sucrose concentration has no detectable effect on the spectrophotometric detection of phytochrome in mitochondrial fractions isolated from mung bean hypocotyls when  $\text{CaCO}_3$  is used as a light scattering agent. In the absence of  $\text{CaCO}_3$  there was a marked effect on the amount of phytochrome detectable.



The elution profile of a G-50 column showing three peaks of  $A_{257}$ ; the first was claimed to correspond to intact plastids, the second to intact plastids and mitochondria and a third to debris and broken plastids.

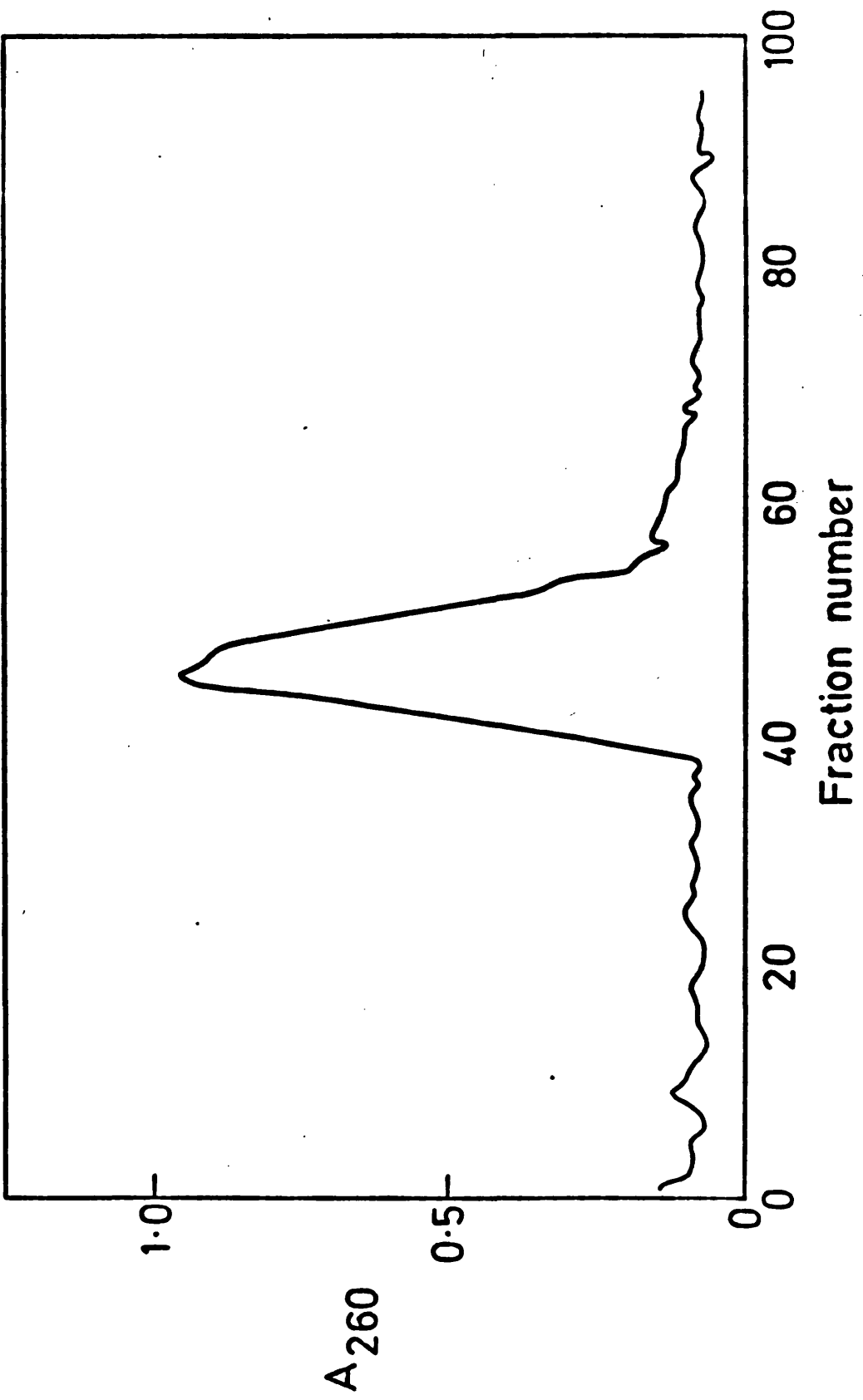
(from Wellburn and Wellburn, 1971)



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The elution profile of a G-50 column showing a single peak of  
 $A_{260}$ .

(from Evans and Smith, 1976a)

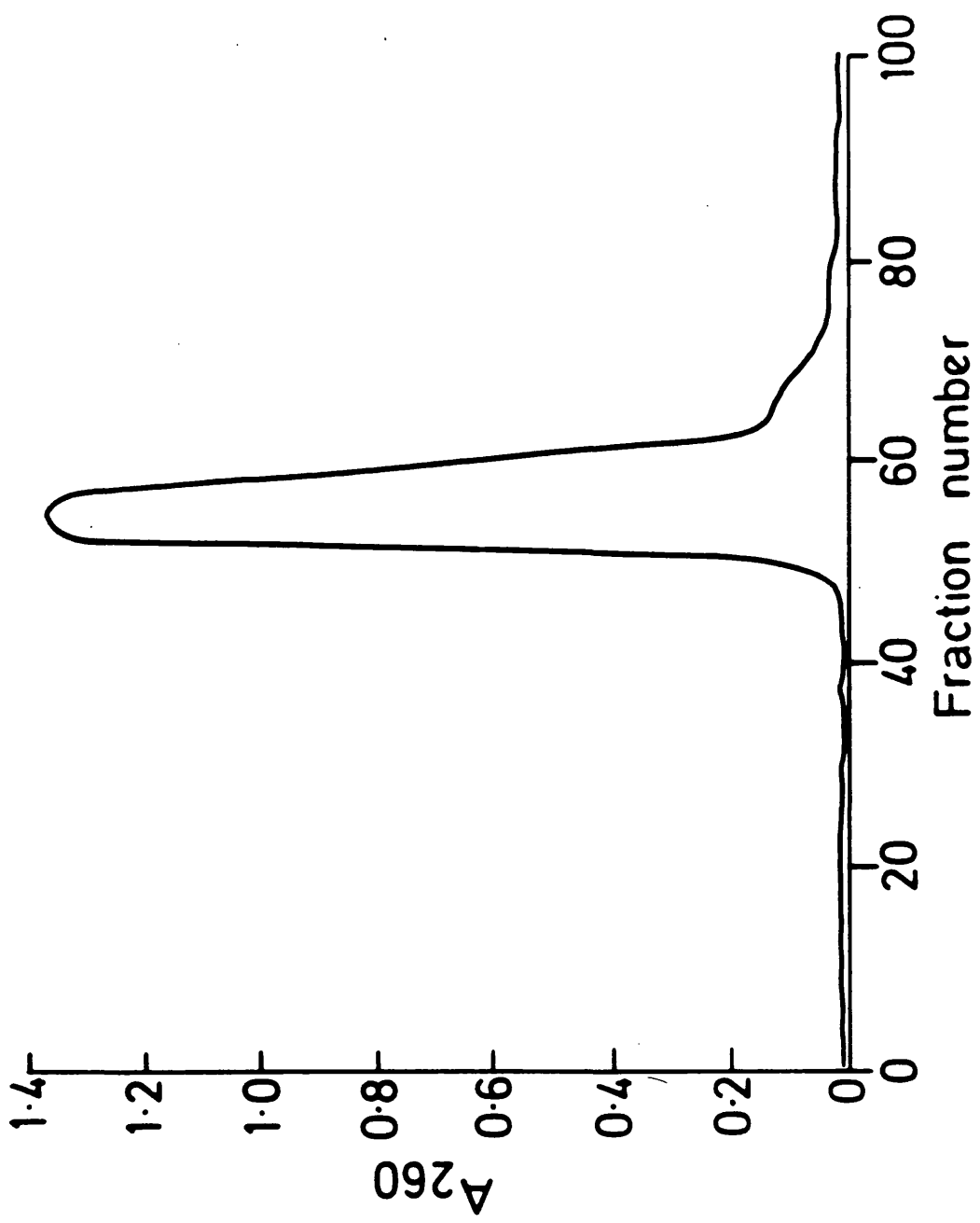


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The elution profile of a G-50 column showing a single elution peak of  $A_{260}$ .

Each fraction was  $0.5 \text{ cm}^3$  in volume.

Data obtained in this work.



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The elution profile of a G-50 column using cytochrome c oxidase and succinate dehydrogenase as marker enzymes of mitochondrial membranes and total extractable carotenoids as a marker of etioplast membranes.

Each fraction was approximately 2 cm<sup>3</sup> in volume.

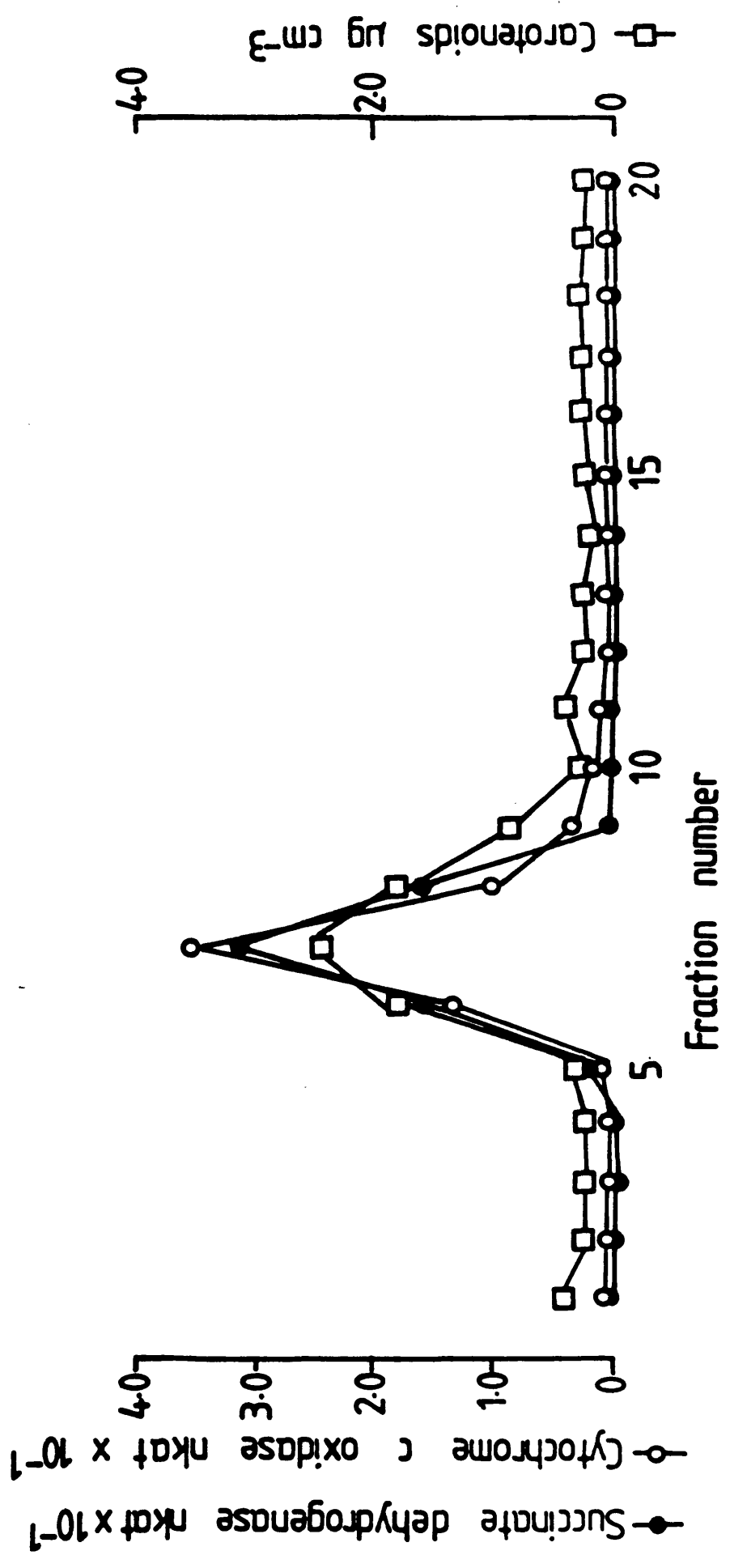




Table 3

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The percentage recoveries of etioplast and mitochondrial membrane markers during G-50 gel filtration.

Total volumes of fractions:

Crude plastid pellet      5 cm<sup>3</sup>

Bulked G-50 fractions      9 cm<sup>3</sup>

|  | Crude plastid pellet | Bulked G-50 fractions | % recovery in G-50 fractions |
|--|----------------------|-----------------------|------------------------------|
| Total carotenoids<br>µg                            | 21.0                 | 5.6                   | 27                           |
| Total succinate dehydrogenase<br>activity<br>n kat | 17.9                 | 6.3                   | 35                           |
| Total cytochrome c oxidase<br>activity<br>n kat    | 3.2                  | 0.7                   | 22                           |

The elution profile of a G-50 column showing spectrophotometrically-detectable phytochrome [ $\Delta(\Delta A)$ ] to have similar elution characteristics to the etioplast membrane marker, total extractable carotenoids.

Each fraction was approximately  $2\text{ cm}^3$  in volume.

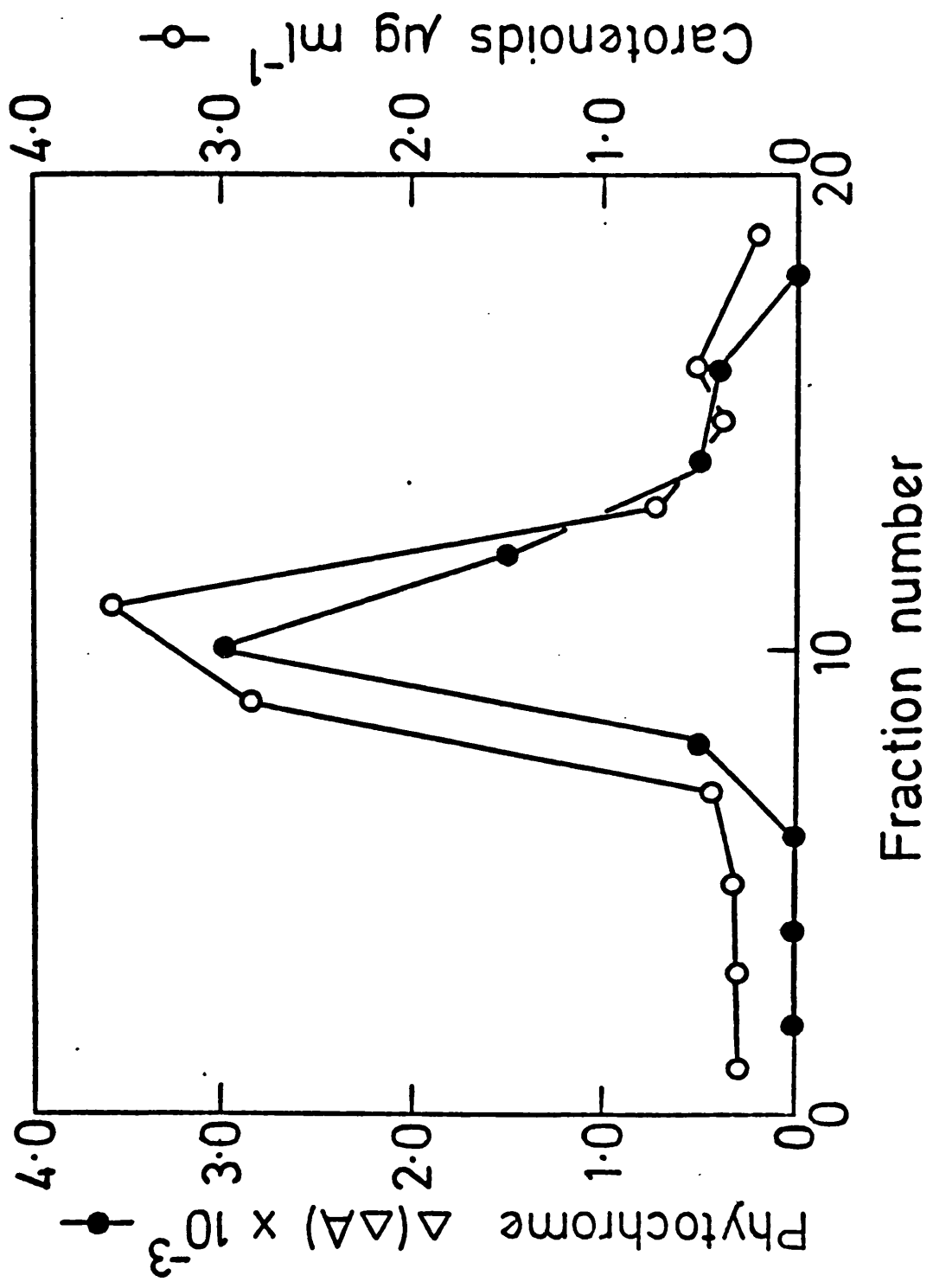


Table 4

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Measurement of phytochrome content at varying stages during the isolation of etioplasts by the G-50 method.

Fractions were diluted in 25 mM MOPS buffer.

| Fraction         | Dilution | $\Delta(\Delta A) \times 10^{-3}$ |
|------------------|----------|-----------------------------------|
| Crude homogenate | 0        | 12.5                              |
|                  | 1:1      | 10.0                              |
| 6KS              | 0        | 11.5                              |
|                  | 1:1      | 6.5                               |
| 6KP              | 0        | 1.0                               |
|                  | 1:1      | 1.75                              |
|                  | 1:4      | 1.0                               |
|                  | 1:20     | 1.0                               |
| G-50 eluate      | 0        | 3.0                               |
|                  | 1:1      | 1.5                               |

Table 5

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The distributions of carotenoids, cytochrome c oxidase and phytochrome in post-G-50 sucrose centrifugation organelle suspensions as a percentage of those present in the initial pooled G-50 eluate.

Each fraction was approximately 9 cm<sup>3</sup> in volume.

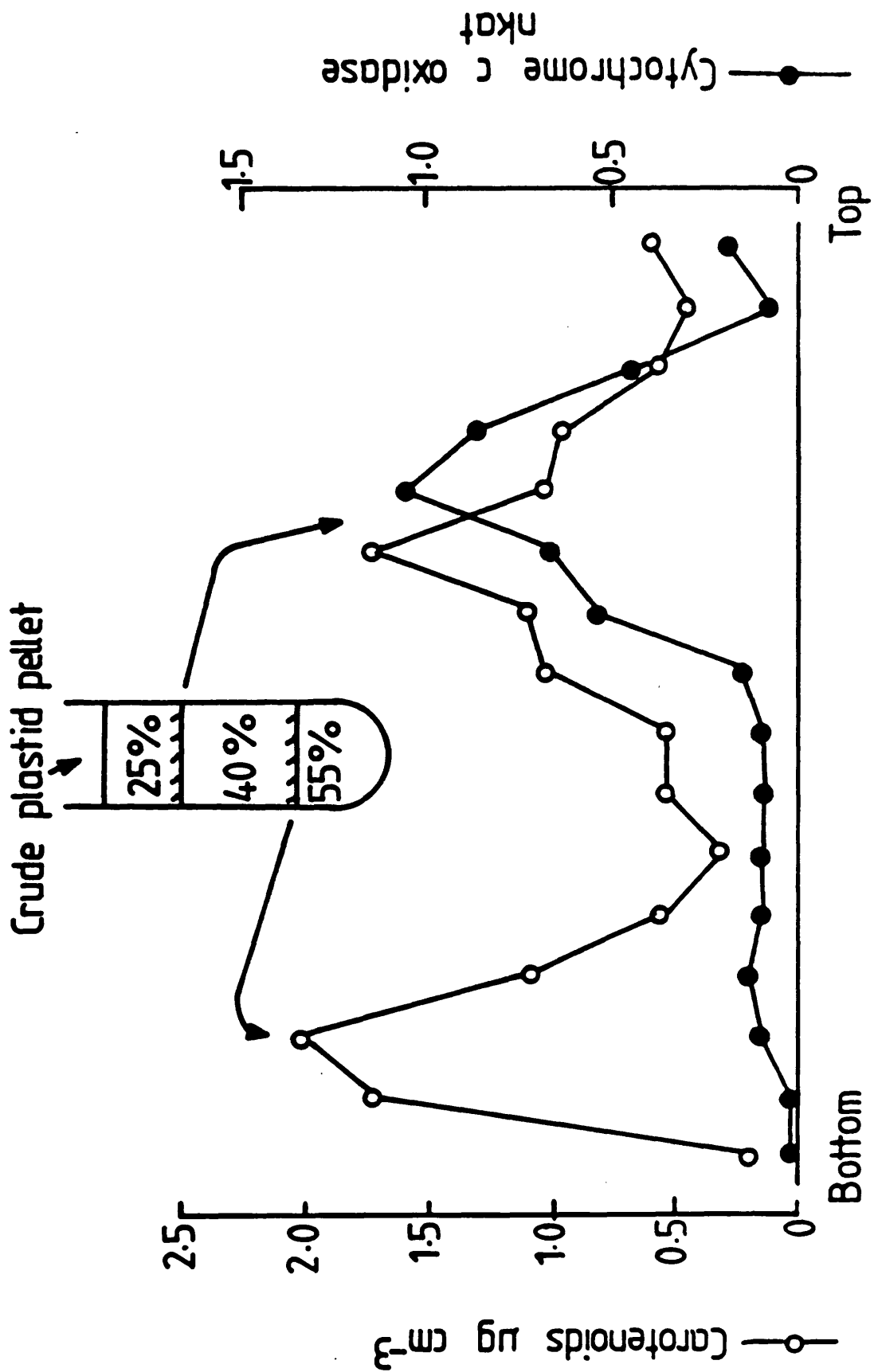
| Fraction                   | Carotenoids<br>% | Gytochrome c<br>oxidase<br>% | Phytochrome<br>%                         |
|----------------------------|------------------|------------------------------|--|
| Plastid-enriched           | 46               | 0                            | 12.5                                     |
|                            |                  |                              | $[0.5 \times 10^{-3} \Delta(\Delta A)]$  |
| Mitochondrial-<br>enriched | 14               | 97                           | 44                                       |
|                            |                  |                              | $[1.75 \times 10^{-3} \Delta(\Delta A)]$ |



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Description of the discontinuous sucrose density gradient and the elution profile obtained using cytochrome c oxidase and total extractable carotenoids as markers of mitochondrial and etioplast membranes respectively. Note the two peaks of extractable carotenoids; the first at the 25%/40% sucrose interface with high associated cytochrome c oxidase activity and the second peak at the 40%/55% sucrose interface with no associated cytochrome c oxidase activity.

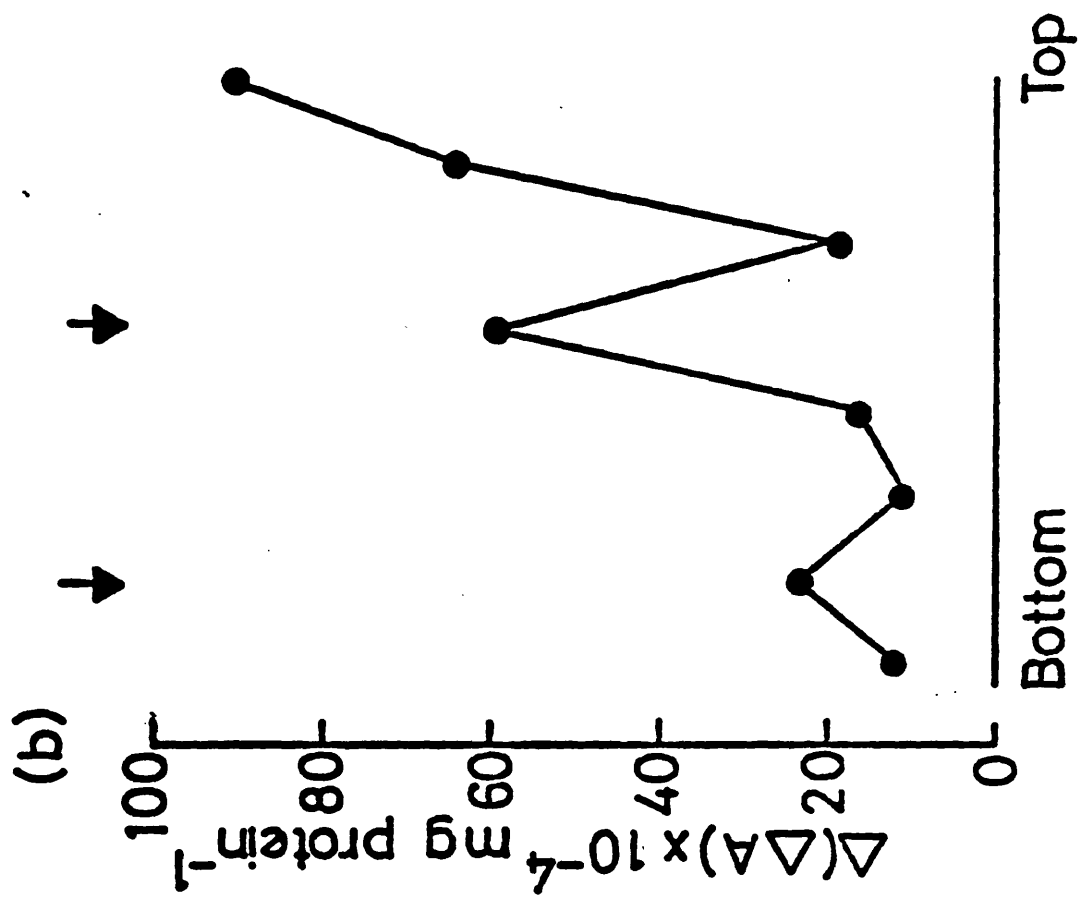
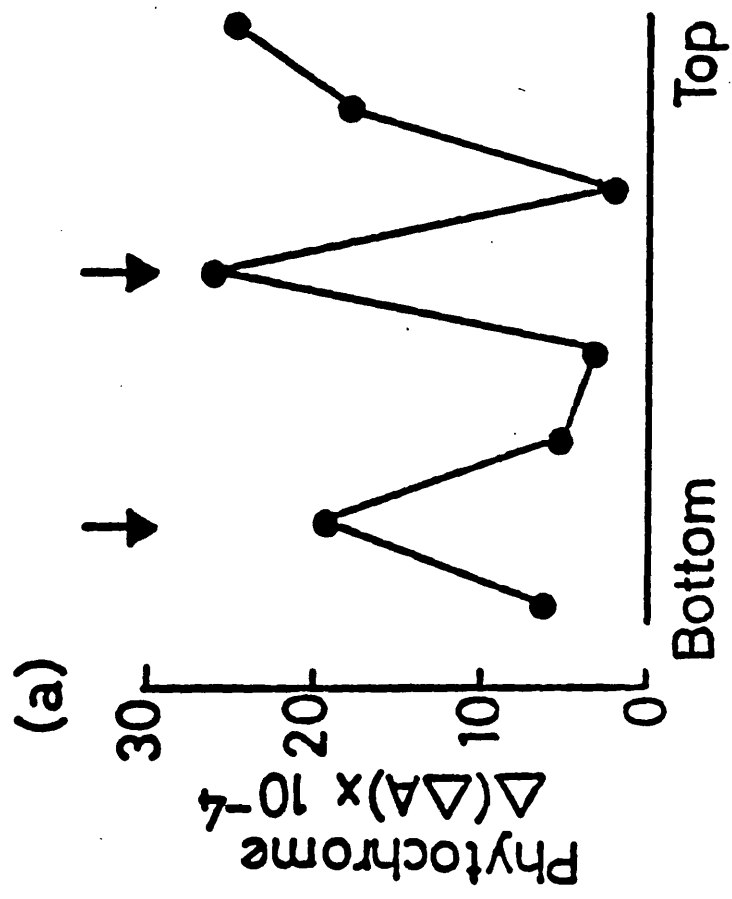
Each fraction was approximately 2 cm<sup>3</sup> in volume.



Elution profile of a discontinuous sucrose density gradient in terms of spectrophotometrically-detectable phytochrome (a), and its specific activity (b). Phytochrome was measureable in association with both the organelle fractions despite approximately 40% of the total phytochrome recovered from the gradient remaining on top of the gradient and was therefore not organelle-associated (i.e. was soluble).

The volume of each fraction isolated from the gradient was approximately 4 cm<sup>3</sup>.

The arrows indicate the banding positions of the organelle fractions subsequently used in light treatment experiments.



(b)(1) The *in vitro* phytochrome mediation of the levels of biologically active GA-LS extractable from organelle suspensions

From the results presented in Chapter 3 part (a) it was possible to obtain several organelle-containing fractions; some were enriched with mitochondria and some were enriched with etioplasts. All the organelle suspensions contained measureable amounts of phytochrome. As previous work on the phytochrome control of gibberellin levels had been performed with the organelle-enriched eluate obtained from a Sephadex G-50 column it was decided to try to repeat these experiments and also to study the effects of light treatment on other organelle-enriched suspensions. In preliminary experiments biological activity extractable from the organelle fractions was determined using both the Tan-ginbozu dwarf rice bioassay (Murakami, 1968) and the barley aleurone bioassay of Jones and Varner, (1967). However, reproducible results were only obtained using the barley aleurone layer bioassay. The main problems involved with using the dwarf rice bioassay were those inherent in the application of the test solution to the plants. It is necessary to apply 1 $\mu$ l droplets to the plants and it proved extremely difficult to make repeated applications. Moreover, the accuracy of doing so was extremely difficult to determine. The use of the lettuce hypocotyl bioassay (Frankland and Wareing, 1960) was precluded as very small differences in hypocotyl elongation between treatments had been observed by Cooke *et al.*, (1975). The accuracy of such measurements is also extremely difficult to determine. When performing bioassays it is far better to use a wide spectrum of bioassay material rather than to use a single bioassay as each bioassay system exhibits a characteristic response spectrum (Reeve and Crozier, 1975). However, for the reasons stated the barley aleurone layer bioassay was the only bioassay system used in all subsequent experiments. The synthesis and secretion of  $\alpha$ -amylase by the aleurone layer of seeds of barley are initiated by GA and the quantity of

the enzyme released is proportional to the concentration of GA applied. This bioassay has the added advantage of being an almost completely sterile experimental system and any problems with contamination from, and by, micro-organisms are reduced to a minimum. Not all barleys are suitable for the barley aleurone bioassay (Reeve and Crozier, 1975). There are considerable varietal variations in the sensitivity of the response to GA. In addition, differences in sensitivity are associated with the season, area of growth and age of the seed. For these reasons the barley variety Himalaya, 1973 was used routinely. Even under these conditions differences were noted between the sensitivity of batches of seed obtained from the supplier at varying times. These differences may have arisen from varying seed storage conditions.

At this stage it is perhaps pertinent to consider quantification of bioassay results. In the literature gross GA levels per tissue are frequently expressed as ng GA<sub>3</sub> equivalents per gram dry weight. Because of the complexity of the GA-inhibitor interactions and the varying activities of the different GA's these estimates are of limited value. Hill and Kimble, (1969) have pointed out that it is only valid to compare estimates of GA-like activity in terms of GA<sub>3</sub> equivalents when parallelism exists between dose/response curves of each of the unknowns and that of GA<sub>3</sub>. The possibility of the existence of such parallelism is remote for two reasons. Firstly, even with pure GA's there is a marked lack of parallelism between dose response curves. Secondly, it is likely that the chromatographic fraction being assayed will be a mixture of several GA's and a number of unknown compounds. The assay response will, therefore, be the net result of the interaction of these compounds. Hence the relationship between the amount of GA actually present and the number of GA equivalents measured will be obscure. (Reeve and Crozier, 1975). Though some authors may argue otherwise, no amount of elegant statistics will overcome this problem unless the

nature of the interacting compounds can be elucidated and measured for every assay performed. Thus, methods for calculating confidence limits for bioassay data (Hill and Kimble, 1969) are of limited value in practice, because they are really only valid for assaying unknown amounts of pure GA's or mixtures of GA's and non-interacting compounds. For these reasons the results presented here are presented as  $\mu\text{g}$   $\alpha$ -amylase induced and are not converted to ng GA<sub>3</sub> equivalents.

From the work of Evans (1975) the conditions required for the maximum levels of methanol-extractable GA-LS were taken as being a 5 min irradiation with red light followed by a 5 min incubation in darkness at 24°C. These conditions were used routinely throughout the following experiments.

GA-LS were extracted into aqueous methanol to a final concentration of 80% (v/v) and the GA-like activity determined in the acidic ethyl acetate fractions following TLC.

Fig. 18a page 59, shows the effects of irradiations with red light and far-red light on the level of biologically-active GA-LS extractable from pooled G-50 eluate organelle fractions. The data closely agree with those previously published (Cooke et al., 1975; Cooke and Saunders 1975; Evans and Smith 1976a). The levels of methanol extractable GA-LS are substantially increased if the organelle suspensions are irradiated for 5 min with red light. However, the levels of activity extractable from samples irradiated with far-red light for 5 min immediately following the red light are closely similar to those extractable from samples maintained in darkness throughout the treatment period. Figs. 18.b and 18.c, page 60 demonstrate the effects of red and far-red light on the levels of GA-LS extractable from the post-G-50 sucrose centrifugation organelle suspensions. The levels of GA-LS extractable from mitochondrial-enriched fractions following light

treatment remain closely similar to that of samples retained in darkness. However, the level of GA-like activity extractable from etioplast-enriched fractions is substantially increased following red light treatment. The red light-mediated increase in activity is prevented if the samples are irradiated with far-red light for 5 min immediately following the red light treatment.

The effects of light treatment on discontinuous sucrose density gradient organelle fractions are shown in Figs. 18.d and 18.e page 61. Biologically-active GA-LS extractable from the upper banding (25%/40% sucrose interface) fraction are unaffected by light treatment whereas the lower banding (40%/55% sucrose interface) fraction shows enhanced levels of methanol-extractable GA-LS following a 5 min irradiation with red light. Again, treatment of the samples with 5 min far-red light immediately following the red light reverses the observed increase in the levels of GA-LS. These data clearly show that phytochrome mediates the levels of GA-LS extractable into aqueous methanol from plastid-enriched fractions but has no detectable effect on the GA-LS extractable from mitochondrial-enriched fractions.



GA-like substances were extracted and fractionated from organelle-enriched suspensions as described on page 21 (s).

All bioassay data was obtained using a modification of the Jones and Varner (1967) barley aleurone layer bioassay for GA-like substances as described on page 22 (t).

Shaded areas on the histograms indicate stimulation of  $\alpha$ -amylase activity above that of the controls that is significant at the 5% level.

The organelle fractions were divided into equal aliquots prior to light treatment. The results presented are therefore directly comparable.

The above information relates to all subsequent figures showing bioassay data. This information will therefore not be presented on subsequent figure headings.

GA-like activity associated with light-treated organelle-enriched suspensions.

Treatments:      10 min in darkness    (DARK)  
                     5 min red + 5 min in darkness    (RED)  
                     5 min red + 5 min far-red    (RED/FAR-RED)

Pooled G-50 eluate organelle suspensions.

