

CONTROL OF
MAIZE SEEDLING GROWTH:
LIGHT AND AUXIN

by

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STATEMENT

This thesis describes the results of research work carried out by myself under the supervision of Professor D.J. Carr, Dr. R. Yu and Professor B.E.S. Gunning in the Department of Developmental Biology, Research School of Biological Sciences, the Australian National University, during the tenure of an ANU postgraduate scholarship.

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CONSTITUTION OF THE THESIS

Essentially chapters of this thesis are each composed of a manuscript, which has been published (Chapters 2 and 3), or accepted (Chapters 4 and 6) or submitted (Chapters 5 and 7) for publication under the same title: the venue of publication is indicated on the title page of each chapter. Exceptions are Chapter 1 (Introductory Review) and Chapter 8 (General Discussion), written specially for this thesis.

Modifications to the published or submitted manuscripts have been made, involving additional tables and figures. Because of the different styles of citation of references in different journals, the bibliography is here assembled in unified fashion and presented at the end of the thesis. Abbreviations have similarly been unified and are presented at the beginning.

ABBREVIATIONS

BHT	Butylated hydroxytoluene
4-Cl-IAA	4-Chloroindole-3-acetic acid
FR	Far-red (in tables and figures)
GC-MS	Combined gas chromatography-mass spectrometry
GLC	Gas-liquid chromatography
HFBI	N-Heptafluorobutyrylimidazole
IAA	Indole-3-acetic acid
i.d.	Inner diameter
IPyA	Indole-3-pyruvic acid
IR	Infrared
5-OH-IAA	5-Hydroxyindole-3-acetic acid
Pfr	Phytochrome in the far-red-absorbing form
Pr	Phytochrome in the red-absorbing form
PVP	Polyvinylpyrrolidone
R	Red (in tables and figures)
RH	Relative humidity
SE	Standard error (in tables and figured)
SIM	Selected ion monitoring
TFAA	Trifluoroacetic acid
TLC	Thin-layer chromatography
UV	Ultraviolet

SYNOPSIS

(1) A review of the literature on the physiology of auxin action in cereal seedlings is presented, showing that the roles of endogenous auxin in their growth are quantitatively not well established, and that important aspects remain unresolved. The following aspects of this field were studied in the work described in this thesis:

(2) Two major technical problems in this field were alleviated; i.e. the unsatisfactory nature of conventional "safelights", and methods for estimating and quantifying endogenous auxin, indole-3-acetic acid (IAA). (a) Dim green light, commonly regarded as "safe", was shown to affect the growth of cereal seedlings, and thus is not safe. Instead, long wave infrared (IR) radiation, under which objects can be visualized using a IR-scope, was found to exert no effect on growth of cereal seedlings. (b) The determination of IAA using the fluorescence of its derivative, indolo- α -pyrone, was improved; the improved procedure enables as little as 0.1 ng IAA to be determined in a whole sample. Difficulties associated with the extraction and purification of IAA, such as its decomposition or conversion of indolepyruvic acid to IAA, are described; the improved procedure provides greater than 90% overall recovery.

(3) The status of endogenous IAA in the shoots of 3-day-old maize seedlings was studied quantitatively, confirming that the coleoptile tip produces IAA; other parts of the coleoptile unit (which includes primary leaves and the shoot apex) also produce some IAA. The mesocotyl

receives IAA from the coleoptile unit and its apical growing region depends almost entirely on this unit for its IAA.

The seed and roots are not immediate sources of IAA in the shoot. Some IAA is decomposed in the coleoptile during its downward transport.

(4) It was found that the major effect of red light on IAA status is to inhibit its biosynthesis, not to affect its transport or conjugation. The conversion of applied [^3H]tryptophan to IAA in the coleoptile tip is also inhibited by red light. Photoreversible phytochrome is a photoreceptor for this response, and the concentration of spectrophotometrically measurable Pfr in the tissue reflects the extent of light action. In addition, IAA production is inhibited by fluences of red light which do not induce any detectable Pfr formation; the threshold fluence for this sensitive response is 5 orders of magnitude less than the minimum fluence required for the detection of Pfr. Far-red light can also induce this sensitive response.

(5) Red light stimulates elongation of the coleoptile of intact seedlings and inhibits that of the mesocotyl. The stimulation is most marked in the apical region of the coleoptile and is not due to the control of IAA level, although it may arise from stimulation of proton excretion by coleoptile cells. Inhibition of mesocotyl elongation is explicable in terms of a reduced supply of IAA from the coleoptile unit. However, the mesocotyl of a seedling from which the coleoptile is removed is also inhibited by red light, without relation to IAA level. Cell division in the mesocotyl is also inhibited by red light.

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CHAPTER 1

INTRODUCTORY REVIEW: AUXIN AND GROWTH IN CEREAL SEEDLING SHOOTS

AUXIN AND GROWTH IN CEREAL SEEDLING SHOOTS

Many of the original findings and subsequent developments in the field of auxin physiology involved the use of cereal seedlings. Nevertheless, information on endogenous auxin in cereal seedlings, and its roles in their growth is still conflicting or lacking, and a clear cut picture has not yet emerged.

1. Auxin: Its Discovery and Chemical Nature

The discovery of auxin resulted from studies of the phototropic curvatures of grass or cereal coleoptiles. About a hundred years ago (1880), Charles Darwin published, in his book "The power of movement in plants", experiments which showed that the tip of the coleoptile is the site of light perception for the phototropic curvature of the entire coleoptile, and concluded that "some influence is transmitted from the upper to the lower part, causing the latter to bend".¹ Following the pioneering work of Boysen Jensen (1911, 1913), Paal (1914, 1919), Söding (1923,

¹ In later work, it was shown that the site of perception is not restricted to the tip of the coleoptile, though it is the most sensitive zone; even the basal region of the coleoptile can perceive the phototropic stimulus to some extent (see Curry 1969, Firn and Digby 1980). Thus, to be quite accurate, separation of the sites of perception and response is not complete. However, Darwin's contribution is historically the most important event leading to the discovery of auxin, since his followers believed in the existence of some key influence or substance transmitted in the coleoptile from tip to base.

1925) and others, the nature of the transmitted "influence" in the coleoptile was believed to be that of a growth-promoting substance, and phototropism was explained in terms of an asymmetrical distribution of this hypothetical substance in the coleoptile.

Conclusive demonstration of the existence of the "growth substance" was provided by F.W. Went (1928). Curvature was induced in a decapitated coleoptile, when he placed a small block of agar, which had previously received diffusates from an excised coleoptile tip, on one side of the cut surface of the coleoptile stump. Went's growth substance was named "auxin" (Kögl and Haagen-Smit 1931). Went's other significant contribution was the establishment of a bioassay for the growth substance, known as the Avena curvature test. Subsequent developments in auxin physiology owed a great deal to this specific and sensitive bioassay method (the high reliability of which will be well supported by the findings of this thesis). The historical background to the development of the concept of plant hormones and eventual discovery of the first hormone, auxin, is well described in the classical text of Went and Thimann, "Phytohormones" (1937).

Indole-3-acetic acid (IAA) was identified, first in human urine by Kögl et al (1934) then by Thimann (1935) in Rhizopus suinus cultures, as a compound having auxin activity. The first isolation of IAA from a higher plant source was made by Haagen-Smit et al (1946) using immature maize seeds. Demonstration of the occurrence of IAA in various plants since then has relied mainly on chromato-

graphic separation and bioassay, but modern physicochemical methods demonstrate it unequivocally (see Bearder 1980).

Compounds other than IAA with auxin activity² have also been shown to occur in higher plants. Chloro-substituted IAA was isolated from immature pea seeds first as the methyl ester (Gandar and Nitsch 1967, Marumo et al 1968a), then as the free acid (Marumo et al 1968b). The chlorine atom was shown to be at the 4-position (Marumo et al 1968a). This compound (4-Cl-IAA) is of special interest because of its high auxin activity (Porter and Thimann 1965). However, its occurrence in plants other than peas has yet to be shown. Engvild et al (1978) developed an identification procedure for 4-Cl-IAA methyl ester using GC-MS, and showed its applicability to immature pea seeds. Use of the method may show the existence of 4-Cl-IAA in a wide variety of plants. Other compounds shown to present in plants are indole-3-propionic acid, indole-3-butyric acid

² The term "auxin" was originally applied to compounds active in the Avena curvature test. However, other bioassays have also been developed since then, and the term "auxin" has been extended to compounds active in these bioassays. Among the bioassays, the slit pea stem test and the straight growth test using Avena or wheat coleoptile segments have found popular usage. It must be borne in mind, however, that a compound active in one assay is not necessarily active in another. The Avena curvature test is especially selective, and in fact many compounds active in other assays are inactive in the curvature test. This is probably because of a clear separation in the curvature test of the sites of application and detection (requiring basipetal and probably polar transport), and a short test time (which necessitates rapid uptake and translocation, and reduces the chance of metabolites of the test compound being active). The best qualification for the application of the term to a compound, especially if chemically unidentified, is that it is active in the curvature test.

and phenylacetic acid (see Schneider and Wightman 1978 for references). Identification of these compounds is based on auxin activity on paper chromatograms and retention times on GLC; their unequivocal characterisation by MS is still awaited.

Apparently IAA is widely distributed in higher plants. It is perhaps the most important auxin in natural plant growth, considering also its specific effects on various morphogenetic processes. The significance of other auxin compounds, known to occur in plants, in natural plant growth is still a task for future study.³

2. Auxin in Cereal Seedling Shoots

Chemical nature. Much attention has been paid to elucidation of the chemical nature of Went's auxin, which diffuses out of the coleoptile tip. Paper or thin-layer chromatographic separation of diffusates from oat or maize coleoptile tips using various solvent systems, followed by bioassay using the Avena curvature test and/or the Avena section test, showed a active zone at the Rf of IAA (Ramshorn 1955, Raadts and Söding 1957, Shibaoka and

³ Use of "auxin" and "IAA" in the thesis: "IAA" is used only for authentic IAA or endogenous IAA after some kind of chemical identification. Even if identification is based only on chromatographic separation and bioassay, "IAA" is used, but with qualification. When there is no attempt at chromatographic separation, such as auxin in tissue diffusates analyzed directly by a bioassay, "auxin" is used. "Auxin" is also used as a generic term for a group of substances with auxin activities.

Yamaki 1959, Bohling 1959, Shen-Miller and Gordon 1966b, Ohwaki 1970b, Greenwood et al 1972). However, activity was not restricted to the zone at the Rf of IAA and one or more active zones well separated from IAA, were found on chromatograms (Ramshorn 1955, Raadts and Söding 1957, Shen Miller and Gordon 1966, Ohwaki 1970b). Shen-Miller and Gordon (1966) and Ohwaki (1970b) detected one or more active zones with the Avena section test, but only one of them, with an Rf corresponding to that of IAA, was active with the Avena curvature test. Shibaoka and Yamaki (1959) also could detect only one zone by the curvature test. Nevertheless, there is no unanimity of opinion and at least one zone or compound other than IAA has been shown to be active with the curvature test (Raadts and Söding 1957, Kuraishi and Muir 1964). The chemical nature of these diffusible non-IAA substances active as auxin is not known. Shen-Miller and Gordon (1966b) showed that one of the non-IAA substances, called auxin P, is converted to IAA by heating. Raadts and Söding (1957) suggested that one non-IAA substance, active in the curvature test, might be an IAA derivative, neutral in nature. Recent use of GC-MS has demonstrated the occurrence of IAA in diffusates from maize coleoptile tips (Greenwood et al 1972). IAA appears to be at least a component, if not the whole, of the auxin which diffuses out of the coleoptile tip. The possibility of the presence of other auxin compounds in coleoptile diffusates should be the subject of future studies. At least the non-IAA compound which is active in the curvature test is likely to be transportable in tissue and it could be of significance in

growth. It is possible, however, that the non-IAA substances are active only after conversion to IAA in the tissue.

Using GC-MS, Bandurski and Shulze (1974) have identified IAA in solvent extracts of oat and maize seedling shoots. IAA has also been identified by GC-MS in maize root extracts (Bridges et al 1973, Elliot and Greenwood 1974, Rivier and Pilet 1974). There seems no doubt that IAA is an endogenous auxin of cereal seedlings.

Distribution and concentration. Thimann (1934) investigated the distribution of chloroform-extractable auxin along the oat seedling. Auxin was shown to be present throughout the seedling. In the shoot, the content of the tip of the coleoptile was the highest and the level declined basipetally. A similar result was obtained by Wildman and Bonner (1948), who used ether to extract auxin. Bandurski and Schulze (1974) estimated IAA concentrations of maize and oat seedling shoots (5 days old) to be 24 and 16 ng/g fresh weight, respectively.

Site of production and its precursor(s). Perhaps the most important question is whether the tip of the coleoptile really produces auxin. Despite its classical background, this question remains unresolved.

It was shown quite early that the amount of diffusible auxin obtained from the coleoptile tip is larger than that which can be obtained from a freshly excised tip by solvent extraction (Thimann 1934, van Overbeek 1941, Wildman and

Bonner 1948). Isotopically labelled tryptophan, logically the most likely IAA precursor in its chemical structure and universal occurrence in plants, has been shown to be converted to IAA by excised oat or maize coleoptiles (Libbert and Silhengst 1970, Kutáček and Kefeli 1970, Black and Hamilton 1971, Erdmann and Schiewer 1971, Heerkloss and Libbert 1976). Supplied tryptophan also enhanced the amount of diffusible IAA obtained from the excised coleoptile tip (Muir and Chang 1974). Although supplied tryptophan produced little effect on the growth of the coleoptile, tryptamine stimulated it considerably (Winter 1966, Thimann and Grochowska 1968). There is thus some controversy as far as the nature of the precursor is concerned, but these results strongly support the classical view of auxin production at the tip.

It was an early suggestion that the auxin of the coleoptile tip comes from the seed (Pohl 1936), and Sheldrake (1973) has strongly supported this hypothesis. He regarded earlier quantitative analyses of auxin, mentioned above, as unreliable, and the demonstration by Skoog (1937) that there is no acropetal supply of auxin as inconclusive. Sheldrake's main evidence in support of the hypothesis was that guttation fluid collected from the decapitated coleoptile contains auxin, and that labelled IAA, applied to the endosperm or cut seedling roots, can move acropetally and accumulate at the tip of the coleoptile. Hall and Bandurski (1978) have shown that IAA can move from the endosperm of the seed to the shoot, although they regarded its amount as inadequate to maintain

IAA levels in the shoot.

Bandurski and co-workers have recently developed a hypothesis that IAA conjugates, which are contained in maize seeds in large amounts (Ueda and Bandurski 1969, Percival and Bandurski 1976, Bandurski and Schulze 1977), are transported into shoots and there serve as major sources of free IAA. Their main evidence is (a) that alkali-labile IAA conjugates (mostly esters) are contained in maize seedling shoots (Bandurski and Schulze 1974), (b) that isotopically labelled indole-3-acetyl-myo-inositol, the main IAA ester in maize seeds, is transported into the shoot when supplied to the cut surface of the endosperm (Epstein et al 1980, Nowachi and Bandurski 1980), and (c) that the supply of the ester to the shoot, calculated from the results of the feeding experiment, is adequate to maintain the IAA production at the coleoptile tip (Nowacki and Bandurski 1980). This hypothesis is not in disagreement with the classical assumption that auxin is produced at the coleoptile tip, but disagrees with the view that tryptophan or tryptamine is its precursor.

All the evidence obtained in precursor studies necessarily results from feeding experiments. Probably, the most straightforward approach to the problem of the site and mode of auxin production would be to reinvestigate the original claim that the amount of diffusible IAA from the excised tip is larger than that of solvent extractable IAA, using a reliable determination method; the amount of conjugated IAA in the tip must also be taken into account as a potential source of diffusible IAA.

Assuming the coleoptile tip to be producing IAA, whether biosynthesized from precursors or released from its conjugates, there still remains a question: is the coleoptile tip the only site of IAA production in the coleoptile or the shoot? Since it is important to know the site and the mode of auxin production in considering the auxin economy, or its role in growth, of the shoot, these problems must be settled sooner or later.

Metabolic fate. It has been suggested that [1-¹⁴C]IAA is decarboxylated in excised coleoptile segments during its basipetal transport (Goldsmith and Thimann 1961). Decarboxylation was also observed following application of [1-¹⁴C]IAA to intact coleoptiles (Menschick et al 1977). Crude, cell-free preparations of maize coleoptiles have been shown to be able to convert added IAA to 3-hydroxymethyloxindole and 3-methylenoxindole (Harger and Schmidt 1968). The natural occurrence of these and other possible oxidative products of IAA (see Sembdner et al 1980) in the coleoptile has yet to be shown.

The enzymatic oxidation of IAA is probably important in the regulation of IAA levels in tissue. It has been suggested that products of IAA metabolism, especially 3-methoxindole, are more important for growth than IAA itself, but such a view has not been supported in later work (see Sembdner et al 1980).

The content of IAA conjugates in maize or oat shoots, already mentioned, may be the result of IAA conjugation in the shoots themselves. This has not been the subject of

experimentation. There is, however, evidence that maize seeds contain enzymes which form esters of IAA with myo-inositol and glucose (Kopcewicz et al 1974).

Transport. The transport properties of auxin have been extensively studied using isolated segments of coleoptiles and donor and receiver blocks of agar (see Goldsmith 1969). It was an early and significant finding that auxin transport in this system is predominantly basipetal (Went 1928, van der Weij 1932, 1934), and thus polar in nature. Polarity of auxin transport applies more or less to most other stem or root tissues studied (see Goldsmith 1969, Schneider and Wightman 1978 for references). The velocity of IAA transport in the coleoptile has also been studied using a similar experimental system. For example, Goldsmith (1967) showed that in maize coleoptiles, the velocity is about 12-15 mm/h.

The question is to what extent polar auxin transport, clearly evident in isolated coleoptile segments, represents the movement in situ of endogenous auxin. Skoog (1937)⁴ could not detect endogenous auxin in agar which had been placed on the cut surface of a decapitated oat coleoptile, even when the seedling was otherwise intact. It can be

⁴ Skoog used his deseeded Avena curvature test, which is about 10 times as sensitive as the standard method. When an agar block which had received diffusates from the apical cut surface of the decapitated coleoptile was placed on the test plant, no curvature was induced within 6 h, but some was induced after about 10 h. Skoog concluded that the late effect was due to conversion of auxin precursors, since if the amount of auxin supplied is too low to induce curvature within 6 h, it does not occur at any time later.

suggested that the movement of endogenous auxin in the coleoptile is also polar and basipetal. Since, however, there is a possibility that auxin can move in the xylem (Sheldrake 1973), acropetal transport of IAA in the coleoptile of an intact seedling cannot be excluded. The problem of transport of endogenous auxin is closely related to that of the production site of auxin, already discussed. Movement of endogenous auxin in a whole plant must be understood as a process of redistribution of auxin produced at a certain place or as a supply of auxin from a certain pool.

3. Roles of Endogenous Auxin in Growth of Cereal Seedling Shoots.

Straight growth of the coleoptile. In pioneering work, Söding (1923, 1925) showed that decapitation of the coleoptile inhibits its longitudinal growth, and replacement of the tip restores its growth considerably. After the discovery of auxin as a growth substance diffusing out of the coleoptile tip, it was shown that inhibition of coleoptile growth following decapitation is reversed by application of auxin to the cut surface (Nielsen 1930). Thimann and Bonner (1933) further showed that the relationship between the concentration of auxin⁵ in agar applied to the cut surface and the growth increment of the

⁵ Auxin was obtained from cultures of Rhizopus suinus, and the concentration was expressed in 'plant units'.

decapitated coleoptile is linear. An increase in the growth rate of the maize coleoptile during early seedling development has been shown to be accompanied by an increase in the yield of diffusible auxin which can be collected from the coleoptile tip (van Overbeek 1938, Briggs 1963). These results support the general conclusion that longitudinal growth of the coleoptile is controlled by auxin supplied by its tip.

This view can also be supported by the phenomenon called "regeneration of the physiological tip". When the tip of the coleoptile is removed the growth of the coleoptile stump is inhibited as already stated, but after a few hours growth resumes and the growth rate is restored to a considerable extent (Dolk 1926). This increase is eliminated by a second decapitation (Dolk 1936). After decapitation the yield of diffusible auxin from the apical part of the coleoptile stump also increases after a few hours (van Overbeek 1941). Thus, in the decapitated coleoptile the apical region of the stump starts to act like the tip of an intact coleoptile and the phenomenon of resumption of growth is explicable in terms of regenerated auxin production.

Curvature of the coleoptile. Auxin-growth relationships have been quite extensively studied in the curvature responses of the coleoptile, i.e. negative-geotropism and positive-phototropism. These tropic responses have long been considered to be due to unequal distribution of auxin induced by gravity or unilateral

illumination. This view is known as the Cholodny-Went theory, following the early work and specific proposals of its two authors (see Went and Thimann 1937).

It has been shown that in the horizontally oriented, excised coleoptile tip of oat or maize, the lower half of the cut surface gives off more auxin than the upper half (Dolk 1936, Gillespie and Briggs 1961). Similar differential yields of auxin were also observed from the lower and the upper halves of the basal cut surface of horizontally oriented, subapical coleoptile segments supplied with auxin at the apical cut surface (Dolk 1936). The latter was further confirmed in similar experiments, using labelled IAA (Gillespie and Thimann 1963). Using labelled IAA, it has also been shown that the lower half of the tissue contains more IAA than the upper half (Gillespie and Thimann 1963, Hild and Hertel 1972). The Cholodny-Went theory is thus well supported in investigations of the geotropic response of the coleoptile. Detailed studies by Gillespie and Briggs (1961) showed that the difference in the diffusible auxin yield was induced without change in the net yield (i.e. the net yield was the same whether the tip is oriented vertically or horizontally) and totally split upper and lower halves of the horizontally oriented coleoptile tip gave off the same amounts of diffusible auxin. These results rule out the possibility that the production or the basipetal transport of auxin is influenced by gravity, but strongly support the view that lateral movement in the direction of gravity is induced. The same conclusions were reached using labelled IAA (Gillespie and

Thimann 1963, Shaw et al 1973).

In experiments on the time course of geotropic curvature of the coleoptile, it has been shown that immediately after the horizontal orientation a slight positive geotropic curvature begins, and then a strong negative curvature commences 15 to 30 min afterwards (Brauner and Zipperer 1961, Hild and Hertel 1972). These two phases of curvature were shown to be correlated positively with the course of redistribution of applied labelled IAA (Hild and Hertel 1972).

In the phototropic response, the Cholodny-Went theory was essentially supported by assays of diffusible auxin (Went 1928, Boysen Jensen 1928, van Overbeek 1933, Briggs et al 1957, Briggs 1963b) and experiments using applied, labelled IAA (Pickard and Thimann 1964, Shen-Miller and Gordon 1966, Gardner et al 1974). The experimental methods are essentially the same as those used in studying the geotropic response. In both system I (first positive) and system III (second positive) phototropic responses (see Briggs 1963c) of maize coleoptiles, it was shown that the effect of unilateral illumination is to induce lateral movement of diffusible auxin toward the shaded side, not to inhibit basipetal transport or to induce photo-oxidation of auxin in the illuminated side (Briggs 1963b). This was essentially supported by Gardner et al (1974). Using micropipettes, they applied high specific activity [^3H]IAA at the maize coleoptile tip as a small spot, and showed its translocation toward the shaded side without inhibition of basipetal transport. However, experiments on oat

coleoptiles presented some complications, and inhibition of IAA transport was also found at the illuminated side in the system I response and at both sides in the system III response.

In the above experiments, the situation appears simpler for maize coleoptiles. However, even with maize coleoptiles, inhibition by light of downward transport of auxin was claimed by Shen-Miller and Gordon (1966a). They obtained lower yields of diffusible auxin from the illuminated side, but the IAA content, estimated by the Avena curvature test after paper chromatographic separation of tissue extracts, was greater in the illuminated side than in the shaded side. They concluded that the effect of unilateral illumination is to inhibit auxin transport at the illuminated side. Shen-Miller et al (1969) have further supported this possibility by showing that downward transport of ^{14}C -IAA applied to the coleoptile tips of intact seedlings is inhibited by equilateral illumination, and that the fluence-response relationships and the action spectrum of this inhibition resemble those for the phototropic response (system I). Light-induced inhibition of IAA transport through excised maize coleoptile segments has also been reported (Naqvi and Gordon 1967, Harger and Schmidt 1968). Harger and Schmidt (1968) showed that the transport inhibition is induced without change in the velocity.