(a)

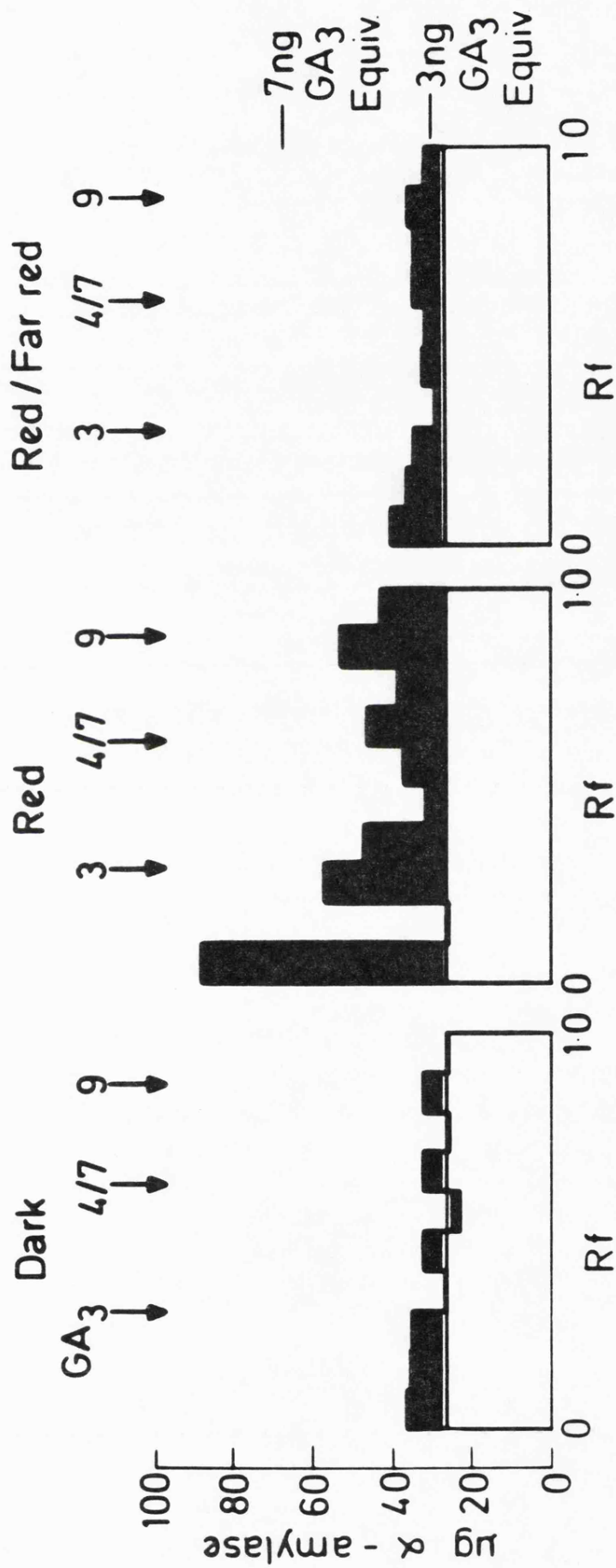


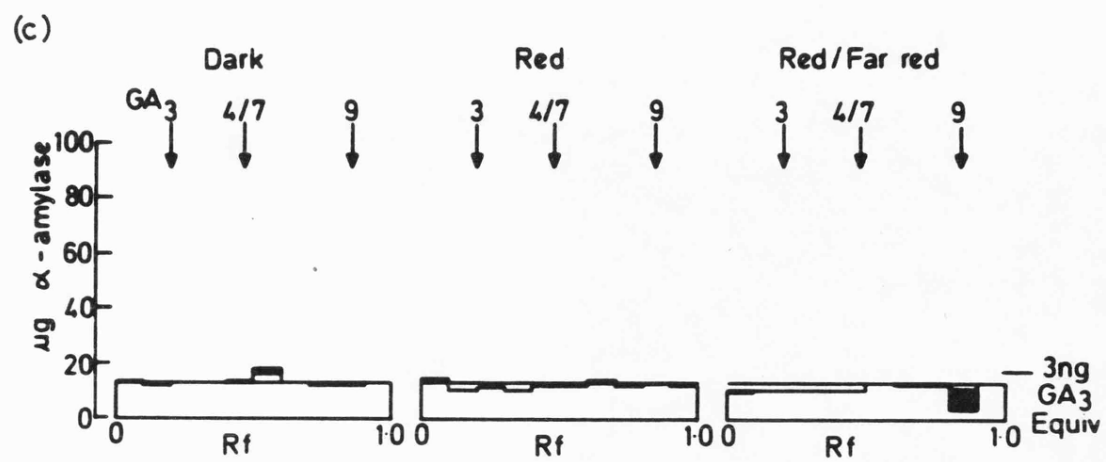
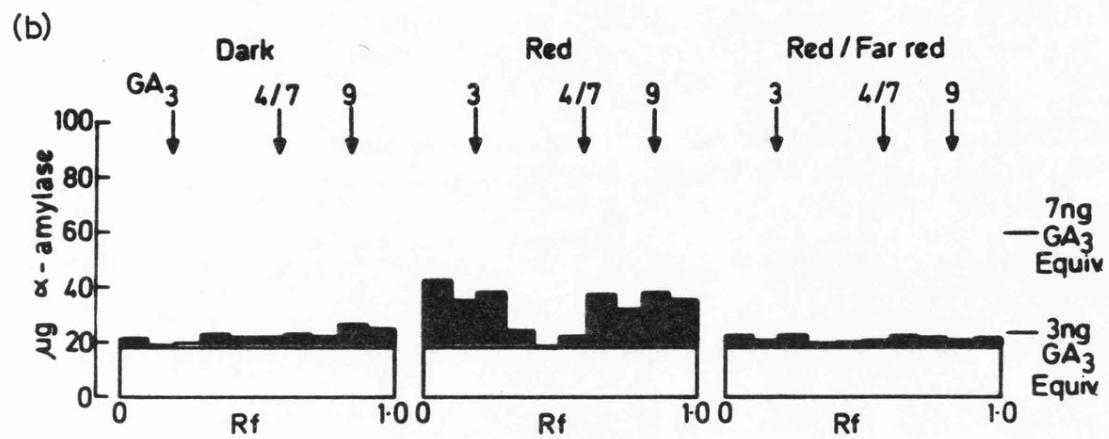
Fig. 18.b and c.

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Post G-50 sucrose centrifugation suspensions.

Fig. 18.b etioplast-enriched fraction

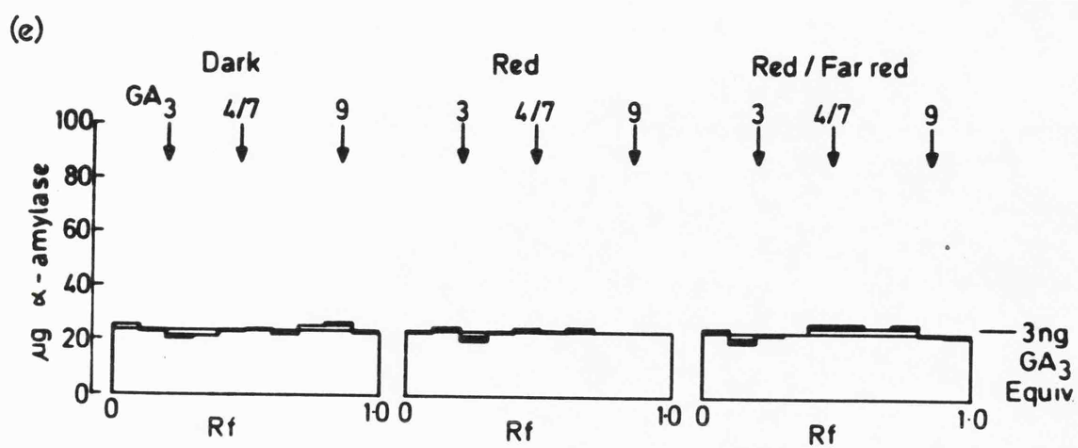
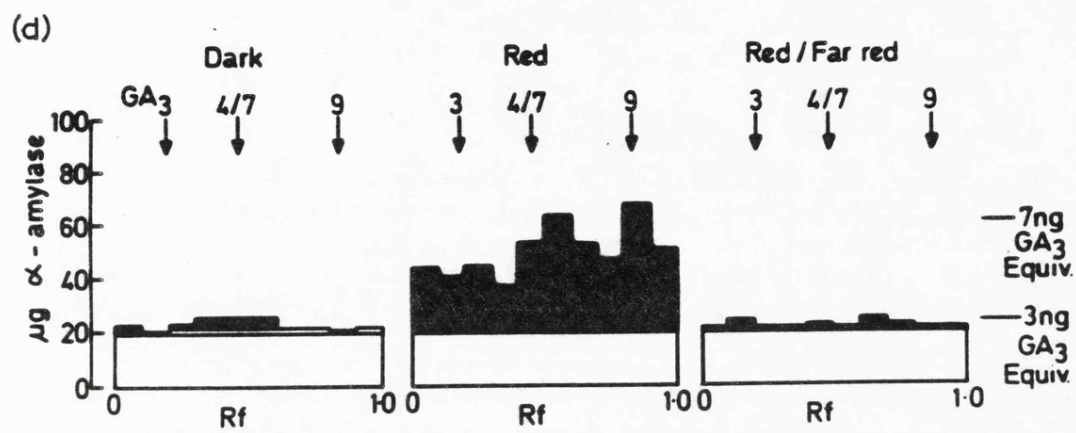
Fig. 18.c mitochondrial-enriched fraction.



Discontinuous sucrose density gradient centrifugation  
organelle suspensions.

Fig. 18.d lower banding (40%/55% sucrose interface)

Fig. 18.e upper banding (25%/40% sucrose interface)



Data of Cooke and Kendrick, (1976) and Evans and Smith, (1976a) showed that the majority of the increase in GA-LS in G-50 eluate organelle suspensions was due to increases in the supernatant fractions when the plastid suspensions were subjected to a post-illumination centrifugation. Indeed, Evans (1975), expressing GA-like activity in ng GA<sub>3</sub> equivalents, showed that 75% of the total GA-like activity was extractable from a 6KP fraction. Similar experiments were performed with the three plastid-enriched suspensions isolated in the course of this work. For the procedure used in these experiments see Fig. 19, page 63. Fig. 20. a, b, and c, page 64 show the results of such experiments. The majority of the phytochrome-mediated increase in the levels of GA-LS in all three cases was extractable from the 6KS fractions. There was, however, a small increase in the GA-like activity extractable from the 6KP fractions. There was also no marked change in the distribution of the peaks of GA-like activity between the 6KP and the 6KS fractions. The results are consistent with an effect of phytochrome either, on the efflux of soluble gibberellins from their site of origin within the plastids, or release of surface-bound gibberellins. Conversely, similar results could have been obtained if the plastids had merely been broken by the post-illumination centrifugation.



General procedure for the fractionation of organelle suspensions following light treatment.

Plastid-enriched organelle suspensions

obtained by:

G-50 gel filtration

Post G-50 sucrose centrifugation

Discontinuous sucrose density gradient  
centrifugation



Light treatments



Centrifugation

6,000g 1min

6KP

6KS

Fig. 20.a ,b, and c.

---

GA-like activity extracted from organelle suspensions centrifuged at 6,000g for 1 min immediately following light treatment.

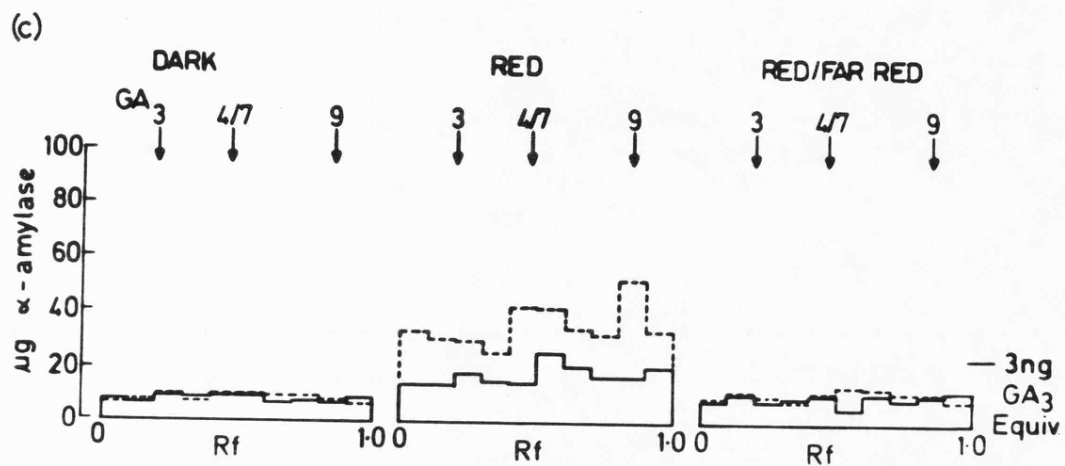
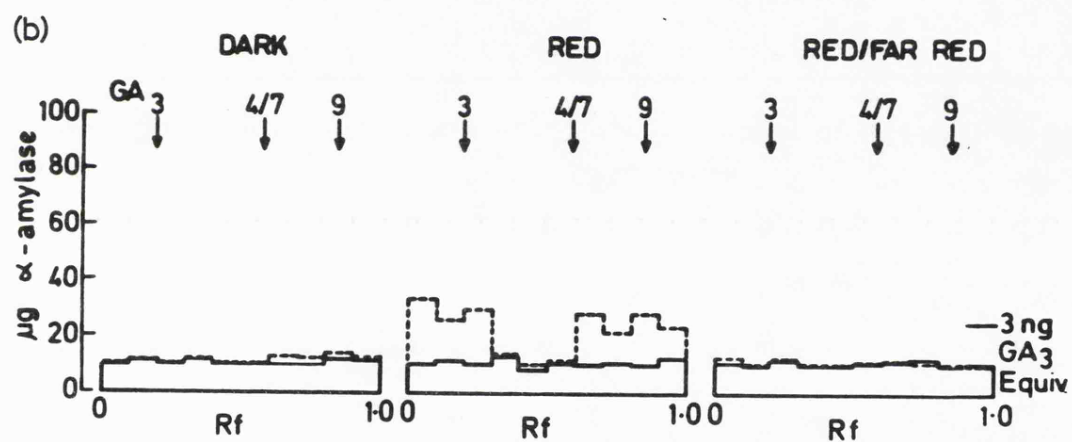
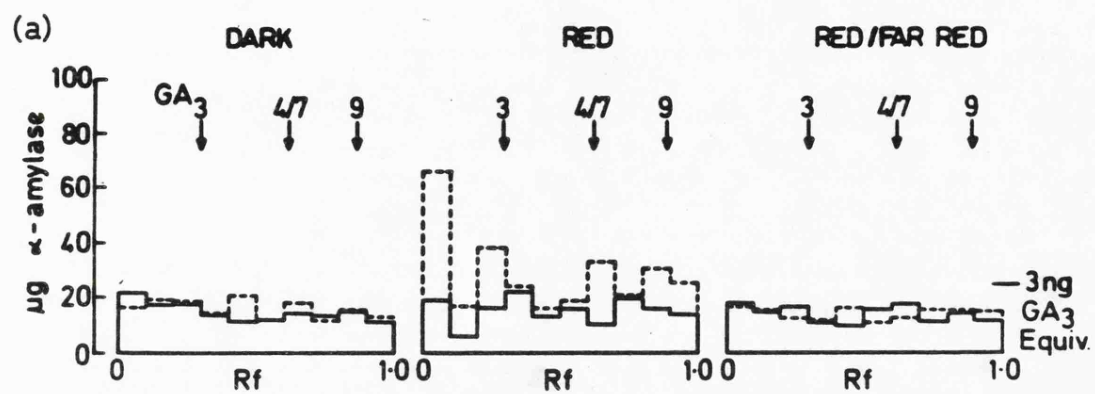
----- 6KS

\_\_\_\_\_ 6KP

Light treatments:

|             |                                  |
|-------------|----------------------------------|
| DARK        | 10 min in darkness               |
| RED         | 5 min red plus 5 min in darkness |
| RED/FAR-RED | 5 min. red plus 5 min far-red    |

|           |  |
|-----------|--|
| Fig. 20.a | Pooled G-50 eluate organelle suspension.   |
| " 20.b    | Post G-50 sucrose centrifugation etioplast-enriched suspension.  |
| " 20.c    | Discontinuous sucrose density gradient lower banding (40%/55% sucrose interface) organelle suspension. |



In an attempt to prevent or minimise such plastid rupture, red light treated aliquots (approximately 6 cm<sup>3</sup> in volume) of G-50 eluates were layered on to a pre-cooled discontinuous sucrose density gradient and, after centrifugation, the gradients were fractionated into soluble and organelle fractions (Fig. 21, page 67). GA-like activity was extracted into methanol and subsequently bioassayed from each fraction. Fig. 22, page 68 demonstrates the movement of [<sup>3</sup>H] gibberellin A<sub>9</sub> into a similar sucrose density gradient. Following centrifugation, the majority of the radioactivity remained in the upper layer of the gradient, thus any GA-like activity remaining in the upper layer must be considered to be truly soluble. Fig. 23, page 69 shows that red-light-mediated increases in GA-like activity were extractable from all three fractions isolated from the gradients. These results are therefore not consistent with the hypothesis that the majority of the increase in the red-light mediated GA levels is due to increases in soluble or non-organelle-associated gibberellins.

Throughout this work an irradiation with 5 min red light followed by a 5 min incubation in darkness at 24°C was used routinely as the conditions necessary to obtain the maximal increase in GA-like activity. (Evans, 1975). However, in order to substantiate the data obtained on the distribution of red-light-mediated increases in GA-like activity between soluble and organelle fractions, a time course of varying periods in darkness following a 5 min. irradiation with red light was performed. Aliquots of G-50 suspensions were irradiated with red light for 5 min and maintained in darkness for 0, 5 or 10 min. After treatment the aliquots were loaded on to a pre-cooled discontinuous sucrose density gradient and treated as described in the previous paragraphs. The results of these experiments are presented in Figs. 24.a, b. and c., pages 71, 72, & 73 Again, the red-light-mediated increases in GA-LS are distributed between the soluble

and the organelle fractions irrespective of time in darkness following the irradiation with red light.

It is clear that the red-light-mediated response is detectable within the first 5 min of irradiation with red light. The levels of GA-LS are very small but were nevertheless repeatable. Without a method of quantitative assessment it is difficult to assess the differences between the GA-like activity associated with increasing time in darkness following red light treatment. What is clear, however, is that there are far higher levels of GA-LS extractable from fraction 2 when a 10 min. period in darkness follows the red irradiation. However, the main aim of the experiment was to differentiate between truly soluble and organelle-associated GA-LS levels. From these results there is no evidence to suggest that the increase in GA-LS is confined to increases in truly soluble GA-LS. The levels of GA-LS present in the soluble phase could possibly be present solely as GA-LS released from plastids following rupture. Indeed, a better experiment (if possible to perform) would have been to isolate a suspension of intact etioplasts only and then investigate the effect of red light on the plastid-associated GA-LS.

Plastids free of soluble GA contamination could have been obtained by using a sucrose density centrifugation technique similar to the one used in these experiments. However, there would still remain the problem involved in transferring the plastids to another sucrose density gradient. This manipulation would have undoubtedly resulted in plastid rupture, and with this, the possible release of GA-LS. The experiments described here do not totally explain the increase in GA-LS but it is difficult to envisage how further data could be obtained. What is clear, however, is that a large proportion of the red-light-mediated increase in GA-LS is due to increases that are associated with the plastids themselves.

Procedure for the discontinuous sucrose density gradient centrifugation of light-treated G-50 organelle suspensions.

Dotted lines indicate the limits of the fractions taken for subsequent extraction of GA-LS.

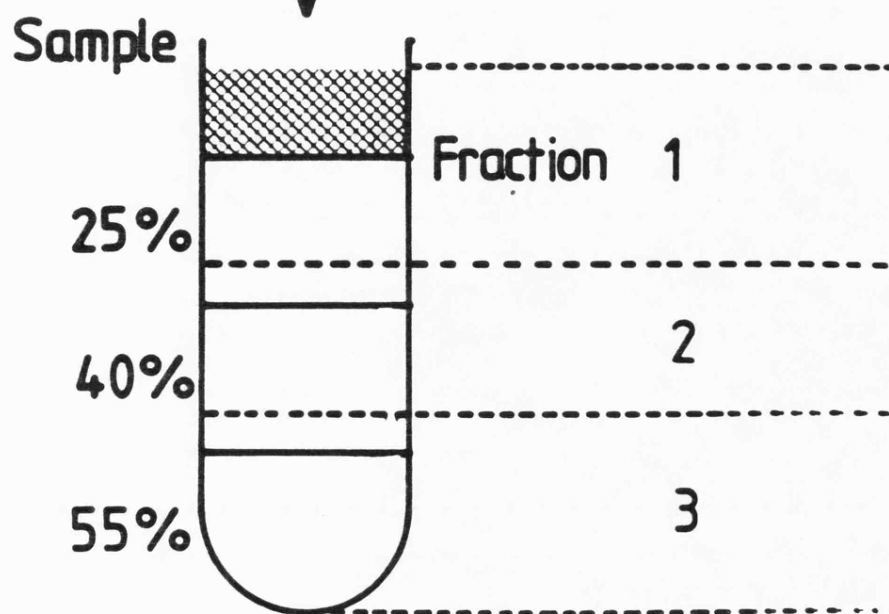
Pooled G-50 eluate  
organelle suspensions



Light treatment



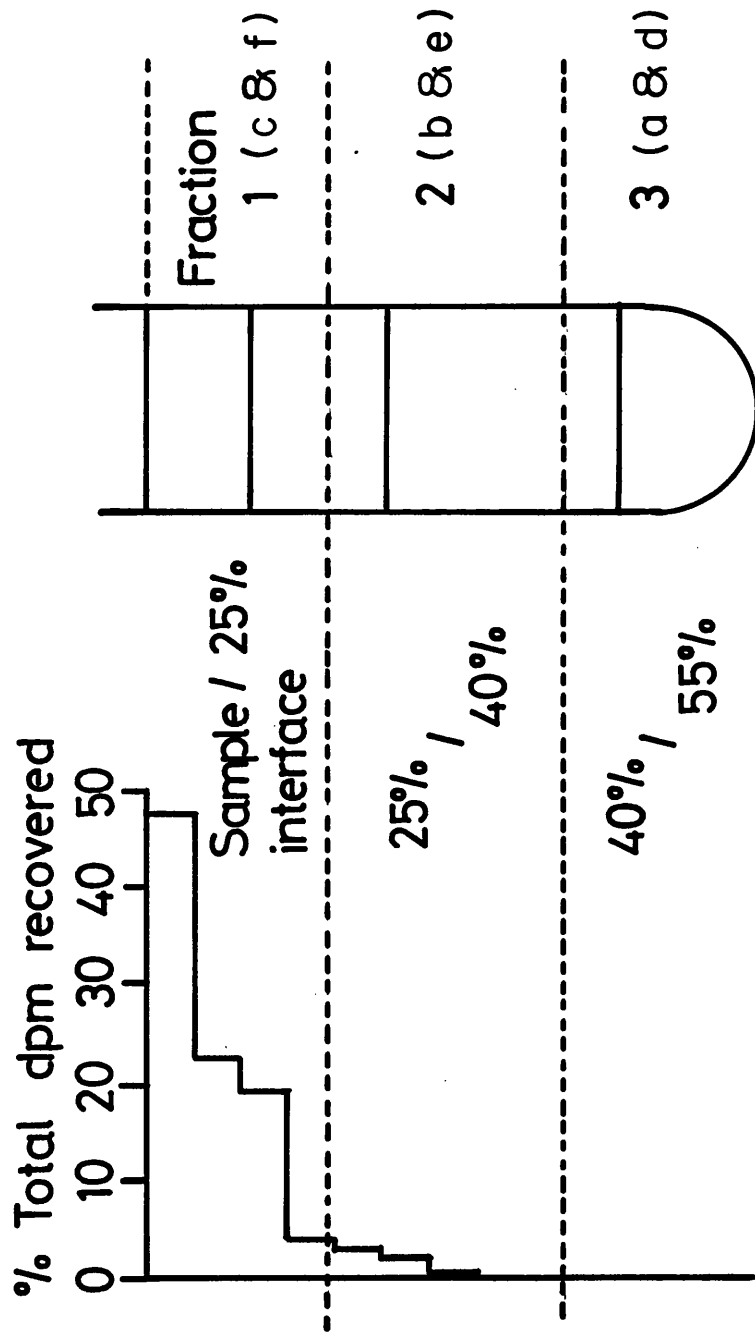
Discontinuous sucrose  
gradient centrifugation





The movement of [ $^3\text{H}$ ] gibberellin  $A_9$  into a discontinuous sucrose density gradient.

Dotted lines indicate the limits of fractions taken for the extraction of GA-LS.



---

GA-like activity extracted from G-50 eluate fractions centrifuged into a discontinuous sucrose density gradient immediately following light treatment. Following centrifugation three fractions were recovered from the gradients as described in Fig. 6 (b), page 32.

---

| Light treatments prior<br>to centrifugation | Fractions recovered from<br>the gradients |
|---|---|
| 5 min red plus 5 min<br>in darkness         | a, b, c (soluble)                         |
| 10 min in darkness                          | d, e, f (soluble)                         |

---

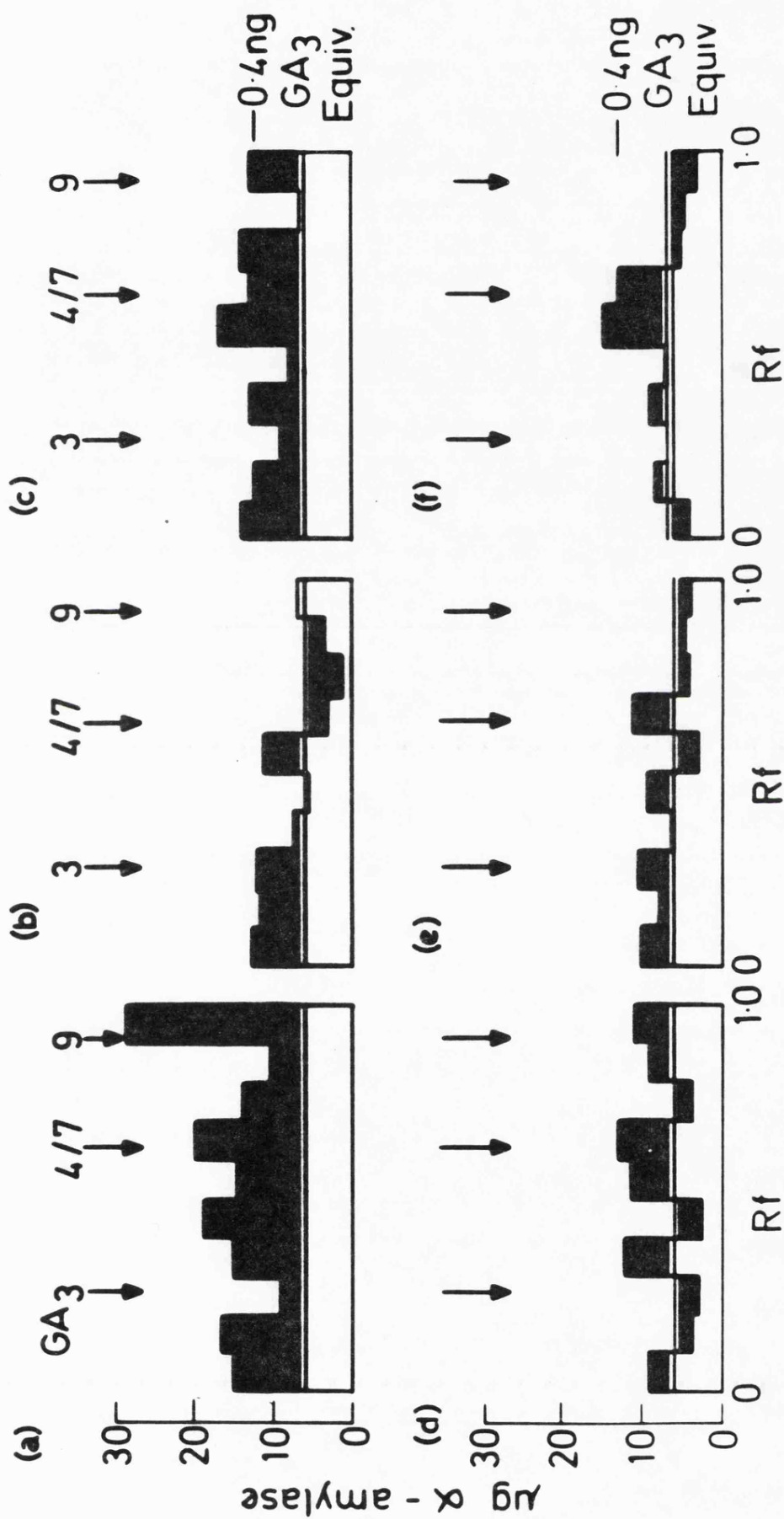


Fig. 24.a, b. and c.

---

GA-like activity extracted from G-50 organelle fractions  
centrifuged into a discontinuous sucrose density gradient  
immediately following light treatment.

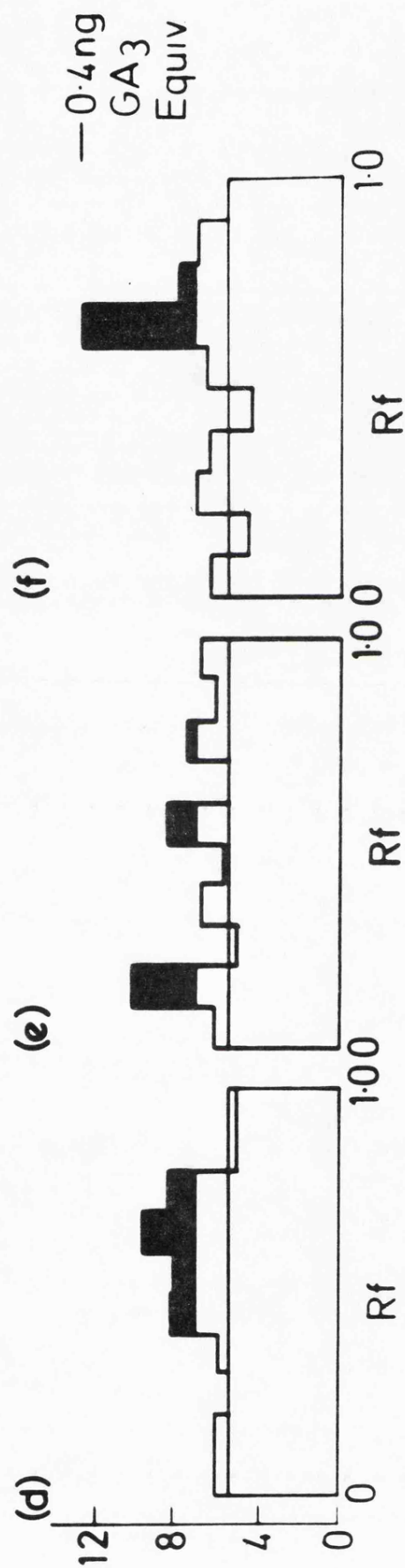
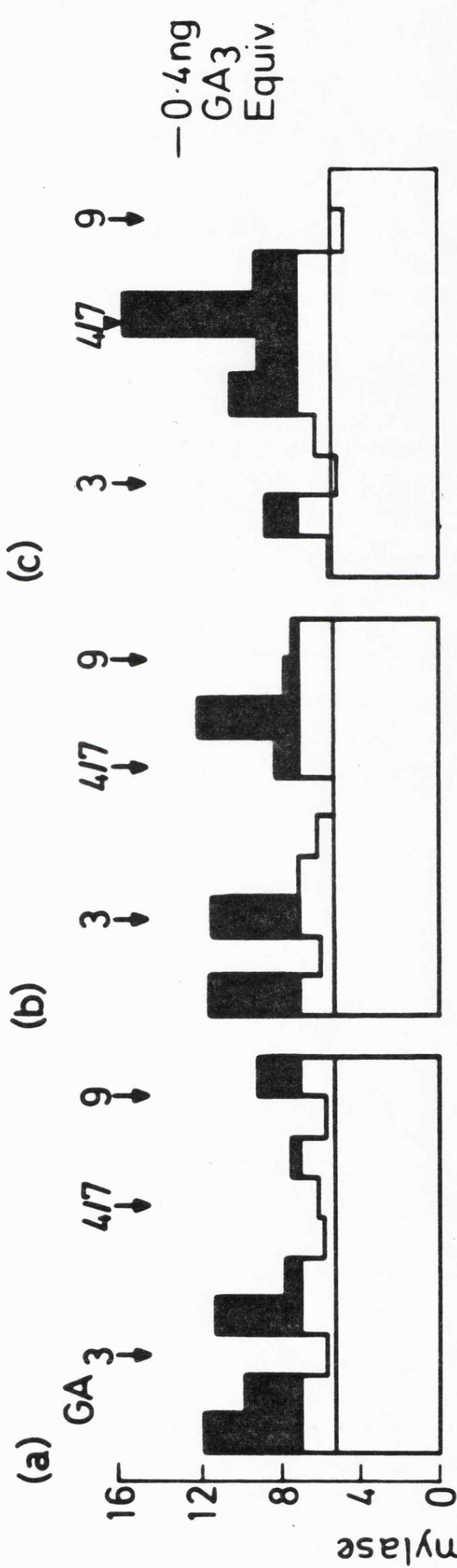
Fractions were recovered from the gradients as described in  
Fig. 6 (b), page 32 .

---

| Light treatments prior<br>to centrifugation | Fractions recovered from<br>the gradients |
|---|---|
| <hr/>                                       |   |
| Fig. 24.a 5 min red plus                    | a, b, c (soluble)                         |
| 0 min in darkness                           |   |
| 5 min in darkness                           | d, e, f (soluble)                         |
| <br>  |   |
| Fig. 24.b 5 min red plus                    | a, b, c (soluble)                         |
| 5 min in darkness                           |   |
| 10 min in darkness                          | d, e, f (soluble)                         |
| <br>  |   |
| Fig. 24.c 5 min red plus                    | a, b, c (soluble)                         |
| 10 min in darkness                          |   |
| 15 min in darkness                          | d, e, f (soluble)                         |

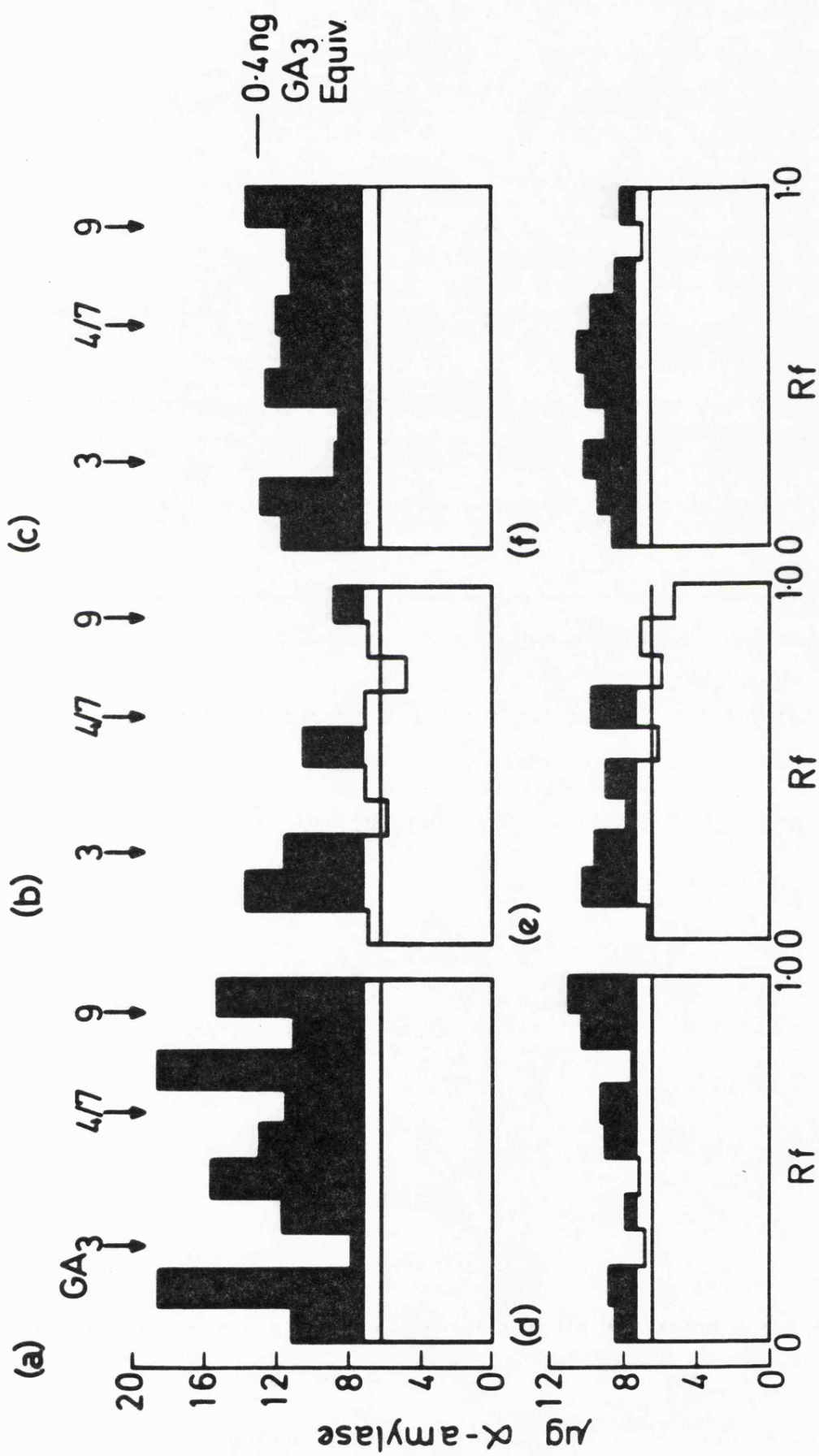
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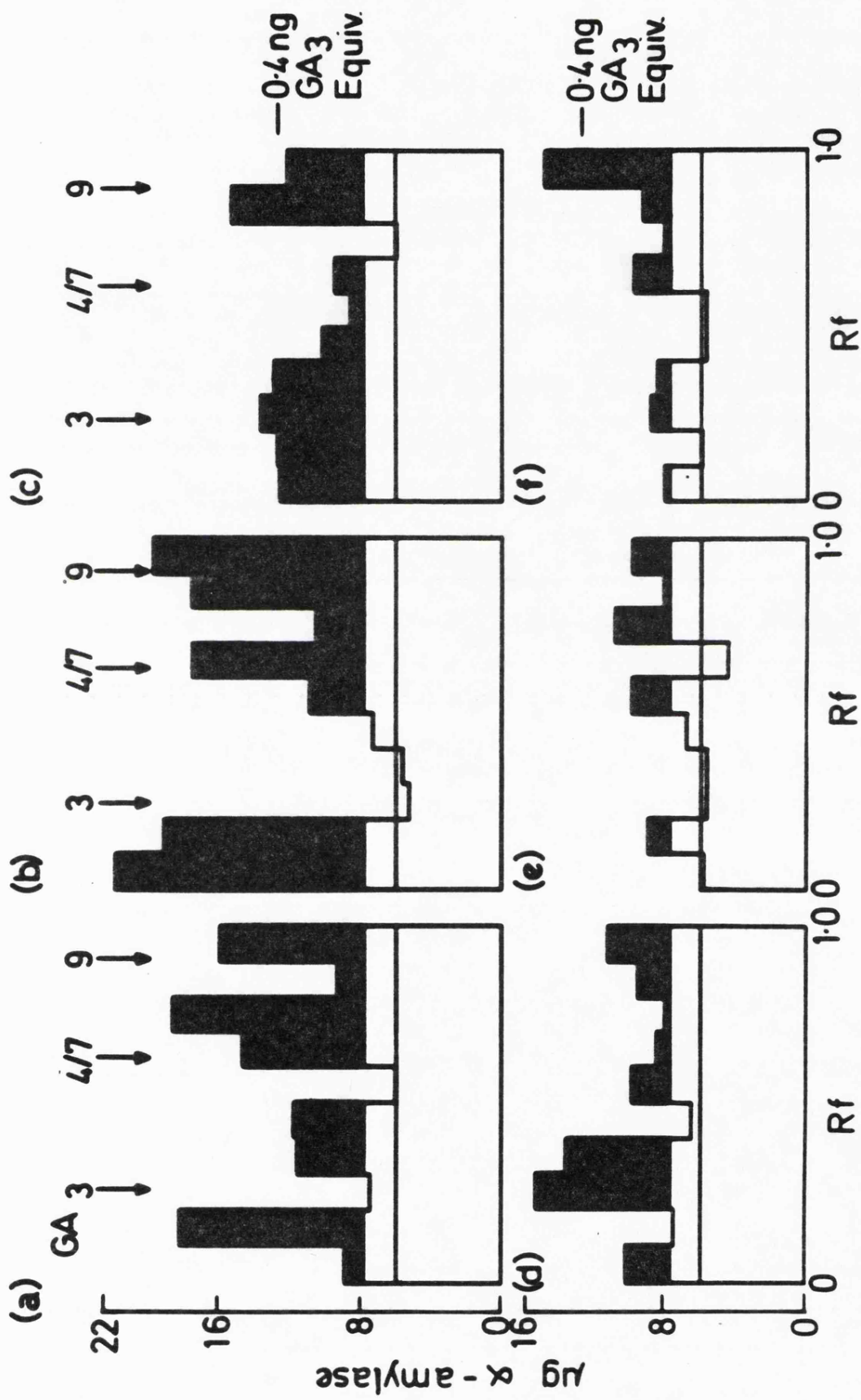












(2) Discussion

Phytochrome has been shown to mediate the levels of GA-LS extractable from etioplast-enriched fractions into aqueous methanol in vitro (Evans and Smith, 1976a; Cooke et al., 1976). When etioplast suspensions were irradiated with red light for 5 min and returned to darkness for a further 5 min there was a marked increase in the biologically-active GA-LS subsequently extractable into aqueous methanol when compared to the activity extractable from etioplast suspensions retained in darkness, or irradiated with 5 min. red light followed immediately by 5 min. far-red light.

Data presented here from experiments in which light-treated etioplast suspensions were centrifuged at 6,000g support the data of Evans and Smith, (1976a) and Cooke and Kendrick, (1976) and show that the majority of the light-mediated increase in GA-LS is extractable from the 6KS fractions. As previously proposed the hypothesis advanced to explain the red light-mediation of GA levels would be that the photoconversion of Pr to Pfr in etioplast suspensions causes a change in the permeability of the etioplast envelope membrane with respect to GA-LS thereby allowing an efflux of these substances out of the plastids into the surrounding medium. The data could also be explained on the basis of release of surface-bound GA-LS from the membrane or, as previously described, by the release of GA's from the plastids by rupture of the organelles themselves. However, data obtained from sucrose density gradient experiments showed that the increase in extractable GA-LS was due to increases in both the soluble and the particulate fractions. A large proportion of the increased GA-like activity was associated with the organelles themselves, and was therefore not due entirely to increases in non-organelle associated or soluble GA's. It is also of interest to note that the increases in GA-LS is an overall increase in GA levels and does not appear to be made up of increases in specific GA-like substances as far as can be judged from bioassay data.

Also, there do not appear to be any specific differences in the type of GA-LS and their distribution between the soluble and the organelle fractions.

In 1976, Cooke and Kendrick advanced another explanation of their results; they proposed that a 5 min irradiation with red light mediated the release of 'bound' GA's present in association with the envelope membranes into 'free' GA's which were then freely soluble. The 'bound' GA's were not extractable into aqueous methanol whereas the 'free' GA's were. They also showed that this response was due entirely to a response of fractions enriched for etioplast envelope membranes. However, the terms 'bound' and 'free' as used by Cooke and Kendrick (1976) are extremely confusing. Free or non-conjugated GA's have a low degree of hydroxylation and therefore a high affinity for membranes. Conversely, bound or conjugated GA's have a high degree of hydroxylation and consequently have a very low affinity for membranes. Conjugated GA's are GA's which are chemically combined with sugar or amino acid residues.

The use of the term 'bound' in relation to a molecule infers that it is adsorbed or non-chemically associated with a macromolecule or a sub-cellular structure. It was following the suggestion of Sembder et al., (1968) that the term conjugated was widely accepted for use as opposed to the term bound. Reasons for the use of this term are easily seen from the present confusion in the literature. The data of Cooke and Kendrick (1976) (Fig. 25, page 78), shows clearly that non-acidic-ethyl-acetate-soluble GA's present in the dark are converted to acidic-ethyl-acetate-soluble GA's following irradiation with red light. It is very surprising that the non-acidic-ethyl-acetate-soluble GA's, having a low affinity for membranes, were found associated exclusively with the envelope membrane enriched fraction. It would seem reasonable to expect these molecules, having a high degree of hydroxylation and/or association with glucosyl moieties to be present

in the soluble components of the etioplasts i.e. in association with the stroma-enriched fraction. Indeed, results presented in another part of this thesis show that metabolites of [ $^3\text{H}$ ] GA<sub>9</sub> with a high degree of hydroxylation are localized in the stroma fraction. However, the conclusions of Cooke and Kendrick (1976) are based on a maximal response in the lettuce hypocotyl bioassay of 1 mm or less. It is therefore questionable whether the results are significant at all. Indeed, in their experiments no R/FR reversal experiments were performed and therefore the involvement of phytochrome in the response can be regarded as no more than extremely tentative.

From data obtained in this work there was no evidence to suggest that polar GA's, which remain on the origin of the TLC plate following development, were converted to less polar GA's by red light treatment. Indeed, there was also no evidence that it occurred between the soluble and the organelle-enriched fractions. In these experiments no R/FR reversal treatments were performed. The involvement of phytochrome in this response can therefore also be regarded as no more than tentative. However, organelle suspensions similar to those used in the experiments have been characterized extensively in terms of the phytochrome mediation of the levels of GA-LS. As the levels of GA-like activity were very low and indeed close to the limits of detection of the bioassay the organelle suspensions were divided into two equal aliquots for light treatment, i.e. dark and red light treatments, and not three equal aliquots, i.e. dark, red and red/far-red light treatments, so that the biological activity per treatment would be higher.

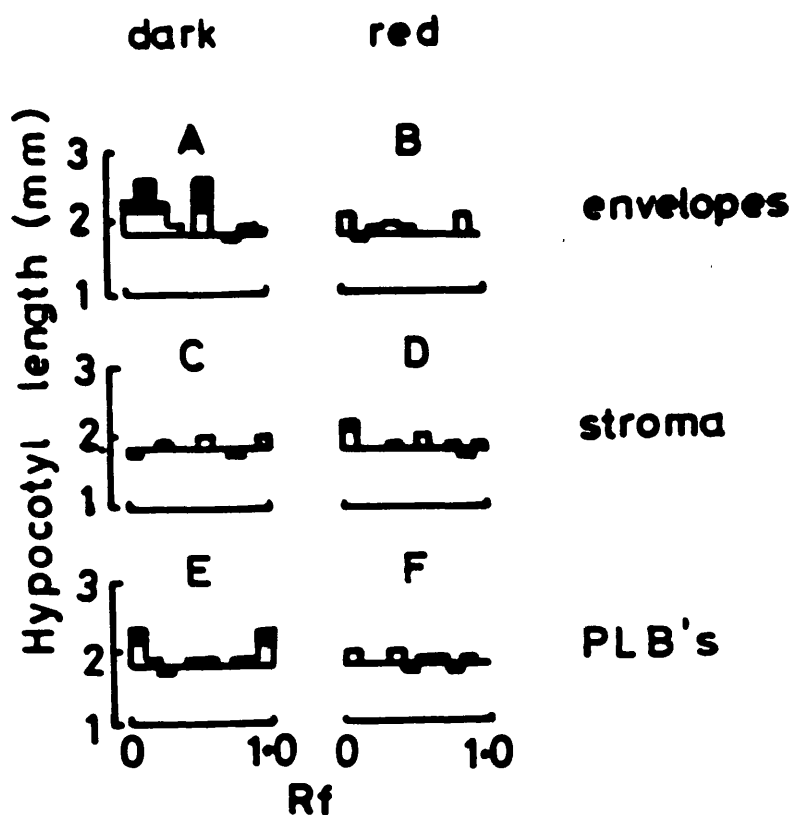
From the results presented in this part of the thesis it appears that etioplast-associated GA-LS may be bound to a sub-etioplast structure in a manner that renders them not readily extractable into aqueous methanol.

Following treatment with red light the binding structure may become modified, in an as yet undermined nature, such that the GA molecules are more readily extractable from the plastids.

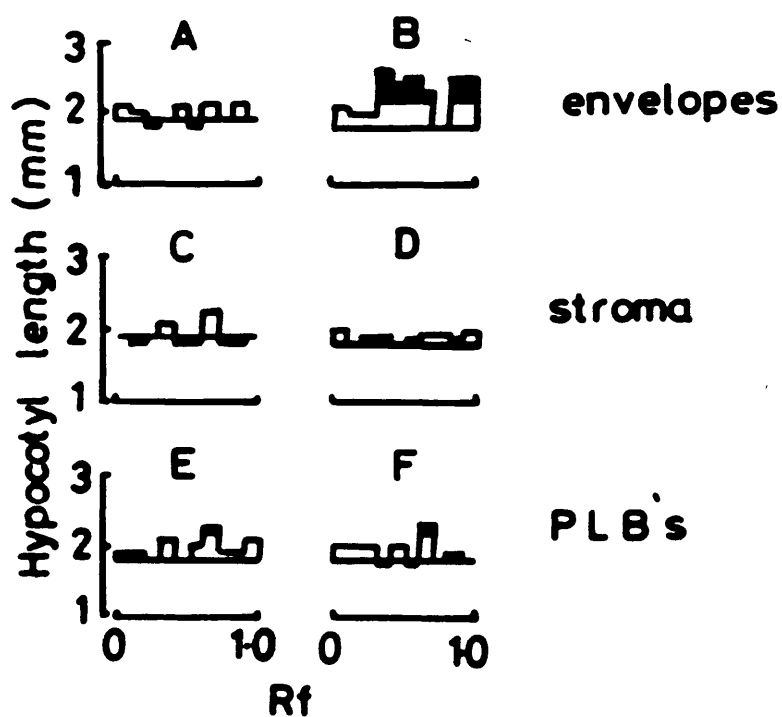
GA-like activity associated with acidic and non-acidic ethyl acetate-soluble fractions extracted from etioplasts following light treatment and sub-fractionation.

(from Cooke and Kenrick, 1976)





non - acidic ethyl acetate fractions



acidic ethyl acetate fractions

- (c) (1) The uptake and metabolism of [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  into detached shoots of Hordeum vulgare L. and the effect of light treatment on plastid fractions subsequently isolated. (For description of method see Fig. 26, page 83).

In a further attempt to study the rôle of phytochrome in the control of etioplast gibberellin levels a series of experiments with a [ $^3\text{H}$ ] gibberellin were performed. Intact shoots of Hordeum vulgare L. seedlings were incubated in a buffered solution of [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  and the effects of light treatments on the distribution of radioactivity in plastid-enriched fractions subsequently isolated from these shoots were studied.

Gibberellin biosynthesis is believed to proceed in the order of increasing hydroxylation (Geissman et al., 1966; Verbiscar et al., 1967; Cross et al., 1968) and  $\text{GA}_9$ , a non-hydroxylated GA could therefore serve as an efficient precursor to other GA's. It was hoped that the use of a radioactive tracer would enable any phytochrome-mediated responses to be presented on a more quantitative basis than by the use of bioassay data alone.

The problems involved in expressing bioassay data have been previously discussed. Long term incubations of detached shoots in [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  were chosen as opposed to short term direct feeding of label to etioplast suspensions to ensure that plastids were capable of taking up the radioactively-labelled compound in vivo.

Whole shoots of dark-grown Hordeum vulgare L. seedlings of varying ages were incubated in a buffered solution of [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  [see Experimental Details, page 18, j(i)] for 24h at  $24^\circ\text{C}$ . At the end of the incubation period radioactivity associated with both the whole shoots and with a total plastid fraction isolated from them [see Experimental Details, page 14, (d) (2.1)] was assessed, Fig. 27, page 84, shows the results of

such measurements. The maximum amount of radioactivity recovered from both a total shoot homogenate and from the plastid-enriched fractions was from six day old plants. Plant material of this age was therefore used routinely in all subsequent experiments.

Fig. 28, page 85, shows the distribution of radioactivity (following TLC) extractable into aqueous methanol from both a total shoot homogenate and from a total plastid fraction. If this figure is compared with Fig. 29, page 86 (the distribution of radioactivity associated with the standard [ $^3\text{H}$ ] gibberellin  $A_9$  following TLC), it is clear that the distribution associated with both fractions has changed markedly from that originally applied. This data suggests, therefore, that a large proportion of the radioactivity associated with the applied [ $^3\text{H}$ ] gibberellin  $A_9$  is now associated to a very large extent with other compounds. The most marked change is the transfer of radioactivity to polar material (i.e. material remaining on the origin of the TLC plate following TLC.) Metabolism of the [ $^3\text{H}$ ] gibberellin  $A_9$  had, therefore, occurred.

Fig. 30, page 87, shows the distribution of radioactivity in zones 1 cm in length isolated from the entire length of the Hordeum vulgare L. shoots. Highest amounts of radioactivity were recovered from the zones nearest the bases of the shoots (i.e. the portions of the shoots nearest the point of immersion in the radioactively-labelled solution). There were, however, considerable amounts of radioactivity associated with all the other zones. Although etioplast suspensions were not isolated from every zone those isolated from segments 3 cm in length, 1 cm from the apex of the shoots had radioactivity associated with them. Indeed, when these etioplast-enriched suspensions were layered onto a discontinuous sucrose density gradient and centrifuged [see Experimental Details page 19 j(ii)] between 15% and 35% of the total radioactivity recovered passed into the gradient. These

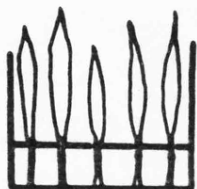
results suggest that a consistently measurable amount of radioactivity was associated with the organelle fractions despite a large proportion remaining on top of the gradient and being truly soluble [Figs. 31(a) and (b), page 88]. When similar etioplast suspensions were further fractionated by the method of Mackender and Leech (1970), only 4% of the radioactivity was associated with the envelope membrane-enriched fraction, 11% was associated with the prolamellar body-enriched fraction and 85% was associated with the soluble components of the stroma-enriched fraction (Table 6, page 89).

Table 7, page 90, shows the results of experiments in which radioactively-labelled etioplast suspensions were subjected to light treatments in vitro at 24°C. Immediately following light treatment the etioplast suspensions were layered onto a pre-cooled discontinuous sucrose density gradient. Following centrifugation, the distribution of radioactivity between two fractions isolated from the gradients was estimated. The results show that a 5 min irradiation with red light has no detectable effect on the distribution of radioactivity between the fractions isolated when compared to similar fractions that were maintained in darkness throughout the treatment period. Similarly, there was no detectable difference in the distribution of radioactivity between the fractions when etioplast-enriched were maintained in darkness for varying periods following a 5 min red irradiation compared to those maintained in darkness throughout the treatment period (Table 8, page 91). When light-treated etioplast-enriched suspensions were extracted into aqueous methanol and subjected to TLC (Fig. 32, page 92) there was only very little apparent change in the distribution of radioactivity between the treatments. The distribution of radioactivity extracted from the red-light-treated samples differed from the dark and the red/far-red treated samples only in the radioactivity associated with the Rf zones 0.8-0.9 and 0.9-1.0. Approximately 10% more of the radioactivity was associated with Rf zone 0.8-0.9 in the

red-light-treated samples. This increase in radioactivity was due to an approximate 5% decrease in the activity of Rf zone 0.9-1.0 and only minor decreases in the amount of radioactivity associated with the remaining zones. The increase could not be accounted for by any loss in radioactivity associated with more polar material.

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General procedure for the investigation of the uptake of radio-activity from a buffered solution containing [ $^3\text{H}$ ] gibberellin A<sub>9</sub> into detached shoots of Hordeum vulgare L. and the effects of light treatment in vitro on etioplast-enriched suspensions subsequently isolated.



Barley shoots incubated in  
a buffered solution of  
[ $^3\text{H}$ ] gibberellin A<sub>9</sub>

Laminae segments isolated

3cm in length, 1cm from the apex

Isolation of organelle fractions

Light treatments at 24°C

Sample

Sucrose 25%  
55%



Discontinuous sucrose gradient  
centrifugation

Fractionation and measurement of  
radioactivity associated with soluble  
and organelle fractions

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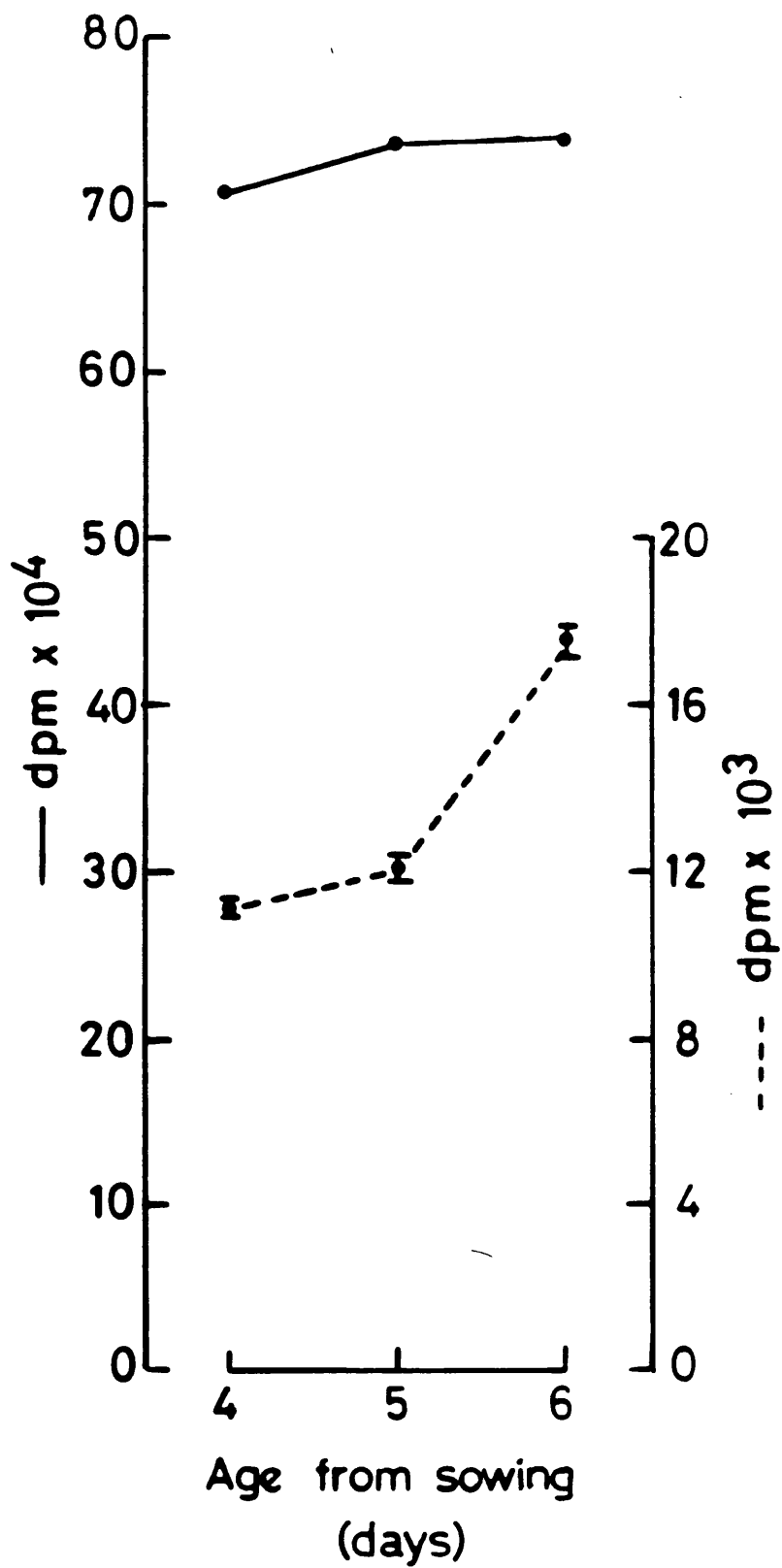
The effect of age of plant material on the uptake of radio-activity from a buffered solution of [ $^3\text{H}$ ] gibberellin  $A_9$  into shoots of Hordeum vulgare L. during a 24h incubation at 24°C in total darkness.

—— uptake into whole shoots

---- uptake into an etioplast-enriched  
suspension subsequently isolated  
from a total leaf homogenate.

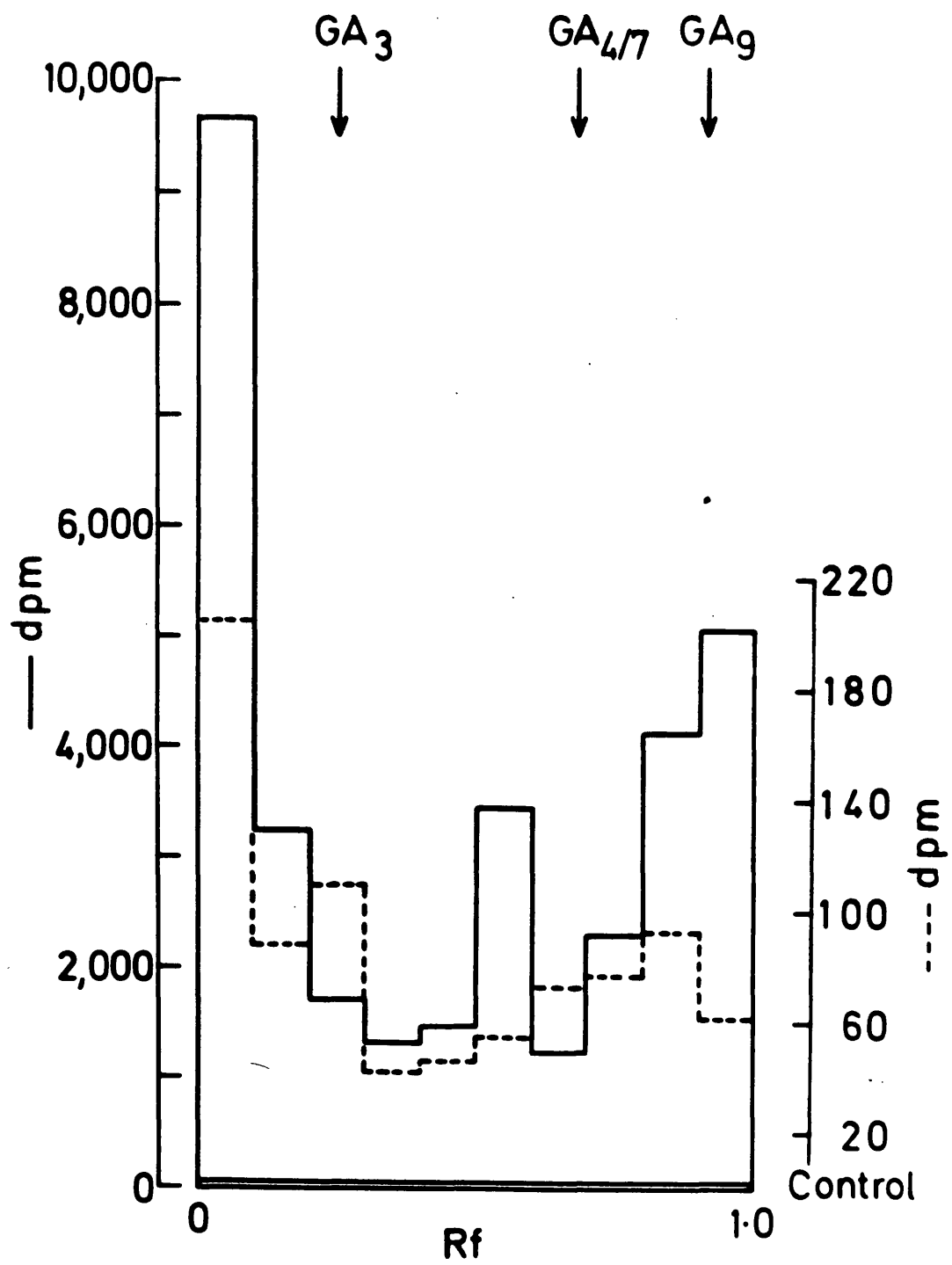
The age of the plant material at the termination of the experiment was age from sowing plus 24h.



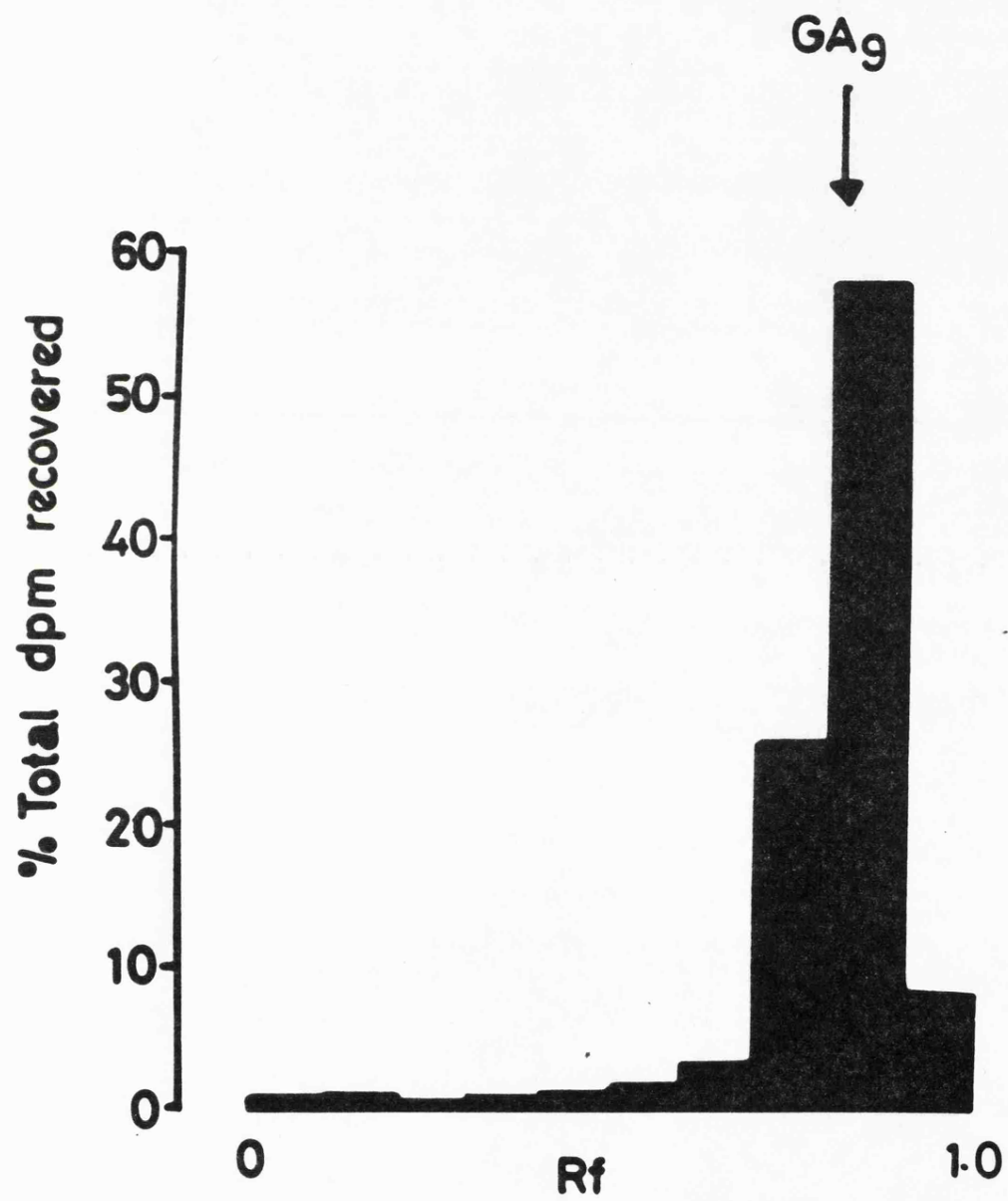


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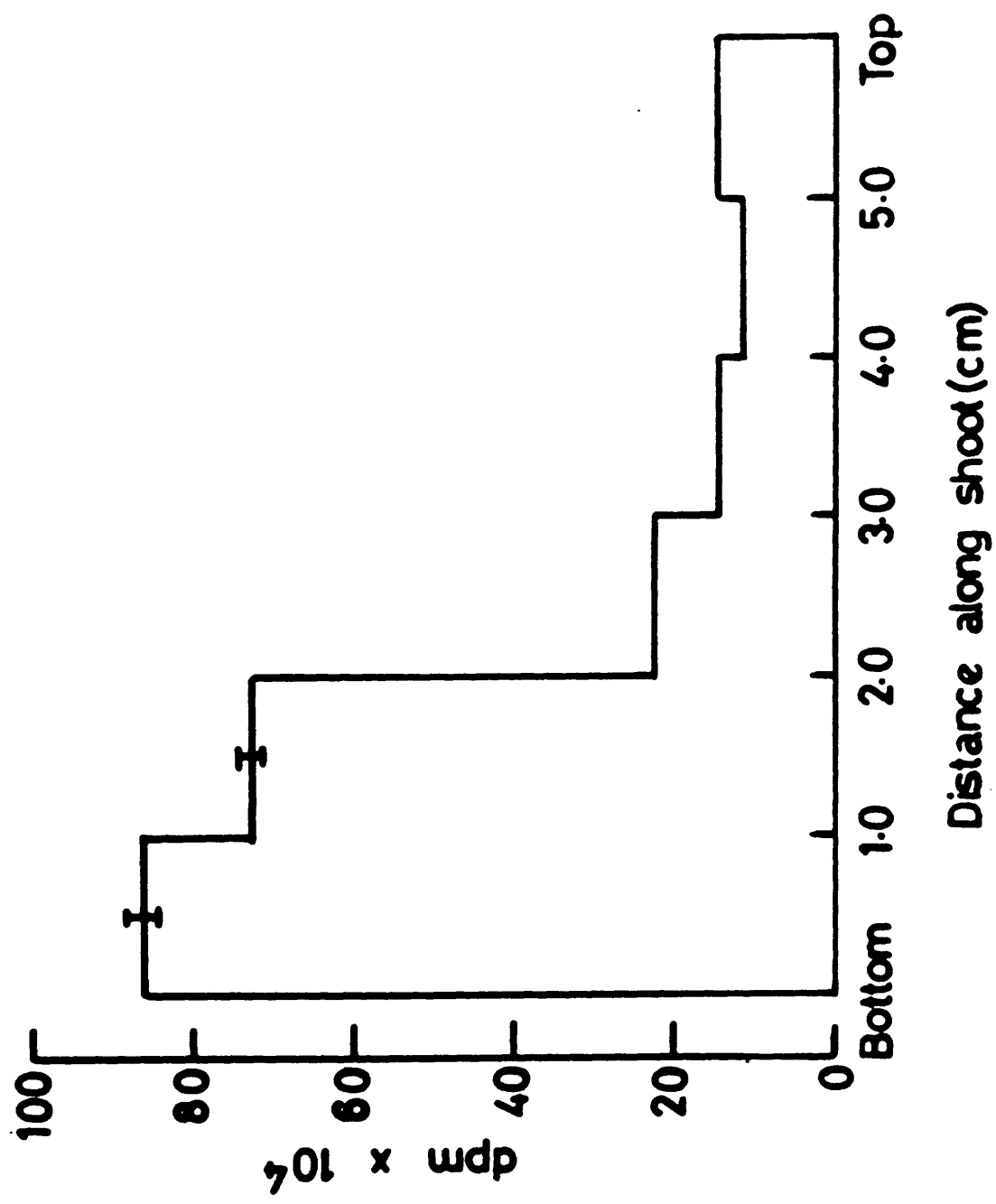
The distribution of radioactivity in a total leaf extract ( ——— ) and an etioplast-enriched extract ( ---- ) following extraction into aqueous methanol and TLC.



The distribution of radioactivity associated with the standard  
[<sup>3</sup>H] gibberellin A<sub>9</sub> following TLC.



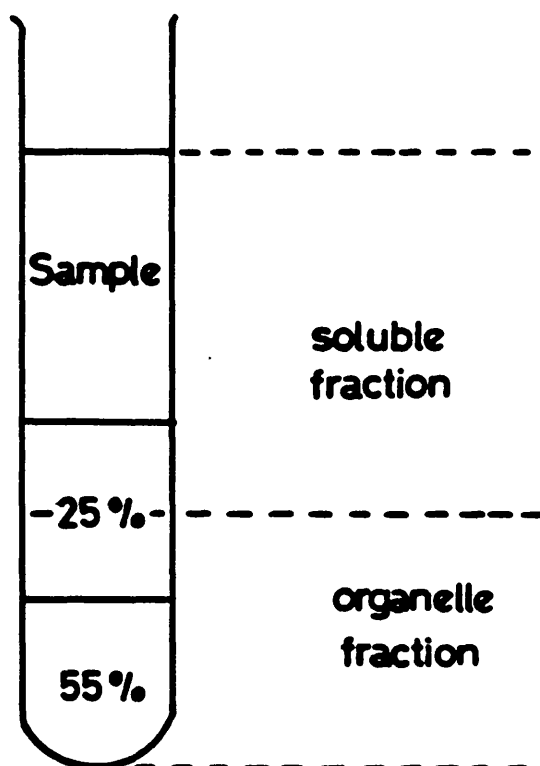
The uptake of radioactivity from a buffered solution containing [ $^3\text{H}$ ] gibberellin  $A_9$  into 1 cm zones isolated from shoots of Hordeum vulgare L. following a 24h incubation at 24°C in total darkness.