The effect of unilateral illumination in producing differential yields of diffusible auxin from the illuminated and shaded sides has been confirmed. However, the

experimental evidence concerning the mechanism is contradictory. We can perhaps exclude participation of light effects on auxin biosynthesis and degradation in the induction of the phototropic responses of the coleoptile. The insignificance of IAA degradation was further attested by Menschick et al (1977) who could see no effect of blue or white light on in vivo decarboxylation of [^{14}C]IAA applied to intact oat coleoptiles. Mechanisms involving auxin transport are those principally supported, giving rise to three possibilities: (1) induction of lateral transport without change in basipetal transport, (2) inhibition of basipetal transport at the shaded side without change in lateral transport, and (3) migration of auxin to the shaded side as a result of inhibition of basipetal transport at the illuminated side. Further information should be sought in investigations of endogenous auxin distribution in tissue under phototropic stimulation.

The recent reviews by Firn and Digby (1976, 1980) questioned the validity of the Cholodny-Went theory. However, the data alleged to contradict the hypothesis may not be valid. Firn and Digby consider especially that the concentration differences induced in the coleoptile by a geotropic or a phototropic stimulus are insufficient to account for the differential growth of the two sides of the coleoptile, on the ground that the effect of IAA applied in the incubation medium on the elongation of excised coleoptile segments, or other tissues, is linear to the log of concentration. However, it had earlier been shown by Thimann and Bonner (1933) that when auxin is applied only to

the cut surface of the decapitated coleoptile, the elongation effect is linear to concentration (not to log concentration). It appears that the quantitative growth promoting effect of IAA differs with mode of application, and Thimann and Bonner's method is probably closer to the intact situation. Thus, the differential distribution of auxin so far observed may be sufficient to induce differential growth. Moreover, since the site of perception of the geotropic or phototropic stimulus is not restricted to the tip of the coleoptile, as Firn and Digby themselves emphasize, the differential distribution so far observed using isolated tips or segments of the coleoptile may be amplified during the long traverse of auxin transported in the intact coleoptile. Firn and Digby also make the point that even the shortest lag time so far observed in the induction of auxin redistribution does not sufficiently precede onset of the geotropic curvature, on the grounds that induction of growth in segments incubated in IAA solution has at least a 10 min lag. However, one can confidently expect the lag time of the action of endogenous IAA to be shorter than that observed with applied IAA.

What we learn from Firn and Digby's reviews is that the hypothesis based on the redistribution of auxin has not yet been conclusively demonstrated. In the near future, kinetic studies of auxin redistribution at short intervals during and following geotropic or phototropic stimulation should be performed. Determinations of the tissue content of endogenous IAA at a given instant, using modern unequivocal and sensitive physicochemical determination methods, are

called for. The situation we face is perhaps summarised in the conclusion of Gardner et al (1974): "Although our study shows unequivocally that lateral transport of IAA occurs in phototropically stimulated coleoptiles of Zea and Avena, the extent to which it is involved in the induction of differential growth, and hence phototropic curvature, has yet to be established. Caution must be exercised in building up a general mechanism for phototropic curvature since different species may utilize different mechanisms to different extents, and the same species may use different mechanisms under different conditions."

Growth of the mesocotyl. The classical assumption that growth of the coleoptile is controlled by auxin produced by its tip has further been extended to growth of the mesocotyl (or first internode) which is located below the coleoptile being separated by a node. Went (1928, p 76) had already outlined the possibility in his early report: "---- das Auswachsen des Mesokotyls bedingt wird durch die Menge des Wuchsstoffes, die dorthin gelangt. Jede Verringerung der Wuchsstoffmenge wird also sein Auswachsen mehr oder weniger verhindern. So kann auch die Wirkung der Belichtung der keimenden Samen vielleicht in dieser Weise erklärt werden, sowie auch die Folge der Dekapitation (Beyer 1927)."⁶

6 "---- the elongation of the mesocotyl is determined by the quantity of growth substance which reaches it (from the coleoptile). Any decrease in the amount of growth substance will more or less reduce its elongation. Perhaps the effect of illuminating the seedling can be thus explained, as also the effect of decapitation (Beyer 1927)."

Van Overbeek has extensively studied the relationships between mesocotyl growth and auxin supply from the coleoptile. He found (1936) that treatments which cause growth inhibition of the oat mesocotyl, such as exposure of the seedlings to heat or light, reduced the yield of diffusible auxin from the coleoptile tip. Using dwarf maize, which produces a short mesocotyl, he also showed (1935, 1938) that the amount of diffusible auxin obtained from the coleoptile tip or the base of the coleoptile is smaller than that from normal maize.

The growth-auxin relationships of the mesocotyl have been studied in relation to light effects in some detail. Following early observations of Beyer (1927) and others that growth of the mesocotyl could largely be suppressed by raising cereal seedlings in the light, or by illuminating them at an early stage in germination, it was shown that red light is most effective (Johnston 1937, Goodwin 1941, Weintraub and McAlister 1942). After the discovery of phytochrome as a red-sensitive photoreversible pigment involved in photomorphogenetic responses, evidence was forthcoming for the participation of this pigment in the light inhibition of mesocotyl growth (Edwards et al 1964, Loercher 1966, Duke et al 1977). Red light has also been shown to inhibit the amount of diffusible auxin obtained both from the coleoptile tip (Briggs 1963 a, Furuya et al 1969, Muir and Chang 1974, Rajagopal and Bulard 1975, Huisinga 1976) and from the base of the coleoptile (Kondo et al 1969); the involvement of the phytochrome system has also been shown in red/far-red reversibility of the response

(Furuya et al 1969, Rajagopal and Bulard 1975). Applied IAA could reverse the light effect (Inge and Loomis 1937, Vanderhoef and Briggs 1978). These results support the view expressed by Went (1928) and van Overbeek (1936) that light inhibits mesocotyl growth by reducing the supply of auxin from the coleoptile.

The hypothesis that auxin participates in mesocotyl growth, however, has not always found support. Schneider (1941) has shown that red light can inhibit growth of even excised mesocotyl segments, and applied IAA does not remove the inhibition. Thus, red light appeared able to inhibit mesocotyl growth directly and without the intervention of the coleoptile. The auxin hypothesis was also contradicted by Mer (1951), who claimed that removal of the oat coleoptile tip followed by successive decapitation of the coleoptile stump (to remove the regenerated physiological tip) did not lead to inhibition of mesocotyl growth. He presented (1951, 1969, 1972) many other experimental results purporting to contradict the auxin hypothesis. Dattaray and Mer (1964) have also shown that heat or light treatment which causes inhibition of mesocotyl growth does not reduce the auxin content of the mesocotyl.

Detailed studies of light effects on the mesocotyl have revealed the complexity of the response. This was indicated in the discontinuity of the dose-response curve (Blaauw et al 1968, Mandoli and Briggs 1981), and a multi-peaked action spectrum in the red region (Goodwin and Owens 1948, Vanderhoef et al 1979). It is suggested that the response is mediated by multiple systems. It is of interest to study

the involvement of auxin in mesocotyl growth in the light of these complex light-growth relationships. It is also of special interest to examine, in relation to auxin status, the very sensitive red light effect on mesocotyl growth, which has been shown to be more sensitive than the classical red/far-red reversible phytochrome response by about 4 orders of magnitude (Blaauw et al 1968, Mandoli and Briggs 1981).

Finally, further healthy development in the field of auxin physiology in relation to natural plant growth must be firmly based on the sort of information discussed in the previous section, i.e. a knowledge of where auxin is produced or pooled and how and where it is distributed in plants. Perhaps it is now time to go back to first principles and try to reconstruct the field on these firm bases. Adequate technologies for such studies are just beginning to be available.

In the present thesis, the status of endogenous IAA in maize seedling shoots has been quantitatively investigated, particularly its production and redistribution. Changes in the status of IAA have then been investigated in relation to the effects of light on growth of the seedling shoot.

CHAPTER 2

SAFELIGHT FOR PHOTOMORPHOGENETIC STUDIES:
INFRARED RADIATION AND INFRARED-SCOPE

IINO, M., CARR, D.J. (1981) PLANT SCI. LETTERS, IN PRESS.

SUMMARY

Green light affects the growth of etiolated cereal seedlings. Green "safelights" are therefore not safe for photomorphogenetic studies. Invisible infrared radiation, in which objects can be viewed using an infrared-scope, is proposed as a safelight. The infrared radiation exerts no measurable effect on the growth of cereal seedlings.

INTRODUCTION

In the field of photomorphogenesis, it is common practice to handle or treat dark-grown seedlings in green light, regarded as "safe" in that it is ineffective in phototropism, or in inducing a detectable transformation of phytochrome from the red-absorbing form to the far-red-absorbing form. Irrespective of such common use of green light, responses of plants to low irradiance green light have long been recognized (Huisinga 1964, Mer 1966, Blaauw et al 1968, Hartmann 1977, Mandoli and Briggs 1981). Blaauw et al. (1968) found that very low fluence of red light can alter the growth of coleoptile and mesocotyl of dark-grown intact oat seedlings, the former being stimulated and the latter inhibited (threshold at about 1 nWs/cm² in both responses). Another significant observation was that even green light of relatively low fluence can induce the same responses. At 519 nm the threshold was around 0.1 μ Ws/cm². These effects of light, including green, of low

fluences on oat seedlings have recently been confirmed by Mandoli and Briggs (1981). Thona and Hartmann (cited in Hartmann 1977 as unpublished data) showed that the threshold for production of anthocyanin in mustard seedlings in response to red light is increased considerably by preirradiation with $23 \mu\text{Ws}/\text{cm}^2$ of green light (519 nm). Since the lowest intensity of green light in which one can work comfortably is around $0.1 \mu\text{W}/\text{cm}^2$, and work is impracticable in intensities lower than $0.01 \mu\text{W}/\text{cm}^2$, these observations suggest that even the best green safelight may not be really safe. In the present work, the effect of green light whose purity has been checked is re-examined. Data given by Goodwin (1941) show that 5 min exposure to IR (above 800 nm) at $2.7 \mu\text{W}/\text{cm}^2$ has no effect on oat mesocotyl elongation. Work on the control of growth in maize seedlings (Chapters 4 to 7) led to the exploration of the use of invisible IR radiation, in which objects can be visualized using an IR scope, as an alternative safelight. The safety of IR radiation whose fluence rate is sufficient to enable an IR-scope to be used is described in the present report. In pioneering work nearly 30 years ago, Mer (1954) used an infra-red image converter, an early form of IR-scope, for the same purpose. The deficiencies of the instrument available at that time, however, made it impracticable and it found no further use in photomorphogenetic studies on plants.

MATERIALS AND METHODS

Light from a green fluorescent tube (Philips, TL/17) was passed through filters consisting of one layer of No. 1 Cinemoid, one layer of No. 20 Cinemoid and 1 cm-thick copper sulfate solution (10% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, W/V) to provide green light. The copper sulphate solution, which absorbs in the far-red range, was placed between sheets of clear acrylic 02 (Cadillac Plastics) 4 mm thick. The spectral energy distribution of the green light, measured from 300 to 800 nm using a spectroradiometer (IL780 Spectroradiometer System, International Light), is given in Fig. 1. The source of IR radiation was a 15 W tungsten lamp enclosed in layers of Cinemoid, 3 layers of No. 1, 2 layers of No. 14 and 6 layers of No. 20. The filter combination allowed no spectrophotometrically detectable transmittance of light between 250 and 750 nm (slit width, 1.0 nm). The fluence rate of green light was measured by an IL700 Radiometer coupled to a SEE100F Detector (International Light); that of IR radiation was estimated using the same radiometer (since the sensitivity of the detector falls with wavelengths longer than 1000 nm, the actual fluence rate of the IR radiation could be higher than the measured fluence rate).

Plant materials used were oats (Avena sativa L. cv. Cooba) wheat (Triticum aestivum L. cv. Gabo) and maize (Zea mays L. cv. GH 390). Oat and wheat seeds were washed in running tap water for a day in the dark. The seeds were then sown, embryo up, on moist paper towels in trays (base measurements 32 x 26 cm) under green light ($0.1 \mu\text{W}/\text{cm}^2$ on

the bench). In each tray, 65 seeds were planted. The tray was then placed in a set of double boxes made of cardboard 1.5 mm thick (seedling boxes) consisting of one base box (32 x 38 x 13 cm) and two cover boxes; the base box is lined with aluminium foil, and the inner cover box has a glass top. The seedling boxes were then kept in the dark. Maize seeds were incubated similarly, but because of size variations it was essential to select seedlings for uniformity. Selection was carried out 48 hours after sowing. Selected seedlings were pooled in distilled water and then planted on moist paper towels in trays, 40 per tray. This transplantation procedure was carried out under green light ($0.05 \mu\text{W}/\text{cm}^2$). The trays were then placed in seedling boxes and kept in the dark. All the manipulations and the incubation were carried out in a dark room at 25° and 80% relative humidity.

Seedlings were exposed to green or to IR radiation at the age of 66 h (oat and wheat) or 72 h (maize). The seedling ages were determined from the time of sowing. The fluences of green light and IR radiation were fixed at 0.1 and $10 \mu\text{W}/\text{cm}^2$ respectively, measured at the base of the seedling box covered with its glass top. Irradiation was carried out by removing the outer dark cover of the seedling box for the given time, exposing the seedlings to the radiation through the glass top. Since the inner box remains closed, conditions such as that of humidity, within the box, should remain unaffected by the irradiation treatment. An IR-scope (Head Mounted "Find-R-Scope", FJW Industries) was used to work in infra-red radiation.

Leakage of weak greenish light from the viewing lens was guarded against by the use of a black cloth covering the head of the viewer. Following irradiation, the outer cover was replaced on the box and the seedlings were incubated further. At 8 h after the start of irradiation the seedlings were moved to a cold room (4°) to terminate growth, and the lengths of coleoptile and mesocotyl (in the case of wheat, only the coleoptile) were measured. The initial length at the time of onset of irradiation was obtained from a batch of seedlings in a replicate tray sacrificed at that time. Dark controls consisted of one (oat, wheat) or two (maize) unirradiated trays of seedlings.

RESULTS AND DISCUSSION

The effects of 3 or 30 min exposure to green light or IR radiation was examined with oat and wheat seedlings, which received no light during germination and growth, except during the sowing of the soaked, ungerminated seeds in dim green light. Even as little as 3 min exposure to green light inhibited the mesocotyl elongation of oats (Fig. 2). The stimulation of oat coleoptile elongation shown by Blaauw (1968) and by Mandoli and Briggs (1981) was not apparent in our experiments; there was however, a significant stimulation of the growth of wheat coleoptiles (Fig. 2). In contrast, IR radiation had no effect on elongation of either coleoptile or mesocotyl (Fig. 2).

Although maize seedlings were transplanted under green light at a very early stage in their growth, green light

given 24 h after transplantation stimulated coleoptile elongation and inhibited elongation of the mesocotyl (Fig. 3). It appeared that as little as 10-100 sec exposure is then sufficient to affect growth. Again, IR radiation did not affect elongation of either the coleoptile or the mesocotyl of this cereal (Fig. 3).

Green light of a fluence rate one tenth or less of that used in these experiments would still be unsafe for cereal seedlings. Such a fluence rate would be too low for working in. It thus appears impossible to carry out experiments involving the manipulation of sensitive monocotyledonous seedlings using green light within the safe fluence range. On the other hand, the fluence rate of IR radiation used here is fully sufficient to visualize the plants using an IR-scope, and at least 30 min exposure is safe.

Effects of green light and IR radiation on the growth of hypocotyls of dark-grown lettuce seedlings (Lactuca sativa L. cv. Pennlake), a dicotyledon commonly used in photomorphogenetic studies, were also investigated. The seedlings were not transplanted, i.e. received no light at all until the experimental irradiation. They were irradiated for 3 min or 30 min at 66 h after sowing, and the hypocotyl length was measured 8 h afterwards. Neither green light nor IR radiation affected hypocotyl elongation (data not shown). Despite this failure to detect an effect of green light on the growth of a dicotyledon, results already referred to (Thoma and Hartman, unpublished) indicate that green light has a desensitizing effect on the formation of anthocyanin by mustard seedlings in response to red light.

Caution must be exercised in using green light in photomorphogenetic studies until its safety has been established with the particular plant material and the experimental procedure to be followed. The use of IR radiation and an IR-scope will make possible certain experiments which cannot otherwise be performed in complete darkness.

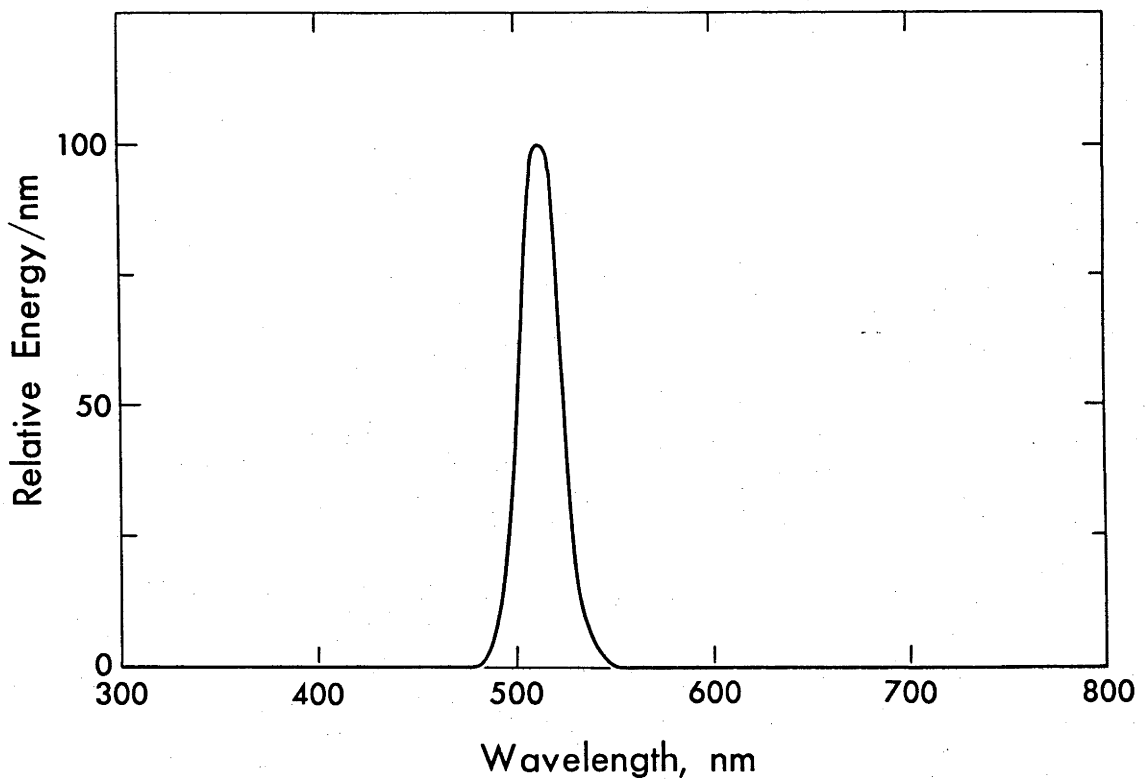


Fig. 1. Spectral energy distribution of green safelight.

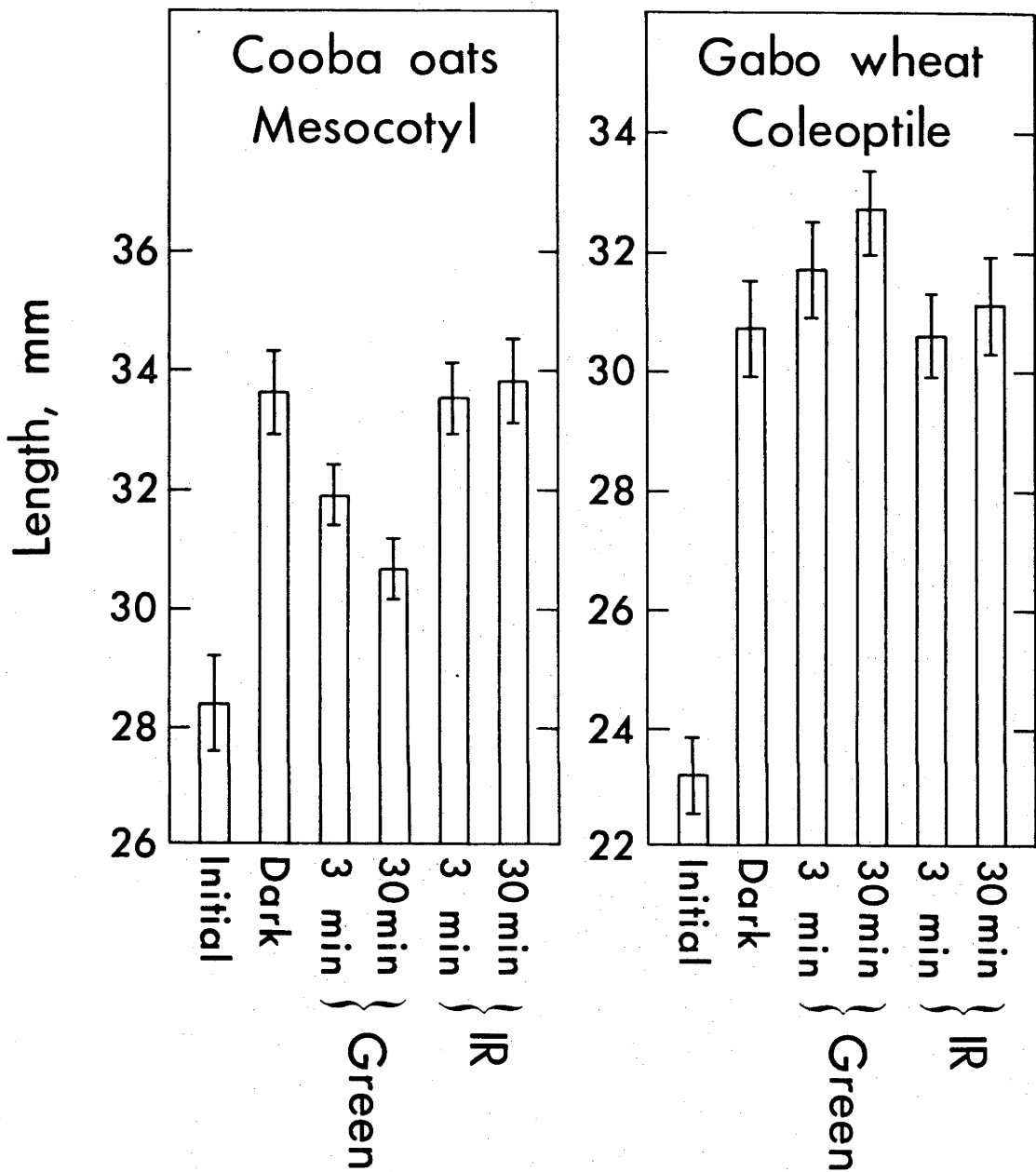


Fig. 2. Effects of irradiation with green and with IR on elongation of etiolated oat and wheat seedlings. Etiolated seedlings of Cooba oats and Gabo wheat, 66 h old, were irradiated with green light ($0.1 \mu\text{W}/\text{cm}^2$) or IR radiation ($10 \mu\text{W}/\text{cm}^2$) for 3 or 30 min. Eight hours after the start of irradiation, the lengths of coleoptile (wheat) or mesocotyl (oats) were measured. Each histogram represents the mean of 65 seedlings. Vertical bars indicate SE.

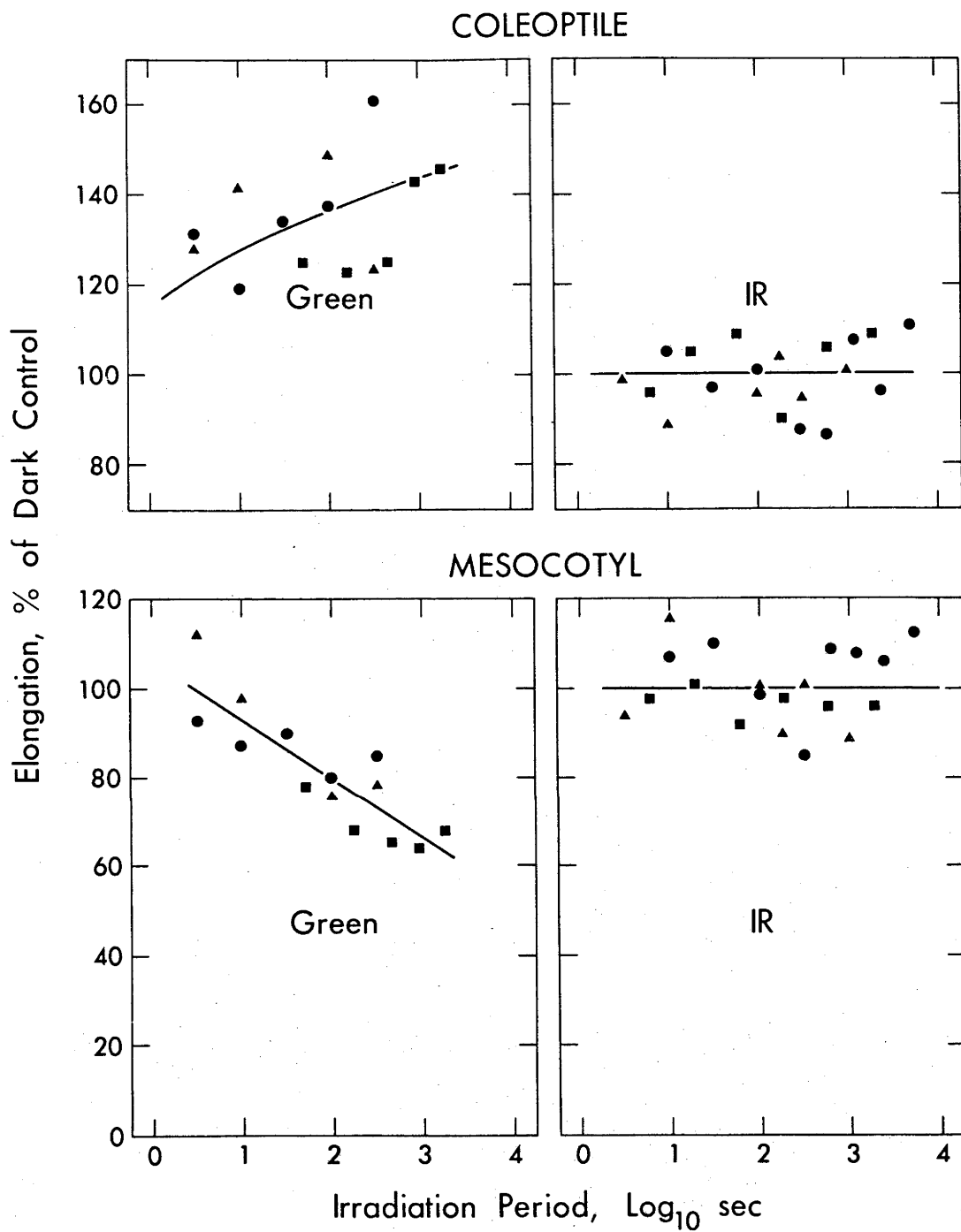


Fig. 3. Effects of irradiation with green and with IR on the shoot elongation of etiolated maize seedlings. Etiolated maize seedlings 72 h old were irradiated with green light or IR (fluence rates as in Fig 2) for a given period. Eight hours after the start of irradiation, the lengths of coleoptile and mesocotyl were measured, and the increments of length expressed as a percentage of dark controls. Different symbols indicate separate experiments. Each point represents the mean of 40 seedlings.

CHAPTER 3

IMPROVED PROCEDURE FOR THE ESTIMATION OF NANOGRAM
QUANTITIES OF INDOLE-3-ACETIC ACID IN PLANT EXTRACTS
USING THE INDOLO- α -PYRONE FLUORESCENCE METHOD

IINO, M., YU, R.S.-T., CARR, D.J. (1980)
PLANT PHYSIOL. 66, 1099-1105

SUMMARY

The indolo- α -pyrone fluorescence method of determining indole-3-acetic acid (IAA) is improved by adding butylated hydroxytoluene (BHT), an antioxidant, to samples: addition of BHT increases the fluorescence intensities and decreases their variability so that amounts of IAA as small as 0.1 to 1 ng become measurable. Interfering compounds, 4-chloro-indole-3-acetic acid and 5-hydroxy-indole-3-acetic acid, can be separated from IAA by thin-layer chromatography using polyamide as the solid support, and benzene-ethyl acetate-acetic acid (70:25:5, v/v) as the developing solvent. Polyamide thin-layer chromatography is also superior in purifying IAA without significant loss or decomposition.

A serious problem in extracting IAA from plant tissues is the considerable loss. Losses can result from decomposition of IAA when its solutions in organic solvents are evaporated to dryness, apparently due to some impurity in the solvents. Decomposition can be eliminated by simple treatments of the solvents, i.e. addition of BHT, washing with water, and passage through cotton wool. The latter is particularly effective in the case of ethyl acetate and ether. Loss of IAA by photo-decomposition is also substantial even in dried samples. Ways to minimize the conversion of indole-3-pyruvic acid to IAA during extraction procedure are also described.

A revised procedure of IAA extraction and determination is proposed. Applied to etiolated shoots of maize seedlings, it enables content to be estimated (18 nanograms per gram fresh weight) with over 90% recovery in terms of [^{14}C] IAA added at the beginning of the extraction.

INTRODUCTION

Several physicochemical methods for determining IAA in the ng range have been proposed in the last decade to replace bioassay systems but, for various reasons, they are of limited use for plant extracts and not readily applicable to physiological experiments (see Sweetser and Swartzfager 1978).

The indolo- α -pyrone fluorescence method, which was first devised by Stoessl and Venis (1970), has been shown to be highly sensitive and specific (Stoessl and Venis 1970, Mousdale et al 1978) and used to determine IAA in crude acidic extracts prepared by liquid partitioning (Stoessl and Venis 1970, Knecht and Bruinsma 1973, Eliasson et al 1976, Kamisaka and Larsen 1977). This procedure suffers from the disadvantage that 4-Cl-IAA and 5-OH-IAA, which occur naturally in plant tissues (West 1959, Gandar and Nitsch 1967, Marumo et al 1969a, b) can produce fluorescence indistinguishable from that of IAA after conversion to indolo- α -pyrone (Böttger et al 1978). The procedure also suffers from the shortcoming that it cannot readily be applied to certain plant tissues, e.g. etiolated tissues of maize or wheat, because of severe interference by impurities present in the extracts (Mousdale et al 1978). If adequate purification steps are included to remove 4-Cl-IAA, 5-OH-IAA, and other interfering substances, the fluorescence method may provide a routine, simple procedure of estimating ng amounts of IAA in plant tissues. This paper describes such a procedure.

The considerable loss of IAA during its extraction has long been a problem (Hamilton et al 1961, Mann and Jaworski 1970, Scott and Jacobs 1974, DeYoe and Zaerr 1976, Little et al 1978). To estimate IAA in plant tissues using the fluorescence method, [^{14}C]IAA can be used as an internal standard to correct for losses (Knegt and Bruinsma 1978). Inasmuch as the losses were attributed to decomposition of IAA, there is a possibility that some breakdown derivatives of labelled IAA contribute to the recovered radioactivity, leading to erroneous estimation of recovery. Here, causes of IAA decomposition are indicated and ways to overcome them are suggested. A potential source of error in the accurate estimation of IAA in plant extracts can also arise from the conversion of IPyA to IAA. Atsumi et al (1976, 1979) considered this to be a serious problem with senescent tissues. Its magnitude is unknown for growing tissues because of ignorance of their content of IPyA. Nevertheless, the biosynthetic pathway from tryptophan to IAA via IPyA has been established (see Schneider and Wightman 1978). IPyA conversion has to be taken into consideration in assessing any extraction procedure not known to effectively prevent or avoid the conversion of IPyA to IAA. We have investigated the occurrence of this conversion during the extraction procedures and suggest ways to minimize it.

We describe improved procedures of IAA extraction and determination which, when applied to etiolated shoots of maize, allowed estimation of IAA with a total recovery of more than 90%.

MATERIALS AND METHOD

TLC. Solid supports used were: silica gel (Silica Gel GF₂₅₄ and HF₂₅₄, Merck), polyamide (MN-Polyamide-DC 6 UV₂₅₄, Macherey & Nagel), and cellulose powder (Cellulose-TLC, Serva; fluorescent indicator, Woelm, was added). TLC plates (20 x 20 cm, 0.3 mm thick) were prepared essentially as recommended by the manufacturers. Silica gel and polyamide TLC plates were washed by developing with ethyl acetate-acetic acid (95:5, v/v), and air-dried until acetic acid was completely vaporized. Cellulose TLC plates were washed with 20% aqueous ethanol and air-dried. Silica gel TLC plates were reactivated at 110°C for 30 min before use.

Elution of IAA from the scraped solid support was carried out using a small column (1.0 x 11 cm) plugged at the end with Cestra cotton wool. When water-saturated ethyl acetate was used as the eluting solvent, anhydrous Na₂SO₄ was placed at the bottom of the column. Other details of TLC are given in the table legends.

Indolo- α -pyrone Fluorescence Method. The fluorimetric assay of IAA was carried out essentially according to Stoessl & Venis (1970), with the modifications suggested by Kamisaka & Larsen (1977) and Böttger et al (1978). A test tube (1.5 x 10 cm) containing a dried sample was placed in an ice bath (ice-water mixture). The sample was dissolved in 0.2 ml of an ice-cooled mixture of acetic anhydride (Pierce) and trifluoroacetic acid (TFAA) (May and Baker) by shaking on a mixer (Vortex-Genie) for a few seconds (the

mixture of acetic anhydride and TFAA was prepared routinely from 2 ml of acetic anhydride and TFAA pre-cooled on an ice bath). The reaction mixture was kept in the ice bath for exactly 15 min, then the reaction was terminated by adding 3 ml of 90% acetic acid and shaking on the mixer for 15 s. Fluorescence was measured with an Aminco-Bowman spectrofluorimeter coupled with an Aminco solid-state, blank-subtract photomultiplier microphotometer and a Minneapolis-Honeywell recorder. The light source was a Xenon arc lamp. Excitation was at 440 nm (half-band-width, 5.5 nm), and fluorescence was measured at 490 nm (half-band-width, 5.5 nm). The reading of fluorescence was started about 30 s after the termination of the reaction, and the relative fluorescence value was obtained.

To determine an unknown amount of IAA, the sample was dissolved in methanol and five aliquots were taken. Equal volumes of methanol containing 0, 0, (x), (2x), (4x) ng IAA were added to the aliquots; they then were evaporated to dryness in a vacuum oven (evacuated by a water pump) at 35°C. One of the aliquots without added IAA was used for a blank, which was prepared by first adding 3 ml of 90% acetic acid, then 0.2 ml of the mixture of acetic anhydride and TFAA. The other four aliquots were reacted according to the procedure described above. The IAA in a sample was determined by constructing a calibration line (see Stoessl and Venis 1970).

Scintillation Counting. Methanol solution of a sample (usually 0.4 ml) was added to 10 ml of scintillation fluid composed of 5 g PPO, 0.3 g POPOP and 1 liter toluene.

A blank was prepared by adding the same volume of methanol to the scintillation fluid. Radioactivity was counted with a Beckman LS-250 liquid scintillation spectrometer, with AQC (automatic quench compensation) function. The X-number (external standard - channels ratio) obtained with this function was recorded to correct for possible quenching of radioactivity. No significant difference in X-number was found with any of the samples counted.

Chemicals and Solvents. Main chemicals used were: IAA (Sigma), [1-¹⁴C]IAA (Radiochemical Centre, Amersham), 4-Cl-IAA (gift from Dr K.C. Engvild), 5-OH-IAA (Sigma), IPyA (Merck), BHT (Merck) and PVP (insoluble, Calbiochem). These chemicals were used without further purification. Main organic solvents used were: methanol (GR grade, Merck), ethyl acetate (AR grade, Ajax Chemicals or May and Baker), diethyl ether (AR grade, Ajax Chemicals), acetone (AR grade, Ajax Chemicals), benzene (AR grade, Ajax Chemicals). These solvents were used after redistillation. Ether was redistilled just before use. The water used was glass-distilled, purified further by a Milli-Q reagent-grade water system (Millipore).

Plant Material. Maize seeds (Zea mays L. cv. GH 390) were washed in running tap water for 24 h. They were then sown, embryo up, on moist paper towels, and grown in a dark growth room at 25°C and 80% RH for 3 days. Shoots were harvested under dim green light, frozen in liquid N₂ and stored at -80°C until use.

RESULTS AND DISCUSSION

1. An Improvement of the Fluorescence Method.

A problem experienced here with the fluorescence method was variation of fluorescence intensities between samples, especially when small amounts of IAA (e.g. 10 ng) were assayed. The poor reproducibility of the fluorescence method was also experienced by Sweetser and Swartzfager (1970). Precooling the reactants before they were mixed (Knegt and Bruinsma 1973) did not overcome this difficulty. However, the problem was effectively overcome by adding BHT, an antioxidant, to the IAA samples. BHT enhances the fluorescence of a given amount of IAA and also reduces variability of fluorescence intensities (Fig. 1). BHT itself produced no fluorescence, even after processing through the entire assay procedure (Fig. 1). Thus, by adding BHT to IAA samples, even 0.1 to 1 ng IAA became measurable with good linearity (Fig. 2). BHT added to the mixture of acetic anhydride and TFAA also enhanced the fluorescence but enhanced it not at all when added to 90% acetic acid (Fig. 3-D and E). It was concluded that BHT prevents oxidation of IAA and/or indolo- α -pyrone during the reaction period, giving a high yield of indolo- α -pyrone. Oxidation of IAA also occurred during sample preparation (Fig. 3, compare B and C). The possibility that some impurity in methanol may be responsible for the oxidation was confirmed from the evidence that the decrease of fluorescence was augmented by increasing the volume of methanol, that distillation of methanol could reduce the

decrease of fluorescence, and that the decrease of fluorescence differed with batches of methanol redistilled at different times. These experiments were carried out by adding 10 ng IAA in 10 μ l methanol to a standard volume of methanol from different batches or to different volumes of a single batch just before evaporation to dryness. It seems most likely that some impurity in methanol caused oxidation of IAA during concentration of the methanol solution.

Originally water was used to terminate the reaction of IAA with acetic anhydride (Stoessl and Venis 1970, Knecht and Bruinsma 1973, Eliasson et al 1976, Monsdale et al 1978), but in our assay procedure, aqueous acetic acid was used instead, following Kamisaka and Larsen (1977) and Böttger et al (1978). This made possible the use of BHT, which is sparingly soluble in water.

2. Purification of Extracts for the Fluorometric Assay.

4-Cl-IAA and 5-OH-IAA are known to interfere with the assay by contributing to the fluorescence intensity; hence, crude acidic extracts need to be purified further to remove at least these compounds before being assayed for IAA. Marumo et al (1971) were able to separate IAA from 4-Cl-IAA by paper chromatography, but found it difficult to separate them by TLC. This difficulty was also experienced by Böttger et al (1978). However, IAA could be separated from both 4-Cl-IAA and 5-OH-IAA by TLC using silica gel, polyamide, or cellulose as the solid support (Table 1). Best resolution was achieved by polyamide TLC using the solvent system, benzene-ethyl acetate-acetic acid.

Polyamide TLC has already been used by Railton (1972) for purification of IAA, specifically to remove phenolic compounds.

Recovery of IAA from TLC plates was examined using [^{14}C]IAA as a monitoring aid (Table 2). Polyamide TLC provided the highest recovery (about 95%). Although the solvent system for the development was the same, recovery from silica gel TLC plates was extremely low, especially when Silica Gel GF₂₅₄ was used. This low recovery was not improved even by eluting the solid support with methanol. The low recovery appeared to result from the decomposition of IAA. The characteristic UV absorption spectrum of IAA underwent considerable change within 20 min after development (Fig. 4). Decomposition of IAA on silica gel TLC plates was also observed even when applied IAA was eluted without development (Fig. 5). Recovery of IAA from silica gel TLC plates is said to approach 80% when particular solvent systems are used (Sagi 1969, McDougall and Hillman 1978). The results here show that silica gel itself is damaging to IAA and, therefore, should not be used for IAA purification, irrespective of the solvent system used. On the other hand, polyamide TLC plates caused no change at all of the absorption spectrum even 4 h after development (Fig. 4). It was confirmed that cellulose TLC provides excellent, although not complete, recovery of IAA (Ohwaki 1970a) (Table 2). IAA is also stable on cellulose TLC plates as indicated by its UV spectrum (data not shown). In comparison, polyamide TLC is far better than cellulose TLC in terms of spot resolution, sample-loading capacity, and developing speed.

Recovery of IAA from polyamide TLC was further enhanced by treating the eluting solvent as described below and by adding BHT to both the samples to be chromatographed and the developing solvent. With these modifications almost complete recovery (99%) of 10 ng IAA was obtained. Since the recovery was estimated from fluorimetric data, it is clear that the decomposition of IAA is minimal. Mann and Jaworski (1970) have already reported the use of an anti-oxidant to reduce loss of IAA during TLC.

3. Decomposition of IAA During Extraction Procedure.

One of the operational steps responsible for the losses of IAA is TLC, especially on silica gel. Losses of IAA were reported to occur also during extraction procedures (Mann and Jaworski 1970). Sweetser and Swartfager (1978) using a single step of Sephadex column chromatography after liquid partitioning steps, found recovery to be only 30 to 50%. We also have had a long experience of inconsistent recovery of IAA (40-80%) with the initial extraction procedure, even using polyamide TLC. There seem to be unknown factors, in the extraction procedure, which cause losses of IAA.

Decomposition of IAA during evaporation to dryness of its solution was found not only with methanol but with all redistilled AR-grade solvents tested, i.e. ethanol, acetone, ethyl acetate and ether. Since ethyl acetate is used to elute IAA from TLC plates and ether to obtain acidic extracts followed by evaporation to dryness, these steps may cause serious decomposition of IAA.

An example of the decomposition (about 30%) of IAA

during evaporation to dryness of its ethyl acetate solution is presented in Table 3. Four ml ethyl acetate is the usual volume of eluate from the scraped thin layer. The percentage decomposition with different batches of redistilled ethyl acetate ranged from 10 to 60%. Redistillation of a particular batch of ethyl acetate which produced 53% decomposition of IAA reduced it to 12%. Decomposition of IAA could be avoided using ethyl acetate distilled several times, a time-consuming procedure, difficult to control. By adding 2 mg BHT to 4 ml ethyl acetate, the decomposition was reduced from 30% to 15%; larger amounts (up to 12 mg) did not reduce the decomposition further (Fig. 6).

When 30 ng IAA was subjected to polyamide TLC, eluted and determined by the fluorescence method, recovery was more than 90%. This seemed paradoxical because the water-saturated ethyl acetate used to elute IAA from the scraped thin layer was prepared from a batch of ethyl acetate which was found to cause about 50% decomposition of IAA. Analysing the detailed steps of the actual operation, the cotton plug used in the column for elution of TLC scrapings was found to prevent significantly the decomposition of IAA. Decomposition of IAA during evaporation of its ethyl acetate solution was reduced to a considerable extent by using ethyl acetate passed through a 0.1 g cotton plug (Table 4-B). Cotton wool seems to adsorb some impurity in ethyl acetate which may contribute to the decomposition of IAA. Ethyl acetate eluate (4 ml) of 0.1 g cotton itself produced fluorescence equivalent to 0.8 ng IAA when processed through the assay procedure. However, the

compound(s) responsible for this fluorescence could be completely washed off by passing 3 ml ethyl acetate through the cotton plug: subsequent eluate did not produce fluorescence. The decomposition of IAA could also be prevented to some extent by using water-saturated ethyl acetate (prepared by shaking four times against water) (Table 4-C). This is not simply due to the water present in ethyl acetate because water-saturated ethyl acetate passed through anhydrous Na_2SO_4 gave the same result (Table 4, compare B, D and E). Some of the impurity seems to be washed off by water. When all these measures for improvement are adopted, decomposition of IAA in ethyl acetate is eliminated (Table 4-F).

Ways of preventing decomposition of IAA in ether were also investigated. A standard volume of ether, 80 ml, was used because it is the maximum volume of the ether acidic extract in the extraction scheme reported here. Addition of BHT could reduce the decomposition (Table 5-B). A further reduction was achieved by using ether passed through cotton and washed by water, as in the case of ethyl acetate (Table 5-C, D). Thus, decomposition was eliminated by combining all these treatments (Table 5-E). Although peroxides are the most likely impurities, passage through an aluminum oxide column was not effective (in Table 5, compare B and F). Mann and Jaworski (1970) observed a substantial loss of radioactivity of $[\text{C}^{14}]$ IAA when its ether solution was evaporated to dryness in vacuo and concluded that some IAA sublimed. It seems more likely that some radioactive products of the decomposition of $[\text{C}^{14}]$ IAA produced during

evaporation of ether are volatile.

Another possible cause of IAA losses could be photo-decomposition. Epstein and Lavee (1975) have reported decarboxylation of [1- 14 C]IAA in aqueous solution by light. Utilization of low-actinic glassware was also reported to reduce losses of IAA (Mann and Jaworski 1970). A substantial loss of radioactivity from dried [1- 14 C]IAA exposed to laboratory lighting (Table 6) was also observed here. Thus not only the extraction but also the handling of dried samples must be carried out under subdued light.

4. Conversion of IPyA to IAA.

Another problem during extraction and purification of IAA is spontaneous oxidation of IPyA to IAA, which can introduce error into determination of IAA in plant tissues. Conversion was shown to occur during paper chromatography (Bentley et al 1956) and IAA extraction procedure (Atsumi et al 1976).

Polyamide TLC was highly effective in separating IAA from IPyA. Two μ g IPyA was developed using polyamide TLC with solvent system I (Table 1), the IAA zone was eluted, and the IAA in it was determined by the fluorescence method [BHT was added to the IPyA sample (8 mg), to the developing solvent (100 mg/l), and to the eluting solvent (1.5/ml)]. The IAA amounts determined were 4 to 6 ng, which represents only 0.4% of the IPyA amount on a molar basis. Separation of IAA and IPyA on polyamide TLC is also adequate: the R_f values of IAA and IPyA are 0.33 and 0.12, respectively. It can be concluded that there is no IPyA in the IAA fraction

after the TLC step.

Analyses of the operations commonly involved in IAA extraction procedure showed that (a) the conversion is greater in aqueous solution than in organic solvents, (b) IPyA is more stable in phosphate buffer than in bicarbonate buffer, (c) pH of the buffer solution (pH 2.5-8.5) has no significant effect on the conversion. In these experiments, IAA was determined by the fluorescence method following polyamide TLC. The conversion of IPyA to IAA in phosphate buffer could be reduced further by lowering the temperature of the solution and adding BHT (Table 7-B, C). The conversion was at its lowest when IPyA was extracted with minimal delay from the phosphate buffer by ether (Table 7-D), indicating that acidification of the aqueous solution and evaporation of the ether solution to dryness (the ether was water-washed, BHT-supplemented, and passed through cotton) caused essentially no conversion. These results show that the conversion of IPyA to IAA during the extraction procedure can be drastically reduced by lowering the temperature, adding BHT, and shortening the duration of extraction.

5. Proposed Scheme for IAA Estimation in Plant Tissues and Application to Maize Seedling Shoots.

Based on the findings described above, a scheme for the estimation of IAA in plant tissues is proposed (Fig. 7). Essential features of the scheme include: (a) minimizing the period of extraction of ground tissues with aqueous acetone - this period must be optimized for each plant material (4 h was determined for etiolated shoots of maize);

(b) application of the aqueous extract directly to a PVP column without prior concentration - further elution is carried out with 0.1 M K_2HPO_4 at pH 8.0 (practically all of a sample of [^{14}C]IAA was recovered in a 10 to 65 ml fraction); (c) partitioning the acidified aqueous solution three times against a 1/3 volume of ether (recovery of [^{14}C]IAA more than 99.5%) - partitioning with petroleum spirit prior to this partitioning (Kamisaka and Larsen 1977, Knecht and Bruinsma 1973) is not included; (d) addition of BHT and pretreatments of solvents (Figure 7); (e) carrying out all manipulations under subdued light.

IAA in etiolated shoots of maize was estimated using the proposed scheme. As shown in Table 8, 18 ng/g fresh weight of IAA was obtained. Recovery of radioactivity from [^{14}C]IAA added at the beginning of the extraction was over 90%.

Although radioactive IAA is the internal standard most commonly used to correct for losses (Knecht and Bruinsma 1973, Little et al 1978, Sweetser and Swartzfager 1978), it should be recognized that the radioactivity recovered may not totally represent the authentic compound originally added (see McDougall and Hillman 1978). Reliability of the proposed scheme in correcting losses of IAA has been assessed by the following experiment. About 20 ng [^{14}C]IAA was processed through the entire procedure in a sham extraction, and the recovery was determined by both radioactivity and fluorimetric assays; the recovery determined based on either parameter was exactly the same. One possibility which remains is that, in the presence of

actual plant extracts, radioactive derivatives of labelled IAA which are co-purified with IAA may be produced.

To achieve a more realistic appraisal of the losses and estimation of IAA, the following suggestions are made: (a) as an internal standard, [^{14}C] IAA seems to be the most suitable radioactive form because $^{14}\text{CO}_2$ evolved upon decarboxylation, one of the possible degradative processes, will not remain in the residues; (b) assays of IAA should be made as soon as possible following the extraction - when storage of samples is inevitable, it is advisable to store them as assay aliquots with added authentic carrier IAA because nonlinearity of the calibration line provides a good indication of breakdown of IAA during the storage period; (c) all aliquots used for the fluorimetric assay and scintillation counting must be taken simultaneously from the sample, freshly extracted and dissolved only once in methanol, because each further evaporation to dryness and dissolution may cause decomposition of IAA.