- (a) Description of the discontinuous sucrose density gradient and the limits of samples fractionated following centrifugation.
- (b) The percentage distribution of radioactivity between soluble and organelle fractions following centrifugation of radioactively-labelled etioplast-enriched suspensions.



(a)



(b)

% Total dpm recovered

| Soluble fraction |    | Organelle fraction |   | Total dpm recovered |
|------------------|----|--------------------|---|---------------------|
| ± SE             |    | ± SE               |   |                     |
| 73               | 4  | 27                 | 1 | 4820                |
| 80               | 11 | 20                 | 4 | 892                 |
| 73               | 11 | 27                 | 5 | 1218                |
| 82               | 12 | 18                 | 4 | 1302                |
| 67               | 3  | 33                 | 2 | 4194                |
| 86               | 13 | 14                 | 3 | 1800                |
| 69               | 9  | 31                 | 5 | 911                 |
| 65               | 3  | 35                 | 2 | 3924                |
| 85               | 13 | 15                 | 3 | 1210                |

Table 6

---

The percentage distribution of radioactivity in etioplast-enriched suspensions following fractionation by the method described by Mackender and Leech (1970).

| Fraction                   | % total dpm recovered | + - SE |
|----------------------------|-----------------------|--------|
| Prolamellar body-enriched  | 11                    | 2      |
| Envelope membrane-enriched | 4                     | 1      |
| Stroma-enriched            | 85                    | 9      |
| Total dpm recovered 797    |                       |        |

Table 7

---

The percentage distribution of radioactivity between the soluble and organelle fractions of a discontinuous sucrose density gradient following light treatments at 24°C and subsequent centrifugation of radioactively-labelled etioplast-enriched suspensions.

The organelle suspensions were divided into two equal aliquots prior to light treatment, the results presented are therefore directly comparable.

| Treatment                 | % Total dpm recovered    |                            |    |   | Total dpm recovered |
|---------------------------|--------------------------|----------------------------|----|---|---------------------|
|                           | Soluble fraction<br>+ SE | Organelle fraction<br>+ SE |    |   |                     |
| 10 min dark               | 73                       | 4                          | 27 | 1 | 4820                |
| 5 min red + 5 min dark    | 67                       | 3                          | 33 | 2 | 4194                |
| 5 min red + 5 min far-red | 66                       | 3                          | 34 | 2 | 3924                |

Table 8

---

The percentage distribution of radioactivity between the plastid and soluble fractions of a discontinuous sucrose density gradient following centrifugation of radioactively-labelled etioplast suspensions irradiated with 5 min red light and maintained in darkness for varying periods at 24°C.

The organelle suspensions were divided into two equal aliquots prior to light treatment. The results presented are therefore directly comparable.

| Treatment               | % Total dpm recovered     |                             |   | Total dpm recovered |
|-------------------------|---------------------------|-----------------------------|---|---------------------|
|                         | Soluble fraction $\pm$ SE | Organelle fraction $\pm$ SE |   |                     |
| 5 min dark              | 69                        | 31                          | 5 | 911                 |
| 5 min red               | 80                        | 20                          | 4 | 892                 |
| 10 min dark             | 85                        | 15                          | 3 | 1210                |
| 5 min red + 5 min dark  | 82                        | 18                          | 4 | 1302                |
| 15 min dark             | 86                        | 14                          | 3 | 1300                |
| 5 min red + 10 min dark | 73                        | 27                          | 5 | 1218                |




The effects of light treatment in vitro on the distribution of radioactivity of etioplast-enriched suspensions following extraction into aqueous methanol and TLC.

The organelle suspensions were divided into three equal aliquots prior to light treatment. The results presented are therefore directly comparable.

Total dpm recovered per treatment:

|             |      |
|-------------|------|
| dark        | 1443 |
| red         | 1362 |
| red/far-red | 1631 |

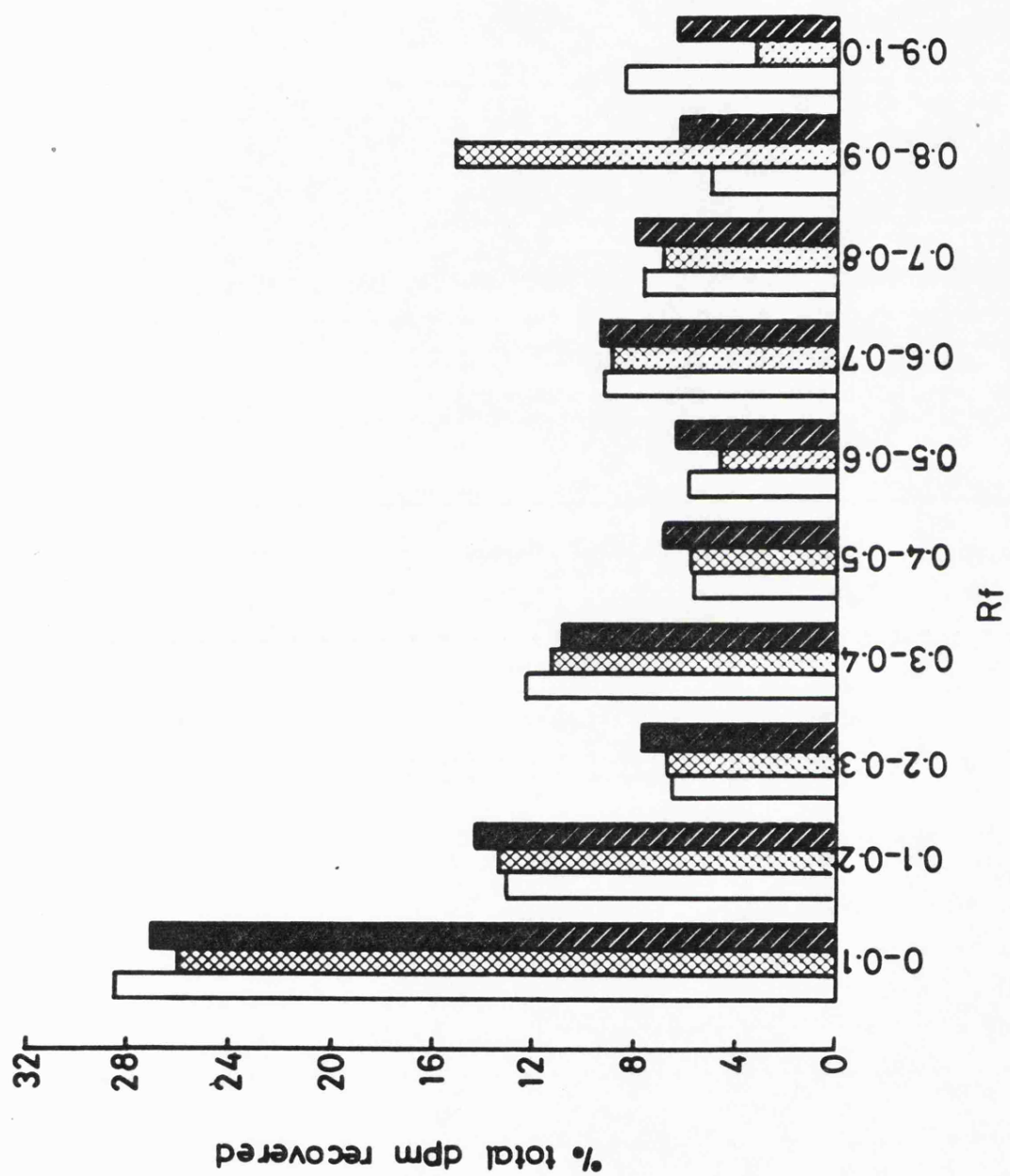
Treatments:

|   |                                  |
|---|----------------------------------|
|  | 10 min in darkness               |
|  | 5 min red plus 5 min in darkness |
|  | 5 min red plus 5 min far-red     |

Rf values of authentic GA's:

|                   |      |
|-------------------|------|
| GA <sub>3</sub>   | 0.23 |
| GA <sub>4/7</sub> | 0.65 |
| GA <sub>9</sub>   | 0.92 |





(2) Discussion

Whole shoots of etiolated seedlings of Hordeum vulgare L. were able to take up radioactivity from a buffered solution of [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  over a 24h incubation period. Some of the radioactivity was associated with etioplast-enriched fractions subsequently isolated from these shoots. The majority of the radioactivity was associated with more polar material (i.e. material remaining at the origin following TLC) than the gibberellin  $\text{A}_9$  originally supplied. There was therefore evidence that metabolism of the gibberellin  $\text{A}_9$  had occurred. In the absence of physical data e.g. GC-MS or GC-RC it was not possible to determine the exact nature of the radioactively-labelled metabolites or, indeed, to confirm that the metabolites were gibberellin-like. However, there is evidence to suggest that [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  does not accumulate in lettuce hypocotyl tissue; after a 2h incubation in [ $^3\text{H}$ ]  $\text{GA}_9$ , 94% of the total tissue radioactivity was no longer associated with  $\text{GA}_9$  (Nash et al., 1978). There was evidence to suggest that a major metabolite is  $\text{GA}_{20}$  and that this and two presumptive glucosides continued to increase up to 24h. In feeding studies with peas, using GC-MS for identification of all products and GC-RC to relate them with the label, Frydman and MacMillan (1975) found that [ $^3\text{H}$ ]  $\text{GA}_9$  was converted to [ $^3\text{H}$ ]  $\text{GA}_{20}$  dihydro - [ $^3\text{H}$ ]  $\text{GA}_{31}$  and a conjugate of the latter. [ $^3\text{H}$ ]  $\text{GA}_{20}$  was converted to [ $^3\text{H}$ ]  $\text{GA}_{29}$  and also to conjugates of both [ $^3\text{H}$ ]  $\text{GA}_{20}$  and [ $^3\text{H}$ ]  $\text{GA}_{29}$ . Conversion of [ $^3\text{H}$ ]  $\text{GA}_9$  to  $\text{H}_2$ -[ $^3\text{H}$ ]  $\text{GA}_{31}$  and [ $^3\text{H}$ ]  $\text{GA}_{20}$  as well as conversion of the latter to [ $^3\text{H}$ ]  $\text{GA}_{29}$  in pea seedlings and germinating peas was shown independently by Railton et al., (1974a, b). They also showed that the radioactive interconversion products produced by the pea from the [ $^3\text{H}$ ]  $\text{GA}_9$  had chromatographic properties similar to biologically-active GA-LS present in etiolated shoots of dwarf pea as assessed by the dwarf pea bioassay. Metabolism of other tritiated GA's

e.g.  $[^3\text{H}] \text{GA}_5$  and  $[^3\text{H}] \text{GA}_1$  to more polar compounds has also been shown to occur in dwarf pea tissue (Jones and Lang, 1968; and Musgrave and Kende, 1970 respectively). It seems reasonable, therefore, on the basis of published data to suggest that many of the radioactively-labelled metabolites present in association with the isolated etioplasts still have a gibbane skeleton and that they may represent those gibberellins naturally-occurring in the etioplasts.

Light treatments had no detectable effect on the distribution of radioactivity between the soluble and organelle fractions. There was therefore no evidence to suggest an effect of phytochrome on the permeability of the etioplast membranes with respect to the radioactively-labelled metabolites. When light-treated etioplast suspensions were extracted into methanol and subjected to TLC there was no marked change in the distribution of radioactivity between treatments. There was, however, a very small increase in the amount of radioactivity associated with the Rf zone 0.8-0.9 in red-light-treated suspensions.

It would seem necessary, in future experiments, to identify the radioactively-labelled compounds by physical methods and attempt to relate these to biological activity. However with a compound of such high specific activity ( $48 \text{ Ci mmol}^{-1}$ ) the mass involved is so small that the use of a bioassay may not prove very fruitful.

- (d) Investigations of the association *in vitro* of 'soluble' phytochrome with organelle fractions and its possible effect on the levels of biologically-active GA-LS.

Currently, the most popular hypothesis for the mechanism of action of phytochrome, proposed by Hendricks and Borthwick (1967), considers phytochrome to modulate directly the properties of some, or all, cellular membranes. The hypothesis has stimulated an extensive search for meaningful associations between phytochrome and plant membranes. From this search, two general categories of phytochrome-membrane associations may be defined:

(a) a small proportion of the total cell phytochrome of etiolated plants is found inherently associated with particulate material (Rubinstein et al., 1969; Cooke et al., 1975; Evans and Smith 1976a and b; Furuya and Manabe, 1976; and in this work) and (b) a larger proportion may be induced to associate with particulate material upon photoconversion of Pr to Pfr with red light in some plant tissues (Quail et al., 1973; Furuya and Manabe, 1976; Marmé et al., 1976; Quail and Gressel, 1976;). As yet there is no evidence that the red-light induced association of phytochrome with particles is related to phytochrome action: however, since relatively large amounts (up to 80%) of the total homogenate phytochrome may be involved the phenomenon is much simpler to investigate and consequently a large body of information has been gathered. Pratt (1977) has comprehensively reviewed the literature on red-mediated phytochrome-membrane associations and describes two main categories: (a) a definite *in vitro* association; and (b) a possible *in vivo* association. The *in vitro* association can be observed in homogenates, is sensitive to high pH and ionic strength, is insensitive to divalent cations (although cations at ca. 10mM are required to pellet the phytochrome-associated particles), has only been found in a few species (Cucurbita, Zea, Sinapis, and Pisum) and probably represents a physiologically non-relevant adsorption of Pfr to

partially degraded ribonucleoprotein material (Quail, 1975; Quail and Gressel, 1976). The so-called in vivo association either occurs upon red irradiation in vivo, or immediately upon homogenization of red-irradiated tissues, requires around 10mM  $Mg^{2+}$  or  $Ca^{2+}$  (presumably for aggregation of membranous material), is relatively insensitive to pH and ionic strength, and has been found in all eleven species so far surveyed. Pratt and Marmé, (1976), Marmé, (1977) and Pratt, (1978) in reviews, conclude that the 'in vivo' associations may be related to the primary mechanism of phytochrome action, but definitive evidence on this point does not yet exist.

An interpretation which has become popular amongst authors in this field is that a specific, membrane-associated, receptor for Pfr exists (e.g. Schäfer, 1975, Steinitz et al., 1976). As Pratt, (1978) points out however, three important criteria need to be fulfilled before such an interpretation can be allowed: (a) the interaction must be specific for phytochrome, as opposed to other proteins; (b) the binding affinity must be high and consistent with the known biological activity of phytochrome; and (c) the interaction of Pfr with the putative receptor must be related to the expression of its biological activity. It is difficult to see how these criteria can be experimentally satisfied in an association which probably takes place only in vivo; what is needed is an in vitro association, free of the artifactual problems outlined by Quail and Gressel (1976) and which may be investigated by the classical techniques of ligand-receptor theory.

In this section experimental results are reported which are a step towards such an in vitro phytochrome-membrane association in which R/FR reversibility in vitro (i.e. the mediation of GA levels) has already been shown to exist.

- (1) Red light-induced phytochrome pelletability in crude homogenates of etiolated *Hordeum vulgare* L. leaves (for description of method see Fig. 33, page 98)

The effects of in vitro irradiations of crude homogenates (filtered through miracloth) of etiolated *Hordeum vulgare* L. leaves on the amount of phytochrome associated with 6KP fractions subsequently prepared from the homogenates were investigated by Dr A Evans at Sutton Bonington, 1975-6. Crude homogenates and 6KP fractions were prepared as described in the Experimental Details on page 12(d)1.1. The results she obtained are set out in Table 9 (a), page 99. A 5 min irradiation with red light increased by approximately 60% the specific activity of the 6KP material. A 5 min irradiation with far-red light given immediately subsequent to the red irradiation did not result in any diminution of the red light-induced pelletability of phytochrome.

The association of phytochrome with the pelletable material is quite stable, both in the Pr and the Pfr form. Fig. 34, page 100, shows the time course of the loss of spectral photoreversibility in a dark-inoculated homogenate, and in a similar homogenate treated with red light and returned to darkness. Loss of total phytochrome follows a similar course in both samples. Dark and red light treated homogenates were subsequently purified by the G-50 method. [See Experimental Details, page 13, (d) 1.2]. The red light treated homogenates showed an approximate 50% enrichment of the specific activity of the phytochrome associated with the column eluate (G-50) when compared to those prepared from dark-maintained homogenates [Table 9 (b), page 99].

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General procedure for the investigation of phytochrome  
pelletability in crude homogenates of etiolated Hordeum vulgare  
L. leaves.

Aliquots of samples marked \* were used in experiments.

Etiolated Hordeum vulgare L. laminae



Homogenization at 3-4°C



Filtration through miracloth at 3-4°C



Light treatments at 25°C



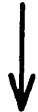
Centrifugation 6000g 1min



\*6KP



6KS



Sephadex G-50 (coarse)  
gel filtration



Pooled  
\* G-50  
eluate



Table 9 (a) and (b)

---

(a) Light-induced phytochrome pelletability of filtered homogenates of etiolated Hordum vulgare L. leaves.

(b) Light-induced phytochrome pelletability of filtered homogenates purified by the G-50 method.

[from Evans, 1975-6 (unpublished results) and subsequently from Smith, Evans and Hilton, 1978 ]

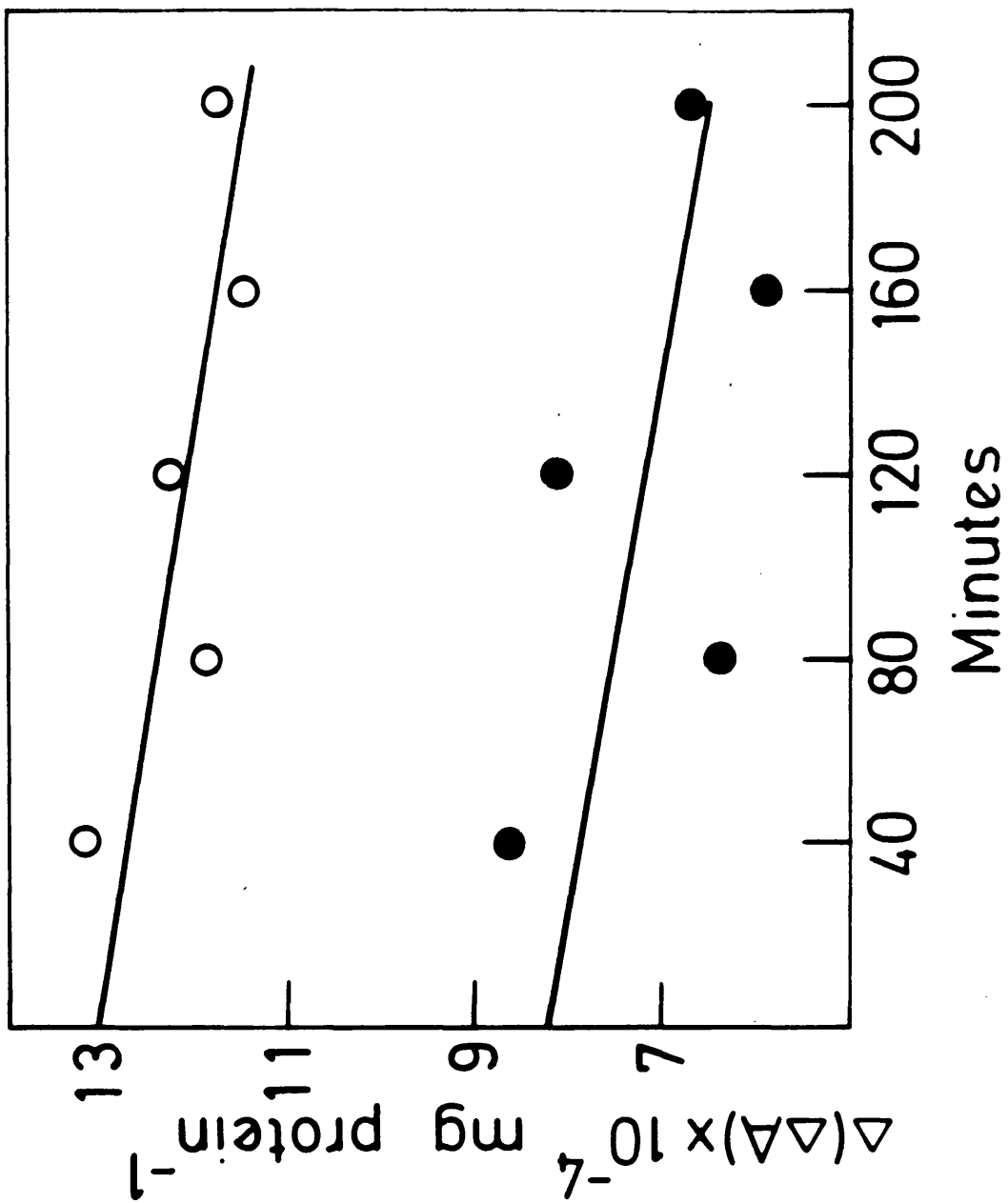
| Fraction | Treatments                       | $\Delta(\Delta A) \times 10^{-4}$<br>S.E. | protein<br>mg | S.E.       | $\Delta(\Delta A) \times 10^{-4}$<br>mg protein <sup>-1</sup> | S.E.       |
|----------|----------------------------------|---|---------------|------------|---|------------|
| (a)      | 6KP Dark                         | 19  | 3.18          | $\pm 0.19$ | 5.97  | $\pm 0.67$ |
|          | 6KP 5 min red                    | 30  | 3.14          | $\pm 0.15$ | 9.55  | $\pm 0.52$ |
|          | 6KP 5 min red +<br>5 min far-red | 37  | 3.24          | $\pm 0.17$ | 11.40   | $\pm 0.66$ |
| (b)      | G-50 Dark                        | 34  | 0.98          |            | 34.7  |            |
|          | G-50 5 min red                   | 48  | 0.92          |            | 52.2  |            |

Time course of the loss of spectral photo-reversibility in freshly-prepared homogenates of etiolated Hordeum vulgare L. leaves.

(from Smith, Evans and Hilton, 1978)

Treatments:

- aliquot given 5 min red light and returned to darkness
- aliquot maintained in darkness



- (2) The association of 'soluble' phytochrome with pelletable material.  
(For description of method see Fig. 5, page 31).

If the red light-mediated increase in phytochrome pelletability found by Evans (1975-6) in crude homogenates represents an association of 'soluble' phytochrome with particulate material, then a similar phenomenon should occur if 'soluble' phytochrome, completely devoid of pelletable material, is mixed with purified organelles. This was tested initially by Evans, and subsequently by myself, by incubating an aliquot of partially-purified organelles obtained by the G-50 method [for Experimental Details see page 13 (d) 1.2] with an aliquot of the 100KS fraction [for Experimental Details see page 17 (f)] which contained substantial amounts of 'soluble' phytochrome but no pelletable material. The aliquots of soluble phytochrome contained an average of 2.02 mg protein and gave  $0.0113 \Delta(\Delta A)$  yielding a specific activity of  $5.59 \times 10^{-3} \Delta(\Delta A) \text{mg protein}^{-1}$ . Table 10, page 102, shows that simple mixing of such aliquots in the absence of light treatment does not yield enhanced pelletability. [Phytochrome pelletability was estimated as outlined in the Experimental Details on page 17 (g)]. On the other hand, if such mixtures are irradiated, after mixing, with red light, the specific activity of the phytochrome detectable in the subsequently prepared 6KP fraction is approximately doubled. A far-red irradiation given immediately subsequent to the red irradiation does not diminish the increased pelletability of phytochrome. In a number of control experiments a red irradiation was found to have no detectable effect on the amounts of pelletable phytochrome in aliquots from the G-50 column. Similarly, aliquots of soluble phytochrome did not contain any phytochrome pelletable at 6,000g when irradiated with red light or maintained in darkness.

Table 10

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The light-induced association of 'soluble' phytochrome with a G-50 organelle fraction.

[from Smith, Evans and Hilton, 1978]

| Fraction       | Treatments                    | $\Delta(\Delta A) \times 10^{-4}$ S.E. | protein mg S.E. | $\Delta(\Delta A) \times 10^{-4}$ mg protein <sup>-1</sup> S.E. |
|----------------|-------------------------------|--|-----------------|---|
| G-50 alone     | Dark                          | 14.0                                   | 0.92            | 15.2  |
| G-50 + soluble | Dark                          | 17.0 $\pm 1.4$                         | 1.08 $\pm 0.17$ | 16.6 $\pm 2.1$  |
| G-50 + soluble | 5 min red                     | 28.3 $\pm 2.2$                         | 0.84 $\pm 0.10$ | 34.1 $\pm 3.2$  |
| G-50 + soluble | 5 min red +<br>5 min. far-red | 27.0 $\pm 1.2$                         | 0.64 $\pm 0.05$ | 42.7 $\pm 1.5$  |

(3) The effect of pre-irradiation on phytochrome pelletability

It appears from the results presented that an irradiation with red light induces 'soluble' phytochrome to associate with the G-50 organelles, and that photoconversion of associated-phytochrome from Pfr to Pr does not lead to a loss of pelletability. Indeed, data of Evans shows that even when samples are maintained in the Pr state after a red/far-red irradiation sequence for several hours, no detectable loss of pelletability was observed, (Fig. 35, page 104). It was important, therefore, to test whether the association of soluble phytochrome with the pelletable material was dependent on the presence of Pfr in either, or both of the components. Table 11(a), page 105, shows data from experiments in which aliquots of 'soluble' phytochrome and G-50 organelle suspensions were treated separately with either red, red followed by far-red or maintained in darkness prior to centrifugation. These results show that the increase in pelletable phytochrome occurs only if both the components of the mixture are irradiated with red light. Table 12, page 106, shows data from separate experiments in which the G-50 organelle suspensions and the soluble phytochrome were subjected to red, or red/far-red treatment separately prior to mixing and centrifugation. It is clear from these results that the requirement for red irradiation represents, in fact, a requirement for the presence of Pfr. Thus, the increase in pelletable phytochrome only occurs if Pfr is present in both the organelle suspensions, and in the soluble phase. Soluble Pfr, therefore, will not pellet with organelles containing only Pr, nor will soluble Pr associate with organelles containing either Pr or Pfr and Pr.



The stability of organelle-associated phytochrome in vitro.

G-50 organelle suspensions were:

incubated in darkness (▲)

irradiated for 5 min with red light (○)

or irradiated for 5 min with red light followed by  
5 min. far-red light (●)

and returned to darkness.

[from Evans, 1975-6 (unpublished results) and subsequently  
Smith, Evans, and Hilton, 1978]

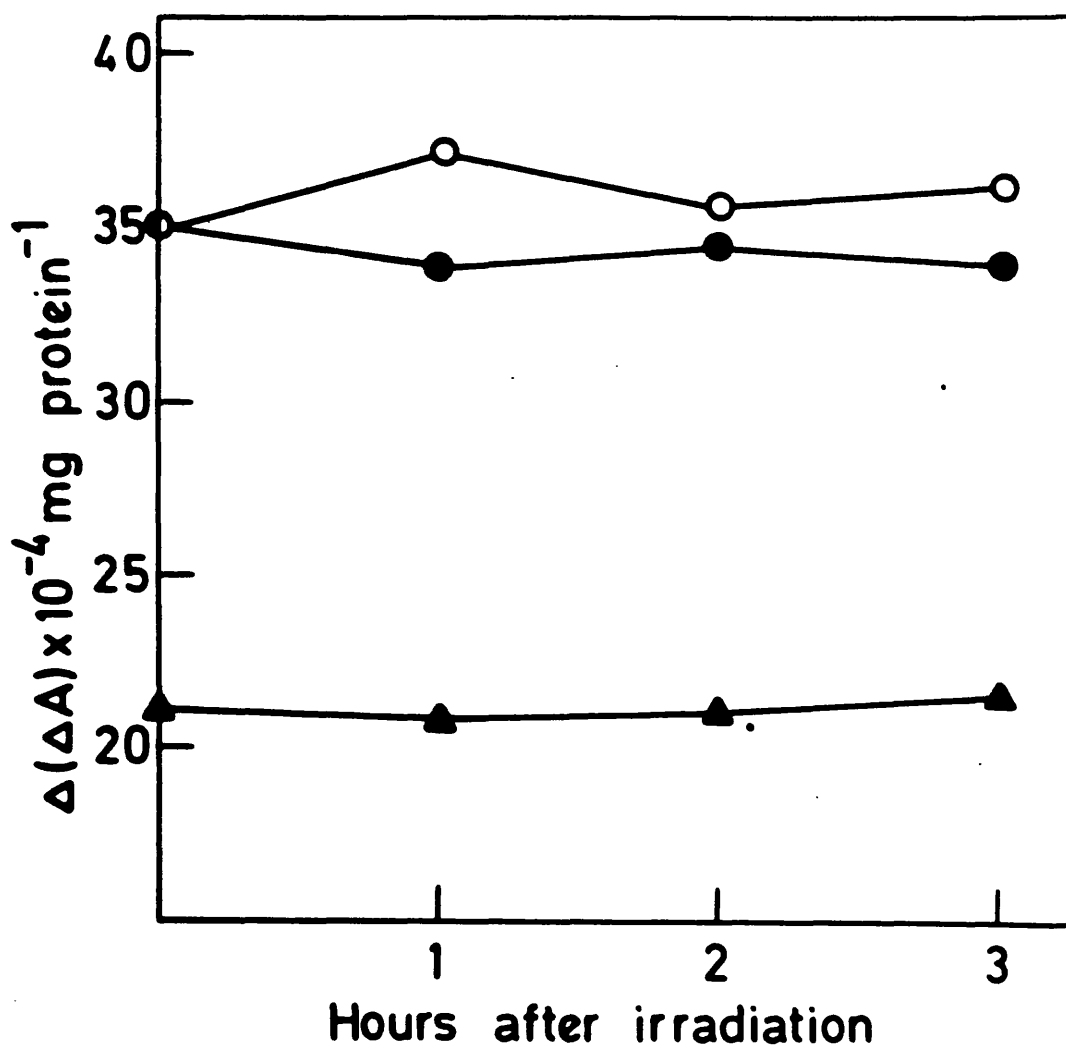


Table 11 (a) and (b)

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The effect of pre-irradiation on the pelletability of phytochrome in subsequently-mixed preparations of 'soluble' phytochrome and G-50 organelle fractions.

- (a) Aliquots of 'soluble' phytochrome (100KS) and purified organelles (G-50) were subjected to the stated light treatments, immediately mixed, incubated in darkness at 24°C for 5 min and phytochrome pelletability determined.
- (b) As a control, an aliquot of soluble phytochrome and one of purified organelles were mixed, irradiated for 5 min with red light and incubated in darkness for a further 5 min before centrifugation and measurement as in (a).

[from Smith, Evans and Hilton, 1978]

| Treatments |  | $\Delta(\Delta A) \times 10^{-4}$ | protein mg | $\Delta(\Delta A) \times 10^{-4}$<br>mg protein <sup>-1</sup> |
|------------|--|-----------------------------------|------------|---|
| G-50       | Soluble                                  |                                   |            |   |
| (a)        | Dark                                     | 11                                | 0.53       | 20.8  |
|            | 5 min. red                               | 10                                | 0.50       | 20.0  |
|            | Dark                                     | 11                                | 0.53       | 20.8  |
|            | 5 min red                                | 18                                | 0.37       | 48.6  |
|            | 5 min red +<br>5 min far-red             | 10                                | 0.51       | 19.6  |
| (b)        | G-50 + 100KS<br>Mixed then,<br>5 min red | 22                                | 0.51       | 43.1  |

Table 12

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The requirement for Pfr in both organelle suspensions and  
'soluble' fractions.

[from Smith, Evans and Hilton, 1978]

| Treatments                   |                              | $\Delta(\Delta A) \times 10^{-4}$ | S.E.      | protein<br>mg | S.E.        | $\Delta(\Delta A) \times 10^{-4}$ | S.E.      |
|------------------------------|------------------------------|-----------------------------------|-----------|---------------|-------------|-----------------------------------|-----------|
| G-50                         | Soluble                      |                                   |           |               |             |                                   |           |
| 5 min. red                   | 5 min red                    | 12.2                              | $\pm 0.8$ | 0.27          | $\pm 0.003$ | 35.3                              | $\pm 3.5$ |
| 5 min red +<br>5 min far-red | 5 min red                    | 6.8                               | $\pm 1.2$ | 0.25          | $\pm 0.017$ | 27.0                              | $\pm 3.5$ |
| 5 min red                    | 5 min red +<br>5 min far-red | 5.9                               | $\pm 0.7$ | 0.25          | $\pm 0.008$ | 23.8                              | $\pm 2.6$ |

(4) The effect of Pfr-induced phytochrome pelletability on GA-LS extractable into aqueous methanol

From the results presented earlier, it is clear that phytochrome associated with G-50 organelle suspensions is active in mediating the levels of GA-LS extractable into aqueous methanol following photoconversion to Pfr. It was therefore of interest to investigate the effects of the Pfr-induced pelletability of 'soluble' phytochrome on the levels of GA-LS subsequently extractable from the organelle suspensions. When an aliquot of G-50 eluate was incubated in red light in the presence of a preparation of 'soluble' phytochrome, there was an inhibitory effect on the levels of GA-LS extractable when compared to those extractable from a similar preparation in which 'soluble' phytochrome was replaced by a similar volume of 25mM MOPS buffer (Fig. 36, page 108). These results could possibly mean that increased amounts of phytochrome associated with the organelles has an inhibitory effect on the levels of GA-LS extractable from them.

On the other hand, although the preparation of soluble phytochrome was devoid of pelletable material, it may contain soluble substances inhibitory to the production of  $\alpha$ -amylase in the barley aleurone layer bioassay; substances inhibitory to the red light-mediated increase in GA-like activity or substances which may nullify the effect of phytochrome photoconversion and/or events leading to the subsequent increase in the extractability of GA-LS. The results obtained may therefore not represent a true effect of the increased amounts of phytochrome associated with the organelles but may reflect the effect(s) of unidentified constituents of the 'soluble' phytochrome preparation. Further experiments would necessitate the use of a more highly-purified preparation of soluble phytochrome or, indeed, radioactively-labelled phytochrome.

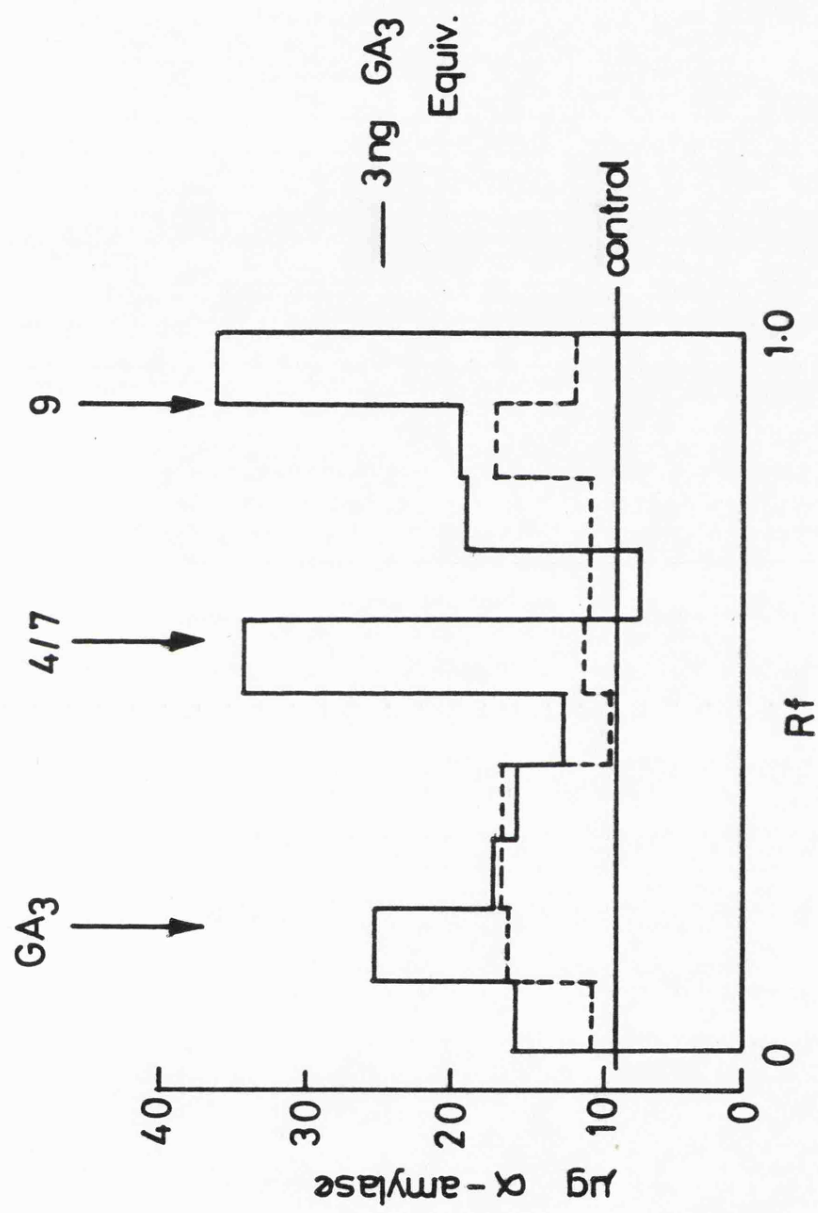
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The effect of Pfr-induced phytochrome pelletability on GA-LS extractable from G-50 organelle suspensions.