It has been suggested by Mousdale et al (1978) that the background values of fluorescence should be subtracted from that of the reacting solution after complete destruction of indolo- α -pyrone by light. With the extracts here, there was no significant difference between background values obtained by adding 90% acetic acid before the addition of the mixture of acetic anhydrous and TFAA or after photo-decomposition of indolo- α -pyrone (Table 9). Nor were there significant increases in background level in extracts (in Table 9, compare Blanks I and II). Although etiolated maize shoot is said to be one of the most difficult materials to work with

owing to reaction-inhibiting impurities in the extracts (Mousdale et al 1978), the inhibition of fluorescence was less than 20%. Thus, IAA samples obtained by the proposed scheme are effectively free of contaminants affecting fluorimetric assay.

The steps in the extraction procedure (Fig. 7) are designed to obtain high recovery of IAA but also to minimize the conversion of IPyA to IAA. However, $8.2 \pm 0.2\%$ (mean of three replicates with SE) of IPyA was still converted to IAA when 2 μ g IPyA was processed through the entire procedure. This degree of conversion seems unavoidable. Atsumi et al (1976) have reported a new liquid partitioning system for IAA (water- Cl_2CH_2 system), which theoretically can remove 82% of IPyA, and recover 91% of IAA. Using this system, IPyA not already converted to IAA before and during the partitioning will be effectively removed.

The aim here was to establish a reliable method of IAA extraction and determination which can be easily utilized for physiological experiments. As it stands, the improved indolo- α -pyrone fluorescence method coupled with the improved extraction method is particularly suitable for such experiments. Minute amounts of IAA in plant extracts (as little as 1 ng) can be determined, and the extraction procedure can be scaled according to the amount of plant material, which can be as little as 0.1 g fresh weight in the case of maize shoots; the entire procedure from homogenization to the determination of IAA can be completed within 10 h, and several samples can be processed simultaneously; and solvents of AR grade are sufficient.

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Table 1. Separation of IAA, 4-Cl-IAA and 5-OH-IAA on TLC. Three spots of each compound (10 µg/spot) were developed on a TLC plate (20 x 20 cm², 0.3 mm thick) for 15 cm at room temperature. Spots were visualized under a UV lamp, and R_f values were measured. Mean R_f values of the three spots are presented. Solvent systems were (I) benzene-ethyl acetate-acetic acid (70:25:5 v/v/v); and (II) butanol-ammonia-water (4:1:1 v/v/v, upper phase).

Solid support	Solvent system	R _f values		
		IAA	4-Cl-IAA	5-OH-IAA
Silica gel GF ₂₅₄	I ^a	0.56	0.50	0.34
Silica gel HF ₂₅₄	I ^a	0.54	0.48	0.33
Polyamide	I ^a	0.33	0.24	0.03
Cellulose	II ^b	0.29	0.37	0.11

^a paper was not placed in the vessel. When a sheet of paper moistened with the developing solvent was placed on the wall, R_f values of the compounds were reduced: 20 to 30% with silica gel plates, and about 10% with polyamide plates.

^b A sheet of paper moistened with the developing solvent was placed on the inside wall of the developing vessel.

Table 2. Recovery of radioactivity from [^{14}C]IAA applied to TLC plates. About 100 ng [^{14}C]IAA loaded on a TLC plate as a 12 cm-long streak was developed for 15 cm, together with authentic IAA marker spots in darkness at 20°C. The marker spots were visualized under a UV lamp but the [^{14}C]IAA area on the plate was protected from UV light by a cover. The IAA zone (3 cm wide on silica gel and polyamide plates, 4 cm wide on cellulose plates) was scraped off, and eluted with water-saturated ethyl acetate or methanol using a small column. Elution was started exactly 5 min (silica gel, polyamide) or 10 min (cellulose) after the development. Radioactivity of the eluate was counted to estimate the recovery.

Solid Support	Eluting solvent	% Recovery ^a
Silica gel GF ₂₅₄	Water-saturated ethyl acetate	7.5
	Methanol	7.1
Silica gel HF ₂₅₄	Water-saturated ethyl acetate	40.8
	Methanol	40.2
Polyamide	Water-saturated ethyl acetate	96.1
Cellulose	Methanol	83.8

^a Recovery in the first 4 ml eluate. Another 4 ml eluate recovered less than 0.2% of this in every case. Mean of two replicates presented.

Table 3. Decomposition of IAA upon evaporation of the ethyl acetate solution to dryness. A given amount of IAA dissolved in 30 μ l ethyl acetate was added to 4 ml ethyl acetate and then evaporated to dryness under N_2 stream at 35°C. The amount of IAA in the residue was determined by the fluorescence method: the residue was dissolved in 2 ml methanol (+ 3 mg/ml BHT) and divided into 5 aliquots, then authentic IAA was added to 3 aliquots for the construction of the calibration line (this IAA was taken from the same ethyl acetate solution added initially to 4 ml ethyl acetate). Although IAA in different volumes of ethyl acetate was added to the 3 aliquots, the calibrations were always linear, i.e. this ethyl acetate had no apparent effect on IAA determination. To check systematic errors of this procedure, the given amount of IAA in 30 μ l ethyl acetate was added directly to 1.97 ml methanol (+BHT) and processed as usual for IAA determination; no systematic errors appeared to be inherent in other steps of the procedure.

Original amount of IAA (ng)	Amount of IAA Recovered ^a	
	ng	%
30	21.6	72
60	42.5	71
90	63.7	71

^a Mean of 2 replicates.

Table 4. Decomposition of IAA upon evaporation of the ethyl acetate solution to dryness: inhibition of decomposition by various treatments of ethyl acetate. Thirty ng IAA in 30 μ l ethyl acetate was added to each of solutions A to F. These solutions then were evaporated to dryness under N_2 -stream at 35°C. The amount of IAA in the residue was determined by the fluorescence method: the residue was dissolved in 2 ml methanol (A-E: + 3 mg/ml BHT, F: - BHT), and divided into five aliquots; then 3, 6, and 12 ng authentic IAA were added to three aliquots respectively for the construction of the calibration line (this IAA was taken from the same ethyl acetate solution added initially to A-F).

Solution	Composition and Treatment Applied	% IAA
	to Ethyl Acetate	Recovered
A	4 ml ethyl acetate	34
B	4 ml ethyl acetate passed through 0.1 g cotton plug ^a	85
C	4 ml water-washed ethyl acetate ^b	58
D	4 ml water-washed ethyl acetate passed through 0.1 g cotton plug ^a	95
E	4 ml water-washed ethyl acetate passed through column ^a of 1 g Na_2SO_4 and 0.1 g cotton plug	95
F	4 ml water-washed ethyl acetate (+1.5 mg/ml BHT) passed through column ^a of 1 g Na_2SO_4 and 0.1 g cotton plug	100

^a pre-washed with 3 ml ethyl acetate.

^b Prepared by shaking 4 times against water, 1/2 volume.

Table 5. Decomposition of IAA upon evaporation of the ether solution to dryness: inhibition of decomposition by various treatments of ether. Sixty ng IAA in 30 μ l methanol was added to each solutions A to F. These solutions then were evaporated to dryness by a rotary film evaporator at 35°C. The amount of IAA in the residue was determined by the fluorescence method: the residue was dissolved in 4 ml methanol (A: + 3 mg/ml BHT, B-F: + 1 mg/ml BHT), and five aliquots of 0.4 ml solution were taken; then 3, 6 and 12 ng authentic IAA were added to three aliquots respectively for the construction of the calibration line (this IAA was taken from the same solution added to A-F).

Solution	Composition and Treatment Applied	% IAA
	to Ether	Recovered
A	80 ml ether	53
B	80 ml ether, 8 mg BHT added	84
C	80 ml water-washed ether ^a , 8 mg BHT added	92
D	80 ml ether passed through 0.25 g cotton plug ^b , 8 mg BHT added	99
E	80 ml water-washed ether passed through column ^b of 12 g Na ₂ SO ₄ and 0.25 g cotton plug, 8 mg BHT added	100
F	80 ml ether passed through column ^c of 15 g aluminum oxide ^d , 8 mg BHT added	87

^a Prepared by shaking 3 times against water, 1/3 volume.

^b Pre-washed with 20 ml ether.

^c Pre-washed with 20 ml ether; column: 2 cm diameter, plugged with glass filter.

Table 6 . Loss of radioactivity from samples of [^{14}C]IAA upon exposure to light. Nine identical 0.2 ml aliquots of a methanol solution of [^{14}C]IAA, about 50 ng/ml, were evaporated to dryness in a vacuum oven at 35°C in the dark. The residues from three aliquots were each taken up immediately in 2 ml methanol and samples, each 0.4 ml, were taken for counting (A). The residues from three other aliquots were exposed to laboratory lighting (daylight-type fluorescent lamps, 1 W/m²) on the bench top at room temperature for 3 hours (B). The remaining aliquots were kept in the dark under otherwise identical conditions (C). Each residue was then taken up into 2 ml methanol, from which a sample of 0.4 ml was taken for counting.

Treatment	Radioactivity ^a	
	cpm	% original activity
A Original	1406 $\pm$ 31	100
B Light	1223 $\pm$ 10	87
C Dark	1414 $\pm$ 11	101

^a Mean value with SE.

Table 7. Conversion of IPyA to IAA in Phosphate Buffer.

Two μg IPyA in 20 μl methanol was added to each of solutions A to D. After standing in darkness, the solutions were acidified to pH 3.0 and partitioned three times against H_2O washed ether (+ 100 mg/l BHT) 0.5 volume. The ether phase was passed through a column of 12 g anhydrous Na_2SO_4 and 0.25 g cotton (prewashed with 20 ml ether), then evaporated to dryness. IAA in the residue was purified by polyamide TLC. (Elution of the scraped IAA zone was carried out using water-washed ethyl acetate containing 1.5 mg/ml BHT). The purified IAA then was assayed by the fluorescence method: a sample was divided into six aliquots and then 5, 10, 20 and 40 ng IAA were added to four aliquots for the construction of the calibration line.

Solution	Incubation	IPyA Converted to IAA (%) ^a
A: 50 ml 50 mM K_2HPO_4 , R.T. ^b	R.T., 1 h	6.3
B: 50 ml 50 mM K_2HPO_4 , 4 C	4 C, 1 h	4.1
C: 50 ml 50 mM K_2HPO_4 (+ BHT ^c), R.T.	R.T. 1 h	4.0
D: 50 ml 50 mM K_2HPO_4 , R.T.	Minimal time	0.7

^a Mean value of two replicates.

^b R.T.: room temperature.

^c 1 mg BHT in 50 μl methanol was added.

Table 8. IAA content of shoots excised from etiolated maize seedlings. IAA was extracted from 60 shoots excised from 3-day-old, etiolated maize seedlings and determined by the fluorescence method according to the procedure shown in Fig. 7. The amount of [^{14}C]IAA added for monitoring recovery was equivalent to 4.8 ng IAA (determined by the fluorescence method), which was subtracted from the total IAA amount after correcting for recovery. The amounts of authentic IAA added to aliquots of the extracted sample for the construction of the calibration line were 10, 20 and 40 ng.

Fresh Wt of Shoots (g)	Recovery of [^{14}C]IAA (%)	IAA Content (Corrected)	
		ng/Shoot	ng/g Fresh Wt
9.88	90.6	2.96	17.9
10.18	92.2	3.01	17.8

Table 9. Background fluorescence in the sample following complete decomposition of indolo- α -pyrone by light. The reacted sample solution (reaction mixture + 90% acetic acid) used in Table 8 was left standing under laboratory lighting (see Table 3) for 2 days to complete decomposition of the indolo- α -pyrone (illumination for 2 days was necessary for complete decomposition of indolo- α -pyrone produced from 60 ng IAA). Relative fluorescence intensities before and after decomposition are presented.

Initial				After 2 days		
Blank	Blank	Reacted		Blank	Reacted	
I ^a	II ^b	No IAA	20ng IAA	II	No IAA	20ng IAA
		Added	Added		Added	Added
8.5	9.9	245	415	9.6	10.2	10.6
8.0	8.6	239	400	8.6	9.6	9.0

^a Blank without sample: prepared by adding 0.2 ml of the mixture of acetic anhydride and TFAA to 3 ml of 90% acetic acid.

^b Blank with sample: prepared by adding 3 ml of 90% acetic acid to sample then 0.2 ml of the mixture of acetic anhydride and TFAA.

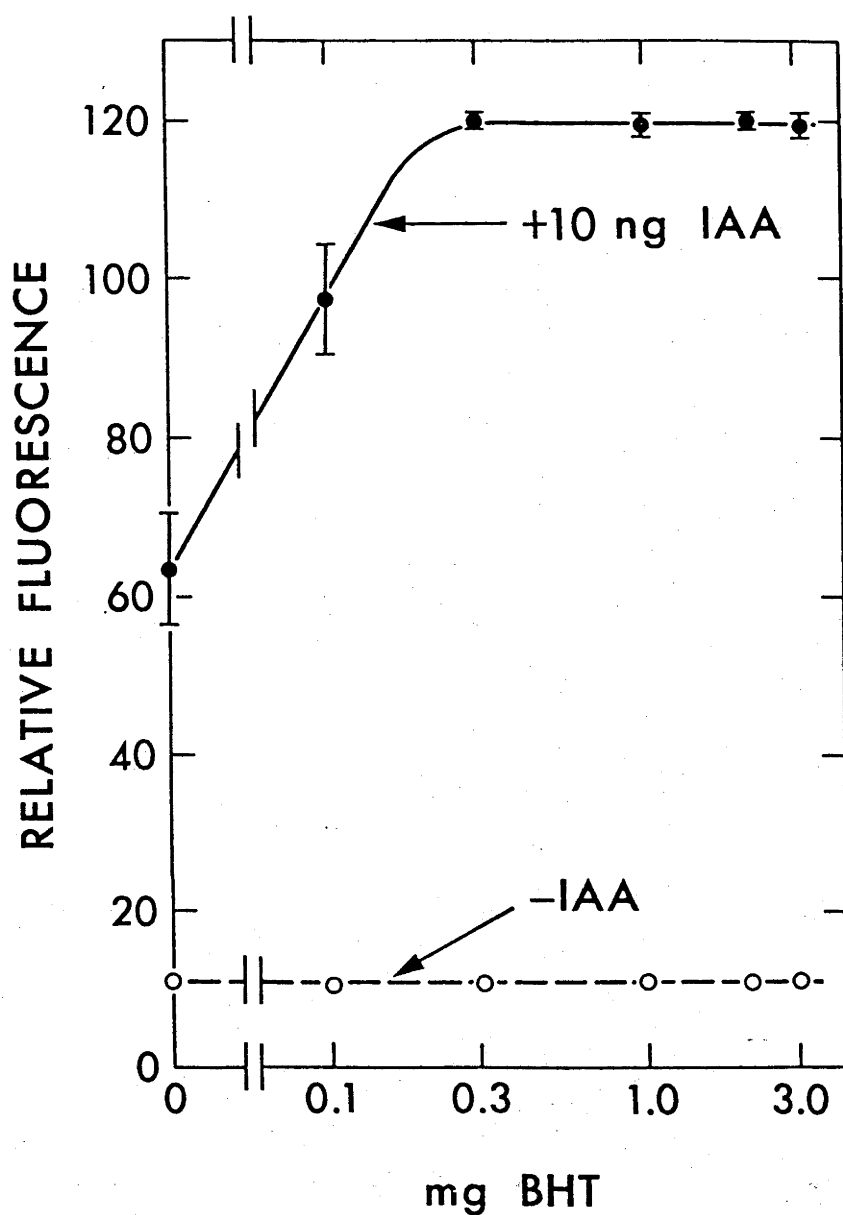


Fig. 1. Effect of BHT on fluorescence intensity in indolo- α -pyrone assay. Ten ng IAA (in 0.2 ml methanol) was added to 0, 0.1, 0.3, 1.0, 2.0, and 3.0 mg BHT (in 0.2 ml methanol) which was immediately evaporated to dryness at 35°C in a vacuum oven. The resultant residues were assayed by the fluorescence method. Minus IAA control for each amount of BHT used was also assayed. Background level was not subtracted from relative fluorescence. Each point in the figure is the mean of four (+ IAA) or two (- IAA) replicates. Vertical bars represent SE.

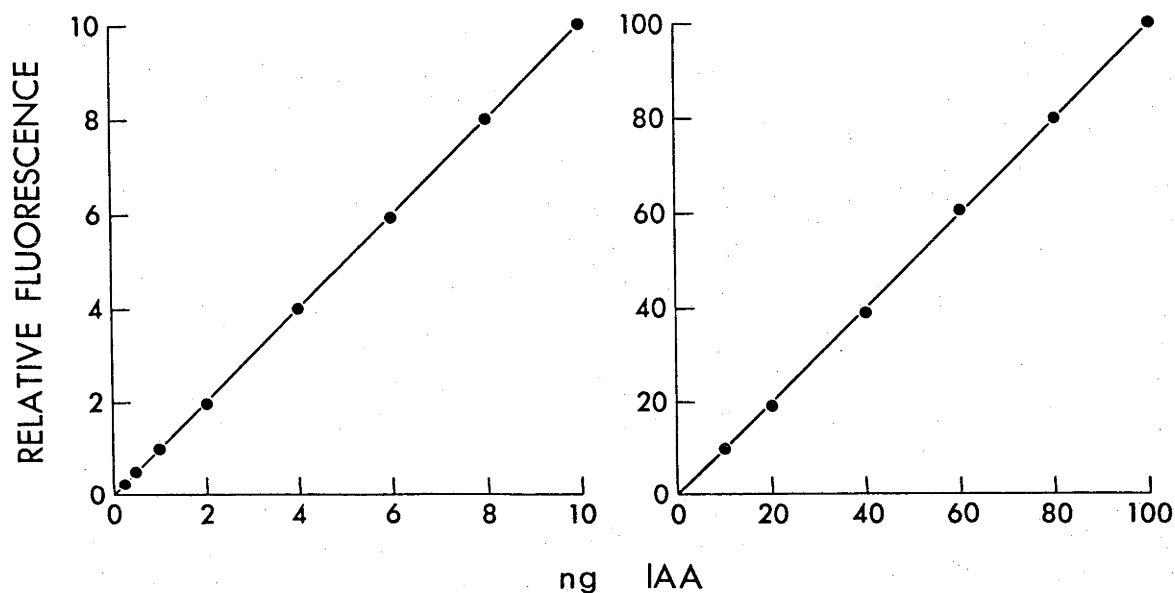


Fig. 2. Standard curves for IAA determination by the indolo- α -pyrone fluorescence method in the presence of BHT. Authentic IAA, taken in 0.4 ml of methanol containing 1 mg BHT, was evaporated to dryness at 35°C in a vacuum oven. The residue was then assayed by the standard indolo- α -pyrone fluorescence method. Relative fluorescence 100 = 100 ng IAA.

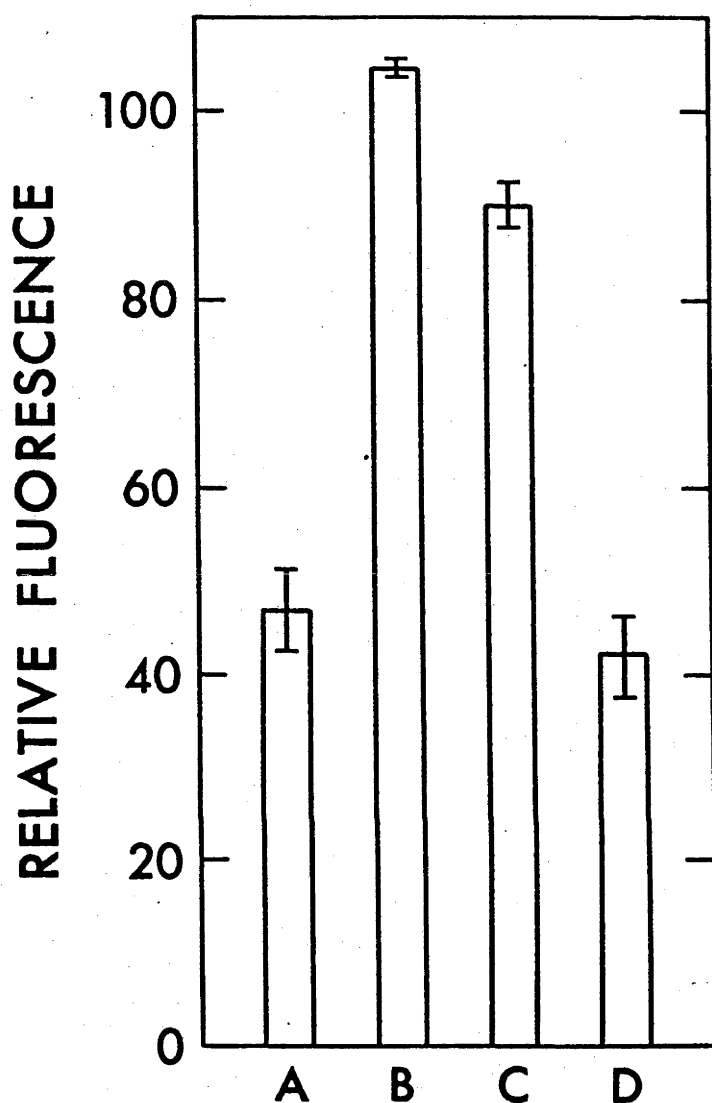


Fig. 3. Effect of adding BHT at different stages of the assay procedure on the fluorescence intensity. Ten ng IAA in 10 μ l methanol was diluted with 0.4 ml methanol. It then was evaporated to dryness at 35°C in a vacuum oven, and the residue was assayed by the fluorescence method. Addition of BHT was as follows: A, BHT was not included at any stage of the procedure; B, BHT was added to the methanol used for diluting the 10 ng IAA sample (1 mg BHT in 0.4 ml); C, BHT was added to the mixture of acetic anhydride and TFAA (1 mg BHT in 0.2 ml); D, BHT was added to 90% acetic acid (1 mg BHT in 3 ml). Vertical bars represent SE.

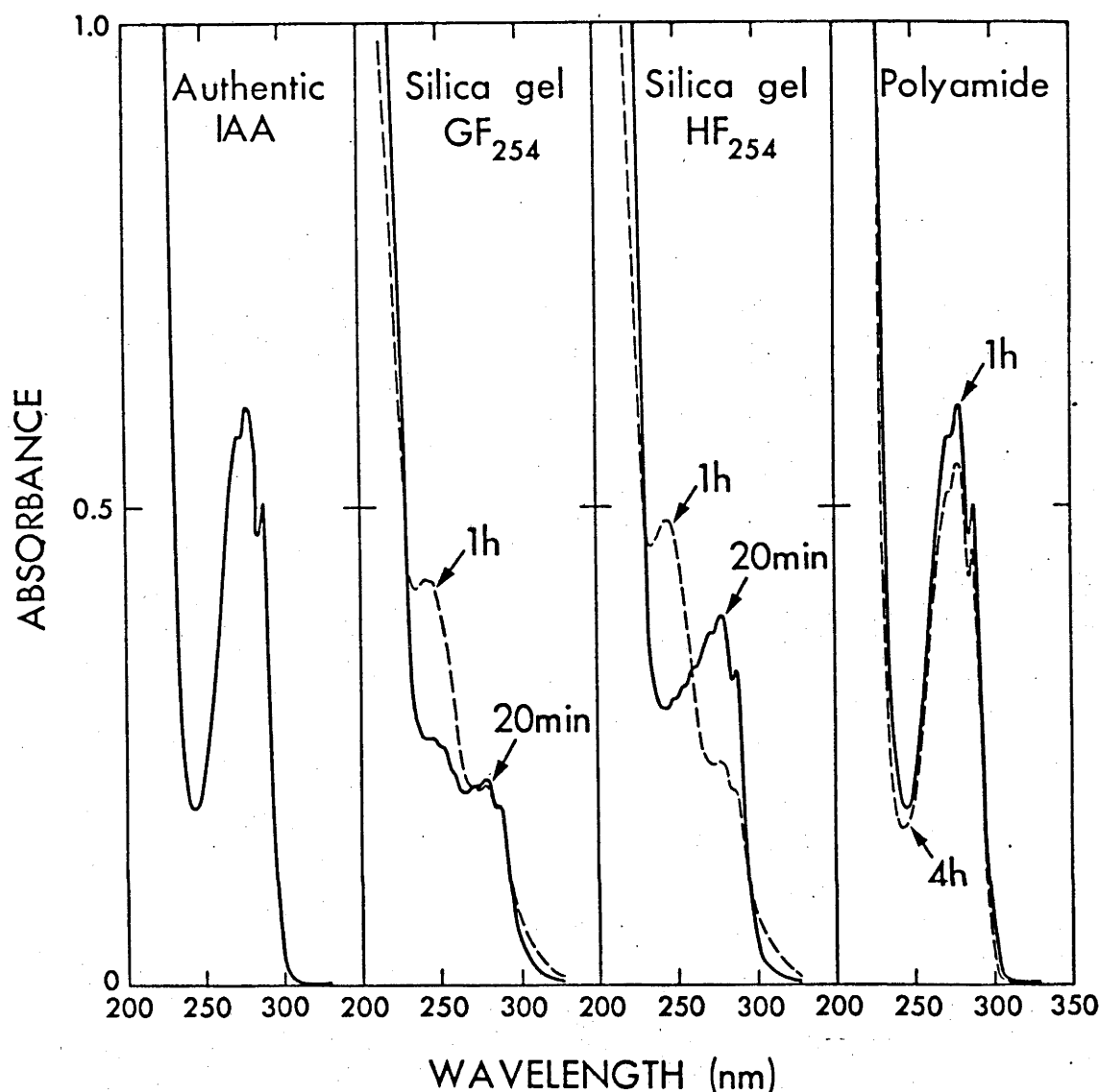


Fig. 4. UV absorption spectra of eluates from IAA zones on TLC plates. Each TLC plate of silica gel or polyamide was divided into two parts by placing a groove vertically at the centre of the plate. Fifty μg IAA in 50 μl methanol was loaded as a 6 cm-long streak on one side of the plate, and developed with the solvent system I (Table 1) for 15 cm in darkness at room temperature. After the development, TLC plate was left standing for a given interval in darkness at room temperature. The IAA band on the plate was marked by visualizing it promptly under a UV lamp. The marked area was scraped off and then eluted with water-saturated ethyl acetate. The eluate was evaporated to dryness. The residue was dissolved in 3 ml ethanol, and a UV spectrum was obtained with a Varian 634 spectrophotometer. A reference solution, prepared from the eluate of the corresponding area on the other side of the plate, did not show a significant UV absorption within the wavelength range used.