Treatments:

- \_\_\_\_\_ G-50 organelle suspension incubated in the presence of 25 mM MOPS
- G-50 organelle suspension incubated in the presence of a preparation of 'soluble' phytochrome.





(5) Discussion

The results indicate that soluble phytochrome will associate in vitro with isolated G-50 organelles, provided Pfr exists in both components. Once associated, the phytochrome remains pelletable for several hours, either in the Pr form or principally in the Pfr state. It is premature to attempt to decide whether this red light-induced increase in pelletability is representative of the in vivo association described in the Introduction, or whether it is another form of the apparently artefactual in vitro association. What can be stated, however, is that the pelletable material contains very little ribonucleoprotein material (Evans, 1975; Quail, Pers. Comm.) and that the red light-induced pelletability does not depend on the presence of divalent cations. It seems unlikely, therefore, to be artefactual in the manner described by Quail and Gressel, (1976). The in vitro association of soluble phytochrome with isolated organelles reported here has some similarities with the phenomenon observed by Georgevitch et al., (1977) who showed that phytochrome binds to particulate fractions and to purified mitochondria from etiolated oat tissues. Although Pfr bound more readily than Pr, Georgevitch et al., (1977) did not test the effects of pre-irradiation in vitro on the binding structures. Red irradiation of oat tissue in vivo yielded particulate fractions which bound lesser amounts of soluble Pfr in vitro, indicating that the binding sites had been saturated in vivo, or immediately upon extraction. In the experiments reported here with barley preparations, the additional pelletable phytochrome was, in all trials, approximately equal to the amount of phytochrome inherently associated with the organelles. A simple explanation, therefore, would be that Pfr molecules may associate to form irreversible dimers. If one of the two phytochrome molecules forming the dimers is a component of an organelle membrane, then subsequent centrifugation would pellet the dimers and enhance the observed pelletability. If such a mechanism does occur, it

would seem important to determine whether or not it is involved in the so-called in vivo pelletability described by Marmé (1977) and Pratt (1978).

When G-50 organelle suspensions were irradiated with red light in the presence of 'soluble' phytochrome, there was a decrease in the levels of GA-LS subsequently extractable into methanol when compared to those extractable from organelle suspensions irradiated with red light in the presence of buffer alone. These results suggest that additional amounts of phytochrome associated with the organelles may inhibit the red-light mediated increase in GA-LS. However, the results may also represent inhibition of the response by unidentified soluble substances present in the 100KS preparation. Another possibility is that the Pfr-induced association of soluble phytochrome with the organelle mediates a change in the conformation of the plastid membranes such that the increase in GA-LS in response to red irradiation cannot be elicited. Conformational changes in the plastid membranes could be operating to block both the movement of GA-LS across the envelope membranes and/or to block the release of GA-LS from the membranes.

The amounts of phytochrome detectable in all the preceding experiments were consistently detectable. They were, however, very close to the limits of detection. Future experiments with highly purified phytochrome, or indeed radioactively-labelled phytochrome may prove fruitful.

(e) Summary

The data presented in this chapter support those obtained by Cooke and Kendrick (1975); and Cooke et al., (1975) and Evans and Smith (1976a and b); and show that phytochrome is present in association with isolated etioplast preparations and that it mediates in vitro the levels of GA-LS extractable from them into aqueous methanol. The data also show that phytochrome is associated with etioplasts following their passage through a sucrose cushion or into a sucrose gradient; it is therefore truly organelle-associated and not present solely as a soluble contaminant. The GA-LS levels extractable from the etioplast preparations which are substantially free of mitochondrial contamination are also controlled by phytochrome in vitro. Simple fractionation data indicating that an irradiation with red light results in an increase in the GA-LS levels extractable from the supernatant (Evans and Smith 1976a; Cooke et al., 1976) has been confirmed; however, data obtained from sucrose density gradient fractionation strongly suggests that the increase in the levels of GA-LS is due to increases in both the organelle and the soluble fractions isolated from these gradients. The distribution of GA-LS between the two fractions was similar irrespective of time of incubation in darkness following the red irradiation. Experiments using [ $^3\text{H}$ ] gibberellin GA<sub>9</sub> demonstrated that shoots of Hordeum vulgare L. seedlings had radioactivity associated with their plastids and that the gibberellin A<sub>9</sub> was metabolised to other radioactively-labelled compounds over a 24h incubation period. When etioplast suspensions were subsequently isolated there was no detectable effect of red light treatment in vitro on the distribution of radioactivity between the organelle and the soluble fractions isolated following sucrose density centrifugation. When the incubation times in darkness following the red irradiation were varied there was also no detectable effect on the distribution of radioactivity between the fractions. There was also no detectable difference

between the dark and red-treated samples when radioactively-labelled substances were extracted into methanol and subjected to TLC.

In experiments with a 'soluble' phytochrome preparation, phytochrome was shown to associate in vitro with a G-50 organelle suspension. The association was detectable only when both the 'soluble' phytochrome and that inherently associated with the organelles was present as Pfr: the association assumed a 1:1 relationship suggesting the possibility of dimer formation upon irradiation with red light.

Increased amounts of phytochrome associated with the organelles markedly reduced the levels of GA-LS extractable from the organelle-suspensions following red light treatment.

Phytochrome and GA-like substances in plastid fractions  
isolated from light-grown plants.

From the results presented in Chapter 3 of this thesis and from data presented elsewhere (Cooke et al., 1975; Cooke and Kendrick, 1976; Evans and Smith, 1976 a and b) there is strong evidence to suggest that phytochrome is associated with, or is intrinsic to, plastids isolated from dark-grown plants and that it mediates in vitro a clearly-defined response (i.e. the mediation of GA levels). In this chapter, the intriguing question of whether phytochrome, (if present), operates similarly in plastids isolated from light-grown plants has been investigated.

(a) The phytochrome mediation of GA-LS levels in a differentiating plastid fraction isolated from light-grown seedlings

There is strong evidence in the literature to suggest that GA-LS are associated with plastids isolated from light-grown plants (see Chapter 1, General Introduction). An opportunity arose to perform some collaborative experiments with Professor R Leech and her colleagues at the University of York on the levels of GA-LS present in association with plastids at different stages of differentiation isolated from Triticum aestivum L. seedlings. They had chosen wheat, a monocotyledonous species, because in the leaves of their young seedlings all cell divisions occur in a basal meristem, resulting in a developmental sequence of cells from the base to the tip of each leaf. Also, the complication of chloroplast dimorphism, as found in maize, was avoided. Dicotyledonous leaves are not an ideal tissue in which to study plastid differentiation since the leaf is a mosaic of cells at different stages of differentiation.

The levels of GA-LS extractable from the wheat shoots varied markedly between the consecutive 5 x 1 cm zones isolated (Fig. 37, page 116). The levels of GA-LS extracted were not corrected for the efficiency of plastid extraction between zones. Generally, chlorophyll content is used as an estimate of the efficiency of plastid isolation but in this case chlorophyll content varied markedly between the zones isolated (Table 13, page 117).

However, the table also shows estimates of plastid number  $\text{cm}^{-3}$ . The plastid number  $\text{cm}^{-3}$  does not vary greatly and any differences could not account for differences between the GA-LS associated with the 0-0.1 cm and the 1.0-2.0 cm zones. Phytochrome was readily detectable spectrophotometrically in plastid fractions isolated from the 0 - 1.0 cm zone and decreased with increasing distance from the base of the shoot (Fig. 38, page 118). However, concomitant with the decrease in spectrophotometrically-detectable phytochrome there was an increase in the chlorophyll content. The decrease in detectable phytochrome may, therefore, be due to increased screening by chlorophyll of the  $\Delta(\Delta A)$  reading obtained (Jose et al., 1977) and may not be due to a true decrease in the amount of phytochrome associated with plastids isolated from zones a greater distance from the base of the leaf.

As phytochrome was present in association with plastids isolated from the lowest zone of the leaf, experiments to investigate the effect of light treatment on the GA-LS associated with this zone were performed. As the 0 - 1.5 cm zone was always beneath the level of the compost plastids were isolated from this zone as opposed to the 0 - 1.0 cm zone only, in order to reduce the amount of material needed to perform the experiments and also to reduce the time taken from excision of the first to the last shoot segments. The levels of GA-LS extractable from plastid fractions isolated from 0 - 1.5 cm zones irradiated with red light in vitro were markedly higher than those extractable from a similar fraction retained in darkness. If a 5 min. irradiation with far-red light immediately followed the red irradiation, the levels remained closely similar to those extractable from the dark control (Fig. 39, page 119).

These data collectively suggest that phytochrome associated with these fractions mediates a change in the levels of GA-LS extractable from them in vitro. Extrapolation of these results can be regarded only as very tentative.



However, the distribution of GA-LS extractable from the fractions following an irradiation with red light in vitro are not too dissimilar to the distribution obtained when GA-LS are extracted from plastids isolated from the adjacent zone. The hypothesis advanced, therefore, is that phytochrome controls the levels of GA-LS associated with differentiating plastids in vivo.

---

GA-like activity extractable from 1 cm zones along the first  
leaves of light-grown Triticum aestivum L. seedlings.

Treatments:

- (a) 0 - 1 cm zone (base)
- (b) 1 - 2 cm zone
- (c) 2 - 3 cm zone
- (d) 3 - 4 cm zone
- (e) 4 - 5 cm zone

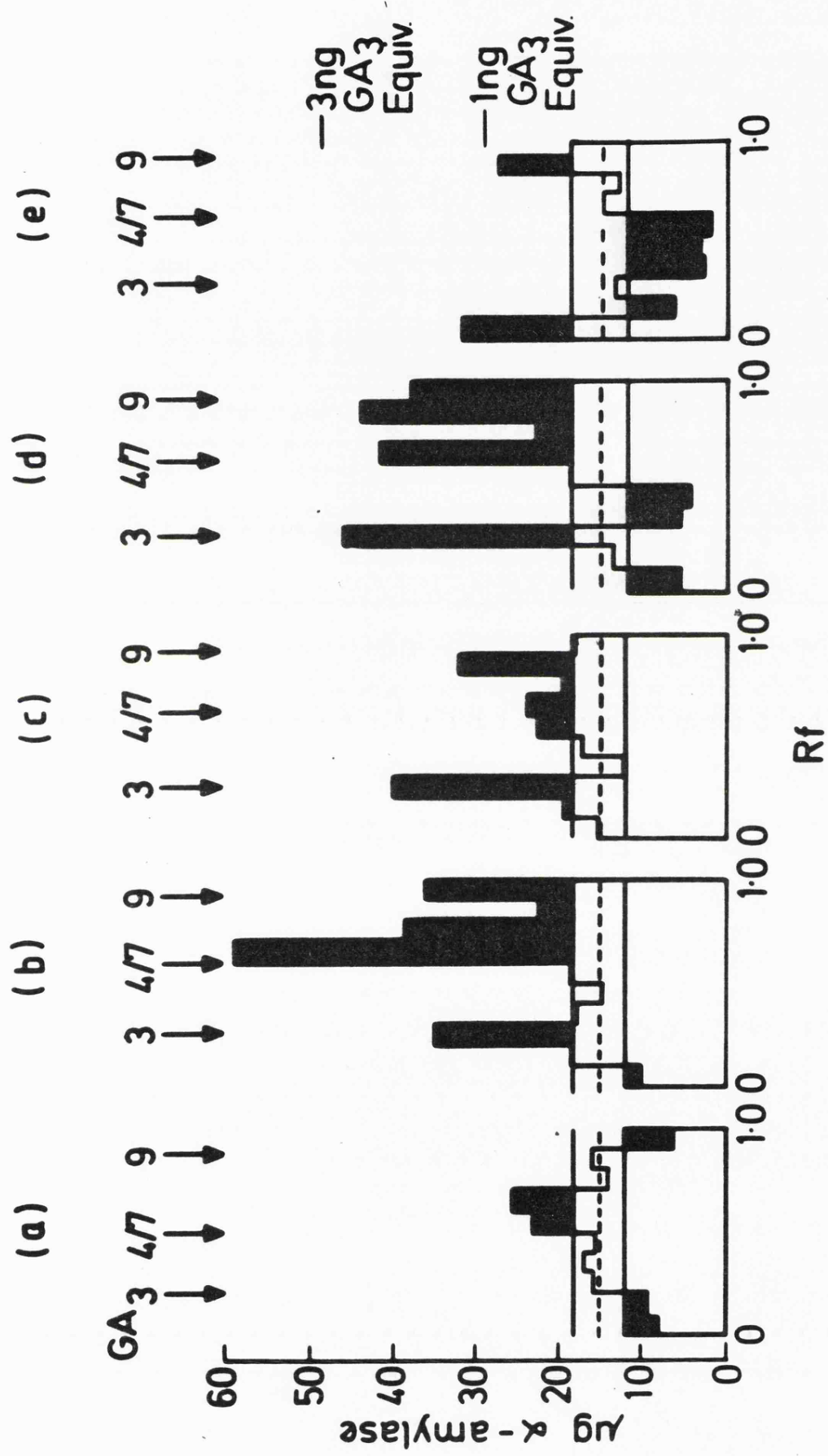


Table 13

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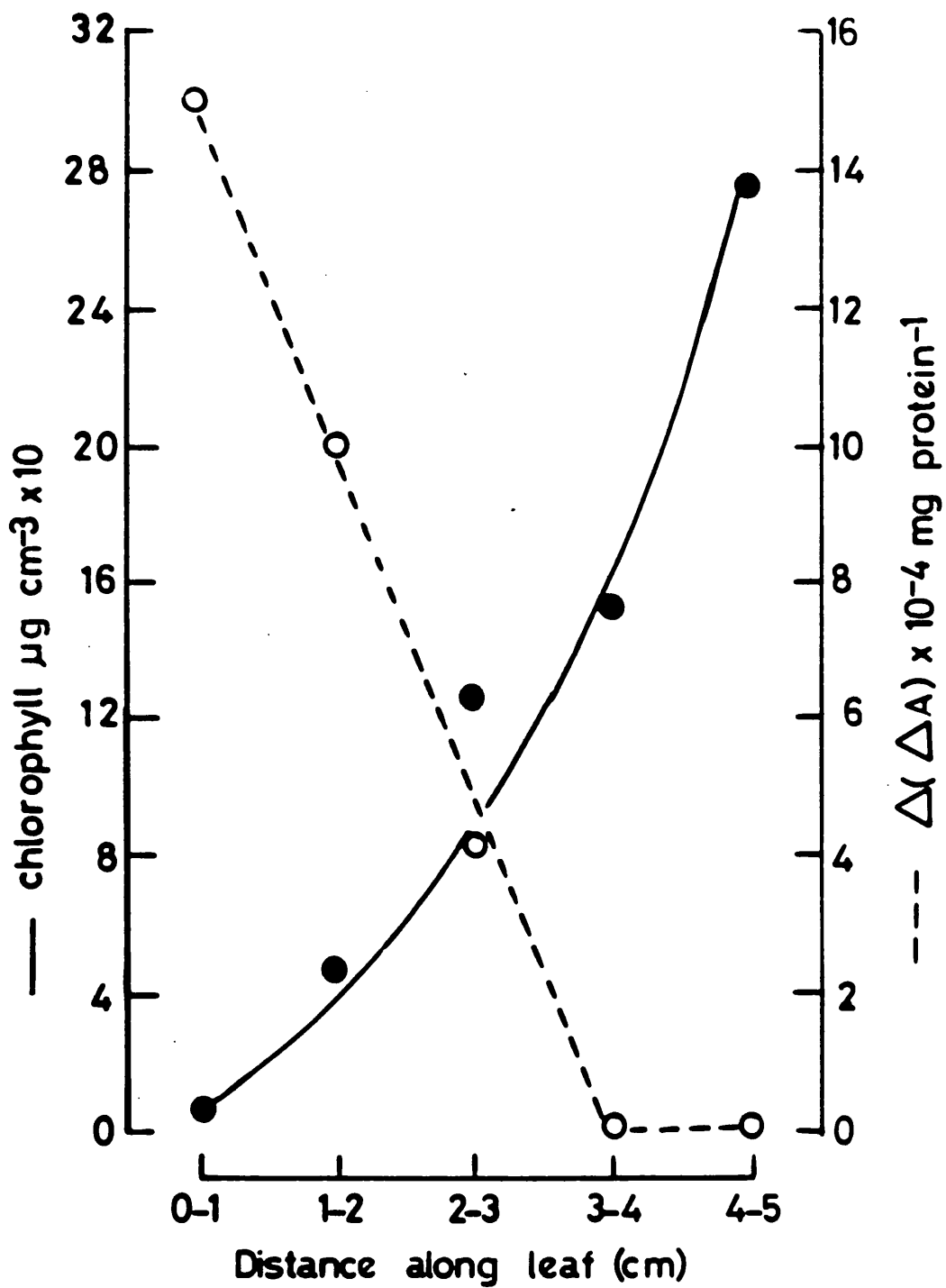
Estimated plastid number  $\text{cm}^{-3}$  of plastid fractions isolated from  
.1 cm zones of the first leaves of light-grown Triticum aestivum L.  
seedlings.

|  | Distance from base of leaf (cm) |       |       |       |       |
|--|---------------------------------|-------|-------|-------|-------|
|  | 0 - 1                           | 1 - 2 | 2 - 3 | 3 - 4 | 4 - 5 |
| Total chlorophyll<br>ug cm <sup>-3</sup>                       | 2.7                             | 14.9  | 37.6  | 118.8 | 229.8 |
| *Estimated chlorophyll<br>per plastid<br>g x 10 <sup>-15</sup> | 5                               | 40    | 80    | 220   | 500   |
| Plastid number cm <sup>-3</sup><br>x 10 <sup>10</sup>          | 5.3                             | 3.7   | 4.7   | 5.4   | 4.6   |

\* figures obtained from R M Leech and colleagues, University of York.

Phytochrome and chlorophyll contents of plastid fractions isolated from successive 1 cm zones of the first leaves of light-grown Triticum aestivum L. seedlings.

[Phytochrome measurements were performed in the absence of  $\text{CaCO}_3$ ; when  $\text{CaCO}_3$  was added there was no increase in the  $\Delta(\Delta A)$  signal obtained]

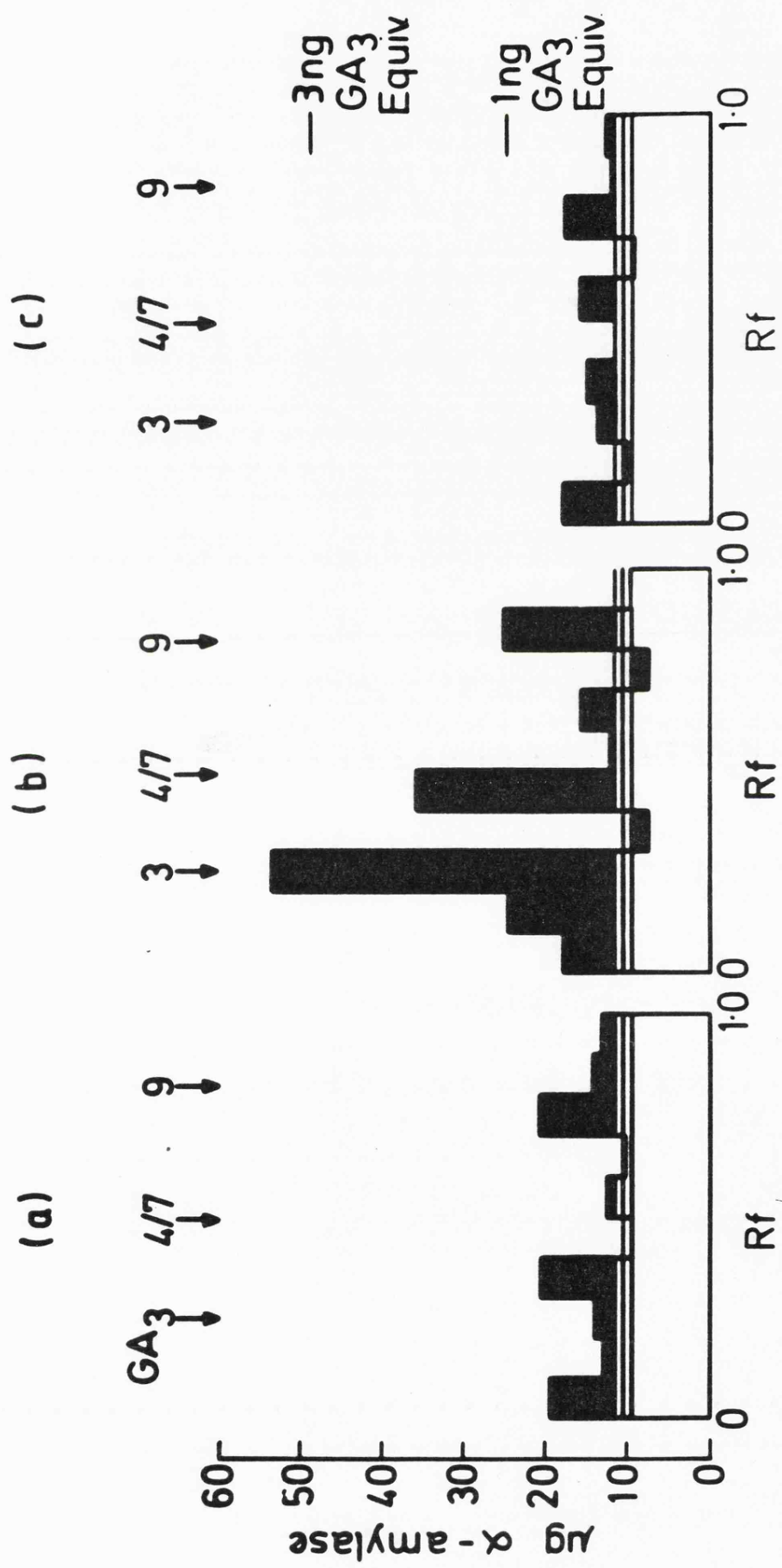


GA-like activity extractable from light-treated differentiating plastid-enriched fractions isolated from the basal 1.5 cm of light-grown Triticum aestivum L. seedlings.

Treatments:

- (a) 10 min. in darkness
- (b) 5 min red plus 5 min in darkness
- (c) 5 min red plus 5 min far-red





(b) The localization of phytochrome in association with chloroplast envelope membranes.

In the previous chapter phytochrome was measured in association with differentiating plastid fractions isolated from light-grown wheat seedlings. It was therefore of interest to investigate whether phytochrome was associated with fully differentiated chloroplasts. As the measurement of phytochrome relies upon absorption changes in the 600-800 nm region such measurement is clearly impossible where significant amounts of chlorophyll are present. An approach to this problem was made by isolating the envelope membranes of spinach chloroplasts, which, when pure, are completely devoid of chlorophyll (Honda et al., 1966). Chloroplasts were isolated from spinach leaves as it is generally accepted that it is far easier to isolate large quantities of chloroplasts from a dicotyledonous species than from a monocotyledonous species; also, the techniques for the isolation of spinach chloroplasts are well documented in the literature. Although the chloroplast envelopes prepared during this work by the method described by Mackender and Leech (1970) had not been further purified, nor characterized rigorously by biochemical markers, the data indicate that they are substantially free of both mitochondrial and thylakoid membrane contamination. Using the method described by Mackender and Leech (1970) in which thylakoid protein contamination is determined from the chlorophyll content, the contamination by thylakoid membranes was shown, in all three samples, to be 6 per cent or less (Table 14, page 122). Also, cytochrome c oxidase, a mitochondrial membrane marker enzyme was present at only very low activities in all samples especially in samples 2 and 3 in which the chloroplasts had been centrifuged through a cushion of sucrose prior to lysis. Phytochrome was readily detectable in all three samples (Table 15, page 123) the values observed being of a similar order of magnitude to those obtained from etiolated subcellular fractions

(Evans and Smith, 1976b). These values may indeed be underestimates due to interference from the small amounts of chlorophyll present (Jose et al., 1977). Further evidence for the presence of phytochrome in the envelope membrane preparations was obtained by the construction of a difference spectrum (Fig. 40, page 124). Although a certain distortion of the spectrum is visible (which may be due to the freezing and thawing of the samples during transport, and subsequent assay, of the samples from York via Sutton Bonington to Reading), the difference spectrum is nevertheless characteristically that of phytochrome with a  $Pr_{\lambda_{max}}$  at 675nm and a  $Pfr_{\lambda_{max}}$  at 735nm. The ratio of the  $\Delta A$  at  $Pfr_{\lambda_{max}}$  to that at  $Pfr_{\lambda_{max}}$  is 0.98, quite close to that found with isolated pure phytochrome.

These data show, therefore, that phytochrome is associated with a membrane fraction isolated from green plants by a method designed to isolate chloroplast envelopes. The membrane fraction is inadequately characterized for this to be more than a preliminary observation. Nevertheless, the similarity of these data to those obtained with etiolated plants provides a sound reason for accepting them as a reliable indication that phytochrome is present in association with chloroplast envelope membranes. The data do not preclude the presence of phytochrome in association with other components of the chloroplast; however, large amounts of chlorophyll in these fractions rendered the spectrophotometric detection of phytochrome impossible.

Table 14

---

The characterization of chloroplast envelope membrane-enriched fractions isolated by the method described by Mackender and Leech (1970).

Samples 2 and 3 were obtained from chloroplast preparation which had been centrifuged through a sucrose cushion prior to lysis.

|          | Cytochrome c<br>oxidase<br>loss $A_{550} \text{ min}^{-1}$ | Total<br>chlorophyll<br>$\mu\text{g}$<br>(A) | Total<br>protein<br>$\mu\text{g}$<br>(B) | B:A | Lamellar<br>protein |
|----------|--|--|--|-----|---------------------|
| Sample 1 | 2.4  | 8.39   | 1000                                     | 119 | 1 per cent          |
| Sample 2 | < 0.02   | 15.76  | 280                                      | 18  | 6 per cent          |
| Sample 3 | < 0.03   | 14.17  | 580                                      | 41  | 2 per cent          |

Table 15

---

Protein and phytochrome  $[\Delta(\Delta A)_{660/730\text{nm}}]$  contents of chloroplast envelope membrane preparations.

|          | Total protein<br>mg | $\Delta(\Delta A)$<br>$\times 10^{-3}$ | $\Delta(\Delta A) \times 10^{-3}$<br>mg protein <sup>-1</sup> |
|----------|---------------------|--|---|
| Sample 1 | 1.00                | 4.5                                    | 4.5   |
| Sample 2 | 0.28                | 1.7                                    | 6.0   |
| Sample 3 | 0.58                | 3.0                                    | 5.0   |

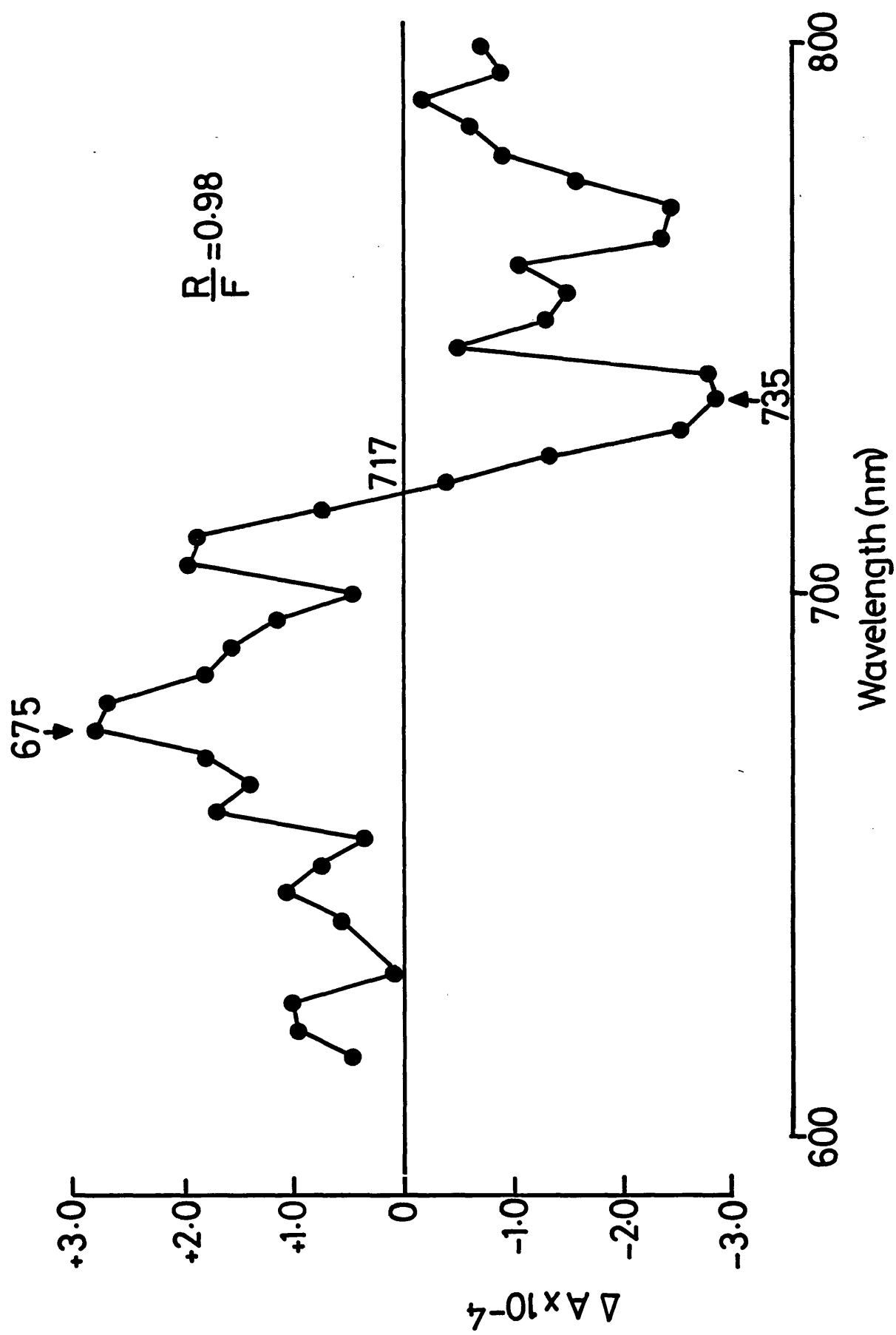
Difference spectrum of chloroplast envelope membrane phytochrome constructed using samples 2 and 3 combined.

$$Pr_{\lambda_{\max}} = 675\text{nm}$$

$$Pfr_{\lambda_{\max}} = 735\text{nm}$$

$$\frac{R}{F} = \text{ratio of } \Delta A \text{ at } 675\text{nm} \text{ and } \Delta A \text{ at } 735\text{nm}.$$





(c) The effect of phytochrome photoequilibria on plastid-associated GA-LS

In a preliminary experiment to determine the effects of phytochrome photoequilibria ( $Pfr/P_{tot}$  or  $\phi$ ) on the levels of GA-LS extractable from chloroplast suspensions, barley seedlings were grown from sowing in specially constructed cabinets in which various red:far-red ratios ( $\zeta$ , or the ratio of the photon fluence rates at 660:730 nm) were established from fluorescent or a combination of fluorescent and tungsten/tungsten halogen sources (Heathcote, Bambridge and McLaren, 1979). As  $\zeta$  has been shown to be related to phytochrome photoequilibrium  $\phi$  (Smith and Holmes, 1977) estimates of  $\phi$  ( $\phi_e$ ) could be derived from the values of  $\zeta$  established in the cabinets.

Fig. 41, page 128, shows the levels of biologically-active GA-LS extractable from plastid fractions isolated from the first leaves of barley seedlings. GA-like activity was converted to ng  $GA_3$  equivalents despite the earlier discussion of the validity of doing so because at that time there appeared to be no other method of comparing the GA-like activity between treatments when the efficiency of plastid isolation was not constant. In retrospect, however, estimates of plastid number  $cm^{-3}$  could have been performed. When the total ng  $GA_3$  equivalents per treatment were estimated and corrected for chlorophyll content (which was used as an estimate of the efficiency of plastid extraction between treatments) it was clear from the data presented in Table 16 and Fig. 42, pages 130 and 131, that as  $\phi_e$  decreased the total levels of GA-LS extractable into aqueous methanol from the plastid suspensions also decreased. As the majority of the GA-LS were associated with the Rf zone 0.2-0.3 zone the chlorophyll-corrected ng  $GA_3$  equivalents of this zone only were also plotted against  $\phi_e$ . Again, it is evident that a decrease in phytochrome photoequilibria is correlated with a decrease in GA-LS associated with this zone (Table 16, page 130).

A decrease in phytochrome photoequilibria was also associated with a decrease in the chlorophyll a/b ratio.

Similar results, showing that a decrease in phytochrome photoequilibria is correlated with a decrease in the chlorophyll a/b ratio of young mustard seedlings have been shown by Whitelam (Pers. Comm.). This is of great interest. Such a modification of the ratio would involve a decrease in the amount of chlorophyll a or an increase in the amount of chlorophyll b. The most likely explanation seems to be an increase in the amount of chlorophyll b. The problem of the origin of chlorophyll b is still open. Shylk (1971) concludes that 'on the strength of all the evidence a verdict is now reached in favour of the sequential scheme of chlorophyll b from chlorophyll a formation'. Virgin (1977) more explicitly holds the view that chlorophyll b is formed only in connection with chlorophyll a formation. Oelze-Karow et al., (1978) suggest that chlorophyll a and chlorophyll b arise from a common precursor, chlorophyllide a, and that chlorophyll b synthesis is controlled by phytochrome. The data presented here support the latter hypothesis, namely that a decrease in the phytochrome photoequilibria (when photosynthetically-active radiation is constant) is correlated with an increase in the amount of chlorophyll b present. Emerson et al., (1957) noted that in far-red light (at wavelengths longer than 680nm) there was a drop in the quantum efficiency of photosynthesis which was counteracted by the addition of supplementary monochromatic illumination at shorter wavelengths. In 1958, Emerson and Chalmers suggested that the longer wavelengths of light are absorbed only by chlorophyll a, the full efficiency of photosynthesis depending on a second light reaction i.e. a simultaneous absorption of light of shorter wavelengths by the accessory pigments, chlorophyll b and the carotenoids. It would seem reasonable to propose that under conditions of added far-red light (i.e. a decrease in the red:far-red ratio or phytochrome

photoequilibria) the efficiency of photosynthesis is maintained by the absorption of more and more light by the accessory pigments and to maintain this requirement it is necessary to form more and more chlorophyll b which is involved in the light absorption. It is generally accepted that chlorophyll b and photosystem II activity are concentrated in the 'heavy' fraction of chloroplasts composed mainly of granal stacks, and the material of the 'light' fraction derived from the intergranal areas catalyse exclusively the reactions related to photosystem I and is depleted in chlorophyll b. As a decrease in the red:far-red ratio is correlated with an apparent increase in the amount of chlorophyll b present it seems reasonable to suggest that there may be an effect of red:far-red ratio on the granal stacks of the thylakoid membrane system of chloroplasts. However, in the absence of electron microscope data such a suggestion can be regarded as no more than merely tentative.

It is of interest to note that the organization of the prolamellar bodies of etioplasts in mustard seedlings decreases conspicuously after approximately thirty-six hours in darkness but this can be prevented if the seedlings are irradiated with far-red light for thirty-six hours after sowing. Following this treatment the etioplasts possess crystalline prolamellar bodies which are much larger than the prolamellar bodies of the etioplasts of the dark-grown seedlings (Kasemir et al., 1975). These results show that there is a strong de novo synthesis of membraneous material because the volume of the etioplast increases considerably after far-red light treatment. It seems reasonable, therefore, on the basis of published data, to propose that de novo synthesis of thylakoid membraneous material may occur in the chloroplasts of seedlings which have developed in light environments with added far-red light. Again, a detailed electron microscope study of the ultrastructure of chloroplasts isolated from seedlings developed under varying phytochrome photoequilibria should prove rewarding.