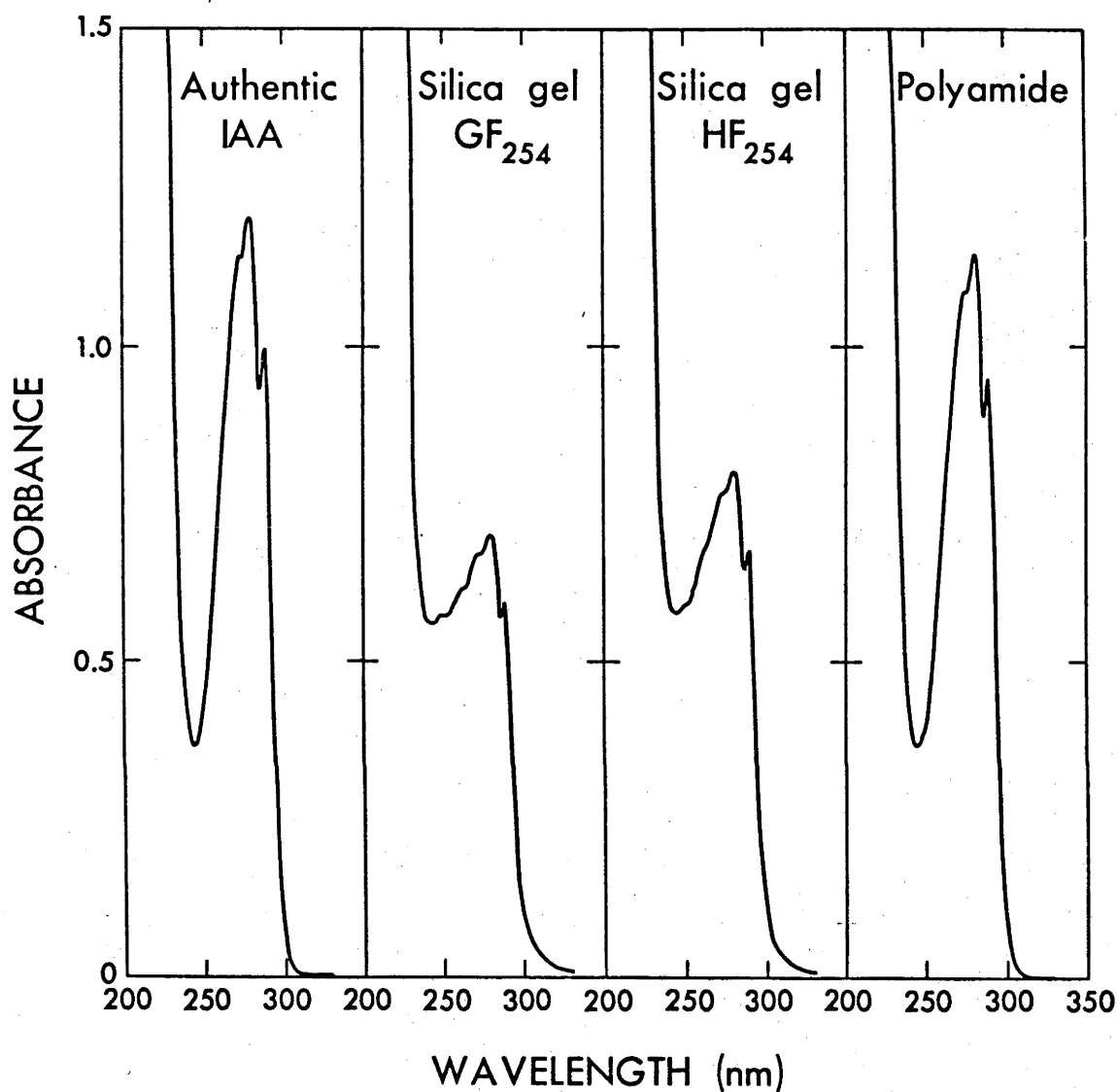


Fig. 5. UV absorption spectra of eluates from IAA-application zones on TLC plates without development. 100 μ g IAA in 100 μ l methanol was loaded on a 1.5 x 10 cm² area of a silica gel or polyamide TLC plate, and left standing in darkness at room temperature. After 2 h the area was scraped off and eluted with water-saturated ethyl acetate. The eluate was then evaporated to dryness and a UV absorption spectrum of the residue was obtained (see Fig. 4). A reference solution was prepared from the eluate of the corresponding area on the same plate.

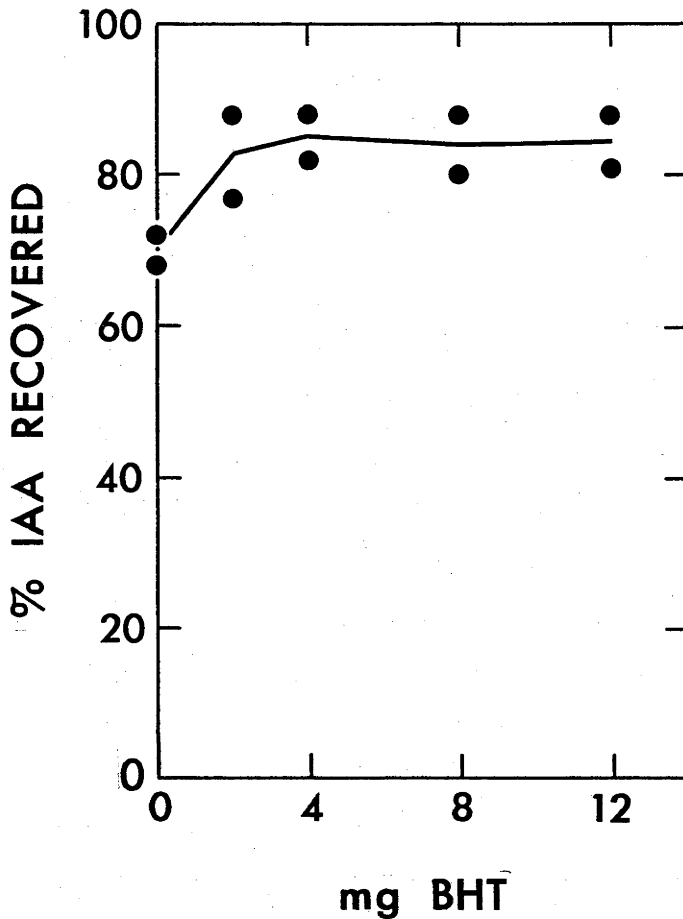
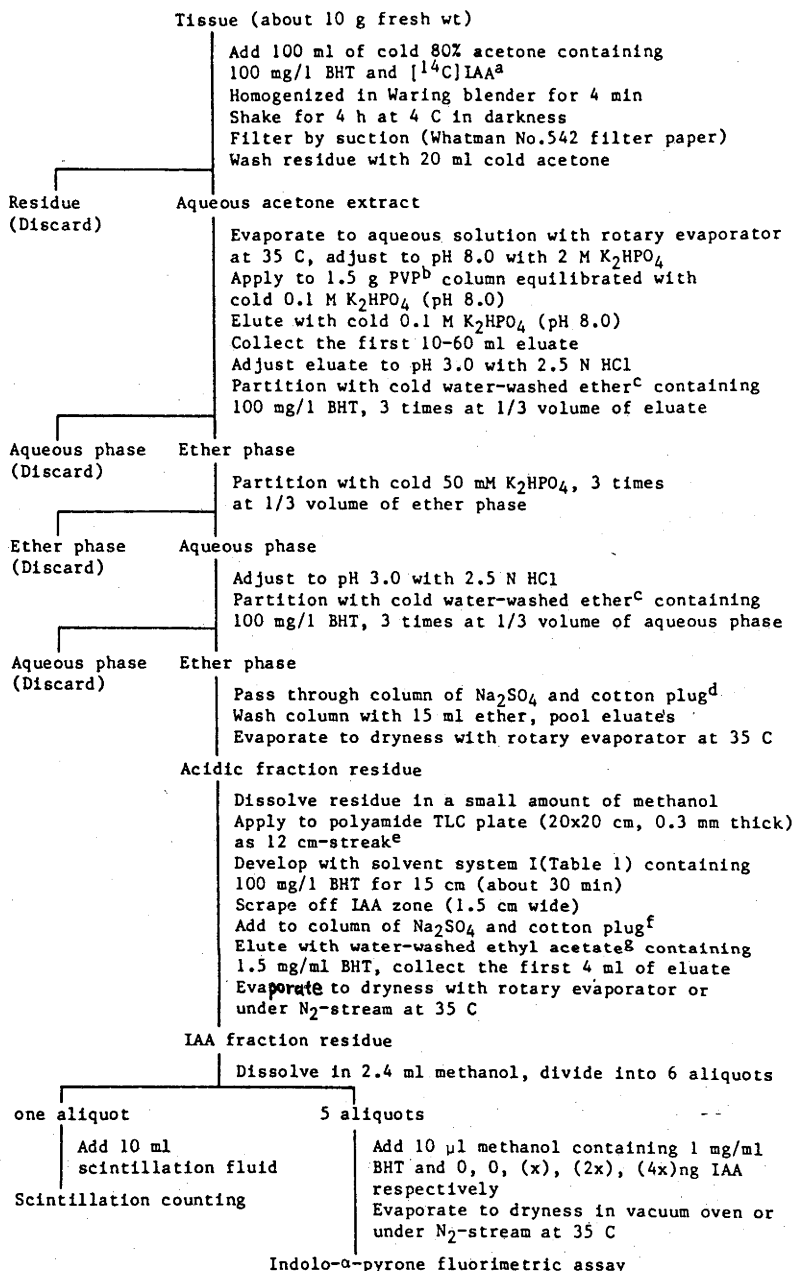


Fig. 6. Decomposition of IAA upon evaporation of the ethyl acetate solution to dryness: inhibition of decomposition by BHT. 30 ng IAA in 30 μ l ethyl acetate was added to 4 ml ethyl acetate containing 0, 2, 4, 8 and 12 mg BHT, and then evaporated to dryness under N_2 -stream at 35°C. The amount of IAA in the residue was determined by the fluorescence method: the sample was dissolved in 2 ml methanol (an appropriate amount of BHT was added so that the final amount of BHT in a sample was 12 mg), and divided into 5 aliquots; then 3, 6 and 12 ng authentic IAA were added to 3 aliquots respectively for the construction of the calibration line (the authentic IAA added was taken from the same ethyl acetate solution added initially to 4 ml ethyl acetate).



- a [¹⁴C]IAA in 0.4 ml acetone containing 1 mg/ml BHT was added to 100 ml of 80% acetone.
- b Insoluble PVP (Calbiochem); fine particles were removed by dispersing in an excess amount of water, leaving it to stand for 10-20 min, and then discarding the supernatant (repeat at least 8 times).
- c Prepared by shaking 3 times against water, 1/3 volume of ether.
- d 12 g anhydrous Na₂SO₄ (powder) and 0.25 g cotton; pre-washed with 20 ml ether.
- e Marker spots were applied on both sides of the sample streak, a groove was placed vertically between the marker spot and the sample streak.
- f 1 g anhydrous Na₂SO₄ (powder) and 0.1 g cotton; pre-washed with 3 ml ethyl acetate.
- g Prepared by shaking 4 times against water, 1/2 volume.

Fig. 7. An improved scheme for IAA extraction and determination.

CHAPTER 4

ESTIMATION OF FREE, CONJUGATED AND DIFFUSIBLE
INDOLE-3-ACETIC ACID IN ETIOLATED MAIZE SHOOTS
BY THE INDOLO- α -PYRONE FLUORESCENCE METHOD

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SUMMARY

Procedures for estimating free IAA (extracted from tissue homogenates by aqueous acetone), conjugated IAA (extracted by aqueous acetone and hydrolysed by 1 N KOH), and diffusible IAA (diffused from the excised tissue into water), in shoots of etiolated 3-day-old maize (Zea mays L.) seedlings are described, the indolo- α -pyrone fluorescence method being used to assay IAA. The reliability of the procedure is shown by comparative IAA determinations of the extracts using the GC-MS method in which the methyl ester, heptafluorobutyryl derivative of IAA is assayed using the selected ion monitoring technique with deuterated IAA as an internal standard. A 3 mm-long coleoptile tip, a coleoptile with its included leaves and nodal region (whole coleoptile) and a mesocotyl each contains 0.2, 1.7 and 1.5 ng of free IAA respectively. The whole coleoptile and the mesocotyl contain slightly less conjugated IAA than their content of free IAA. IAA diffuses from the coleoptile tip at the rate of 1.0 ng/tip/h; from the base of the whole coleoptile and a set of leaves excised from a coleoptile, IAA diffuses at the rate of 0.62 and 0.17 ng/plant part/h respectively. The data obtained support the classical assumption that the coleoptile tip produces IAA. It is also suggested that some IAA is decomposed during its downward transport in the coleoptile.

INTRODUCTION

Improved procedures for estimating endogenous IAA in etiolated maize shoots have already been reported (Iino et al 1980, Chapter 3). The endogenous IAA dealt with there was so-called free IAA, which is extractable by a solvent (in our case, aqueous acetone). This contribution extends

the procedures to the estimation of so-called conjugated and diffusible IAA. Conjugated IAA here implies IAA liberated from aqueous acetone extracts by 1 N KOH. As was shown by Bandurski and Schulze (1974), such alkali-labile, presumably esterified, IAA is the major form of conjugated IAA in maize shoots. Diffusible IAA is that which diffuses from the cut surface of excised tissue into water.

The assay procedure used here is the indolo- α -pyrone fluorescence method (Stoessl and Venis 1970) which was improved by Kamisaka and Larsen (1977) then by Iino et al. (1980, Chapter 3). The most serious problem of this method is the presence in the extracts of compounds which interfere by producing fluorescence identical to that produced by IAA upon reaction with acetic anhydride. The most likely compounds are 4-chloro-IAA and 5-hydroxy-IAA (Böttger et al 1978). Polyamide TLC was introduced for the separation of these compounds from IAA (Iino et al 1980, Chapter 3). However, one cannot exclude the possible existence in extracts of other such interfering compounds which could co-purify with IAA. The extent of this problem is evaluated here by making parallel IAA determinations by the fluorescence method and by a less equivocal GC-MS method.

MATERIALS AND METHODS

Plant Material. Etiolated seedlings of maize (Zea mays L. cv. GH 390: a field corn produced at the Agricultural Plant Breeding Station, Grafton N.S.W.) were prepared as previously reported (Iino and Carr 1981, Chapter

2). Three-day-old seedlings were used throughout. The materials for free and conjugated IAA extraction were harvested and frozen in liquid N₂ under dim green light (0.05 μ W/cm² on bench, light source as described in Iino and Carr 1981, Chapter 2) and stored if necessary at -80°C until extraction.

Preparation of Free IAA. Free IAA was obtained essentially as previously reported (Iino et al 1980, Chapter 3), but on a reduced scale. Fifty ml of 80% aqueous acetone containing about 3000 cpm [1-¹⁴C]IAA (58 mCi/mmol) was added to the tissue, less than 1 g fresh weight, in a Waring blender jar (a stainless steel semimicro jar with a screw cap) which was chilled beforehand by adding liquid N₂ and then discarding it; the tissue was homogenized for 4 min; the jar was washed with 10 ml of 80% acetone and the wash was added to the homogenate. The homogenate was then incubated for 4 h at 4°C on a shaker (Metabolyte Water Bath Shaker, New Brunswick Scientific Co., Inc.) at 200 rpm, and then filtered by suction through Whatman No. 542 filter paper. The residue was washed by passing through another 10 ml absolute acetone. The filtrate was evaporated to aqueous solution in a rotary evaporator. The aqueous solution was adjusted to pH 8.0 by adding 2 M K₂HPO₄. It was then passed through a PVP column (0.15 g insoluble PVP, equilibrated with 0.1 M K₂HPO₄ at pH 8.0, the column i.d. 11 mm), then 8 ml 0.1 M K₂HPO₄ (adjusted to pH 8.0) was added to the column. All the eluate was collected and acidified to pH 3.0 with 2.5 N HCl. This PVP treatment was

carried out in a cold room (4°C). The acidified aqueous solution was shaken twice against 9 ml ether. The resulting ether phase was shaken twice against 9 ml 0.05M K_2HPO_4 (pH not adjusted) and the ether discarded. The aqueous phase was adjusted to pH 3.0 with 2.5 N HCl and again shaken twice against 9 ml ether. The ether phase was passed through a column (i.d. 14 mm) consisting of 6 g anhydrous Na_2SO_4 plugged with 0.2 g absorbent cotton wool (Johnson & Johnson Pty. Ltd.); the column was pre-washed with 15 ml ether. The remainder of the ether phase in the column was washed off by adding an additional 5 ml ether. The ether phase was then evaporated to dryness in a rotary evaporator. When the fresh weight of plant tissue to be extracted was more than 1 g (but did not exceed 10 g), the tissue was homogenized in 100 ml of 80% acetone containing [^{14}C]IAA (about 8000 cpm) and after evaporation of the filtrate to the aqueous phase, an aliquot (usually 9 ml) was taken for further purification. To all the organic solvents used, BHT was added (80% acetone and absolute acetone, 100 mg/l; ether, 200 mg/l). Ether was washed by shaking it 3 times against a half volume of water (ether used to pre-wash the column was hardened at -20°C to remove excess water). All the solvents so prepared were chilled to 4°C before use.

Preparation of Conjugated IAA. Extracts for the estimation of conjugated IAA by subtraction of the accompanying amount of free IAA, were prepared following essentially the method devised by Bandurski and Schulze (1974, 1977). Plant tissue, 1-10 g fresh weight, was

homogenized with 100 ml of 80% acetone containing [^{14}C]IAA (about 8000 cpm), and the aqueous extract was obtained as described for free IAA preparation. A 9 ml aliquot was taken and its free IAA extracted. From the remaining solution a 7 ml aliquot was taken, made up to 1 N KOH by adding 10 N KOH, and incubated at 25°C for 1 h. After incubation the pH of the solution was adjusted to 8.0 by first adding 1 ml KH_2PO_4 -saturated water, then 5 N HCl. The solution was passed through a PVP column and the ether-soluble acidic fraction was obtained as described for free IAA. This sample contained free IAA plus IAA liberated from conjugation.

Preparation of Diffusible IAA. The seedlings were sprayed with water, then parts of the shoots were excised and placed in vials, cut surfaces in contact with water. After 1 h, the incubation water was collected. These procedures were carried out in darkness at 25°C, using an IR-scope and physiologically safe IR radiation during manipulations (Iino and Carr 1981, Chapter 2). The duration of the incubation was measured from the middle of the time required for the excision, which in any case did not exceed 10 min, and was usually about 5 min.

Details of the procedure for each of the shoot parts to be investigated were as follows:

[a] The coleoptile tip: Coleoptile tips 3 mm long, without included leaves, were incubated, 20 tips to 1 ml water in a vial (standard scintillation vial), shaken at 150 rpm. After incubation the water was pipetted into an

ice-cold 10 ml test tube containing 25 μ l of [14 C] IAA methanol solution (about 4000 cpm). The tips were washed twice with 0.5 ml of ice-cold 0.1 M K_2HPO_4 solution and the washes added to the test tube. When the same tips were incubated further, they were washed with water and 0.1 ml 1 M K_2HPO_4 was added afterwards to the test tube.

[b] The whole coleoptile: Coleoptiles (with their included primary leaves) were excised at about 1 mm below the coleoptilar node and 8 placed vertically on 0.4 ml water in a small vial (i.d. 1 cm, height 4.5 cm). Three vials were prepared for each sample. After incubation, the water in the three vials was taken up, and added to an ice-cold test tube containing [14 C] IAA as was done for coleoptile tips. The coleoptiles in each vial were washed twice with 0.2 ml of ice-cold 0.1 M K_2HPO_4 .

[c] The primary leaves: A set of primary leaves from within a coleoptile was obtained by cutting about 1 mm above the coleoptilar node and removing the coleoptile. The single set of leaves so obtained consists of leaves 1 to 3. Diffusible IAA was collected as described for the whole coleoptile, except that each vial contained ten sets of leaves.

The aqueous solution collected accordingly was then shaken twice against 2 ml ether. The ether phase was discarded using a Pasteur pipette. The aqueous phase was adjusted to pH 2.5-3.0 by adding 1 drop of 2.5 N HCl, and shaken 4 times against 1 ml ether. The ether phase was passed through a column (i.d. 11 mm), pre-washed by passing through 6 ml ether, of 2 g anhydrous Na_2SO_4 plugged with 0.15 g cotton wool; the remaining ether in the column was washed off by adding a further 1 ml ether. The ether extract collected was then evaporated to dryness in a rotary evaporator. All the ether used here was water-washed, BHT-added (1 mg/ml), and hardened at $-20^\circ C$. The ether thus prepared was placed on ice and used for partitioning.

Polyamide TLC. All the extracts prepared above were developed on polyamide TLC plates using the solvent system, benzene:ethyl acetate:acetic acid (70:25:5, v/v). A 20 x 20 cm plate was used to develop two samples; a marker spot of authentic IAA was applied at the centre of the plate, between two samples (grooves were placed vertically both sides of the marker spot). The scrapings of the IAA zone were loaded on a column of 1 g anhydrous Na_2SO_4 plugged with 0.1 g cotton wool, and eluted with water-washed ethyl acetate containing 1 mg/ml BHT; 2.5 ml eluate was collected and evaporated to dryness. The other details were as previously reported (Iino et al 1980, Chapter 3).

Estimation of IAA content by the Fluorescence Method. The residue obtained as described above was dissolved in 2 ml methanol containing 1 mg/ml BHT. An aliquot of 300 μl was added to 10 ml scintillation fluid for estimation of the recovery of [^{14}C]IAA by scintillation counting, and aliquots of 150 μl were taken into test tubes for assay of IAA by the indolo- α -pyrone fluorescence method (five aliquots are necessary for one determination, and usually duplicate sets were prepared). Recovery in 300 μl aliquot was calculated as a percentage of the counts originally added. This original count was obtained directly from control samples of [^{14}C]IAA, identical with that added at the beginning of the procedure. The IAA in a 150 μl aliquot was determined by the fluorescence method essentially as previously reported (Iino et al 1980). The amounts of authentic carrier IAA added to the samples for the

construction of a calibration line, were 1, 2 and 3 ng (these were taken up in 150 μ l methanol containing 1 mg/ml BHT; the same volume of methanol was added to the samples without added carrier IAA, including the blank).

From the percentage [^{14}C]IAA recovered in the 300 μ l aliquot and the amount of IAA in the same aliquot (double that determined in a 150 μ l aliquot), the total content of IAA in the original extract was calculated. It is necessary to subtract from this figure the relatively substantial amount of [^{14}C]IAA added before homogenization of the tissue (the amount of [^{14}C]IAA was usually 10 to 50% of the endogenous IAA). This amount was determined by direct measurement of the IAA content of identical aliquots of the [^{14}C]IAA added, using the fluorescence method.

GC-MS Method. Determination of IAA by GC-MS was carried out using SIM technique (Rivier and Pilet 1974, Caruso et al 1978, Little et al 1978, Allen Baker 1980, Magnus et al 1980). Deuterated IAA was used as an internal standard, and the mixture of deuterated and endogenous IAA was derivatized before GC-MS assay.

Derivatization: The methyl ester-HFB-IAA was prepared essentially according to Rivier and Pilet (1974). A sample residue in a 10 ml test tube was dissolved in an ether:methanol mixture (4:1, v/v) containing diazomethane, left to react for 15 min at room temperature, and the sample solution was evaporated to dryness under a N_2 -stream. The residue was then dissolved in 100 μ l HFBI (Pierce), the test tube was filled up with dry N_2 , and sealed with a ground glass stopper. It was allowed to react at 80°C for 2 h.

After the reaction, the test tube was cooled on ice, and 1 ml of ice-cold 0.1 M K_2HPO_4 , then 1 ml n-hexane were added; the test tube was vibrated thoroughly on a Vortex mixer. The resulting aqueous phase was discarded. The remaining hexane layer was washed 4 times with 0.5 ml water, and hardened at $-20^\circ C$. The hexane layer was transferred to a Reacti-vial (Pierce) and evaporated to dryness under a N_2 -stream. The Reacti-vial was then filled with N_2 , sealed and stored at $-20^\circ C$ (storage did not exceed two days). The residue was taken up in n-hexane and used for GC-MS analysis.

Internal standard: Deuterated IAA (d_2 -IAA, two deuterium atoms at the C-2' position of the side-chain; Stohler Isotope Chemicals) was used as an internal standard. The d_2 -IAA was purified by polyamide TLC before use (BHT was not added at any stage).

Instruments and conditions: GC-MS analysis was carried out using a VG Micromass 70/70 F mass spectrometer equipped with a Digital Multiple Ion Detection system (DIGMID MK 1), coupled to a 1400 Varian gas-liquid chromatograph. GLC was carried out on a glass column (6 ft x 0.125 in) packed with 2% OV-17 on 80-100 mesh Gas Chrom Q, using Helium as the carrier gas (flow rate 25 ml/min), with the column temperature programmed to rise from $120^\circ C$ at $10^\circ C/min$ (injector temperature, $220^\circ C$). MS conditions were: ion source temperature $220^\circ C$, electron impact ionizing energy 70 eV, trap current 200 μA . The jet separator and the connecting line were maintained at $270^\circ C$. Selected ion currents were recorded using a multichannel pen recorder.

Retention time and mass spectrum: Monitoring the total ion current on GC-MS after an injection of the derivative of authentic IAA (Sigma, used without further purification), the peak found at the retention time 3.5 min was confirmed to be that of the IAA derivative by obtaining mass spectra over this peak. The mass spectrum showed the major ions to be at m/e 385 (molecular ion), 326 (base peak) and 129, the relative intensity (% of base peak) being 48, 100 and 53 respectively (Fig. 1).

Isotopic purity of d_2 -IAA: The mass spectrum of the d_2 -IAA derivative showed this standard to be essentially free of nondeuterated IAA (ion abundance at m/e 385 or 326, 2% or less of that at 387 or 328). However, the derivatized standard appeared to contain a significant amount of d_1 -component (ion abundance at m/e 386 or 327 was about 15% of that at m/e 387 or 328).

Determination of IAA in extracts: Molecular ions (m/e 385, 387) and base peaks (m/e 326, 328) corresponding to the derivatives of IAA and d_2 -IAA standard added to the extract were monitored simultaneously. IAA was determined by obtaining the peak height ratios, 385/387 and 326/328, and then referring each ratio to a corresponding calibration curve. Each sample was injected at least 3 times to obtain a mean value. The calibration curve was obtained by running mixtures of authentic nondeuterated IAA and d_2 -IAA on GC-MS. Solutions of these authentic compounds were prepared in ethanol (the concentration of d_2 -IAA was calibrated using UV absorbance at 280 nm and referring to that of standard solutions of nondeuterated IAA). They were mixed before derivatization, the amount of d_2 -IAA in each sample being fixed at 50 ng, and the amount of IAA varied (10, 30, 50, 100 and 150 ng). The molecular and base ions were monitored, and the peak ratios, 385/387 and 326/328, were plotted against the actual molar ratio on a linear scale. The plots were linear with the extrapolated lines passing through the origin (Fig. 2).

RESULTS

1. Reliability of IAA Estimation Using the Fluorometric Assay; Evaluation by GC-MS Assay.

Free IAA, free-plus-conjugated IAA, and diffusible IAA were extracted and purified using the standard procedures ([^{14}C] IAA was not added). Free IAA and free-plus-conjugated IAA were extracted from the whole shoots, and

diffusible IAA was collected using the whole coleoptile system. Each purified extract was dissolved in 3 ml methanol. Five 150 μ l aliquots were taken, and the IAA determined by the fluorescence method. A 60 ng $\underline{d}_2$ -IAA standard (taken in 150 μ l methanol) was added to the remaining aliquot; IAA in it was then purified by another step of polyamide TLC (BHT was not added to solvents used for this purification) and determined by the GC-MS method. The carrier IAA used to construct the calibration line in the fluorometric assay was also taken from the same $\underline{d}_2$ -IAA standard solution after proper dilution (the amounts of carrier IAA were 2, 4 and 6 ng).

The profile of selected ion currents obtained from the purified extracts ($\underline{d}_2$ -IAA added) showed a peak for each of the ions monitored simultaneously, i.e. molecular ions: $\underline{m/e}$ 385 (IAA) and 387 ($\underline{d}_2$ -IAA), base peaks: $\underline{m/e}$ 326 (IAA) and 328 ($\underline{d}_2$ -IAA), at the same retention time. The retention time also corresponded to that of authentic IAA. An example of the profile, obtained from a sample of free-plus-conjugated IAA, is given in Fig. 3. Thus, it is unlikely that the selected ion currents under investigation are subject to interference by impurities of the same mass number.

The comparative fluorescence and GC-MS results are presented in Table 1. There is good accord between the determinations with all types of IAA tested. It thus appears that the extraction and purification procedures proposed here can provide IAA samples free of compounds likely to interfere with the fluorometric assay.

2. Free IAA and Conjugated IAA.

A 4 h incubation of the homogenized tissue with aqueous acetone was sufficient to recover most of the free IAA and the conjugated IAA, i.e. further incubation did not significantly increase the recovery (Table 2).

The excised seedling shoot was cut into two parts at about 1 mm below the coleoptilar node, and the free IAA content of these parts was estimated. The upper part, i.e. the coleoptile with its included leaves and its nodal region (whole coleoptile), contained 28 ng/g fresh weight of free IAA; the lower part, i.e. the mesocotyl, contained 15 ng/g fresh weight (Table 3). The distribution of free IAA along the shoot has been investigated in greater detail (Fig. 4). The coleoptile tip is richest (49 ng/g fresh weight) and the level declines basipetally. The free IAA content of the apical 1 cm of mesocotyl, the most actively growing region of the etiolated seedling, was 20 ng/g fresh weight. The leaves inside the coleoptile also contain some IAA (14 ng/g fresh weight). These estimates of IAA content are highly reproducible. The free IAA content of the coleoptile tip (3 mm long), estimated from the results of three separate experiments, was 0.211 ± 0.007 ng/tip (mean and S.E.). That of the apical region of the mesocotyl (1 cm long) also estimated from the results of three separate experiments was 20.5 ± 0.4 ng/g fresh weight. The conjugated IAA content of the whole coleoptile and the mesocotyl is shown in Table 4. In each case, it was slightly less than that of free IAA.

3. Diffusible IAA.

The amounts of diffusible IAA collected for 1 h from the coleoptile tip, the whole coleoptile and the primary leaves are shown in Table 5. From the coleoptile tip and the whole coleoptile, 1.0 ng and 0.62 ng of IAA were obtained respectively. A single set of primary leaves yielded 0.17 ng IAA. When the coleoptile tips were incubated for several hours, changing the incubation water every hour, IAA diffused out at the same rate for 3 h but the rate declined afterwards (Fig. 5).

Accurate estimation of diffusible IAA depends not only on the accuracy of estimation of IAA in the extracts, but also on the diffusion technique itself. Errors may arise from decomposition of IAA at the cut surface of the tissue or by enzymes released from it, as has long been recognized (Wetmore and Morel 1949, Steeves et al 1953, Briggs et al 1955, Goldsmith and Thimann 1961, Zenk and Müller 1964, Iversen and Aasheim 1970, Aasheim and Iversen 1971, Bruinsma et al 1975). Re-uptake of diffused IAA by the tissue may also have to be considered. These possibilities were tested by incubating the coleoptile tips, or the whole coleoptiles, in [^{14}C]IAA solution for 1 h as for the collection of diffusible IAA, and then estimating the recovery of [^{14}C]IAA by radioactivity measurements. The amount of [^{14}C]IAA added to the incubation water in a vial was 7.5 ng (coleoptile tips) or 2.5 ng (whole coleoptiles). The recovery was estimated after purification of [^{14}C]IAA in the incubation water, employing essentially the procedure used to purify diffusible IAA. However, to correct for

losses of [^{14}C]IAA during purification, authentic "carrier" IAA (200 μg) was added to the incubation water immediately after incubation; the recovery of the carrier IAA in the purified extract was estimated from measurements of UV absorbance at 280 nm using that at 320 nm as background. Background effects of the extracts on the UV absorbance determination were checked by measuring the differential UV absorbance, $A_{280}-A_{320}$, of extracts obtained by the same methods but without added carrier IAA; the differential absorbance was negligible (less than 1% of the absorbance due to 200 μg authentic IAA). It thus appears that the procedure for correction of losses using UV absorbance of the carrier IAA is not subject to interference by other compounds in the extract. BHT was not added to any of the solvents used, since it absorbs UV.

The data thus obtained are presented in Table 6. Losses of [^{14}C]IAA due to its incubation with coleoptile tips did not appear to be significant. Losses were higher when whole coleoptiles were incubated, but were well within 10%. Thus it appears that decomposition of IAA diffused into incubation water and re-uptake of IAA by the tissue are not serious problems in estimating diffusible IAA. There is a possibility that some IAA diffusing from the tissue might be decomposed in crossing the injured tissue at the cut surface. However, such a possibility would not be easy to test.

Production of IAA by contaminating bacteria is another problem which might introduce errors in the estimation of diffusible IAA (Libbert et al 1969). The possible

contribution of bacteria to the yield of diffusible IAA was tested by comparing yields obtained with and without streptomycin (0.1 mg/ml) in the incubation water. Seedlings to be incubated with streptomycin were also sprayed with streptomycin (same concentration) just before excision of their coleoptiles; controls were similarly sprayed with water. The yield of diffusible IAA was slightly less in the presence of streptomycin, but the difference was not significant (Table 7).

IAA diffuses from the excised coleoptile tip at a constant rate for about 3 h (Fig. 5). IAA also diffuses out from the whole coleoptile at a constant rate for at least 3 h (data not shown). These results suggest that the experimental treatments do not affect the IAA status of the tissue for at least a few hours after excision. A further check of this was made by examining the IAA levels in the whole coleoptile immediately before and after 1 h incubation, for possible changes in IAA status induced by experimental treatments. As shown in Table 8, 1 h incubation appeared to enhance the IAA level slightly, but the change was not significant.

DISCUSSION

The IAA estimation procedure using the indolo- α -pyrone fluorescence method reported previously (Iino et al 1980, Chapter 3) has been extended to the estimation of conjugated IAA and diffusible IAA. Its reliability has been demonstrated in comparative determinations on samples of the

same extract by the GC-MS method.

The content of free and conjugated IAA in etiolated maize shoots has already been investigated by Bandurski and co-workers. Using 5-day-old seedlings, they showed the free IAA content to be around 24 ng/g fresh weight (Bandurski and Schulze 1974, Bandurski et al 1977), a measurement in good accord with ours (Table 3, also see Iino et al 1980, Chapter 3). Earlier they had reported the conjugated IAA content to be 330 ng/g fresh weight (Bandurski and Schulze 1974) which is much higher than our measurement, but in later work (Bandurski et al 1977) they published a much lower figure (45 ng/g fresh weight, estimated from their published data on free and free-plus-conjugated IAA contents), closer to ours. Their most recent estimate, obtained using a GC-MS technique, of free-plus-conjugated IAA is 102 ng/g fresh weight (Magnus et al 1980). Although still two to three times as high as our figure, the difference could possibly be due to the use of a different variety of maize or of seedlings of a different age.

As Thimann (1934), van Overbeek (1941), and Wildman and Bonner (1948) have shown, the amount of auxin obtained from the coleoptile tip by diffusion is larger than that obtained by solvent extraction, supporting the classical assumption that the coleoptile tip produces auxin (see Went and Thimann 1937). The data obtained by these authors were later critically examined and questioned by Sheldrake (1973). The results obtained here, however, clearly show that IAA diffuses from the excised coleoptile tip in amounts far exceeding its initial content. From a 3 mm-long coleoptile

tip about 3 ng IAA diffuses out in 3 h, more if diffusion is allowed to continue (Fig. 5), whereas the freshly-excised coleoptile tip contains only 0.2 ng of free IAA (fig. 2). Moreover, after 1 h-diffusion of about 1 ng IAA, the content of free IAA in the excised tip was maintained at the original level (data not shown).

Bandurski and co-workers (Epstein et al 1980, Nowacki and Bandurski 1980) have concluded from feeding experiments using radioactive indole-3-acetyl-myo-inositol that this IAA conjugate, which is contained in the maize seed in large amounts, is transported into the shoot and there serves as a major source of free IAA. It is, however, unlikely that an acropetally moving supply of conjugated IAA could be a source of IAA produced at the coleoptile tip. A whole coleoptile, with its included leaves, contains only about 1.4 ng conjugated IAA (Table 4), and even this small amount of conjugated IAA is not restricted to the coleoptile tip (a 10 mm-long coleoptile segment cut 3 mm below the tip, without included leaves, contained 0.63 ng conjugated IAA). The conjugated IAA content is thus too low to account for the high yield of IAA which can be collected by continuous diffusion from the excised coleoptile tip, which, following excision, is cut off from the hypothetical acropetal supply of conjugated IAA. The results also indicate that the pool size of endogenous IAA (whether free or conjugated) at the coleoptile tip is kept very small, so that the IAA produced there moves out of the tip immediately after its production.

The yields of diffusible IAA from a coleoptile tip and a set of leaves from inside a coleoptile were 1.0 and 0.2 ng/h respectively. However, the yield of diffusible IAA from the base of the whole coleoptile, which includes the tip and leaves as sources of IAA, was only 0.6 ng/h. As far as our examination goes, problems of IAA decomposition induced by cutting the tissue and IAA production by bacteria did not give rise to serious discrepancies (see Results). As already indicated by Goldsmith and Thimann (1961), IAA may be decomposed or immobilized during its downward transport in the coleoptile. In seedlings of the age selected for use, there is a gradual increase of free and conjugated IAA content of the whole coleoptile with time (see Chapter 5). However, the increase is too small to compensate for the losses of IAA during its downward transport. Immobilization either in the free state or by conjugation is not likely. Decomposition appears to be the major process by which loss occurs.

The plants used in the present study were not raised aseptically. Libbert and co-workers have shown that many bacteria can produce IAA and claim that the existence of such bacteria on plant surfaces can falsify estimates of extractable auxin content (e.g. Libbert et al 1968, 1969). Their own data rely on bioassay methods and require confirmation using less equivocal physico-chemical methods. However, the objections they raise must be borne in mind until the contribution of contaminant bacteria to estimates of the IAA content of plants is clarified.

Table 1. Determination of endogenous IAA: Comparison of fluorometric and GC-MS assays. Free IAA, free-plus-conjugated IAA or diffusible IAA in aliquots of each purified extract was determined using both the fluorometric and the GC-MS assays. For the GC-MS assay, ion currents of molecular ions (m/e 385, 387) and base peaks (m/e 326, 328) derived from IAA and d_2 -IAA (added to the extract as an internal standard) were monitored. Two sets of determinations were obtained from the peak height ratios of m/e 385/387 and m/e 326/328, each with reference to a corresponding calibration line. The numbers in the table represent total IAA in each purified extract.

Source of IAA	ng IAA		
	Fluorometric Assay	GC-MS/MID Assay	
		385/387	326/328
(A) Free IAA	113	113	117
in Whole Shoot ^a	145	150	152
	142	147	146
(B) Free - plus -	143	149	153
Conjugated IAA in	178	183	183
Whole Shoot ^b			
(C) Diffusible IAA	52	54	53
from Whole	37	39	39
Coleoptile ^c			

^a 60 shoots were used for each sample.

^b 40 shoots were used for each sample.

^c 64 coleoptiles were used for each sample.

Table 2. Efficiency of aqueous acetone in extracting free and free-plus-conjugated IAA. Etiolated maize shoots, 30 or 60, were homogenized in 100 ml 80% acetone, and incubated for 4 h at 4°C. The aqueous acetone extract was filtered and the tissue residues reincubated at 4°C in two further 100 ml lots of 80% acetone, the first for 4 h, the second for 16 h. Free IAA and free-plus-conjugated IAA in the filtrate obtained from each incubation were estimated; [^{14}C]IAA was added to the filtrate to obtain a correction for losses of IAA during its purification.

No. of Shoots	Fresh Wt (g)	ng IAA Extracted					
		Free			Free + Conjugated		
		4h	→ 4h	→ 16h	4h	→ 4h	→ 16h
60	8.48	156	2.0	1.3	237	3.9	1.7
60	8.55	159	1.5	0.8	248	3.6	1.2
30	4.23	81	1.1	0.8	136	2.3	0.7
30	4.22	68	1.7	1.2	106	2.5	1.2

Table 3. Free IAA content of coleoptiles and mesocotyls. Coleoptiles (each including the primary leaves and the coleoptilar node) or mesocotyls excised from 30 etiolated maize seedlings were used in each estimation. Means of 8 estimates obtained in separate experiments are presented with SE.

	Tissue Weight (mg Fresh Wt/ Plant Part)	IAA Content	
		ng IAA/Plant Part	ng IAA/g Fresh Wt
Coleoptile	59.2 $\pm$ 1.6	1.66 $\pm$ 0.09	28.0 $\pm$ 1.2
Mesocotyl	101.0 $\pm$ 2.1	1.54 $\pm$ 0.07	15.2 $\pm$ 0.5

Table 4. Conjugated IAA content of coleoptiles and mesocotyls. Free IAA and free-plus-conjugated IAA was extracted from coleoptiles (including the primary leaves and the coleoptilar node) or mesocotyls excised from 60 etiolated maize seedlings. From these estimates, conjugated IAA content was obtained by subtraction.

	Tissue Weight (mg Fresh Wt/ plant Part)	ng IAA/g Fresh Wt (ng IAA/ Plant Part)		
		Free	Free + Conjugated	Conjugated
Coleoptile	64.8	26.0	47.4	21.4(1.39)
	63.3	26.8	49.3	22.5(1.42)
Mesocotyl	107.5	16.1	27.5	11.4(1.23)
	105.5	15.9	27.9	12.0(1.27)

Table 5. Amounts of diffusible IAA collected from excised coleoptile tips, whole coleoptiles and primary leaves. The coleoptile tip 3mm long was excised without the included primary leaf; the coleoptile including the primary leaves and the coleoptilar node (whole coleoptile) was obtained by cutting at about 1 mm below the node; a set of primary leaves was obtained by cutting the coleoptile at about 1 mm above the node and removing the coleoptile. Twenty coleoptile tips were floated on 1 ml water in a vial and shaken; eight whole coleoptiles or ten sets of the primary leaves were stood vertically on 0.4 ml water in a vial. They were incubated for 1 h, and diffusible IAA collected in the incubation water was estimated. For each estimation, IAA collected in one vial (coleoptile tips) or 3 vials (whole coleoptiles, primary leaves) were used. The means and SE of 5 (coleoptile tips, whole coleoptiles) or 3 (primary leaves) estimates obtained in separate experiments are presented.

	ng IAA Diffused/Plant Part/h
Coleoptile Tip	1.03 $\pm$ 0.03
Whole Coleoptile	0.615 $\pm$ 0.016
Primary Leaves	0.168 $\pm$ 0.016

Table 6. Losses of [^{14}C]IAA during 1 h-incubation of coleoptile tips and whole coleoptiles. Excised coleoptile tips or whole coleoptiles were incubated with [^{14}C]IAA in water using the procedures given in the legend to Table 5. Amount of [^{14}C]IAA added to the incubation water in a vial was 7.5 ng (coleoptile tips) or 2.5 ng (whole coleoptiles). After incubation, [^{14}C]IAA in the incubation water was extracted into ether and purified by polyamide TLC. Recovery of [^{14}C]IAA was estimated from the recovery of radioactivity. The figure for recovery was corrected for losses of [^{14}C]IAA due to extraction and purification, estimated in terms of the UV absorbance (at 280 nm) of carrier IAA (200 μg) added to the incubation water immediately after incubation.

	% [^{14}C] IAA Recovered
Coleoptile Tip	96.8 $\pm$ 0.4
Whole coleoptile	93.2 $\pm$ 0.7

Table 7. Effect of streptomycin on the yield of diffusible IAA. Excised coleoptile tips and whole coleoptiles were incubated in water for 1 h with or without streptomycin (0.1 mg/ml) using the procedures given in the legend to Table 5. Diffusible IAA yield was then estimated. Means and SE of 3 replicates are presented.

	Streptomycin	ng IAA Diffused/Plant Part/h
Coleoptile Tip	-	1.00 $\pm$ 0.03 ^a
	+	0.91 $\pm$ 0.06 ^a
Whole Coleoptile	-	0.63 $\pm$ 0.03 ^b
	+	0.56 $\pm$ 0.02 ^b

^a Means not significantly different by t-test ($p > 0.2$).

^b Means not significantly different by t-test ($0.2 > P > 0.1$).

Table 8. Effect of incubation on IAA content of coleoptiles. Excised whole coleoptiles were incubated for 1 h using the procedures given in the legend to Table 5. IAA content of the coleoptile before (i.e. at the time of excision) and after incubation was estimated. Means and SE of 3 replicates are presented.

	ng IAA/g Fresh Wt	ng IAA/Plant Part
Initial	25.0 $\pm$ 0.9 ^a	1.69 $\pm$ 0.09 ^b
After Incubation	27.1 $\pm$ 0.6 ^a	1.78 $\pm$ 0.12 ^b

^a Means not significantly different by t-test ($p > 0.2$).

^b Means not significantly different by t-test ($p > 0.2$).

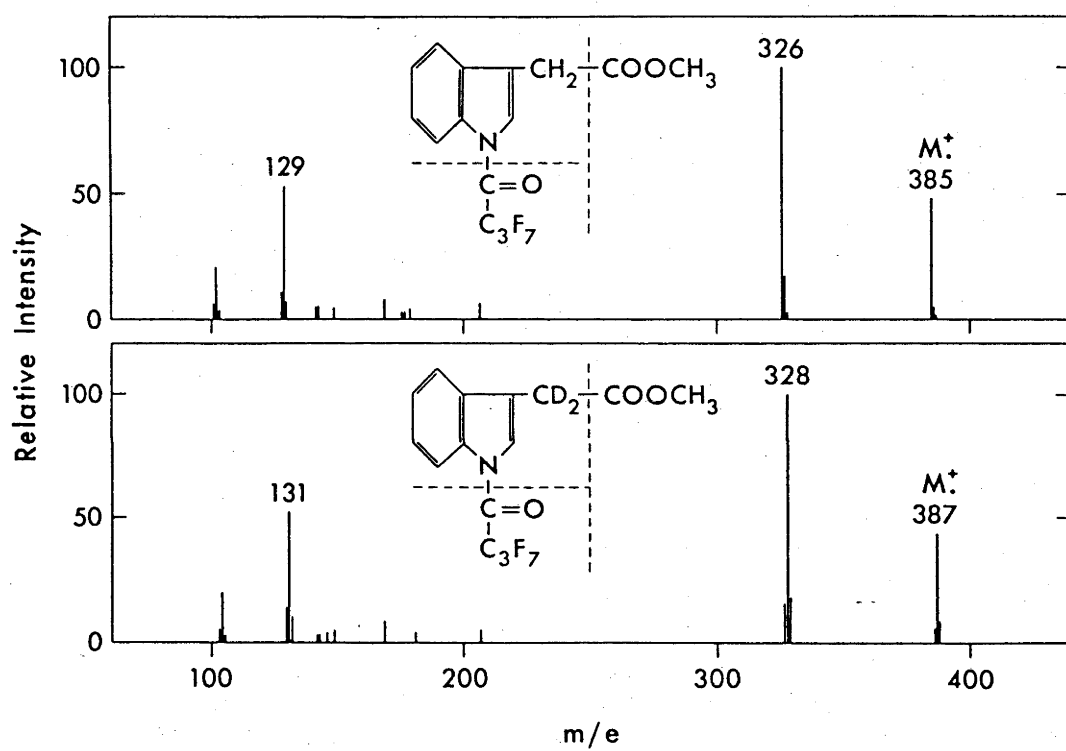


Fig. 1. Mass spectra of IAA and d_2 -IAA derivatives.