GA-like activity extractable from plastid-enriched fractions isolated from Hordeum vulgare L. seedlings grown under light sources which established varying phytochrome photoequilibria ( $\phi$ ).

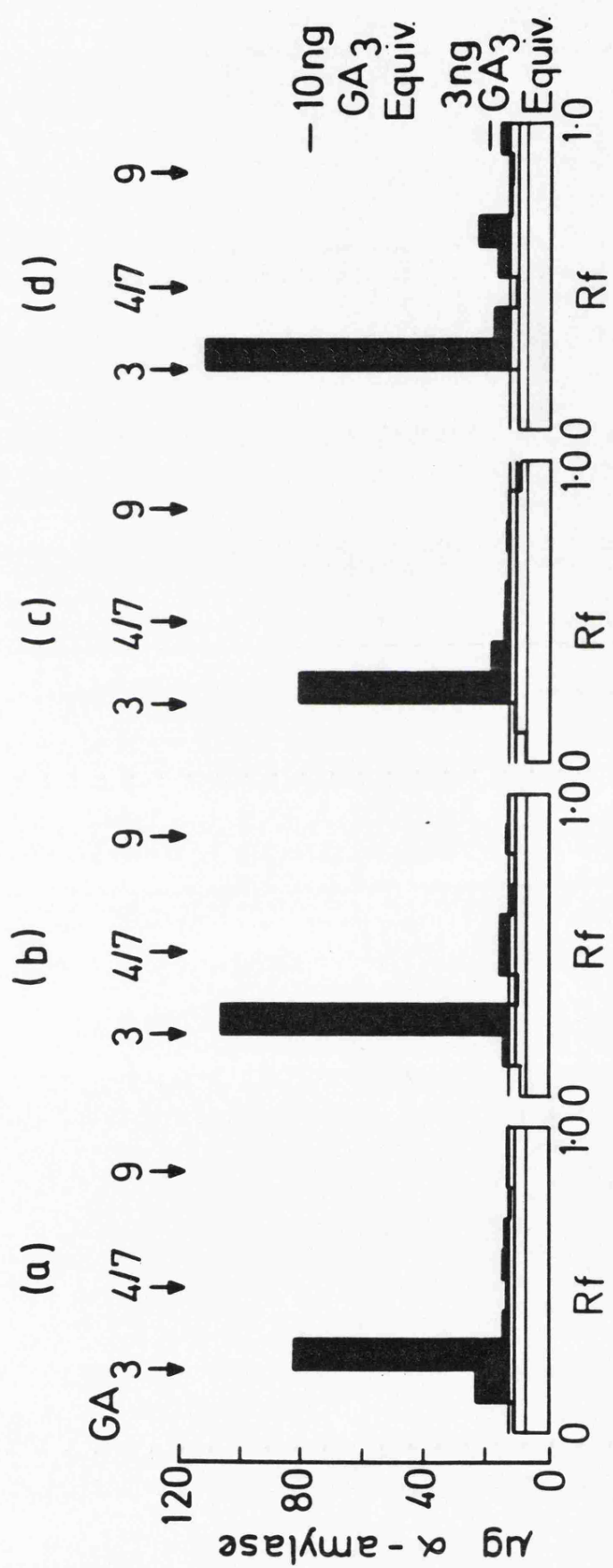
Treatments:

(a)  $\phi$  0.26

(b)  $\phi$  0.42

(c)  $\phi$  0.56

(d)  $\phi$  0.69



Chlorophyll a/b ratios and GA-LS levels (converted to ng GA<sub>3</sub> equivalents) associated with chloroplast fractions isolated from Hordeum vulgare L. seedlings grown under light sources which established varying phytochrome photoequilibria ( $\emptyset$ ).

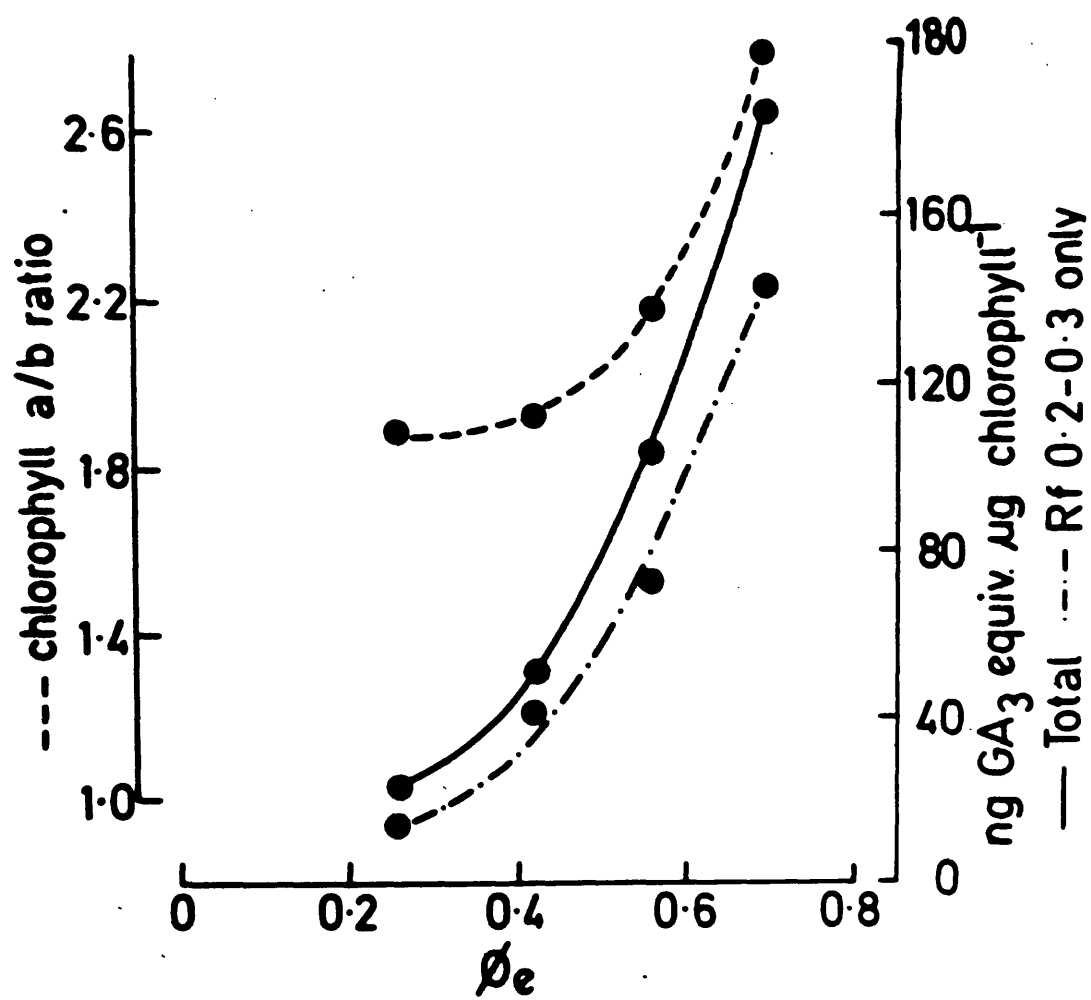
Table 16

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| ng GA <sub>3</sub> equivalents                                 |       |                                |        |        |
|--|-------|--------------------------------|--------|--------|
|  | 0.26  | 0.42 <sup>ø</sup> <sub>e</sub> | 0.56   | 0.69   |
| Rf 0.0 - 0.1   | 0.76  | 0.15                           | 0.04   | 0.12   |
| 0.1 - 0.2  | 2.60  | 0.64                           | 0.38   | 0.12   |
| 0.2 - 0.3  | 10.80 | 19.00                          | 10.80  | 21.50  |
| 0.3 - 0.4  | 0.57  | 0.66                           | 1.47   | 1.00   |
| 0.4 - 0.5  | 0.48  | 0.79                           | 0.62   | 0.23   |
| 0.5 - 0.6  | 0.58  | 0.80                           | 0.56   | 0.86   |
| 0.6 - 0.7  | 0.56  | 0.40                           | 0.50   | 2.37   |
| 0.7 - 0.8  | 0.40  | 0.35                           | 0.55   | 0.36   |
| 0.8 - 0.9  | 0.46  | 0.53                           | 0.44   | 0.33   |
| 0.9 - 1.0  | 0.50  | 0.46                           | 0.11   | 0.64   |
| Total  | 17.81 | 23.78                          | 15.47  | 27.53  |
| Total chlorophyll<br>µg cm <sup>-3</sup>                       | 0.78  | 0.47                           | 0.15   | 0.15   |
| Total  | 22.83 | 50.60                          | 103.15 | 183.53 |
| ng GA <sub>3</sub> equivalents<br>µg chlorophyll <sup>-1</sup> |       |                                |        |        |
| Rf 0.2-0.3<br>only   | 13.85 | 40.43                          | 72.00  | 143.33 |
| chlorophyll a/b ratio  | 1.88  | 1.92                           | 2.18   | 2.79   |





(d) The uptake and metabolism of [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  in detached *Hordeum vulgare* L. shoots and the effects of light treatment on chloroplast-enriched suspensions subsequently isolated. (For description of method see Fig. 26, page 83)

Data presented in previous sections indicate that phytochrome is associated with plastid fractions isolated from light-grown plants and that it mediates the levels of GA-LS extractable from them.

In a further attempt to study the rôle of phytochrome in the control of plastid GA levels a series of experiments with [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  were performed. It was proposed that the monitoring of radioactivity would enable any light-mediated effects to be expressed on a more quantitative basis than from the use of bioassay data alone. As stated previously when referring to similar experiments with etioplast-enriched suspensions (page 79)  $\text{GA}_9$  is a non-hydroxylated gibberellin and as biosynthesis is believed to proceed in order of increasing hydroxylation it was thought that  $\text{GA}_9$  could serve as an efficient precursor to other GA's thereby giving a wider spectrum of radioactively-labelled compounds within the tissue. For reasons similar to those discussed on page 79 a buffered solution of [ $^3\text{H}$ ]  $\text{GA}_9$  was fed to whole shoots of light-grown *Hordeum vulgare* L. seedlings and not to isolated chloroplast suspensions as it was of interest to see if radioactivity could be taken into the shoots and ultimately into the plastids in vivo.

As there were reports from previous work with etioplasts to suggest that phytochrome mediated a change in the permeability of the envelope membrane with respect to GA-LS it was of interest to investigate any phytochrome-mediated effects on chloroplast membranes.

Fig. 43, page 136, shows the effect of age of plant material on the uptake of radioactivity, over a 24h incubation period, into detached whole shoots and also into a total plastid fraction subsequently isolated from the

shoots. As the maximum amount of radioactivity was present in both the total shoot and the total plastid fraction isolated from the shoots of plants five days old from sowing, plant material of this age was used routinely in all subsequent experiments. Fig. 44, page 137, shows the distribution of radioactivity (following extraction and TLC) extractable from a total leaf homogenate and a total plastid fraction. If this figure is compared with Fig. 29, page 86, the distribution of radioactivity associated the standard [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  following TLC it is clear that the distribution has changed markedly in both fractions indicating that metabolism of the [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  has occurred. Fig. 45, page 138, shows the distribution of radioactivity recovered from 1 cm zones isolated along the entire length of the Hordeum vulgare L. shoots. Highest amounts of radioactivity were recovered from the base of the shoots (i.e. the zones nearest the point of immersion of the shoot in the buffered [ $^3\text{H}$ ] gibberellin  $\text{A}_9$ ), nevertheless, there were considerable amounts of radioactivity recoverable from all the other zones isolated. Although plastid suspensions were not isolated from all zones those isolated from segments 3 cm in length, 1 cm from the apex had radioactivity associated with them. Indeed, when these chloroplast suspensions were layered onto a sucrose density gradient and centrifuged, between 15 and 48% of the total radioactivity recovered passed into the gradient suggesting, therefore, that a consistent amount of radioactivity was associated with the organelle fractions themselves despite a large proportion remaining on top of the gradient and being truly soluble (Fig. 46, page 139). When similar chloroplast suspensions were fractionated as described by Mackender and Leech, (1970) only 4% of the radioactivity was associated with the envelope membrane-enriched fraction, 11% was associated with the prolamellar body-enriched fraction and 85% was associated with the soluble components of the stroma fraction (Table 17, page 140).

Table 18, page 141, shows the results of experiments in which radioactively-labelled chloroplast suspensions were subjected to light treatments in vitro. Immediately following light treatment the chloroplast suspensions were layered onto a sucrose density gradient and, following centrifugation, the distribution of radioactivity between the organelle and soluble fractions was estimated. The results show that a 5 min irradiation with red light has no detectable effect on the distribution of radioactivity between the two fractions when compared with similar fractions maintained in darkness throughout the treatment. Similarly, there was no detectable difference in the distribution of radioactivity between the organelle and soluble fractions when chloroplast suspensions were maintained in darkness for varying periods of time following a 5 min irradiation with red light when compared to those maintained in darkness throughout the treatment period.

When radioactively-labelled compounds were extracted into aqueous methanol from light-treated chloroplast suspensions and subjected to TLC there was no detectable difference between the distribution of radioactivity extractable from the dark and the red treated samples (Fig. 47, page 142). However, when radioactively-labelled substances were extracted from chloroplast suspensions irradiated with 10 min far-red light followed by a period of 5 min in darkness, there was a redistribution of radioactivity when compared with the distribution obtained from those samples retained in darkness for the whole treatment period (Fig. 48, page 143). Following a 10 min irradiation with far-red light approximately 6% more of the total dpm recovered was present associated within the Rf zone 0-0.1 than was associated with the corresponding zone of dark maintained samples; also the GA-LS present in association with Rf zones 0.5-0.9 were consistently lower than those associated with samples maintained in darkness. These results suggest, therefore, that a 10 min irradiation with far-red light

has an effect either on the metabolism, or on the extractability of radioactively-labelled compounds.

In a preliminary follow-up investigation chloroplast suspensions were isolated from Hordeum vulgare L. leaves and subjected to a 10 min irradiation with far-red light followed by 5 min in darkness. The GA-LS were extracted into aqueous methanol immediately following irradiations. Control suspensions were maintained in darkness for the entire treatment period.

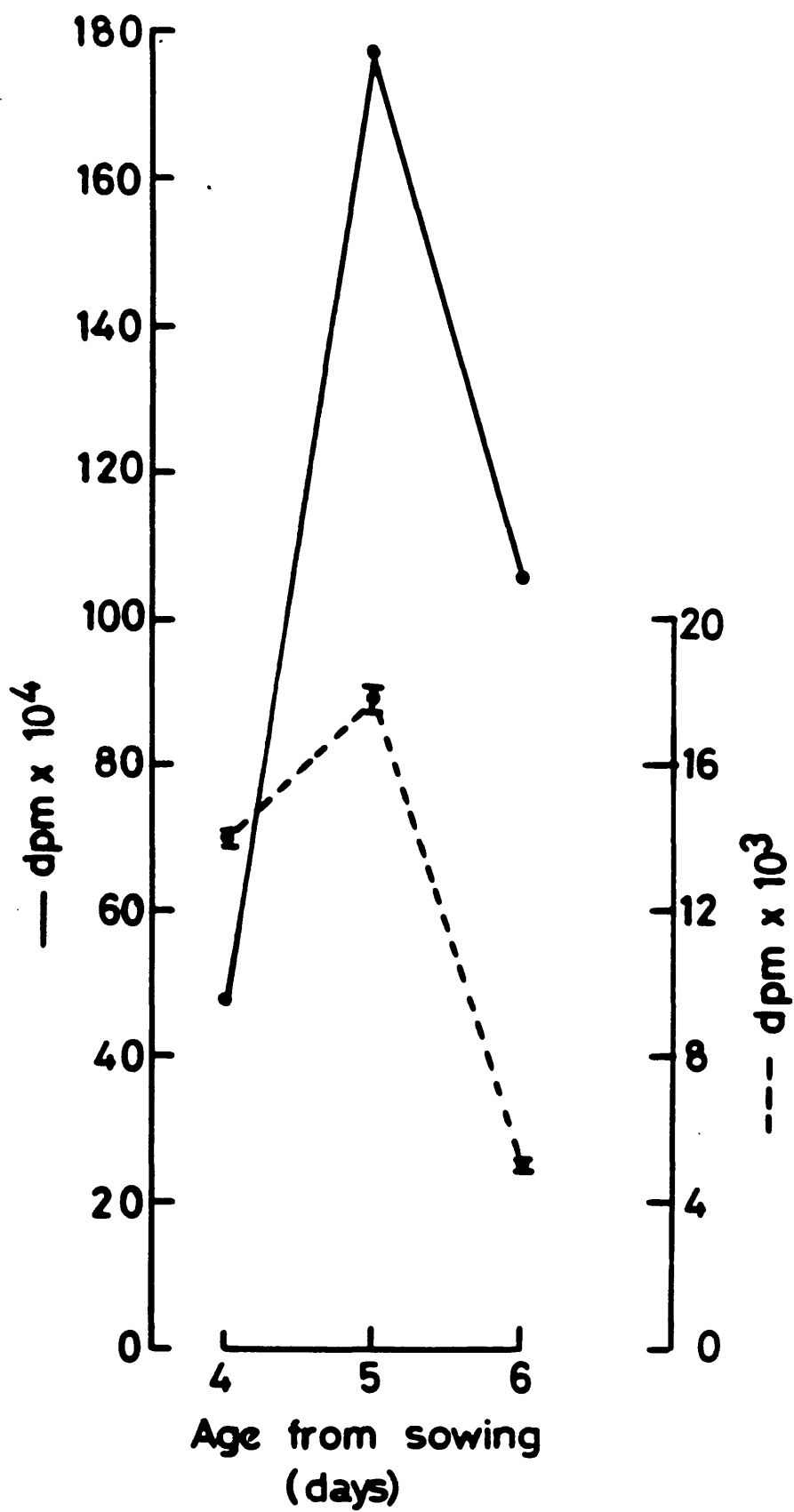
The results shown in Fig. 49, page 144, show that an irradiation with far-red light mediates a marked diminution in the levels of GA-LS that can be extracted from chloroplast suspensions into methanol when compared to those extractable from suspensions maintained in darkness.

The effect of age of plant material on the uptake of radio-activity into Hordeum vulgare L. shoots during a 24h incubation in buffered [ $^3\text{H}$ ] gibberellin  $\text{A}_9$  at  $24^\circ\text{C}$  in constant light.

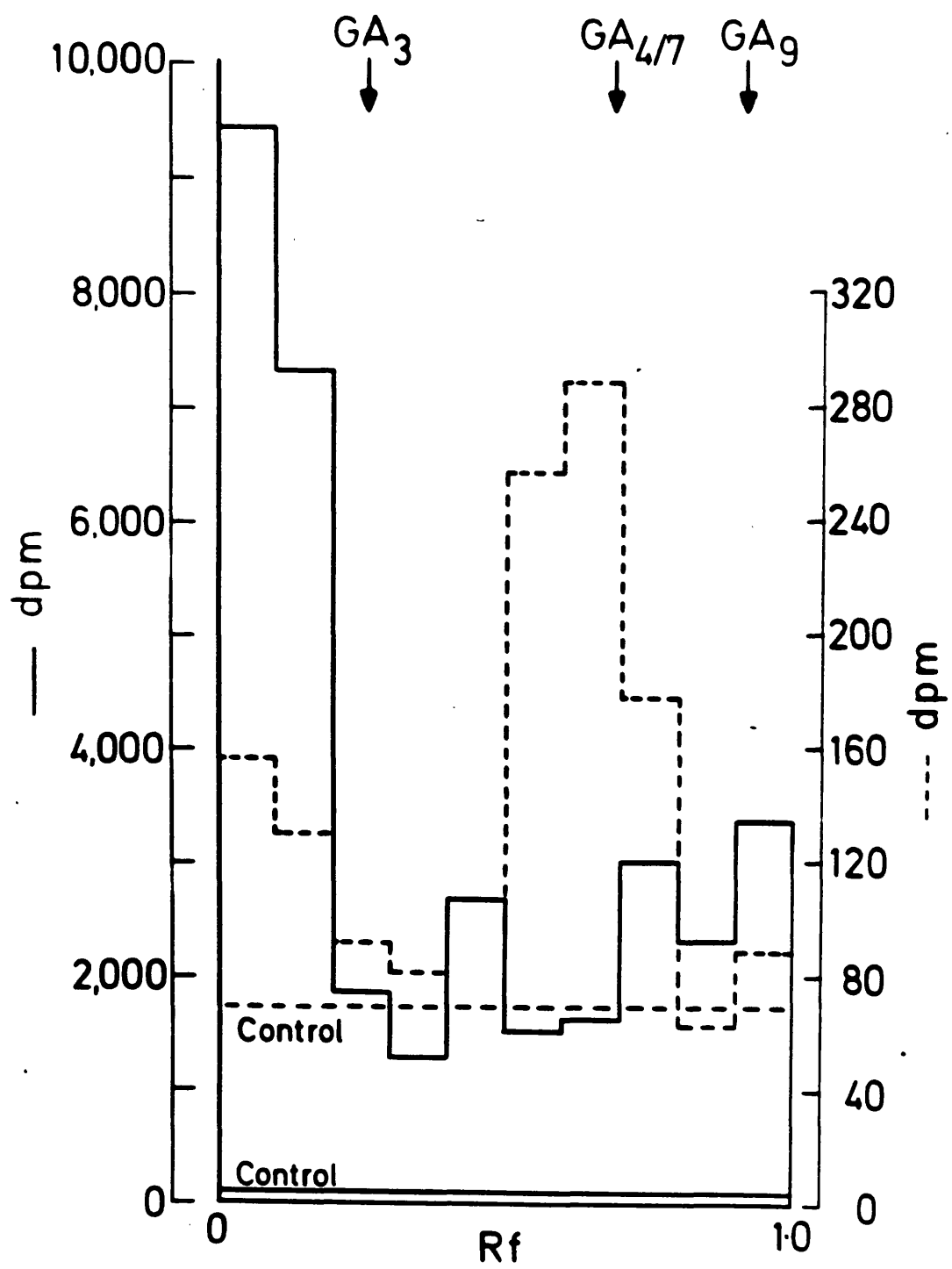
- uptake into whole shoots
- uptake into a plastid-enriched fraction isolated from a total shoot homogenate.

The age of the plant material at the termination of the experiment was age from sowing plus 24h.

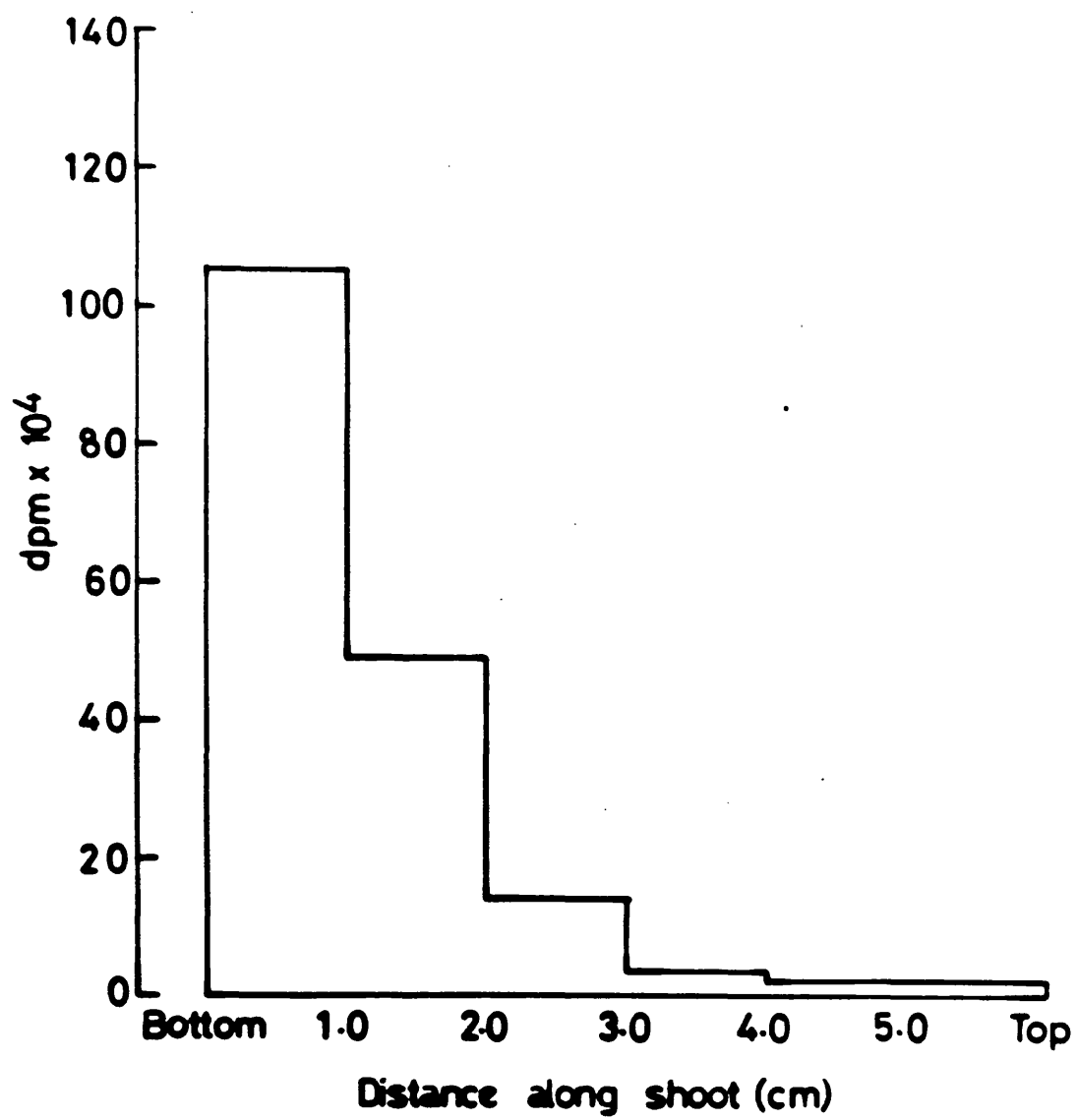




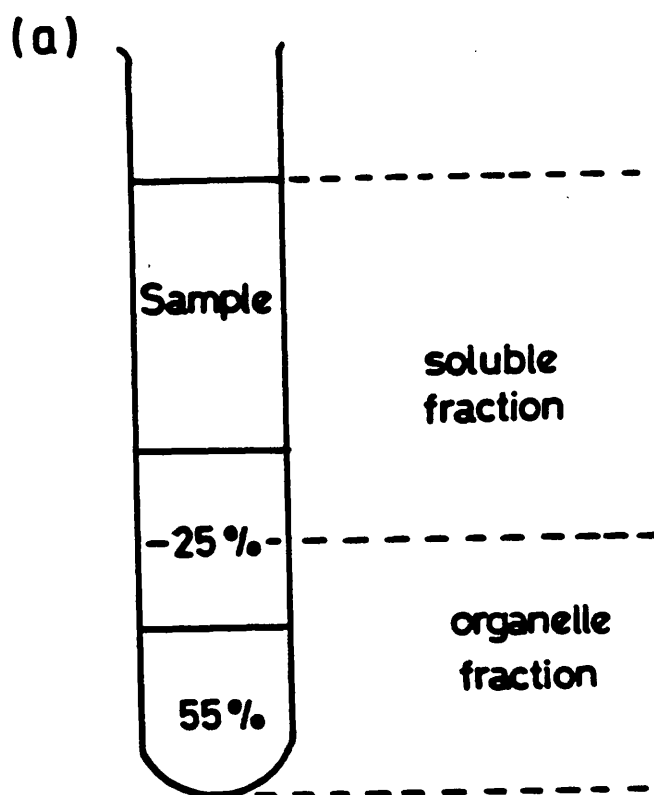
The distribution of radioactivity in a total leaf homogenate ( — ) and a plastid-enriched extract ( --- ) following extraction into aqueous methanol and TLC.



The uptake of radioactivity into 1 cm zones isolated from shoots of Hordeum vulgare L. following a 24h incubation in a buffered solution containing [<sup>3</sup>H] gibberellin A<sub>9</sub>.



- (a) Description of the sucrose density gradient and the limits of samples fractionated following centrifugation.
- (b) The percentage distribution of radioactivity between the soluble and organelle fractions following centrifugation of radioactively-labelled chloroplast-enriched suspensions.



(b)

% Total dpm recovered

| Soluble fraction |    | Organelle fraction |   | Total dpm recovered |
|------------------|----|--------------------|---|---------------------|
| $\pm$ SE         |    | $\pm$ SE           |   |                     |
| 79               | 6  | 21                 | 3 | 1059                |
| 88               | 11 | 12                 | 2 | 1695                |
| 76               | 15 | 24                 | 5 | 606                 |
| 79               | 6  | 21                 | 3 | 1014                |
| 52               | 5  | 48                 | 5 | 1396                |
| 81               | 6  | 19                 | 2 | 774                 |
| 73               | 15 | 27                 | 5 | 569                 |
| 78               | 14 | 22                 | 5 | 1072                |
| 57               | 11 | 43                 | 9 | 715                 |
| 78               | 16 | 22                 | 4 | 583                 |
| 84               | 11 | 16                 | 3 | 1884                |
| 85               | 15 | 15                 | 3 | 686                 |
| 83               | 6  | 17                 | 2 | 1208                |

Table 17

---

The percentage distribution of radioactivity in chloroplast-enriched suspensions following fractionation by the method described by Mackender and Leech, (1970).



| Fraction                   | % dpm recovered | ± SE |
|----------------------------|-----------------|------|
| Thylakoid-enriched         | 21              | 4    |
| Envelope membrane-enriched | 5               | 1    |
| Stroma-enriched            | 74              | 11   |

Total dpm recovered 712

Table 18

---

The percentage distribution of radioactivity between the soluble and the organelle fractions of a discontinuous sucrose density gradient following light treatments at 24°C and centrifugation of radioactively-labelled chloroplast-enriched suspensions.




The organelle suspensions were divided into two equal aliquots prior to light treatment. The results presented are therefore directly comparable.

% Total dpm recovered

| Treatment                   | Organelle fraction | ± SE | Soluble fraction | ± SE | Total dpm |
|-----------------------------|--------------------|------|------------------|------|-----------|
| 5 min dark                  | 78                 | 14   | 29               | 5    | 1072      |
| 5 min red                   | 83                 | 15   | 17               | 3    | 1208      |
| 10 min dark                 | 62                 | 7    | 38               | 4    | 898       |
| 5 min red + 5 min dark      | 67                 | 7    | 33               | 3    | 877       |
| 15 min dark                 | 85                 | 15   | 15               | 3    | 686       |
| 5 min red + 10 min dark     | 81                 | 14   | 19               | 4    | 774       |
| 15 min dark                 | 83                 | 6    | 17               | 2    | 1355      |
| 10 min far-red + 5 min dark | 82                 | 6    | 18               | 2    | 1492      |

The effects of light treatment on the distribution of radio-activity associated with chloroplast-enriched suspensions following extraction into aqueous methanol and TLC.

Treatments:-

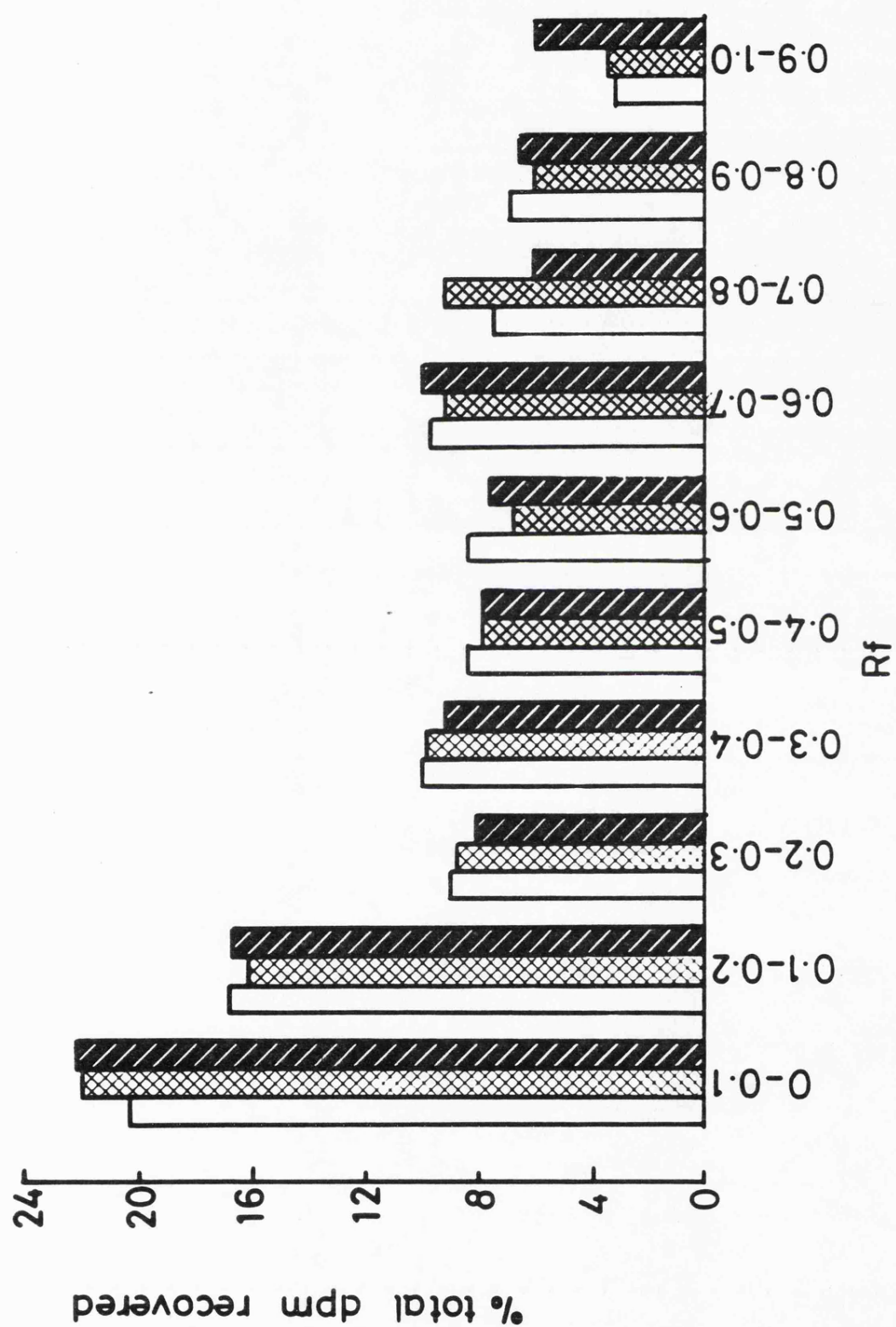
|   |                                     |
|---|-------------------------------------|
|  | 10 min. in darkness (756)           |
|  | 5 min red + 5 min in darkness (764) |
|  | 5 min red + 5 min far-red (750)     |

The total dpm recovered per treatment are shown in brackets.

Rf values of authentic GA's:-


|                   |      |
|-------------------|------|
| GA <sub>3</sub>   | 0.23 |
| GA <sub>4/7</sub> | 0.65 |
| GA <sub>9</sub>   | 0.92 |


The organelle suspensions were divided into three equal aliquots prior to light treatment. The results presented are therefore directly comparable.



The effects of light treatment on the distribution of radio-activity associated with chloroplast-enriched suspensions following extraction into aqueous methanol and TLC.

Treatments:-

 15 min in darkness (756)

 10 min. far-red plus 5 min. in darkness (811)

The total dpm recovered per treatment are shown in brackets.

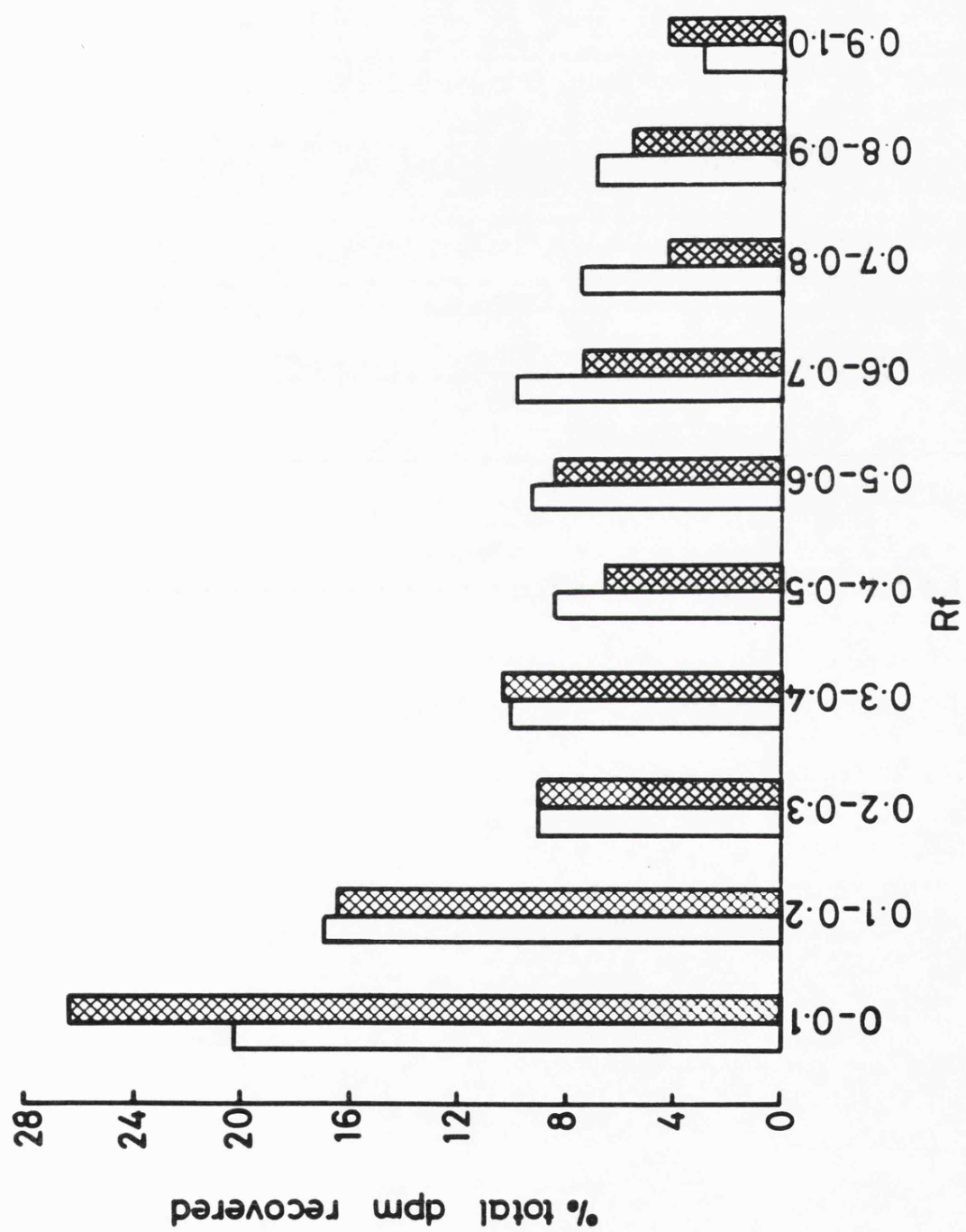
Rf values of authentic GA's:-

GA<sub>3</sub> 0.23

GA<sub>4/7</sub> 0.65

GA<sub>9</sub> 0.92

The organelle suspensions were divided into two equal aliquots prior to light treatment. The results presented are therefore directly comparable.

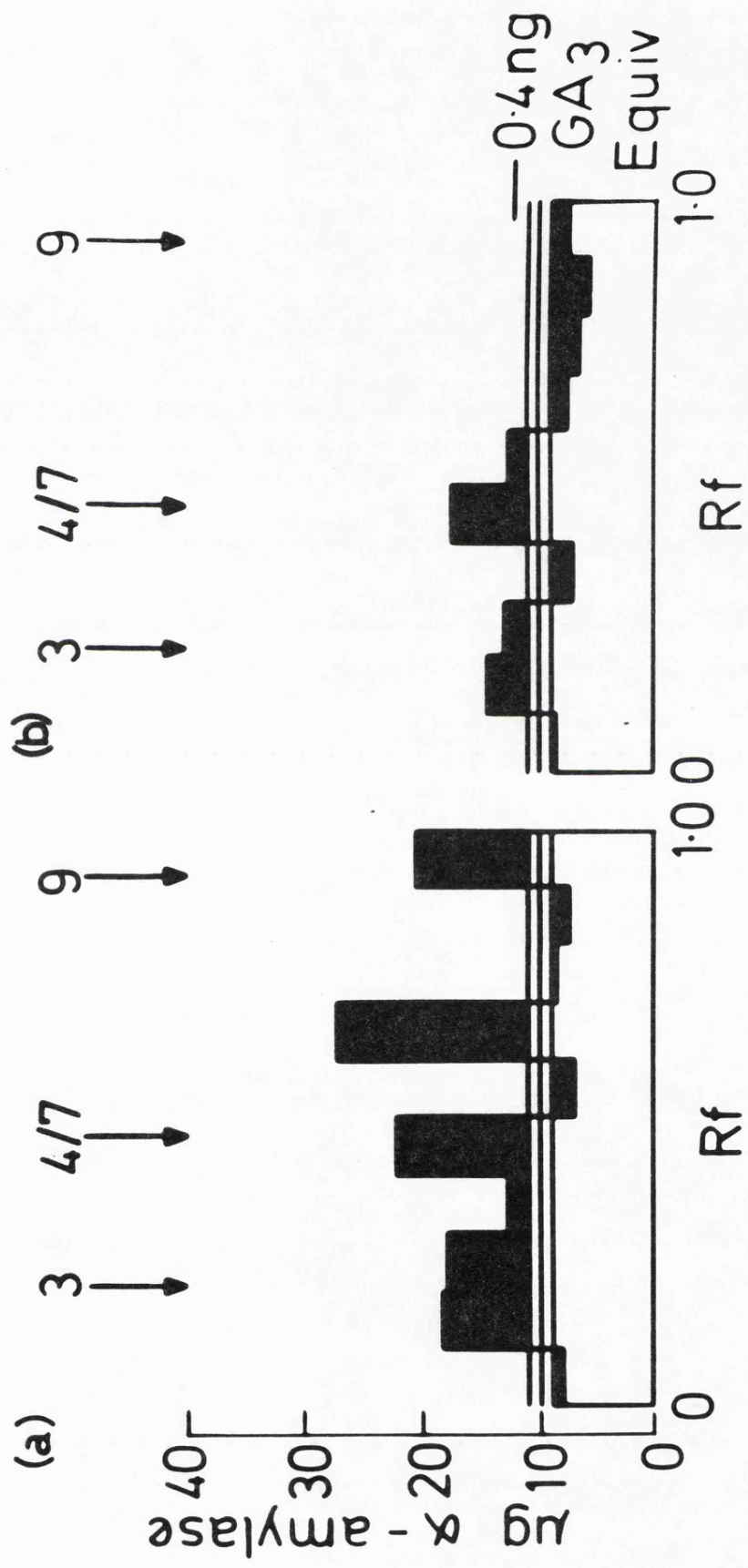


GA-like activity extractable from light-treated chloroplast-enriched suspensions.

Treatments:-

- (a) 15 min in darkness
- (b) 10 min far-red plus 5 min in darkness.





(e) Summary

Results presented in this chapter suggest that phytochrome is associated with an envelope membrane-enriched fraction when chloroplasts are sub-fractionated. These results do not preclude the presence of phytochrome in association with the other components of the chloroplast, but the presence of large amounts of chlorophyll in these fractions prevented the spectrophotometric detection of phytochrome.

When developing plastid fractions, isolated from light-grown seedlings, were irradiated with red light for 5 min there was a marked increase in the levels of GA-LS extractable into aqueous methanol. Conversely, when a far-red irradiation immediately followed the red irradiation the levels of GA-LS extractable were closely similar to those extractable from similar fractions maintained in darkness.

When chloroplast-enriched suspensions were isolated from seedlings grown under light environments maintaining varying phytochrome photoequilibria there was a correlation between the levels of GA-LS extractable into aqueous methanol and the amount of far-red light added to the light environment during growth of the seedlings. There was also a correlation between the chlorophyll a/b ratio of the chloroplasts and the phytochrome photoequilibria; the lower the phytochrome photoequilibria the lower the chlorophyll a/b ratio.

Chloroplasts isolated from shoots incubated in the light in a buffered solution containing [ $^3\text{H}$ ] gibberellin A<sub>9</sub> were able to take up radioactivity and metabolize it over a 24h incubation period. The majority of the radioactivity was associated with the soluble components of the stroma when the chloroplasts were sub-fractionated. There was, however, no significant effect of red light treatment on the distribution or radioactively-labelled compounds between the soluble and the organelle fractions

when the plastids were further fractionated. Also, there was no effect of this light treatment on the distribution of radioactivity following extraction and TLC of the radioactively-labelled organelle suspensions. However, when chloroplast suspensions were isolated and irradiated with far-red light there was a small re-distribution of the radioactively-labelled compounds extractable from far-red light treated suspensions when compared to the distribution of radioactivity associated with plastids maintained in darkness.

In preliminary experiments to determine the effect of far-red light on the extractability of GA-LS from chloroplasts in vitro, chloroplast-enriched suspensions were irradiated with far-red light for 10 min and returned to darkness for 5 min. The levels of GA-LS extractable from far-red light treated suspensions were markedly lower than those extractable from those maintained in darkness throughout the treatment period.

General Discussion

As discussed in the General Introduction there is strong evidence in the literature to suggest that there are two types of phytochrome in dark-grown cells; one that is associated in very small quantities with particulate material and another, (the bulk of the detectable phytochrome), that is freely soluble. There is also evidence to suggest that the latter type can become associated (and therefore becomes pelletable) with pelletable material upon photoconversion of Pr to Pfr.

In this, and in other work, phytochrome has been shown to be associated with etioplasts. Although etioplast-enriched fractions contain a high proportion of phytochrome that is non-organelle associated or is soluble, results from sucrose density gradient experiments reported in this thesis suggest that a small proportion of the phytochrome is truly organelle associated. The nature of this association of phytochrome with etioplasts has yet to be determined. The amounts of phytochrome involved are quite low and close to the limits of detection with the instruments available. Evans and Smith (1976b) reported that etioplast phytochrome was associated exclusively with an envelope membrane-enriched fraction when G-50 etioplast fractions were further fractionated. However, the etioplast preparations they used were obtained by the G-50 method and, as discussed previously, were undoubtedly contaminated by mitochondria. The validity of these results are therefore questionable. From the results presented in this thesis there is strong evidence to suggest that phytochrome is present in association with an envelope membrane-enriched preparation isolated from chloroplasts of light-grown spinach plants. As previously discussed these results do not preclude the presence of phytochrome in association with other components of the chloroplast but the presence of large quantities of chlorophyll rendered the spectrophotometric detection of phytochrome impossible in these fractions. The possibility still exists that the small amounts of phytochrome detectable in association with organelle/

membrane fractions could be an artefact of the extraction technique. Soluble phytochrome could be induced to associate with membranous material upon homogenization and/or centrifugation. However, the results obtained with immunocytochemical techniques also suggest that phytochrome is associated with membranous/organelle material and that soluble phytochrome, present over wide areas in the cell, becomes associated into discrete areas following irradiation of dark-maintained tissue with red light. The results of experiments performed jointly with A. Evans show that a preparation of 'soluble' phytochrome can be induced to associate with an organelle-enriched G-50 suspension. 'Soluble' phytochrome can associate with the organelles in an approximately 1:1 relationship provided that both the organelle-associated phytochrome and the 'soluble' phytochrome are present as Pfr. If either of the two types of phytochrome is present as Pr no increased association of the 'soluble' phytochrome with the organelles can be detected. In future experiments the use of highly purified phytochrome may enable the nature of this light-mediated association to be determined. There have been lengthy discussions into the physiological significance of light-mediated associations of phytochrome with organelles and membranes. Although there are innumerable reports of red light-induced associations with membrane fractions there are, at present, no reports of the occurrence of a physiological response as a result of such an association.

There is now a considerable amount of evidence showing that phytochrome mediates the levels of GA-LS in etioplast-enriched fractions in vitro. As quite large quantities of soluble phytochrome are present in association with G-50 etioplast suspensions it is possible that irradiation with red light causes a proportion of the phytochrome to associate with the etioplasts which could subsequently mediate the increase in GA-LS levels. However, throughout the course of this work no increased association of

phytochrome with the organelles has been detected following irradiation with red light. Furthermore, etioplast-enriched suspensions isolated from sucrose density gradients, devoid of soluble phytochrome, also exhibit a phytochrome mediation of the levels of GA-LS. It seems reasonable to propose, therefore, that it is the phytochrome that is inherently associated with the etioplasts that is responsible for the observed response. When G-50 suspensions were incubated with a preparation of 'soluble' phytochrome under conditions known previously to induce an increased association of phytochrome with the organelles there was no detectable enhancement of the GA-LS levels subsequently extractable. Indeed, the levels were markedly reduced compared to those extractable from preparations incubated with buffer only. As discussed previously this effect may have been due to other factors and may not have been a true reflection of the effect of the increased association of phytochrome with the organelles. Again, the use of highly purified phytochrome in future experiments may be invaluable in determining a physiological significance of the increased association of phytochrome with the organelles.

Previously, it had been generally accepted that photoconversion of etioplast-associated phytochrome to Pfr mediated a change in the permeability of the etioplast envelope membrane with respect to GA-LS thereby allowing an efflux of GA-LS out of the etioplasts into the surrounding medium. However, the results presented in this thesis suggest that a large proportion of the red-light-mediated increase in GA-LS is retained within the plastids themselves. Throughout this work the GA-LS extracted were acidic ethyl-acetate-soluble i.e. the so-called 'free' or non-glucosylated GA's. These molecules have a low degree of hydroxylation and therefore have a high affinity for membranes. It is reasonable to assume therefore, that the GA-LS extracted and assayed throughout this work were associated with membranes. The nature of the association of GA-LS with membranes, and with which particular membranes remains undetermined. The GA-LS could be

associated with the membranes of the prolamellar body complex or may be associated with the envelope membranes. Wherever the GA-LS are localized, and indeed, wherever the phytochrome is localized it is hypothesized from the results presented here that when the phytochrome is photoconverted in vitro to the Pfr form the GA-LS are subsequently more readily extractable into aqueous methanol. Conversely, when phytochrome is present as Pr (i.e. in the dark or following a R/FR irradiation sequence in vitro) the GA-LS are less readily extractable into aqueous methanol. This hypothesis is strengthened by the fact that there were increases over a wide spectrum of GA-LS following red light treatment; furthermore, there did not appear to be increases in particular GA-LS in any of the experiments reported here. However, it is clear that the use of bioassay techniques for the detection of GA-LS is far from ideal and is severely limited, despite giving information on biological activity of the GA-LS concerned. It will be essential in future experiments, therefore, to employ physical methods to determine more exactly the nature of the GA-LS involved in the response.

Although the results of experiments designed to study the effect of phytochrome photoequilibria on chloroplast-associated GA-LS are only very preliminary they provide evidence to suggest that light, operating via the phytochrome system, also has an effect on the extractability of GA-LS from chloroplasts. When chloroplast-enriched fractions were isolated from plants grown in light environments supplemented with varying amounts of far-red light the GA-LS subsequently extractable into aqueous methanol were correlated with the amount of supplementary far-red light. The higher the amount of added far-red light the lower the levels of GA-LS subsequently extractable. There was also an effect of phytochrome photoequilibria on the chlorophyll a/b ratios of the chloroplasts; chloroplasts isolated from plants grown under high supplementary far-red light had a



lower chlorophyll a/b ratio than those grown under low supplementary far-red light. On the basis of published data this lowering of the chlorophyll a/b ratio may be due to an increase in the amount of chlorophyll b. It is interesting to speculate on the results that may be obtained from an electron microscope study of these chloroplasts. From published data it is possible that there may be a difference in the organization of the thylakoid membrane system such that the granal stacks may be smaller in the chloroplasts of plants maintained under high levels of supplementary far-red light. This may be an advantage in terms of maintaining photosynthetic efficiency under conditions of high far-red illumination.

Further evidence in support of the rôle of phytochrome in the extractability of GA-LS comes from the results of experiments in which chloroplasts were irradiated with far-red light in vitro; lower levels of GA-LS were extractable from chloroplasts irradiated with far-red light than from those maintained in darkness. It appears, therefore, that an irradiation with far-red light renders the GA-LS in chloroplasts less readily extractable into aqueous methanol.

In conclusion, the results presented in this thesis strongly suggest that phytochrome is present in close association with both etioplasts and chloroplasts and that it mediates the levels of GA-LS extractable from them into aqueous methanol. The nature of this response is not fully understood but may involve phytochrome-mediated conformational changes of the plastid membranes.

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## An in Vitro Association of Soluble Phytochrome with a Partially Purified Organelle Fraction from Barley Leaves

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**Abstract.** Red light treatment in vitro increases the pelletability of phytochrome in homogenates of etiolated barley (*Hordeum vulgare* L. cv. 'Julia') leaves. When mixtures of soluble phytochrome ( $100,000 \times g$  supernatant) and partially-purified organelles (Sephadex G-50 eluate) are irradiated the amount of pelletable phytochrome increases by a factor of two. Pre-irradiation treatments show that phytochrome in both components of the mixture must be in the Pfr form for increased pelletability to be observed. Once associated, photoreversion of Pfr to Pr does not result in decreased pelletability. The results are consistent with a non-artifactual in vitro association of soluble phytochrome to organelle membranes. One possible explanation is that Pfr molecules associate to form dimers.

**Key words:** *Hordeum* – Membranes – Organelles – Phytochrome.

### Introduction

Currently, the most popular hypothesis for the mechanism of action of phytochrome is that originally proposed by Hendricks and Borthwick (1967) in which phytochrome is considered to modulate directly the properties of some, or all, cellular membranes. This hypothesis has stimulated an extensive search for meaningful associations between phytochrome and plant membranes. From this search, two general categories of phytochrome-membrane associations may be defined: (a) a small proportion of the total cell phytochrome of etiolated plants is found inherently associated with particulate material; and (b) a larger proportion may be induced to associate with particulate material upon photoconversion of Pr to Pfr with red light.

In the former case, the amounts of phytochrome inherently associated with membranous material is often very small and near the limits of detection using optical methods, (Rubinstein et al., 1969), and yet in certain cases, it mediates a defined R/FR photoreversible response in vitro. For example, Manabe and Furuya (1974) observed R/FR modulation of NADP<sup>+</sup> reduction by isolated pea mitochondria which contained only about 3 per cent of the total homogenate phytochrome (Furuya and Manabe, 1976). Cooke and Saunders (1975) and Evans and Smith (1976) reported R/FR control of gibberellin levels in organelles prepared by Sephadex G-50 fractionation of crude wheat and barley leaf homogenates respectively. Evans (unpublished) has shown that only about 2 per cent of the total homogenate phytochrome is recovered in the G-50 eluate, although it should be remembered that the recovery of the organelles present in the G-50 eluate is also very low. Thus, in these instances, a small fraction of the total cell phytochrome is associated with a reasonably well-defined particle fraction in which it mediates a specific response.

As yet, there is no evidence that the red-light induced association of phytochrome with particles is related to phytochrome action; however since relatively large amounts (up to 80%) of the total homogenate phytochrome may be involved the phenomenon is much simpler to investigate and consequently a large body of information has been gathered. Pratt (1977) has comprehensively reviewed the literature on R-mediated phytochrome-membrane associations and describes two main categories: (a) a definite in vitro association; and (b) a possible in vivo association. The in vitro association can be observed in homogenates, is sensitive to high pH and high ionic strength, is insensitive to divalent cation concentration, (although cations at ca. 10 mM are required to pellet the phytochrome-associated particles), has only been

found in a few species (*Cucurbita*, *Zea*, *Sinapis*, and *Pisum*) and probably represents a physiologically non-relevant adsorption of Pfr to partially degraded ribonucleoprotein material (Quail, 1975; Quail and Gressel, 1976). The so-called *in vivo* association either occurs upon R-irradiation *in vivo*, or immediately upon homogenization of R-irradiated tissues, requires around 10 mM  $Mg^{2+}$  or  $Ca^{2+}$  (presumably for aggregation of membranous material), is relatively insensitive to pH and ionic strength, and has been found in all eleven species so far surveyed (Pratt and Marmé, 1976). Pratt (1977) and Marmé (1977), in reviews, conclude that the "in vivo" associations may be related to the primary mechanism of phytochrome action, but that definitive evidence on this point does not yet exist.

An interpretation which has become popular amongst many authors in this field is that a specific, membrane-associated, receptor for Pfr exists (e.g. Schäfer, 1975, Steinitz et al., 1976). As Pratt (1977) points out however, three important criteria need to be fulfilled before such an interpretation can be allowed: (a) the interaction must be specific for phytochrome, as opposed to other proteins; (b) the binding affinity must be high and consistent with the known biological activity of phytochrome; and (c) the interaction of Pfr with the putative receptor must be related to the expression of its biological activity. It is difficult to see how these criteria can be experimentally satisfied in an association which probably takes place only *in vivo*; what is needed is an *in vitro* association, free of the artifactual problems outlined by Quail and Gressel (1976), and which may be investigated by the classical techniques of ligand-receptor theory. In this paper, we report a step towards such an *in vitro* phytochrome-membrane association in an experimental system in which R/FR reversibility *in vitro* has already been shown to exist. In addition, the phenomena described here bring together inherently membrane-associated phytochrome with a R-mediated association of phytochrome and membranes.

## Materials and Methods

### Preparation of Plant Material

Seedlings of *Hordeum vulgare* L. cultivar Julia (Stevens, Shardlow, Derbyshire, England) were grown on moist cotton wool at 24°C for 6 days in total darkness. Harvesting, and all subsequent procedures, were carried out under a dim green safe light. Segments of the first leaf were cut 3 cm long, 1 cm from the apex and immediately chilled on ice. All further procedures were carried out at 2–4°C except where stated.

### Cell Fractionation

Partially-purified etioplasts were isolated by a modification of the method of Wellburn and Wellburn (1971). The laminae were finely chopped with a razor blade and gently homogenized in 25 mM N-morpholino-3-propanesulfonic acid (MOPS) containing 3 mM EDTA (disodium salt), 14 mM 2-mercaptoethanol and 250 mM sucrose, adjusted to a final pH of 7.5. A tissue to buffer ratio of 1.2 was used. The homogenate was gently exuded through 12 layers of cheese cloth. The resulting filtrate had a pH of 7.1 which was maintained throughout the isolation procedure. The filtrate was centrifuged at  $6000 \times g$  for 1 min in a Sorvall model RCB-2 Superspeed centrifuge, and the pellet was washed and recentrifuged. The final crude plastid pellet derived from 80 g fresh weight of leaf tissue was resuspended in 4 ml of buffer and loaded onto a loosely-packed Sephadex G-50 (coarse) gel filtration column, 50 cm long and 1.1 cm diameter. Fractions, 0.5 cm<sup>3</sup> in volume, were eluted from the column, and one-drop fractions were taken at intervals for routine measurement on a Unicam SP 1800 ultraviolet spectrophotometer. Fractions composed largely of intact etioplasts (see Evans and Smith, 1976) were pooled and used in the subsequent experiments. Although this procedure does not yield wholly pure etioplasts (Quail, 1977), in our hands the main contaminants are mitochondria which are present in smaller numbers than etioplasts; since etioplasts have an average volume approximately 100–200 times that of mitochondria, the greater part of the G-50 eluate is composed of etioplasts. Virtually no other membranous material is found in this fraction.

### Preparation of Soluble Phytochrome

The first  $6000 \times g$  supernatant from the above procedure was centrifuged at 34,000 rpm ( $100,000 \times g$ ) in the Type 75 Ti rotor of a Beckman L5-65 ultracentrifuge for 60 min, and the supernatant used as "soluble phytochrome".

### Treatments

Preparations were brought to 24°C and, if appropriate, mixed by pipetting particulate suspensions via a wide bore pipette into soluble phytochrome solutions. Usually, 5 cm<sup>3</sup> of particulate suspensions and 10 cm<sup>3</sup> of soluble phytochrome were used. Premixed samples were irradiated for the stated time periods and immediately centrifuged at  $6000 \times g$  for 1 min. When samples were irradiated separately prior to mixing, the mixture was allowed to incubate for 5 min before centrifugation. Pellets were resuspended in 2.5 cm<sup>3</sup> 25 mM MOPS buffer, 14 mM mercaptoethanol, pH 7.5, for phytochrome and protein assays. Where possible 3–5 replicates of each treatment were performed, although in some cases this was not possible within experiments. Repeated experiments gave qualitatively similar results.

### Light Sources

The red light source consisted of four 15 W 'warm white' fluorescent tubes filtered through 1 layer of no. 1 Yellow Cinemoid and 1 layer of no. 14 Ruby Cinemoid (Rank Strand Electric, Kingsway, London, England). The resulting photon fluence rate was 2.3  $\mu\text{mol m}^{-2}\text{s}^{-1}$  at the sample surface. The source of far-red light consisted of four 250 W single coil tungsten bulbs filtered through 10 cm of running water, one layer of no. 5A Deep Orange and one layer of no. 20 Deep Blue Primary Cinemoid. The photon fluence rate at the sample surface was 4.6  $\mu\text{mol m}^{-2}\text{s}^{-1}$ .



### Phytochrome Assay

Total phytochrome was measured at 25° C using a Perkin Elmer 156 Dual Wavelength Spectrophotometer. Calcium carbonate was used to increase effective path length using a plastic cuvette. Particulate preparations were serially diluted such that recorded  $\Delta(\Delta A)$  values were proportional to concentration. Actinic and measuring beams were set at 660 nm and 730 nm. All samples were pre-irradiated with sufficient red light to effect maximum conversion of protochlorophyllide to chlorophyllide prior to measurement. Actinic irradiations were of 60 s duration. The absolute values of  $\Delta(\Delta A)$  were low in these experiments, but the relatively low standard errors illustrate the reliability of the measurements. Specific activities [i.e.  $\Delta(\Delta A) \cdot \text{mg protein}^{-1}$ ] differed by a factor of ca. 2 between samples prepared on different days. This variation is not great for this type of work, but it clearly makes quantitative comparisons *between* experiments impossible, although such comparisons *within* experiments are valid.

### Protein Assays

All samples were precipitated in 10% TCA at 0° C for 30 min, washed, dissolved in NaOH and protein determined by the Lowry et al. (1951) method using bovine serum albumen as a standard.

## Results

### Red-Light Mediated Phytochrome Pelletability in Crude Homogenates

In preliminary experiments, the effects of irradiation of homogenates in vitro on the amounts of phytochrome found associated with a subsequent  $6000 \times g$  pellet were investigated. Table 1 shows that 5 min R irradiation increased by between 60 and 100% the specific activity of the material which pelleted at  $6000 \times g$ . FR given subsequent to the R did not result in any diminution of the R-induced phytochrome pelletability. Purification of pellets from R and dark-treated homogenates gave an approximate 5 fold enrichment of phytochrome and showed that the pellet-

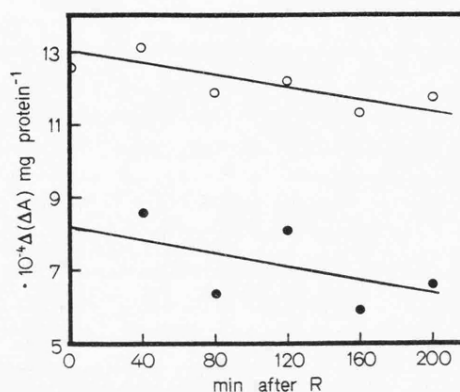


Fig. 1. Time-course of the loss of spectral photo-reversibility in freshly-prepared homogenates of etiolated barley leaves. Open symbols, aliquot given 5 min R and returned to darkness; closed symbols, aliquot maintained throughout in darkness

able phytochrome was associated with the organelles fractionated by this procedure, i.e. etioplasts and mitochondria.

The association of phytochrome with the pelletable material is quite stable, both in the Pr and Pfr form. Figure 1 shows the time course of the loss of spectral photoreversibility in a dark-incubated homogenate, and in a similar homogenate treated with R light and returned to darkness. Loss of total phytochrome follows a similar course in both samples.

### Association of Soluble Phytochrome with Pelletable Material

If the R-mediated increase in pelletability found in crude homogenates represents an association of soluble phytochrome with particulate material, then a similar phenomenon should occur if soluble phytochrome, completely devoid of pelletable material, is

**Table 1.** Light-induced phytochrome pelletability in filtered homogenates of etiolated barley leaves. Homogenates prepared as in Materials and Methods were filtered through Miracloth and aliquots treated as described. Immediately after treatment the preparations were centrifuged at  $6000 \times g$  for 1 min, the pellets resuspended and total phytochrome and protein contents assayed (6 KP). The samples and the resuspended pellets were purified through the G-50 column before phytochrome and protein assays (G-50). Data presented are means  $\pm$  S.E.

| Fraction | Treatment          | Mean<br>$\times 10^{-4} \Delta(\Delta A)$ | Mean<br>protein (mg) | Mean<br>$\times 10^{-4} \Delta(\Delta A) \text{ mg}$<br>protein <sup>-1</sup> |
|----------|--------------------|---|----------------------|---|
| 6 KP     | Dark               | 19 $\pm$ 1.22                             | 3.18 $\pm$ 0.19      | 5.97 $\pm$ 0.67   |
| 6 KP     | 5 min R            | 30 $\pm$ 2.65                             | 3.14 $\pm$ 0.15      | 9.55 $\pm$ 0.52   |
| 6 KP     | 5 min R + 5 min FR | 37 $\pm$ 2.89                             | 3.24 $\pm$ 0.07      | 11.4 $\pm$ 0.66   |
| G-50     | Dark               | 34  | 0.98                 | 34.7  |
| G-50     | 5 min R            | 48  | 0.92                 | 52.2  |

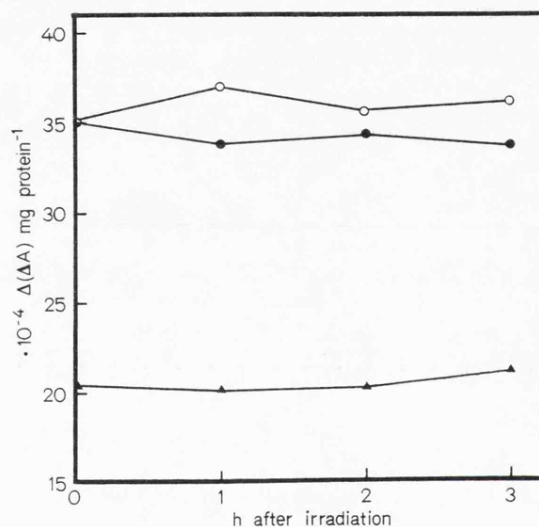
**Table 2.** Light-induced association of soluble phytochrome with partially-purified organelle fraction. Aliquots ( $5 \text{ cm}^3$ ) of organelle fractions purified via the G-50 column were mixed with  $10 \text{ cm}^3$  soluble phytochrome ( $100,000 \times g$  supernatant) (100 KS), raised to  $24^\circ \text{C}$  and subjected to the stated light treatments. Immediately after the treatment the samples were centrifuged at  $6,000 \times g$  and the resuspended pellets assayed for phytochrome and protein. The aliquots of soluble phytochrome contained an average of  $2.02 \text{ mg}$  protein and gave  $0.0113 \Delta(\Delta A)$ , yielding specific activity of  $5.59 \times 10^{-3} \Delta(\Delta A) \text{ mg protein}^{-1}$

| Fraction      | Treatment          | $\times 10^{-4} \Delta(\Delta A)$ | mg protein      | $\times 10^{-4} \Delta(\Delta A) \text{ mg protein}^{-1}$ |
|---------------|--------------------|-----------------------------------|-----------------|---|
| G-50 alone    | Dark               | 14.0                              | 0.92            | 15.2  |
| G-50 + 100 KS | Dark               | $17.0 \pm 1.4$                    | $1.08 \pm 0.17$ | $16.6 \pm 2.1$  |
| G-50 + 100 KS | 5 min R            | $28.3 \pm 2.2$                    | $0.85 \pm 0.10$ | $34.1 \pm 3.2$  |
| G-50 + 100 KS | 5 min R + 5 min FR | $27.0 \pm 1.2$                    | $0.64 \pm 0.05$ | $42.7 \pm 1.5$  |

mixed with purified organelles. This was tested by incubating an aliquot of partially-purified organelles (mainly etioplasts) from the G-50 column with an aliquot of a  $100,000 \times g$  supernatant, which contains substantial amounts of phytochrome but no pelletable material. Table 2 shows that simple mixing of such aliquots in the absence of light treatment does not yield enhanced pelletability. On the other hand, if such mixtures are given R irradiation, the amount of phytochrome found in the subsequent pellet is more than doubled; FR given after R does not diminish the increased pelletability of phytochrome. In a number of control experiments, R-irradiation was found to have no detectable effect on the amounts of pelletable phytochrome in aliquots from the G-50 column; similarly, aliquots of soluble phytochrome did not contain any phytochrome pelletable at  $6000 \times g$  when R-irradiated or incubated in darkness.

#### Effect of Pre-Irradiation on Phytochrome Pelletability

It seems clear from the above that R-irradiation induces soluble phytochrome to associate with the partially-purified organelles, and that photoconversion of associated-phytochrome from Pfr to Pr does not lead to loss of pelletability. Indeed, even when samples are maintained in the Pr state after a R/FR irradiation sequence for several hours, no detectable loss of pelletability was observed (Fig. 2). It was important, therefore, to test whether the association of soluble phytochrome with the pelletable material was dependent on the presence of Pfr in either or both of the components. Table 3 shows data from experiments in which aliquots of soluble phytochrome and partially-purified organelles were treated separately with either R, R followed by FR, or left in darkness, and then mixed and incubated before pelleting. These



**Fig. 2.** Stability of organelle-associated phytochrome in vitro. Organelle samples prepared by the G-50 column technique were either incubated in darkness (▲), given 5 min R and returned to darkness (○), or given 5 min R followed by 5 min FR and returned to darkness (●)

results show that the increase in pelletable phytochrome only occurs if both components are R-irradiated.

Table 4 shows data from separate experiments in which the partially-purified organelles and the soluble phytochrome were subjected to R or R/FR treatment separately prior to mixing and centrifugation. It is clear from these results that the requirement for R-irradiation represents, in fact, a requirement for the presence of Pfr. Thus, the increase in pelletable phytochrome only occurs if Pfr is present in both the partially-purified organelles, and in the soluble phase. Soluble Pfr, therefore, will not pellet with organelles containing only Pr, nor will soluble Pr associate with organelles containing either Pr or Pfr.

**Table 3.** Effect of pre-irradiation on the pelletability of phytochrome in subsequently-mixed preparations of soluble phytochrome and purified organelle fractions. **a** Aliquots of soluble phytochrome (100 KS) and purified organelles (G-50) were given the stated light treatments, immediately mixed, incubated in darkness at 24° C for 5 min, and then centrifuged at 6000 × *g* and the pellets assayed for phytochrome and protein. **b** As a control, an aliquot of soluble phytochrome and one of purified organelles were mixed, given 5 min R and incubated in darkness for 5 min before centrifugation and measurement as in **a**. (Other details as in caption to Table 2)

| Treatments                         |                    | $\times 10^{-4} \Delta(A)$ | mg protein | $\times 10^{-4} \Delta(A) \text{ mg protein}^{-1}$ |
|------------------------------------|--------------------|----------------------------|------------|--|
| G-50                               | 100 KS             |                            |            |  |
| <b>a</b>                           |                    |                            |            |  |
| Dark                               | Dark               | 11                         | 0.53       | 20.8   |
| 5 min R                            | Dark               | 10                         | 0.50       | 20.0   |
| Dark                               | 5 min R            | 11                         | 0.53       | 20.8   |
| 5 min R                            | 5 min R            | 18                         | 0.37       | 48.6   |
| 5 min R + 5 min FR                 | 5 min R + 5 min FR | 10                         | 0.51       | 19.6   |
| <b>b</b>                           |                    |                            |            |  |
| G-50 + 100 KS, mixed, then 5 min R |                    | 22                         | 0.51       | 43.1   |

**Table 4.** The requirement for Pfr in both organelles and the soluble fraction. Details of the experiments are as in the caption to Table 3.

| Treatments         |                    | $\times 10^{-4} \Delta(A)$ |                | mg protein |                  | $\times 10^{-4} \Delta(A) \cdot \text{mg protein}^{-1}$ |                |
|--------------------|--------------------|----------------------------|----------------|------------|------------------|---|----------------|
| G-50               | 100 KS             | samples                    | average        | samples    | average          | samples   | average        |
| 5 min R            | 5 min R            | 11.1                       | $12.2 \pm 0.8$ | 0.265      | $0.27 \pm 0.003$ | 41.7  | $45.3 \pm 3.5$ |
|                    |                    | 14.0                       |                | 0.269      |                  | 52.0  |                |
|                    |                    | 11.6                       |                | 0.275      |                  | 42.3  |                |
| 5 min R + 5 min FR | 5 min R            | 5.3                        | $6.8 \pm 1.2$  | 0.253      | $0.25 \pm 0.017$ | 21.0  | $27.0 \pm 3.5$ |
|                    |                    | 9.1                        |                | 0.276      |                  | 33.1  |                |
|                    |                    | 5.9                        |                | 0.218      |                  | 27.1  |                |
| 5 min R            | 5 min R + 5 min FR | 5.0                        | $5.9 \pm 0.7$  | 0.255      | $0.25 \pm 0.008$ | 19.5  | $23.8 \pm 2.6$ |
|                    |                    | 7.4                        |                | 0.258      |                  | 28.7  |                |
|                    |                    | 5.4                        |                | 0.233      |                  | 23.1  |                |

## Discussion

The results indicate that soluble phytochrome will associate, in vitro, with isolated organelles, as long as Pfr exists in both components. Once associated, the phytochrome remains pelletable for several hours, either in the Pr or principally in the Pfr state. It is premature to attempt to decide whether this R-induced increase in pelletability is representative of the in vivo association described in the Introduction, or whether it is another form of the apparently artifactual in vitro association. What can be stated, however, is that the pelletable material contains very little ribonucleoprotein material (Evans and Smith, 1976; Quail, pers. comm.) and that the R-induced pelletability does not depend on the presence of divalent cations. It seems unlikely, therefore, to be artifactual in the manner described by Quail and Gressel (1976). The G-50 column technique was used here, in spite of its admitted incomplete success in purifying or-

ganelles (Quail, 1977), because it is very rapid, and highly reproducible in our hands and because the fractions so purified display a R/FR mediated response in vitro (Cooke and Saunders, 1975; Evans and Smith, 1976). It is not fully satisfactory, however, and subsequent work will be undertaken with preparations purified in other ways.