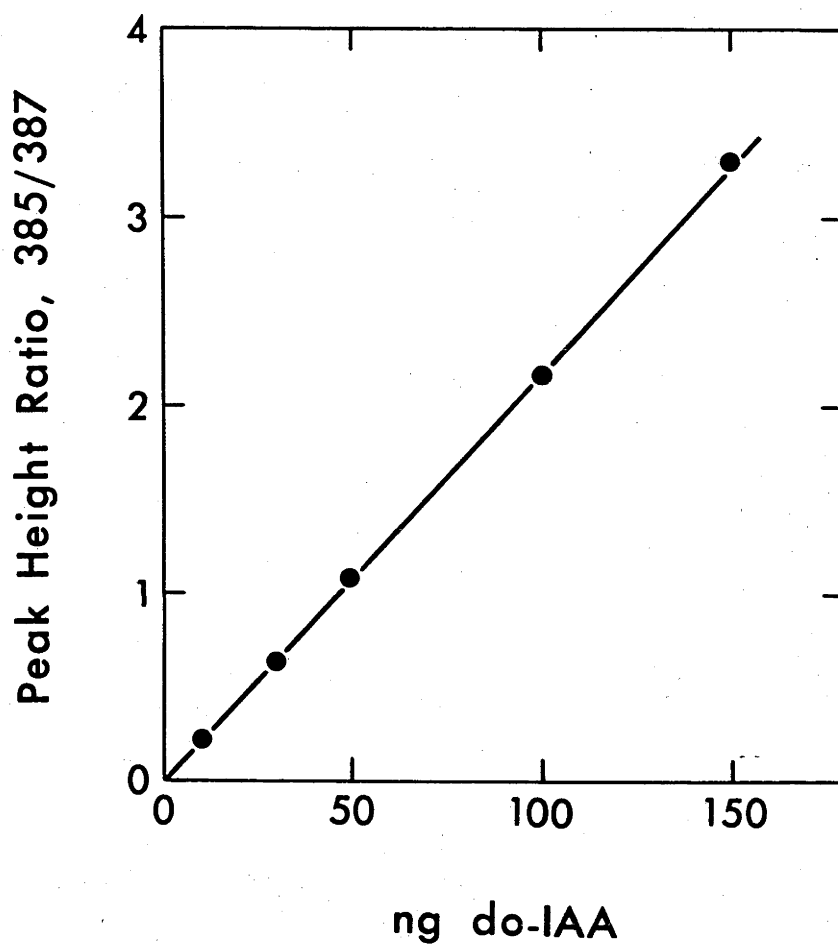


Fig. 2. An example of a standard curve in IAA determination by GC-MS. The amount of d_2 -IAA in each sample was fixed (50 ng) and that of IAA varied (10, 30, 50, 100 ng). The mixtures of IAA and d_2 -IAA were then derivatized and analyzed by GC-MS. Peak ratios, m/e 385/387 (molecular ions of IAA and d_2 -IAA derivatives) are plotted against the molar ratios, IAA/ d_2 -IAA.

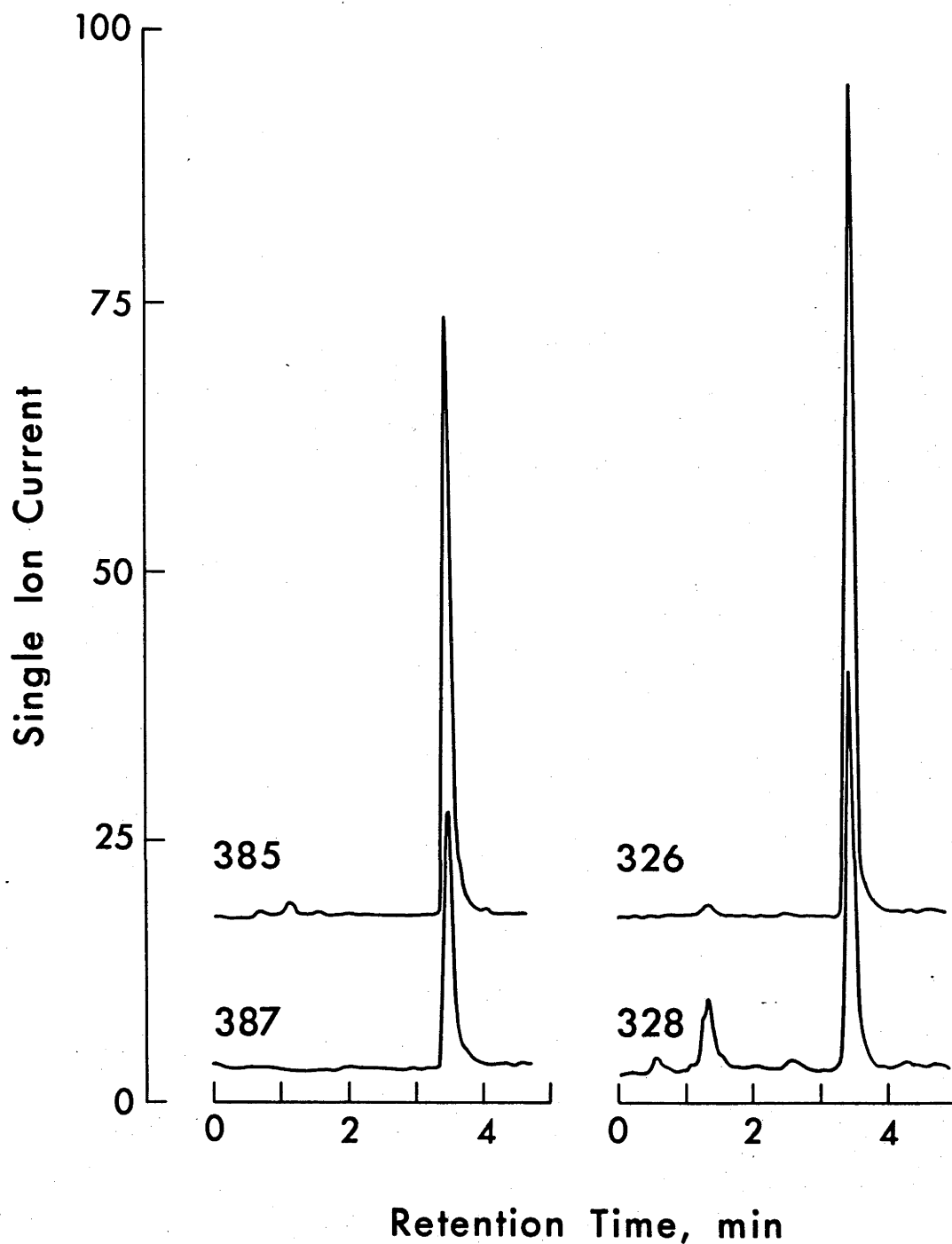


Fig. 3. Selected ion monitor traces of the derivatives of endogenous IAA and d_2 -IAA standard. Free-plus-conjugated IAA was extracted from 60 shoots excised from etiolated maize seedlings. After addition of 60 ng d_2 -IAA, the extract was reacted to form methyl ester-heptafluorobutryl derivatives and subjected to GC-MS. Selected ions corresponding to the molecular ions (m/e 385, 387) and the base peaks (m/e 326, 328) of derivatized IAA and d_2 -IAA were monitored simultaneously.

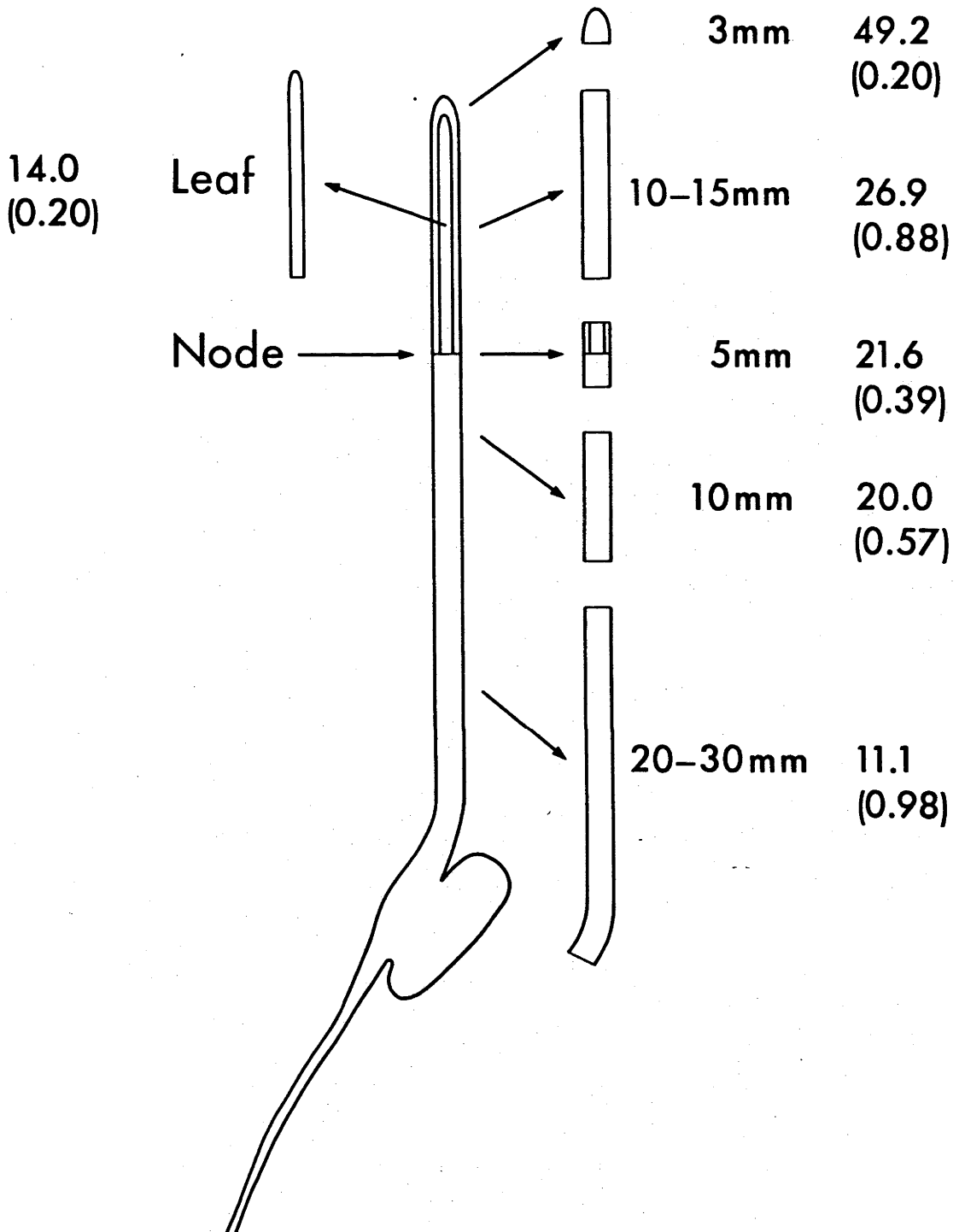


Fig. 4. Distribution of free IAA in an etiolated maize shoot. Free IAA content of various parts excised from the seedling shoots, as shown in the figure, was estimated. At least 60 of each part were used. The estimates are shown as ng/g fresh weight, or ng/plant part (in parentheses).

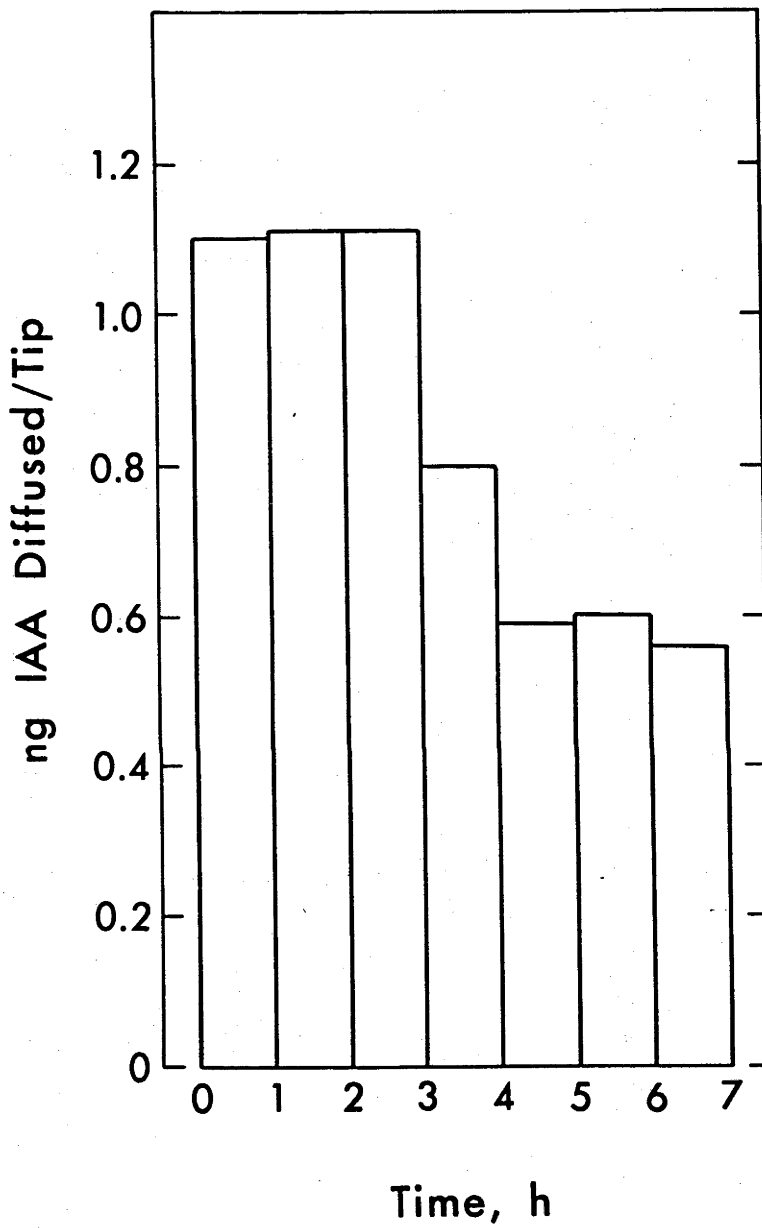


Fig. 5. Diffusible IAA yield from coleoptile tips. Diffusible IAA was collected from twenty excised 3 mm-long coleoptile tips in 1 ml water. The water was renewed every hour, and IAA in each collection was estimated.

CHAPTER 5

LIGHT ACTION ON IAA STATUS AND GROWTH IN COLEOPTILES OF ETIOLATED MAIZE SEEDLINGS

IINO, M. (1980) PLANTA, SUBMITTED FOR PUBLICATION

----- SUMMARY -----

Brief irradiation of intact etiolated seedlings of maize (Zea mays L.) with red light ($30 \mu\text{W}/\text{cm}^2$, 10 min) results in reduced yields of diffusible IAA from excised coleoptile tips and reduced amounts of free IAA in the same tissues. The effect is transient, the lowest level being achieved at about 3 h after irradiation. The free IAA content of the whole coleoptile and the diffusible IAA yield from the base of the same organ are similarly reduced, whereas the conjugated IAA content of the coleoptile is not affected. These results do not support the hypotheses that red light inhibits transport or stimulates conjugation of IAA but support the view that red light inhibits the production of IAA. Furthermore, red light is shown to inhibit biosynthesis of [^3H]IAA from [^3H]tryptophan supplied to the coleoptile tip.

Studies on relations between light fluences and the yield of diffusible IAA indicate the participation of two photoreactive systems. One has the threshold at $1 \text{ nWs}/\text{cm}^2$ of red light, 5 orders of magnitude less than the minimum required for the appearance of spectrophotometrically measurable Pfr in vivo, and shows a linear response to log fluence over at least 5 orders of magnitude. Far-red light can also induce this response, though it is about 100 times less effective. Interpretation of this system in terms of the Pfr concentration appears to be difficult. The other system is only apparent with red light. Its response is far-red reversible and related to the Pfr level of total photoreversible phytochrome. Both systems inhibit biosynthesis of IAA from tryptophan.

Elongation of the coleoptile is stimulated by red light; the stimulation is most apparent in the apical region, and is saturated with a fluence with which no detectable Pfr is formed. Far-red light can also saturate this response. It is concluded that the stimulation of coleoptile elongation is not due to changes in IAA levels.

INTRODUCTION

It is a well-confirmed observation that diffusible auxin, collected from the cut surfaces of excised coleoptile tips and assayed by the Avena curvature test, decreases in yield following exposure of intact seedlings to red light (van Overbeek 1936, Briggs 1963a, Furuya et al 1969, Muir and Chang 1974, Huisinga 1976). This response can be induced by brief irradiation (Furuya et al 1969, Muir and Chang 1974, Huisinga 1976), and is at least partially under phytochrome control (Furuya et al 1969).

How red light or the phytochrome system acts to reduce the yield of diffusible auxin is, however, not understood. The classical assumption that the coleoptile tip produces auxin has been questioned by Sheldrake (1973). Nevertheless, Iino and Carr (1982, Chapter 4) have provided quantitative estimates of diffusible and solvent extractable IAA which show that the coleoptile tip must be producing IAA at a high rate. Thus, though there is no crucial evidence for it, an obvious possibility is that red light inhibits the production of auxin.

Other interpretations have also been put forward. Huisinga (1964, 1967) proposed a hypothesis, based on observations of growth responses of oat seedlings, that red light inhibits auxin transport. The main evidence is that growth of the coleoptile tip is stimulated but that of the more basal region of the shoot is inhibited. Huisinga (1976) claimed further support for this hypothesis by showing that the amount of diffusible auxin obtained from

the excised subapical segments of oat coleoptiles was increased upon irradiation of intact seedlings. Bandurski et al (1977) showed that red light reduces the content of free IAA and increases that of esterified IAA in maize seedling shoots, and suggested that the decrease in diffusible IAA is related to the conjugation of free IAA. Red light might also induce or stimulate the decomposition of auxin, but no evidence has so far been provided (see also Furuya et al 1969).

To elucidate whether all of these mechanisms, or a particular one, is involved, a direct approach was made, carrying out quantitative determinations of endogenous IAA and measurements of IAA biosynthesis from radioactive tryptophan. Phytochrome control was also examined in detail. The results were then related to growth responses.

MATERIALS AND METHODS

Plant Material. Etiolated seedlings of maize (Zea mays L. cv. GH 390) were raised on moist paper towels in cardboard seedling boxes, 36 to 40 seedlings per box, as previously reported (Iino and Carr 1981, Chapter 2), and used when 3 days old. A box of seedlings was used as a unit of treatment, unless the number required for a treatment exceeded 40. All the procedures, from the sowing of seeds to the light treatment of the seedlings and subsequent manipulations of the seedlings or excised parts, were carried out in dark rooms kept at 25° C and 80% relative humidity. The seedling boxes were opened only when

required, and on those occasions the humidity was raised to about 90%.

Light Sources and Irradiation. Red light was obtained by passing light from red fluorescent tubes (Philip) through a layer each of No. 1 and No. 14 Cinemoid, and 1 cm-thick copper sulfate solution (5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ plus 0.2% conc. H_2SO_4); far-red light was obtained by passing light from tungsten lamps through a layer each of No. 5A and No. 20 Cinemoid, and 1 cm-thick ferrous ammonium sulfate solution (30% $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ plus 1% conc. H_2SO_4). The copper sulfate and ferrous ammonium sulfate solutions were held between 4 mm-thick sheets of clear acrylic 02 (Cadillac Plastic). The combined light sources and filters were enclosed in light-proof cabinets, which were placed in the dark room. The spectral energy distribution of the red and far-red light, measured from 300 to 800 nm using a spectroradiometer (IL780 Spectroradiometer System, International Light), is given in Fig. 1.

Intact seedlings were irradiated, unless otherwise specified, through the glass top of the seedling box (see Iino and Carr 1981, Chapter 2). Fluence rates of light were measured using an IL700 Radiometer coupled to a SEEl00F Detector (International Light).

IAA Estimation. Free, conjugated (alkali-labile), and diffusible IAA were estimated as previously reported (Iino and Carr 1982, Chapter 4; see also Iino et al 1980, Chapter 3).

Biosynthesis of [^3H]IAA from [^3H]Tryptophan.

The incubation medium was 10 mM potassium phosphate buffer (pH 6.5) containing 10^7 dpm/ml L-[5- ^3H]tryptophan (The Radiochemical Centre, Amersham) and 0.1 mg/ml streptomycin. This medium was initially adjusted to pH 3.0 and washed 5 times with an equal volume of ether, just before use. Ether contaminating the medium was removed by a rotary evaporator.

Intact seedlings were sprayed with 0.1 mg/ml streptomycin. Coleoptile tips, 3 mm-long, were then excised and floated on 1 ml of incubation medium in a standard scintillation vial, 25 to 30 tips per vial. During incubation, the vial was shaken at 150 rpm (Metabolite Water Bath Shaker, New Brunswick Scientific Co.). Incubation was terminated by pipetting the medium into a ice-cold test tube containing 200 μg carrier IAA (in 20 μl methanol). The coleoptile tips remaining in the vial were washed twice with 0.5 ml ice-cold water containing 0.1 mg/ml streptomycin, and the washes added to the test tube. The collected medium was then adjusted to pH 2.5-3.0 by adding 0.5 N HCl and shaken with 3 aliquots of 1 ml ice-cold ether. The collected and pooled ether phase was washed twice with 1 ml of ice-cold 10 mM KH_2PO_4 , and then passed through a column (i.d. 11 mm) of 2 g anhydrous Na_2SO_4 . The remaining ether phase in the column was collected by adding a further 1 ml fresh ether. The ether extract thus obtained was evaporated to dryness in a rotary evaporator.

The extract was then developed on polyamide TLC plates (Macherey-Nagel Polyamid DC 6 UV₂₅₄) using the solvent system, benzene:ethyl acetate:acetic acid (70:25:5).

Three samples were developed simultaneously on a 20 x 20 cm plate, each loaded as a 4.5 cm-long line. After development, the IAA zone was marked promptly under a UV lamp. Scrapings of this zone were put on 1 g Na₂SO₄ in a column (i.d. 11 mm), and were extracted with water-saturated ethyl acetate. The collected ethyl acetate extract, 2.5 ml, was evaporated to dryness, and dissolved in 0.8 ml ethanol. A 0.4 ml aliquot was taken and added to 10 ml of scintillation fluid (for components, see Iino et al 1980, Chapter 3), and counted for radioactivity. The remaining aliquot was diluted with 2 ml ethanol, and the difference in UV absorption, $A_{280\text{nm}} - A_{320\text{nm}}$, was measured. This UV absorption difference was then used to assess losses of IAA during extraction and purification (for details of this correction procedure, see Iino and Carr 1982, Chapter 4).

A problem encountered with the procedure was that freshly-prepared incubation medium gives rise, even in the absence of incubating tissues, to some radioactivity in the IAA fraction: 300 to 500 dpm per ml of the medium (the counts were corrected for losses using recovery of carrier IAA, see above). Furthermore, the radioactivity showed an increase with time following medium preparation (about 50 dpm per ml per hour; the medium was kept at 25° C in the dark). This increase in radioactivity may be due to auto-decomposition of [³H] tryptophan to [³H]IAA and/or to the bacterial conversion of [³H]tryptophan to [³H]IAA. The radioactivity obtained from 1 ml of the medium after 3 h-incubation of 30 coleoptile tips was over 10⁴ dpm, which is high enough to regard the background counts as negligible. However, time

course experiments required short incubation periods, and the medium had to be kept up to 12 h until used. In such experiments, the radioactivity was necessarily corrected for the background value, which was assessed using a time-course calibration of counts incorporated into the blanks.

In vivo Phytochrome Measurement. Excised tissues were collected on an ice-cold Petri-dish, and packed in an ice-cold cylindrical metal cuvette (i.d. 1 cm, quartz bottom). In the case of the coleoptile tip, 45 excised tips (3 mm long) were used in each estimation. The relative Pfr level was estimated (see Spruit 1972) using a dual wavelength spectrophotometer (Ratiospect model R-2, Agricultural Specialty Co., Maryland; see Marmé 1969). The in vivo photostationary state of Pfr under saturating red light was taken as 75% of the total photoreversible phytochrome (Platt 1975, 1978).

Safelights. During experimental treatments, seedlings were handled in the dark, using physiologically safe infrared radiation and an infrared-scope (Iino and Carr 1981, Chapter 2). Dim green light (Iino and Carr 1981, Chapter 2) was, however, used when seedlings or excised segments were no longer incubating; i.e. when harvesting and freezing plant parts for estimation of free or conjugated IAA content, and on the termination of incubation.