The in vitro association of soluble phytochrome to isolated organelle membranes reported here has some similarities with the phenomenon observed by Georgevitch et al. (1977) who showed that purified phytochrome binds to particulate fractions and purified mitochondria from etiolated oat tissues. Although Pfr bound more readily than Pr, Georgevitch et al. (1977) did not test the effects of pre-irradiation in vitro of the binding structures. R-irradiation of oat tissue in vivo yielded particulate fractions which bound lesser amounts of soluble Pfr in vitro, indicating that the binding sites had been saturated in vivo, or immediately upon extraction. In the experiments

reported here with barley preparations, the additional pelletable phytochrome was, in all trials, approximately equal to the amount of phytochrome inherently associated with the organelles. A simple explanation, therefore, would be that Pfr molecules associate to form irreversible dimers. If one of the two phytochrome molecules forming the dimers is a component of an organelle membrane, then subsequent centrifugation would pellet the dimers and enhance the observed pelletability. If such a mechanism does occur, it would seem important to determine whether or not it is involved in the so-called in vivo pelletability described by Pratt (1977) and Marmé (1977).

The in vitro phytochrome pelletability phenomenon described here appears to be largely free of the artifacts described by Quail and Gressel (1976) and thus provides an opportunity to carry out competition-binding experiments, similar to those performed by Georgevitch et al. (1977), to assess the specificity of the association. The use of labelled, purified phytochrome will also allow the stoichiometry, and thus the question of dimer formation, to be assessed, together with an investigation of the kinetics of binding. Finally, the fact that phytochrome modulates a defined response in the isolated organelles, i.e. the levels of extractable gibberellins (Cooke and Saunders, 1975; Evans and Smith, 1976), may provide a basis for relating the characteristics of the phytochrome-membrane interaction to its observed biological effect.

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# The Presence of Phytochrome in Purified Barley Etioplasts and Its In Vitro Regulation of Biologically-Active Gibberellin Levels in Etioplasts

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**Abstract.** Data are presented confirming that purified barley etioplasts contain, or have associated with them, consistently detectable amounts of photoreversible phytochrome. Etioplasts, separated from mitochondrial contamination by sucrose gradient centrifugation, respond in vitro to red light treatment by an increase in the level of extractable gibberellin-like substances. It is concluded that earlier reports of the phytochrome regulation of biologically-active gibberellin levels in crude plastid fractions represent responses of the etioplast alone.

**Key words:** Etioplast – Gibberellin – *Hordeum* – Phytochrome.

theless failed to separate mitochondria from the etioplasts purified by this method. He also suggested that the spectrophotometrically-detectable phytochrome present in G-50 organelle suspensions is present solely as a soluble contaminant and that it is not co-pelletable with the organelles. In view of these comments we have re-examined the previously reported phytochrome-mediated regulation of biologically active GA-like substances in etioplast fractions.

In this communication we present data to show that organelle-associated phytochrome is present in G-50 organelle suspensions and, further, that etioplast fractions free of mitochondrial contamination exhibit a phytochrome-mediated regulation of the levels of gibberellin-like substances extractable into aqueous methanol. Fractions enriched with mitochondria do not.

## Introduction

Cooke et al. (1975) and Evans and Smith (1976a, b) detected phytochrome spectrophotometrically in purified etioplast preparations and Cooke and Saunders (1975), Cooke et al. (1975) and Evans and Smith (1976a) have demonstrated its regulation in vitro of the levels of biologically-active gibberellin-like substances extractable into aqueous methanol. They used modifications of the Wellburn and Wellburn (1971) Sephadex G-50 (coarse) gel filtration technique to isolate etioplast-rich fractions from homogenates of etiolated cereal leaves. In 1977 Quail extensively and critically examined G-50 eluate fractions he obtained from *Avena* shoots using both electron microscopy and marker enzymes. He concluded that the Sephadex G-50 column, despite removing endoplasmic reticulum and other membrane fragments, never-

## Materials and Methods

### Preparation of Plant Material

Seedlings of *Hordeum vulgare* L. cultivar Julia (Rothwell Plant Breeders, Lincoln, England) were grown in moist vermiculite at 24° C for 6 days in total darkness. Harvesting and all subsequent procedures were performed under a green safe-light. Segments of the first leaf were cut 3 cm long, 1 cm from the apex.

### Isolation of Organelle Fractions

(a) *Preparation of the Crude Plastid Pellet.* Barley laminae segments were finely chopped with a razor blade and gently homogenized in a pestle and mortar in 25 mM N-morpholino-3-propanesulphonic acid (MOPS) containing 3 mM EDTA (di-sodium salt), 14 mM 2-mercaptoethanol and 250 mM sucrose, adjusted to a final pH of 7.5. A tissue to buffer ratio of 1:2 was used. The homogenate was gently exuded through 12 layers of cheese cloth and the resulting filtrate had a pH of 7.1 which was maintained throughout the isolation procedure. The filtrate was centrifuged at 6,000 g for 1 min in a Sorvall model RCB-2 Superspeed centrifuge and

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the pellet derived from 80 g fresh weight of leaf tissue was re-suspended in 5 cm<sup>3</sup> initial extraction buffer.

Sucrose solutions subsequently used were made up in extraction buffer minus sucrose.

(b) *Sephadex G-50 (Coarse) Gel Filtration*. A modification of the Wellburn and Wellburn (1971) gel filtration technique described by Evans (1975) was used to isolate etioplasts. In brief, a crude plastid pellet approximately 5 cm<sup>3</sup> in volume was loaded on to a loosely-packed Sephadex G-50 (coarse) gel filtration column, 50 cm long and 1.1 cm diameter. Fractions 0.5 cm<sup>3</sup> in volume were eluted from the column with extraction buffer. Prior to light treatments, the organelle-containing fractions eluting from the column were pooled. The resultant organelle suspension was approximately 9 cm<sup>3</sup> in volume.

(c) *Post G-50 Sucrose Centrifugation*. 1 cm<sup>3</sup> aliquots of the pooled G-50 organelle suspensions were layered on to an equal volume of 20% (w/v) sucrose and centrifuged in an MSE bench centrifuge at approximately 500 g for either 1 or 1.5 min. Following a 1 min centrifugation, the pellets derived from separate aliquots were re-suspended in approximately 9 cm<sup>3</sup> original extraction buffer and used as an etioplast-enriched organelle fraction. The supernatants remaining after a 1.5 min centrifugation were further centrifuged at 27,000 g for 10 min in a Sorvall model RCB-2 Superspeed centrifuge. The pellets were re-suspended in approximately 9 cm<sup>3</sup> of original extraction buffer and used as a mitochondrial-enriched organelle fraction.

(d) *Sucrose Gradient Centrifugation*. A crude plastid pellet approximately 5 cm<sup>3</sup> in volume was layered on to a discontinuous sucrose gradient of 7.5 cm<sup>3</sup> 55% (w/v) sucrose, 12.0 cm<sup>3</sup> 40% (w/v) sucrose and 7.5 cm<sup>3</sup> 25% (w/v) sucrose and centrifuged in an SW 27 swing-out rotor in a Beckman L2-65B ultracentrifuge for 20 min at approximately 9,350 g. Two fractions, those banding at the upper, i.e. the 55/40% interface and the lower, i.e. the 40/25% interface were fractionated from the gradient, diluted with original extraction buffer and subsequently used as the organelle fractions. Each fraction was approximately 9 cm<sup>3</sup> in volume.

#### Light Sources

The red light source consisted of four 15 W 'warm white' fluorescent tubes filtered through 1 layer of no. 1 Yellow Cinemoid and 1 layer of no. 14 Ruby Cinemoid (Rank Strand Electric, Kingsway, London, England). The resulting photon fluence rate was 2.3  $\mu\text{mol m}^{-2}\text{s}^{-1}$  at the sample surface. The source of far-red light consisted of four 250 W single coil tungsten bulbs filtered through 10 cm of running water, 1 layer of no. 5A Deep Orange and 1 layer of no. 20 Deep Blue Primary Cinemoid. The photon fluence rate at the sample surface was 4.6  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Safe lights consisted of one 15 W 50.8 cm fluorescent tube filtered through 2 layers of no. 39 Primary Green Cinemoid.

#### Light Treatments

Organelle suspensions were divided into three aliquots of similar volume and treated separately. One aliquot was irradiated with red light for 5 min and returned to darkness for a further 5 min (RED); another aliquot was irradiated with red light for 5 min and immediately irradiated for a further 5 min with far-red light (RED/FAR RED), and a third aliquot was maintained in darkness for the entire 10 min treatment period (DARK). All treatments were performed at 24° C in a water bath.

#### Carotenoid Assay

Total carotenoids were extracted into acetone to a final concentration of 80% (v/v) and estimated as outlined by Davies (1976).

#### Cytochrome c Oxidase Assay

Cytochrome c oxidase was assayed according to the method of Smith (1955). All fractions to be assayed passed through at least one cycle of freezing and thawing to effect disruption of the mitochondrial membranes.

#### Succinate Dehydrogenase Assay

Succinate dehydrogenase was assayed according to the method of Slater and Bonner (1952).

#### Phytochrome Measurement

Total phytochrome was measured at 25° C using a Perkin Elmer 156 dual wavelength spectrophotometer. Calcium carbonate was used to increase effective path length over a 1 cm path length plastic cuvette. Actinic and measuring beams were set at 660 nm and 730 nm. All samples were pre-irradiated with sufficient red light to effect maximum conversion of protochlorophyllide to chlorophyllide prior to measurement. Sucrose gradient fractions were centrifuged at 27,000 g for 10 min in a Sorvall model RCB-2 Superspeed centrifuge and pellets re-suspended in original extraction buffer in order to obtain similar sucrose concentrations prior to phytochrome measurement.

#### Protein Assay

All samples were precipitated in 10% (w/v) trichloroacetic acid at 0° C for 30 min and centrifuged in an MSE bench centrifuge at approximately 500 g for 5 min. The pellets were washed and re-dissolved in the original volume of 0.1 M NaOH and protein determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

#### Extraction and Fractionation of GA-Like Substances

After light treatment, aliquots of organelle suspensions were immediately homogenized in ice-cold methanol to a final concentration of 80% (v/v). After extraction, the homogenates were centrifuged at 2,000 g for 5 min in an MSE bench centrifuge and the supernatants decanted, reduced to the aqueous phase in vacuo at 35° C and dissolved in 0.5 M phosphate buffer pH 8.5. The solutions were partitioned three times against equal volumes of re-distilled ethyl acetate after which the pH was lowered to 2.5 with 4 M HCl. The pooled organic phases following similar partition against re-distilled ethyl acetate at low pH were reduced to dryness in vacuo and re-dissolved in a small volume of re-distilled ethanol:ethyl acetate, 1:1. Thin-layer chromatography was carried out on 0.25 mm layers of silica gel (Macherey-Nagel and Co., Duren, F.R.G.) using chloroform:ethyl acetate:acetic acid, 60:40:5 as the developing solvent which was allowed to run 10 cm from the point of sample application. After being thoroughly dried, zones scraped from the TLC plates were eluted three times in 0.5 cm<sup>3</sup> re-distilled ethanol:ethyl acetate, 1:1. The pooled eluates were reduced to dryness under a stream of nitrogen and re-dissolved in 1 cm<sup>3</sup> of sterile water prior to bioassay.

### Estimation of GA-Like Activity

GA-like activity was estimated using a modification of the Jones and Varner (1967) barley half-seed bioassay. Half-seeds of *Hordeum vulgare* L. cultivar Himalaya, 1974 (Professor J.D. Maguire, Washington State University, USA) were used. Embryo-less halves of the barley seeds were sterilized for 20 min in 1% (v/v) sodium hypochlorite. At the end of the sterilization period, the grains were washed at least three times with sterile water and transferred to sterile filter papers in sterile petri dishes so that the cut ends of the half seeds were not in contact with the paper nor were they in contact with each other. The half-seeds were imbibed for 48 h at room temperature, ensuring that the filter papers were moist, but not flooded, with sterile water. At the end of the imbibition period 10 sterile half-seeds were transferred to sterile conical flasks containing 0.5 cm<sup>3</sup> sterile 0.01 M, succinate buffer, pH 4.8, 0.5 cm<sup>3</sup> sterile 0.1 M calcium chloride, 1 drop chloramphenicol (0.5 mg cm<sup>-3</sup>) and 1 cm<sup>3</sup> test solution. Conical flasks were incubated in an orbital shaker at 25°C for 24 h at approximately 50 oscillations min<sup>-1</sup>. After incubation the liquid was decanted, the half-seeds rinsed with 2 cm<sup>3</sup> distilled water and the combined solutions centrifuged at 2,000 g for 5 min in an MSE bench centrifuge. The supernatant was used as the enzyme solution and the  $\alpha$ -amylase activity was estimated using starch solution as the substrate and iodine/hydrochloric acid as the terminator of the reaction.  $\alpha$ -amylase activity induced in the half-seeds by the test solution was calculated from the absorbance of the resulting solutions at 620 nm.

## Results

### Characterisation of the Sephadex G-50 (Coarse) Eluate

The Sephadex G-50 (coarse) elution profile of Wellburn and Wellburn (1971) shows three peaks of  $A_{256}$  (Fig. 1). The first is claimed to correspond to intact plastids, the second to intact plastids and mitochondria, and the third to debris and broken plastids. Fig. 2a and b, the Sephadex G-50 elution profiles obtained by Evans and Smith (1976a) and in this

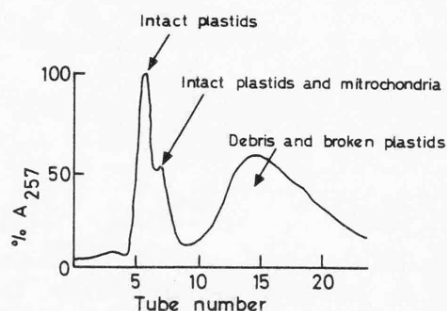


Fig. 1. Elution profile of Sephadex G-50 (coarse) gel filtration column showing three peaks of  $A_{257}$ ; the first claimed to correspond to intact plastids, the second to intact plastids and mitochondria and the third to debris and broken plastids. (After Wellburn and Wellburn 1971)

work show only single elution peaks. Fig. 3 shows the profiles of extractable carotenoids and cytochrome c oxidase/succinate dehydrogenase activities measured through a G-50 column eluate. The carotenoids and the mitochondrial membrane marker enzymes elute together. The percentage recovery of these markers in a pooled G-50 eluate compared to that of the initial crude plastid pellet is shown in Table 1. The data closely agree with those of Quail (1977) and support the conclusion that etioplasts prepared by the G-50 method have a significant level of mitochondrial contamination. Both Evans and Smith (1976a) and Quail (1977) have showed spectrophotometrically-detectable phytochrome to have similar elution characteristics to the single elution peak they obtained from the G-50 column. We have obtained similar results (Fig. 4). However, Quail (1977) was unable to measure phytochrome in a re-suspended organelle pellet, obtained after further low-speed centrifugation of the pooled G-50 organelle fractions and suggested that the phytochrome was present solely as

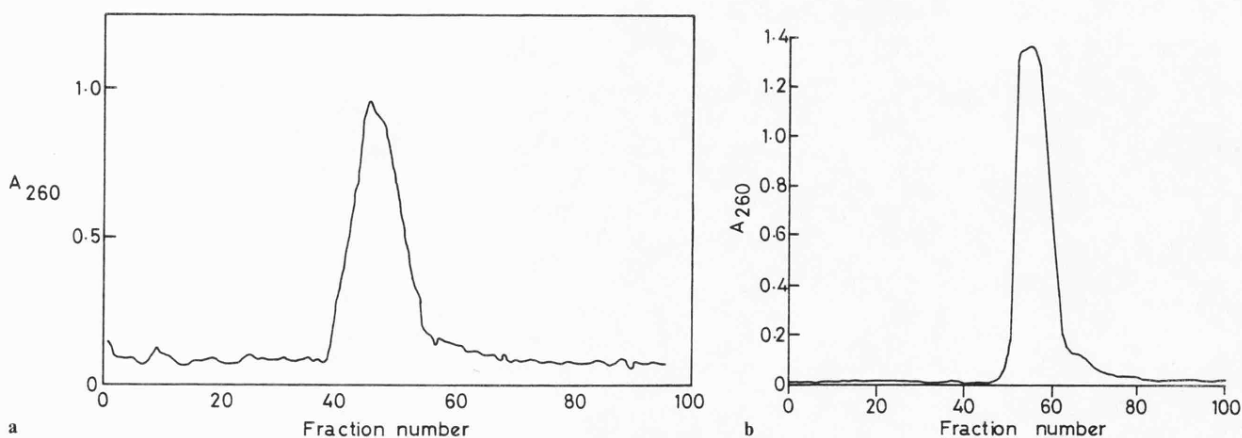
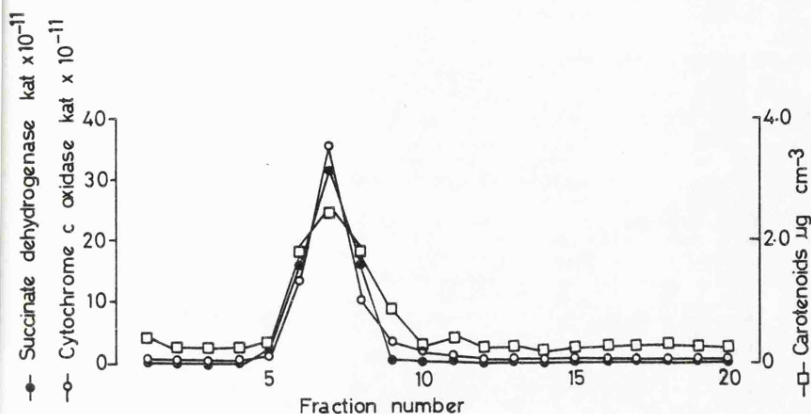


Fig. 2a and b. Elution profiles of Sephadex G-50 (coarse) gel filtration showing single peaks of  $A_{260}$  a after Evans and Smith, 1976a, and b obtained in this work. Each fraction was 0.5 cm<sup>3</sup> in volume

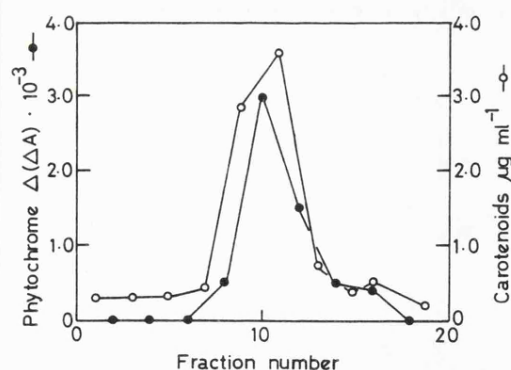




**Fig. 3.** Elution profile of Sephadex G-50 (coarse) gel filtration column using cytochrome c oxidase and succinate dehydrogenase as markers of mitochondrial membranes and carotenoids as a marker of etioplast membranes. Each fraction was 0.5 cm<sup>3</sup> in volume

**Table 1.** Percentage recoveries of etioplast and mitochondrial membrane markers during Sephadex G-50 (coarse) fractionation

|   | Crude plastid pellet | Bulked G-50 fractions | % recovery in G-50 fractions |
|---|----------------------|-----------------------|------------------------------|
| Carotenoids $\mu\text{g cm}^{-3}$                   | 10.5                 | 2.8                   | 27                           |
| Succinate dehydrogenase $\text{kat} \cdot 10^{-10}$ | 179.4                | 63.3                  | 35                           |
| Cytochrome c oxidase $\text{kat} \cdot 10^{-11}$    | 321.4                | 70.3                  | 22                           |



**Fig. 4.** Elution profile of Sephadex G-50 (coarse) gel filtration column showing that spectrophotometrically detectable phytochrome [ $\Delta(\Delta A)_{660/730\text{nm}}$ ] has similar elution characteristics to the etioplast membrane marker, carotenoids. Each fraction was approximately 2 cm<sup>3</sup> in volume

a soluble contaminant running in the void volume of the Sephadex column. In contrast, we were able to measure small, but nevertheless, consistent amounts of phytochrome in both the supernatant [ $20 \cdot 10^{-4} \Delta(\Delta A)$ ] and the organelle-containing fraction [ $15 \cdot 10^{-4} \Delta(\Delta A)$ ] following centrifugation of a pooled G-50 eluate at 6,000 g for 1 min, suggesting that at least part of the G-50 phytochrome is truly organelle-

associated, despite a large proportion of it being non-organelle associated or soluble, as suggested by Quail (1977). Kraack and Spruit (Pers. Comm.) have also been able consistently to measure pelletable or organelle-associated phytochrome in G-50 fractions isolated from maize shoots in complete darkness or under green safelight.

#### Characterisation of the Post G-50 Sucrose Centrifugation Fractions

The two fractions obtained after centrifuging an equal volume of pooled G-50 eluate through 20% sucrose exhibited very distinct characteristics in terms of marker enzymes and of measurable phytochrome. The etioplast-enriched fraction contained 45% of the extractable carotenoids, 12.4% of the total measurable phytochrome [ $5 \cdot 10^{-4} \Delta(\Delta A)$ ] and no cytochrome c oxidase activity, whereas the mitochondrial-enriched fraction contained 14% of the carotenoids, 44% of the measurable phytochrome [ $17.5 \cdot 10^{-4} \Delta(\Delta A)$ ] and 97% of the cytochrome c oxidase activity. We were therefore able to obtain an etioplast suspension containing measurable amounts of phytochrome but no cytochrome c oxidase activity, indicating the absence of mitochondrial contamination, and a further fraction enriched with mitochondria.

#### Characterisation of the Discontinuous Sucrose Gradient Fractions

The elution profile obtained after centrifuging a crude plastid pellet through a discontinuous sucrose gradient (Fig. 5) shows two distinct peaks of extractable carotenoids, one at the 25%/40% interface with high associated cytochrome c oxidase activity and another at the 40%/55% interface with no associated cytochrome c oxidase activity. Figure 6 shows the distribu-

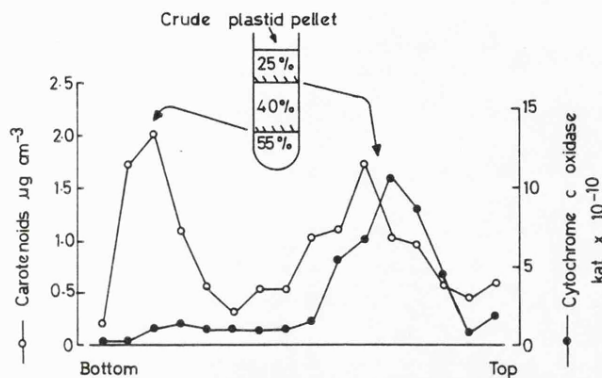


Fig. 5. Description of discontinuous sucrose gradient and the elution profile obtained using cytochrome c oxidase and total extractable carotenoids as markers of the mitochondrial and etioplast membranes respectively. Note the two peaks of extractable carotenoids. The first at the 25/40% interface is associated with a peak of high cytochrome c oxidase activity whereas the second peak has no associated cytochrome c oxidase activity. Each fraction was approximately 2 cm<sup>3</sup> in volume

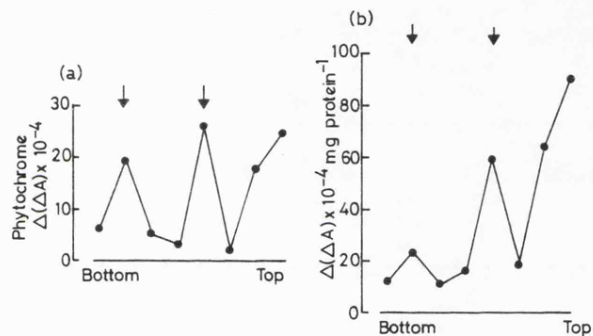


Fig. 6a and b. Elution profile of a discontinuous sucrose gradient in terms of spectrophotometrically-detectable phytochrome (a) and its specific activity (b). Phytochrome is measurable associated with both the organelle fractions despite approximately 40% of the total phytochrome recovered remaining on top of the gradient which is not organelle-associated (i.e. is soluble). The volume of each fraction isolated from the gradient was approximately 4 cm<sup>3</sup>. The arrows indicate the banding positions of the organelle fractions subsequently used in light treatment experiments

tion of phytochrome expressed in absolute units (a) and also specific activity (b) through a sucrose gradient. A large proportion (approximately 40%) of the total phytochrome recovered from the gradient is not organelle-associated (i.e. is soluble) and remains on the top of the gradient. The phytochrome passing into the gradient is associated with both of the organelle fractions, a large amount being associated with the upper banding fraction but a small, consistent, amount being associated with the lower banding fraction.

#### Phytochrome-Mediated Changes in Gibberellin-Like Substances

Figure 7 (a) demonstrates the effects of red and far-red light on the level of biologically-active GA-like

substances extractable from G-50 eluate organelle fractions. The data closely agree with those previously published (Cooke et al., 1975; Cooke and Saunders, 1975; Evans and Smith, 1976a). The level of GA-like activity is substantially increased when the suspensions are irradiated for 5 min with red light but the levels of activity extractable from samples irradiated with far-red light immediately following red light, are similar to those extractable from the dark controls.

Figures 7b and c demonstrate the effects of red and far-red light on the levels of GA-like substances extractable from post-G-50 sucrose centrifugation suspensions. The level of GA-like substances extractable from mitochondrial-enriched fractions following light treatment remains closely similar to that of samples retained in darkness. However the level of GA-like activity extractable from etioplast-enriched samples is substantially increased following light treatment. The red light mediated increase in activity is prevented if the samples are irradiated with far-red light immediately following red light.

The effects of light treatments on the discontinuous sucrose gradient fractions are shown in Fig. 7d and e. Biologically-active GA-like substances extracted from the upper banding fractions are unaffected by light treatments whereas the samples obtained from the 40%/55% interface show enhanced extraction of GA-like substances following a red light treatment. Again, far-red light given immediately after the red light reverses the enhanced bioassayable activity of GA-like substances.

#### Discussion

It is clear from these data and those of Quail (1977) that the eluate obtained from a Sephadex-G-50 (coarse) column is composed of both intact etioplasts and a significant level of mitochondrial contamination. Both Evans (1975) and Quail (1977) observed the complete absence of membrane fragments and vesicles in electron micrographs of G-50 eluate pellets. These suspensions exhibit a phytochrome-mediated regulation of the levels of GA-like substances extractable into aqueous methanol. If purified preparations of etioplasts free of mitochondrial contamination are obtained by further purification of the G-50 eluate, a phytochrome-mediated increase in the level of GA-like substances can again be demonstrated, strongly supporting previously published data. Conversely, fractions enriched with mitochondria exhibit no R/FR reversible increase in extractable GA-like activity following light treatment. Etioplast-enriched fractions obtained from a discontinuous sucrose gradient also show a phytochrome-mediated regulation of biologically-active GA-like substance levels. Again, mito-



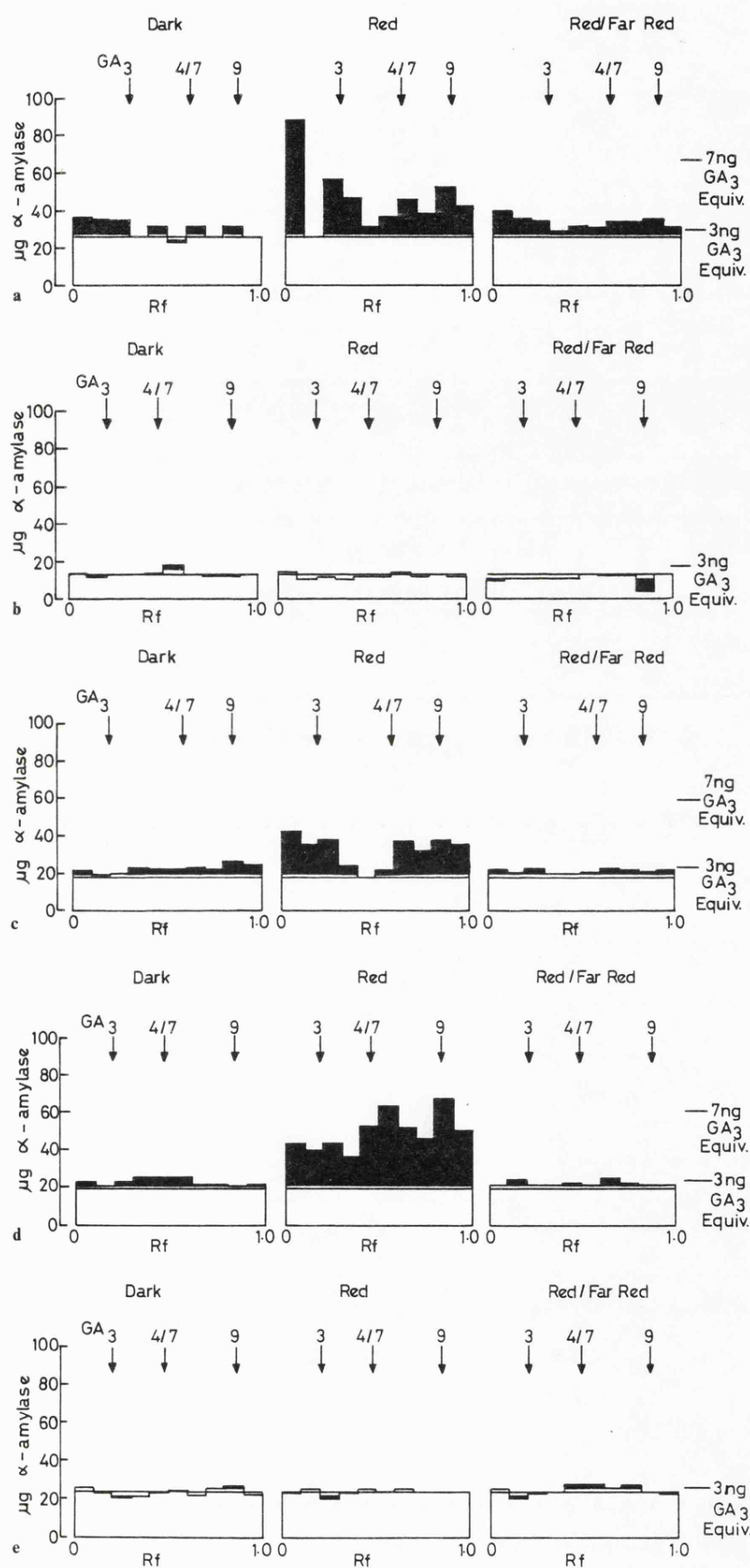


Fig. 7a-e.  $\alpha$ -amylase activity induced in a modification of the Jones and Varner (1967) barley half seed bioassay for  $\text{GA}$ -like substances by thin-layer chromatographed acidic ethyl acetate fractions of organelle suspensions. **a** Bulked G-50 eluate fraction; **b** Post G-50 sucrose centrifugation mitochondrial-enriched fraction; **c** Post G-50 sucrose centrifugation etioplast-enriched fraction; **d** Discontinuous sucrose gradient lower banding fraction; **e** Discontinuous sucrose gradient upper banding fraction.  $\blacksquare$  = significant promotion at the 5% level of probability. Light treatments and all subsequent procedures were performed with aliquots of the same organelle suspensions and are therefore directly comparable.

chondrial-enriched fractions exhibit no such phytochrome-mediated response. These discontinuous gradient fractions are, as yet, uncharacterised in terms of electron microscopy but the data obtained from these fractions are consistent with those obtained from G-50 fractions and substantiate previous reports of an *in vitro* regulation of GA levels by phytochrome in etioplast-rich fractions.

We were able to detect phytochrome in etioplast-enriched fractions from both the post G-50 sucrose centrifugation and the discontinuous sucrose gradient centrifugation procedures. The phytochrome present in these preparations is organelle-associated, as is that present in the pellet when a pooled G-50 eluate is subsequently centrifuged. The absence of membrane fragments and vesicles from the G-50 eluate (Evans, 1975; Quail, 1977) indicates that the phytochrome present in these eluates cannot be a contaminant of the endoplasmic reticulum or the plasmalemma.

With the information available at the present time we are unable to distinguish between phytochrome that may be loosely, or peripherally, and therefore possibly artifactually, associated with the organelles or that which may be intrinsic to the organelle membranes. What is certain, however, is that the photo-conversion of Pr→Pfr in these fractions is associated with increased levels of biologically-active GA-like substances. Thus, active phytochrome must be present in, or on, the etioplasts and mediates a well-defined response *in vitro*. Clearly, of course, it should go without saying that we do not claim that a large proportion of the total cellular phytochrome is associated with the etioplasts.

We have recently shown (Smith et al., 1978) that incubation of G-50 eluate fractions with a soluble phytochrome preparation leads to enhanced levels of phytochrome associated with the organelles following red light treatment. The phytochrome-mediated regulation of biologically-active GA-like substance levels, together with the light-mediated association of soluble phytochrome to etioplasts, may provide an opportu-

nity to study the mode of action of phytochrome in a clearly defined cell free system.

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Phytochrome control of plastid gibberellin levels.

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Previously published work has shown that phytochrome is present in association with etioplast-enriched organelle suspensions and that it mediates in vitro the levels of gibberellin-like substances (GA-LS) extractable from them into aqueous methanol. However, the technique used to isolate the organelle suspensions has recently been critically examined, both biochemically and with the use of the electron microscope and found to purify mitochondria to a similar extent to etioplasts. The validity of these results is therefore questionable.

This thesis presents results of experiments designed to study further the presence of phytochrome in association with etioplast-enriched suspensions and also re-examines the rôle of phytochrome in the mediation of GA-LS levels. When etioplasts are prepared free of mitochondrial contamination (as judged by biochemical marker enzymes) phytochrome remains in association with the etioplasts and also mediates the levels of GA-LS extractable from them. Suspensions enriched with mitochondria also contain spectrophotometrically-detectable phytochrome but exhibit no phytochrome mediation of extractable GA-LS levels.

It was previously hypothesized that photoconversion of etioplast-associated phytochrome to Pfr mediated a change in the permeability of the etioplast envelope membranes with respect to GA-LS thereby allowing an efflux of GA-LS out of the etioplasts into the surrounding medium. The results presented in this thesis show that a large proportion of the red light-mediated increase in GA-LS is retained within the etioplasts. The results suggest, therefore, that phytochrome photoconversion has an effect on the extractability of GA-LS from etioplasts possibly via conformational changes of the etioplast membranes.

Results of preliminary experiments with chloroplast-enriched organelle suspensions are also presented and suggest that phytochrome is detectable in association with chloroplasts (namely the envelope membranes) and that it mediates the levels of GA-LS extractable from them.