RESULTS AND DISCUSSION

1. Red Light and Endogenous IAA.

Exposure of intact seedlings to 10 min red light resulted, during subsequent incubation of the seedlings in the dark, in reduced yields of diffusible IAA collected from excised coleoptile tips (Fig. 2-A). The decrease observed in the first few hours was followed by an increase, and the final yield approached that of unirradiated controls. The lowest yield (point of inflection, Fig. 2-A) was obtained at about 3 h after irradiation. Thus red light-induced reduction in the diffusible auxin yield, which previously had been demonstrated using a bioassay method, was confirmed for IAA determined by a physicochemical method. The result agrees in principle with the reports of Furuya et al (1969) on rice and of Huisinga (1976) on oats.

A similar effect was also seen on diffusible IAA collected from the base of the whole coleoptile, a coleoptile unit obtained by cutting at about 1 mm below the coleoptilar node (Fig. 2-B). By comparing the time courses obtained (Fig. 2-A and B), it can be seen that the relative amounts of diffusible IAA from the coleoptile tip reflect those from the base of the whole coleoptile with a delay of about 2 h. The results suggest that the basipetal transport of IAA is not affected by red light. Using oat seedlings, Kondo et al (1969) found a similar effect of red light on the yield of diffusible auxin obtained from the base of the coleoptile, which had been excised above the node. However, when the coleoptile with attached node was used, the

increase following the initial decrease in the yield was not observed. Thus, they claimed that IAA is immobilized within the nodal region, upon exposure to red light. This phenomenon was not evident in the present results using maize. Not all the diffusible IAA obtained from the base of the whole coleoptile comes from the coleoptile tip (Iino and Carr 1982, Chapter 4). However, at least the site acted on by red light may be located at the IAA-producing coleoptile tip.

The effect of red light on the free IAA content of the coleoptile was then investigated. Were the action of red light to be the inhibition of IAA transport, the content of IAA of the coleoptile, or at least its apical part, as was predicted by Huisinga (1967, 1976), would increase. It is clear from Fig. 3-A that, on the contrary the free IAA content of the coleoptile is reduced by irradiation, showing a time course pattern similar to that of the diffusible IAA (see Fig. 2-A). A similar reduction was also seen in the free IAA content of the coleoptile tip (Fig. 3-B). This latter result recalls the observation of Blaauw-Jansen (1958), who showed a reduction (about 50%) in IAA content of oat coleoptile tips (IAA in purified hot-water extracts was assayed by the Avena curvature test) 1.5 h after irradiation of seedlings with red light. These results, together with those obtained for diffusible IAA (see above), do not support the hypothesis that red light inhibits auxin transport. Thornton and Thimann (1967) also were not able to observe an effect of red light on the transport of applied radioactive IAA through excised coleoptile

cylinders.

The action of red light must be on a mechanism which can result in reduction of the levels of both diffusible and free IAA. The conjugation of free IAA is one of the possibilities, supported by Bandurski et al (1977, see also Bandurski 1980). However, the content of conjugated IAA in the coleoptile unit is not affected by irradiation (Fig. 4). The effect on conjugated IAA content of the coleoptile tip was not tested. If the decrease of diffusible IAA yield from the coleoptile tip is due to conjugation of IAA, the amount of IAA conjugated in the first 4 h after irradiation would be expected to be over 1 ng (see Fig. 2-A). Such a rate of conjugation should be reflected in the conjugated IAA content of the whole coleoptile, which is relatively low, about 1 ng (see Fig. 4). Thus conjugation is not likely to be the process causing the decrease in diffusible IAA.

Decomposition of IAA might be induced or stimulated by red light. If this were so, the red-sensitive process of decomposition must be located at the extreme tip of the coleoptile. Huisinga (1976) observed a considerable reduction in diffusible auxin in oat coleoptile tips only 1.5 mm long. Moreover, decomposition of IAA appears to proceed at a considerable rate even in the unirradiated, non-tip tissues of the coleoptile (Iino and Carr 1982, Chapter 4). These considerations make a decomposition hypothesis very unlikely. Menschick and Hild (1976) and Menschick et al (1977) were unable to perceive any effect of white light on the decarboxylation of [^{14}C]IAA by

etiolated, intact oat coleoptiles. Since the coleoptile tip is a very active site of IAA production in the coleoptile (Iino and Carr 1982, Chapter 4), the process on which red light is most likely to act appears to be production of IAA.

Experimental findings concerning the effects of red light irradiation of excised coleoptile tips are conflicting. Briggs (1963) claimed that red light does not affect diffusible auxin yield when the tips of the maize coleoptiles are irradiated after their excision, unlike Huisinga (1976) who found a decrease using oats. There is clear evidence in the result presented in Fig. 5, that the yield of diffusible IAA from the maize coleoptile tips is reduced even when irradiated after excision (dark controls show a decrease with a lag period of a few hours, but red light induced the decrease with very little lag). The lowest yield was obtained about 3 h after irradiation as in the case when intact seedlings were irradiated (see Fig. 2-A). It can be concluded that all the components of a red-sensitive system responsible for the reduction of the diffusible IAA yield are contained in the coleoptile tip.

2. Red Light and IAA Biosynthesis.

Although tryptophan is generally believed to be the endogenous precursor of IAA in higher plants (see e.g. Schneider and Wightman 1978), it has been regarded by some as not the precursor in etiolated coleoptiles (Winter 1966, Thimann and Grochowska 1968, Bandurski 1980). Nevertheless, incorporation of radioactivity into IAA from isotopically labelled tryptophan has been shown (Kutáček and Kefeli 1970,

Black and Hamilton 1971, Erdmann and Schiewer 1971, and Heerkloss and Libbert 1976). Muir and Chang (1974) were able to increase the yield of diffusible IAA from the coleoptile tip by applying tryptophan. Tryptophan is likely to be at least one of the IAA precursors in the coleoptile.

Incorporation of tritium from [^3H]tryptophan into IAA was first investigated. Excised coleoptile tips were incubated for a few hours in the medium containing [^3H]tryptophan, and an ether extract was obtained from the incubation medium (for details, see legend to Fig. 6). The extract was developed on polyamide TLC, and radioactivity on the plate was scanned. As shown in Fig 6-A, only one peak was found, its R_f value corresponding to that of the authentic IAA marker. No such peak was found in the ether extract obtained from the medium alone, minus incubating tissue (Fig. 6-B). Radioactivity in the blanks measured by scintillation counting (see Materials and Method) was not detected by the scanner. The ether extract, methylated by diazomethane, was developed on TLC. A peak was again found at the position of the authentic Me-IAA marker (Fig. 7). The results support the view that the radioactive peak on TLC is due to [^3H]IAA, produced from [^3H]tryptophan.

At the position of tryptophan on the TLC plate (the origin), there was no apparent peak of radioactivity (Fig. 6). Thus the extraction procedure employed is very effective in removing most of the [^3H]tryptophan present in the incubation medium, and after the ether extraction, arrests its auto-decomposition to produce [^3H]IAA or other radioactive compounds which might interfere with measurement

(see Materials and Methods).

The ether extract, developed on TLC, contains acidic and neutral compounds, but the only radioactive peak found was at the position of IAA. This probably means that, of the many metabolites produced from tryptophan, including IAA intermediates, IAA is selectively exported from the tissue.

The rates of [^3H]IAA production during a few hours incubation were examined. There was some incorporation of tritium into IAA in the first 30 min, and the rate increased with time (Fig. 8). This result is taken as evidence that the [^3H]IAA produced was not due to contaminating bacteria. If it were, the rate of [^3H]IAA production would show no such increase in the course of the renewal of the medium, which contained streptomycin. The most probable explanation for the increase in the rate of production is the tissue content of [^3H]tryptophan, which is expected to increase by continuous uptake.

The time course of red light effects on the formation of [^3H]IAA from [^3H]tryptophan was then examined. Intact seedlings were irradiated with red light, in the usual manner, for 10 min. After scheduled dark intervals, coleoptile tips were excised and incubated with [^3H]tryptophan. After 30 min the medium was replaced with a fresh aliquot, and the tips were incubated for a further 1 h. [^3H]IAA present in the second incubation medium was determined. Effects due to excision of the coleoptile tip on the IAA production are considered to be minimal within this incubation period, since the initial rate of IAA diffusion is maintained constant for a few hours after the

excision (Iino and Carr 1982, Chapter 4; see also the dark control in Fig. 5). As shown in Fig. 9-A, red light reduced the yield of [^3H]IAA, with a time course very similar to that obtained for diffusible IAA (Fig. 2-A).

In the present studies, the [^3H]IAA was recovered from the incubation medium, not from the tissue. From the determination of diffusible and free IAA (Figs. 2-A and 3-B), it can be concluded that, following exposure to red light, the level of diffusible IAA parallels that of free IAA. Moreover, the content of free IAA in the coleoptile tip is low (0.2 ng per/3 mm-long unirradiated tip) compared to the yield of diffusible IAA (1.0 ng/tip/h) (Iino and Carr 1982, Chapter 4; see also Fig. 2-A and Fig. 5 for diffusible IAA). The rate of [^3H]IAA diffusion probably reflects very closely the rate of [^3H]IAA production in the tissue. It should also be noted that the radioactivity of [^3H]IAA diffusing out of an unirradiated coleoptile tip is about 200 dpm, corresponding to only 5.6 pg of [^3H]IAA (calculated from the specific activity of the [^3H]tryptophan used). The assumption is made that the exogenously supplied [^3H]tryptophan does not, e.g. by increasing the pool size of tryptophan, alter the status quo of IAA production.

The possibility that the decrease in [^3H]IAA formation might be caused by inhibition of [^3H]tryptophan uptake by the tissue is negated by the fact that radioactivity in the coleoptile tips, collected at the end of the incubation period was considerably increased by red light (Fig. 9-B). Thus red light can affect uptake of tryptophan, but not so as to inhibit [^3H]IAA formation.

These results confirm that red light acts to reduce the yield of diffusible IAA, or the level of free IAA, by changing the metabolism of IAA. Although stimulation or induction of IAA decomposition cannot be totally excluded from the possibilities, the most likely metabolic process on which red light acts is the synthesis of IAA, as already discussed (see Red light and endogenous IAA). It should also be noted that the results obtained here support the view that tryptophan is a physiologically important precursor of IAA in the maize coleoptile tip.

3. Phytochrome and IAA Metabolism.

The involvement of phytochrome in the photo-reactions studied here was next investigated. Fig. 10-A shows fluence-response curves for the reduction in diffusible IAA yield from the coleoptile tip. Various fluences of red and far-red light were obtained by means of a number of light sources, neutral-density filters and by varying the duration of irradiation. The duration was varied within time in which the response obeys the reciprocity law (Fig. 11). The yield of diffusible IAA was reduced by both red and far-red, the latter being less active. The thresholds were around 1 nWs/cm² with red and 100 nWs/cm² with far-red. The response to red light was linear with log fluence up to 0.1 mWs/cm² and at this point linearity was interrupted by a sudden change in slope, seen as a fall in the response curve (Fig. 10-A). With far-red, the response was linear within the range examined (i.e. up to about 320 mWs/cm²), and was parallel to that of red light, before the fall.

The linear response to far-red with increasing fluences, however, was maintained beyond the response level where, with red light, the change in slope commences. The simplest interpretation of these fluence-response curves is that the reduction in diffusible IAA is controlled by two photoreactive systems. One has its threshold at very low fluences and is linear with respect to log fluence over a wide range; the action of the other is apparent only with red light above 0.1 mWs/cm^2 . The yield of diffusible IAA from the base of the whole coleoptile responded similarly to red and far-red light (Fig. 10-B), and no additional complication was found.

These fluence-response relations were compared to in vivo photo-transformation of phytochrome. Intact seedlings were exposed to red light, and, immediately afterwards, the Pfr level of excised coleoptile tips was determined spectrophotometrically. Pfr was found to be detectable at about 0.1 mWs/cm^2 and photoconversion was saturated above about 18 mWs/cm^2 (Fig. 10-A). Phytochrome in other parts of the seedlings, e.g. the middle of the coleoptile or the apical part of the mesocotyl, gave similar responses (not shown). The reduction in diffusible IAA is obviously induced at very low fluences of red light with which no spectrophotometrically detectable Pfr is formed (Fig. 10); the difference in the two thresholds was five orders of magnitude. The sudden fall in the curve at around 0.1 to 1 mWs/cm^2 corresponds well to the appearance of detectable Pfr.

The pigment system, which responds to both red and far-red, having a threshold at a very low fluence and saturation at a very high fluence (separated at least 6 to 7 orders of magnitude, see the curve for far-red, Fig. 10), cannot be explained simply in terms of the amount of Pfr formed. If Pfr is the responsible pigment for this system, Pfr of only a very small portion of the total phytochrome must be considered to be active. This assumption is further complicated. The graph of reduction of diffusible IAA by far-red light is linear up to 320 mWs/cm^2 , and at such a high fluences, although no detectable Pfr is formed, far-red is more effective than red light of 0.1 mWs/cm^2 , which produces a detectable amount of Pfr (Fig. 10). The result is therefore not in agreement with established views on the molecular mechanism of phytochrome action in which Pfr is the active form of phytochrome. If the above result is still to be explained in terms of the established level of Pfr, one must postulate a population of phytochrome molecules which behaves differently from total photo-reversible phytochrome, e.g. a minute proportion which has a very low quantum efficiency for Pfr transformation or is heavily screened from light, thus requiring a high light fluence for the establishment of the photostationary state. Such a population is, however, not apparent in the total photoreversible phytochrome (see Pratt 1978). The simplest interpretation would be that the reaction is induced by a phytochrome (or phytochrome analogue) whose action is not dependent on the established level of Pfr, or by a totally different kind of pigment.

The response to red light at above 0.1 mWs/cm^2 , seen as a fall in the curve (Fig. 10) is, however, likely to be induced by Pfr. This is suggested from the agreement with the appearance of detectable Pfr. Further support was obtained in photo-reversibility experiments. As shown in Fig. 12, the reduction in diffusible IAA induced by 10 mWs/cm^2 red light, which almost saturates the Pfr formation, was reversed, by immediately subsequent irradiation with far-red, close to the level induced by far-red alone. Nearly total reversal was achieved by 320 mWs/cm^2 far-red light. The reversal was also in good accord with the transformation of Pfr to Pr (Fig. 12). The extrapolated background response to red light, shown as a dotted line in Fig. 10-A, is very close at 10 mWs/cm^2 to the response induced by 320 mWs/cm^2 far-red light. Thus, reversal up to the predicted background could be almost total with 320 mWs/cm^2 far-red light. The response to red light, seen as a fall in the response curve, is accordingly explicable in terms of the Pfr level, which is reflected in the total photoreversible, or so-called bulk, phytochrome.

Furuya et al (1969) using rice seedlings showed that the reduction in the yield of diffusible IAA was also induced by far-red light, although less effectively than by red light, and that far-red given after red could reverse the response to the level induced by far-red alone. Their results are consistent with those of the present study. However, once reversed by far-red, the response could not be induced again by a second red light treatment (Furuya et al 1969). This aspect warrants further investigation.

The fluence-response curves presented here are clearly relevant to the data of Blaauw et al (1968) and Mandoli and Briggs (1981) on the growth of intact oat seedlings (inhibition of mesocotyl elongation and stimulation of coleoptile elongation). Although both groups showed stepwise responses to red light with increasing fluences, each achieving saturation before the next (Blaauw et al characterized 3 steps), the threshold value obtained in the present study corresponds best with that of the most sensitive step, and the sudden fall in the response curve to the 2nd step. Both groups were able to show photoreversible phytochrome control only in this 2nd step, as was the case in the present study. The saturation of the 1st step before the 2nd step was, however, not apparent in the present results. It can be noted, nevertheless, that the effect on diffusible IAA studied here may be a simpler manifestation of the photoreactive systems, than the more complex growth responses, previously characterized (Blaauw et al 1968, Mondoli and Briggs 1981).

On the basis of the above findings, effects of red and far-red light on the formation of [^3H]IAA from applied [^3H]tryptophan were investigated. The results are summarized in Table 2. Red ($18 \mu\text{Ws}/\text{cm}^2$) and far-red ($320 \mu\text{Ws}/\text{cm}^2$), which give rise to no spectrophotometrically detectable Pfr formation, considerably inhibited [^3H]IAA production. The response to red of a high fluence ($18 \text{mWs}/\text{cm}^2$), which saturates Pfr formation, is reversed by subsequent exposure to far-red light, known to reverse most of the Pfr to Pr, close to the level induced by far-red

alone. Therefore, both pigment systems, characterized as reducing the yield of diffusible IAA, appear to act in some way on biosynthesis of IAA from tryptophan.

4. Red Light and Growth.

As shown in Fig. 13, exposure of intact seedlings to red light (18 mWs/cm²) resulted in stimulation of coleoptile elongation; the stimulation was actually of the apical region rather than of the more actively growing basal region. The effect of red light on apical elongation appeared to be saturated at a fluence (18 uWs/cm²) which causes no detectable Pfr formation (Table 3). The response was also induced by far-red light and no far-red reversible phytochrome control was apparent (Table 3). The results are comparable to those which have been obtained using oat seedlings. Huisinga (1967) observed a similar stimulatory effect on the apical elongation of oat coleoptiles. Fluence-response relationships in intact oat seedlings (Blauuw et al 1968, and Mandoli and Briggs 1981) indicate that the stimulation of overall elongation of the coleoptile is induced by very low fluences of red light, which apparently cause no detectable Pfr formation. A difference found was that the far-red reversible phytochrome control of coleoptile growth shown by Mandoli and Briggs at higher fluences of red light was not apparent in the present study using maize.

The results show that the content of IAA is reduced by red light throughout the coleoptile (Fig. 3). The transport hypothesis of Huisinga (1964, 1967, 1976) already mentioned

cannot be maintained for maize. The possibility that the IAA content is in the inhibitory range in the apical region of the unirradiated coleoptile is not supported, since IAA applied in solution to isolated 5 mm-long tips (tested from 0.1 to 100 μ M, incubated 6 h or shorter) showed only stimulation at the higher concentrations (not shown). Stimulation of coleoptile elongation by red light must be due to some mechanism other than its effects on IAA levels.

Inhibitory effects of red light on the rapidly growing middle or basal regions of the coleoptile have been found in wheat (Roesel and Haber 1963, Lawson and Weintraub 1975), barley (Lawson and Weintraub 1975) and rice (Furuya et al 1969), and can be explained in terms of a reduced supply of auxin from the coleoptile tip (Furuya et al 1969, Masuda et al 1970). However, the inhibition of elongation was not apparent in oat (Huisinga 1967) or maize (Fig. 13), although a red-induced reduction of diffusible IAA from the coleoptile tip has been shown in these cereals (van Overbeek 1936, Briggs 1963, Muir and Chang 1974, present study). In the basal region of the oat or maize coleoptile, even the reduced IAA level following red irradiation may still be within the saturating range for growth. An alternative explanation is that, in the basal region, the inhibitory effect arising from reduced supply of auxin is nullified by the stimulatory effect, which can readily be observed in the apical region (see above).

Without exception, red light inhibits the elongation of the mesocotyl (e.g. Huisinga 1967, Blaauw et al 1968, Duke et al 1977, Vanderhoef et al 1979, Mandoli and Briggs

1981). It is most likely that reduction of IAA supply from the coleoptile tip is involved in this growth response.

Similarity in the fluence-response curves for inhibition of mesocotyl elongation (Blauuw et al 1968, Mandoli and Briggs 1981) and for reduction in the yield of diffusible IAA has already been mentioned.

Table 1. Effect of red light on levels of free and free-plus-conjugated IAA in coleoptiles. See Fig. 4.

Time after Irradiation (h)	IAA Content			
	ng/g Fresh Wt		ng/Coleoptile	
	Free	Free + Con- jugated	Free	Free + Con- jugated
Dark Control				
0	26.8	45.6	1.21	2.06
1.5	24.4	46.1	1.20	2.28
3	23.5	46.1	1.24	2.43
5	22.0	45.3	1.38	2.85
Irradiated				
1	22.2	43.5	1.00	1.95
2	16.3	36.0	0.85	1.88
3	12.1	33.4	0.64	1.76
4	12.1	34.8	0.74	2.12
5	12.7	32.7	0.85	2.20

Table 2. Effects of red and far-red light on the formation of [^3H]IAA from [^3H]tryptophan. Intact seedlings were irradiated briefly, and 1 h after the onset of irradiation, 20-30 coleoptile tips were excised and incubated in 1 ml of the medium containing [^3H]tryptophan. After 1 h, the medium was replaced with a fresh aliquot and incubated another 1 h. IAA in the second lot of the medium was extracted and its radioactivity measured. Other details as in Fig. 8. Results of 3 separate experiments are given. R: red light, FR: far-red light, R $\rightarrow$ FR: red light immediately followed by far-red light.

Irradiation (mWs/cm ²)	[^3H] IAA Produced/Tip			
	% of Dark Control			Mean
R (18 x 10 ⁻³)	-	88.9	67.9	78.4
R (18)	34.3	48.0	39.7	40.7
FR (320)	58.8	69.0	51.4	59.7
R (18) $\rightarrow$ FR (320)	59.9	54.1	47.7	53.9

Table 3. Effects of red and far-red light on the apical elongation of the coleoptile. Coleoptiles of intact seedlings were marked at 5 or 10 mm from the tip with Indian ink, and immediately afterwards they were irradiated briefly with red or far-red light of a given fluence. Eight hours after marking, the length of the apical marked portion of the coleoptile was measured as described in Fig. 13. As a unit of treatment, 25 to 30 seedlings were used. Each column shows results of separate experiments. Means are presented with SE. R: red light, FR: far-red light, R→FR: red light immediately followed by far-red light.

Irradiation (mWs/cm ²)	Growth increment (mm)			
	a	a	b	b
	I	II	III	IV
Dark Control	0.72±0.06	0.65±0.06	2.27±0.09	2.17±0.10
R (18 x 10 ⁻³)	1.49±0.10	1.28±0.07	-	-
R (18)	1.38±0.05	1.33±0.08	3.52±0.15	3.59±0.09
FR (320)	1.38±0.06	1.38±0.09	3.57±0.13	3.40±0.10
R (18) → FR (320)	1.46±0.06	1.51±0.05	3.40±0.12	3.35±0.12

^a Initial length: 5 mm, ^b Initial length: 10 mm.

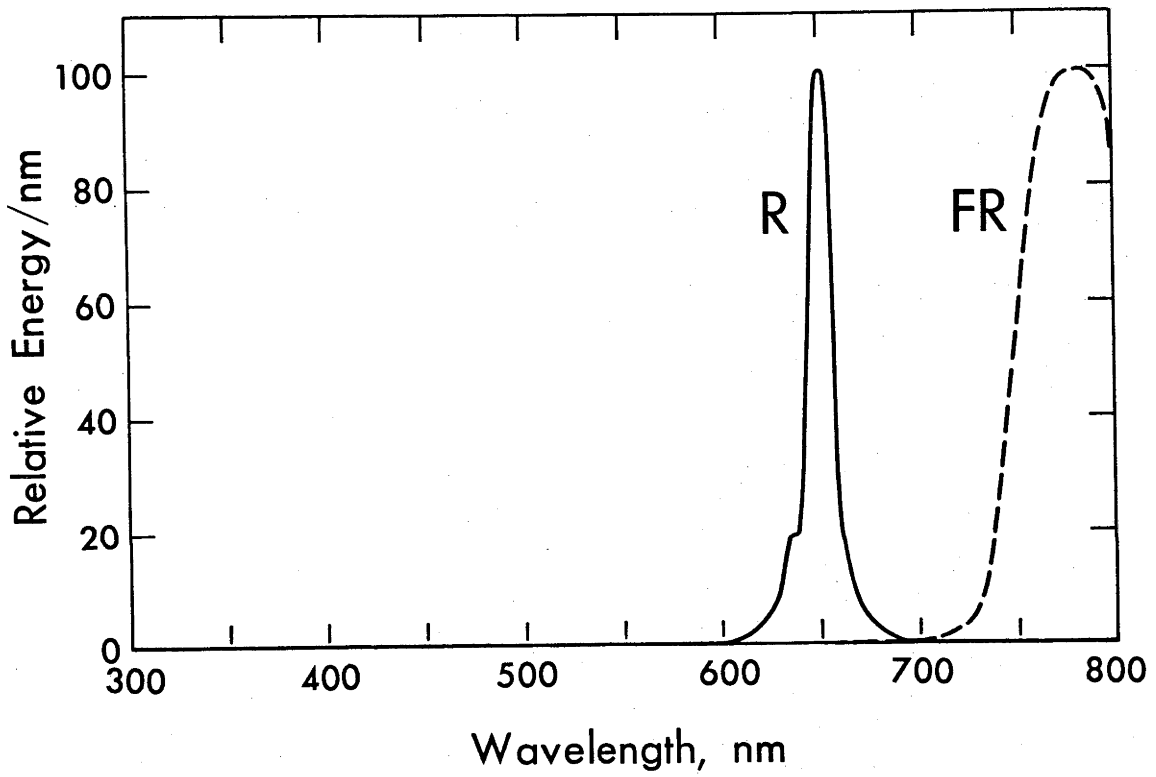


Fig. 1. Spectral energy distribution of red and far-red light used in the experiments.

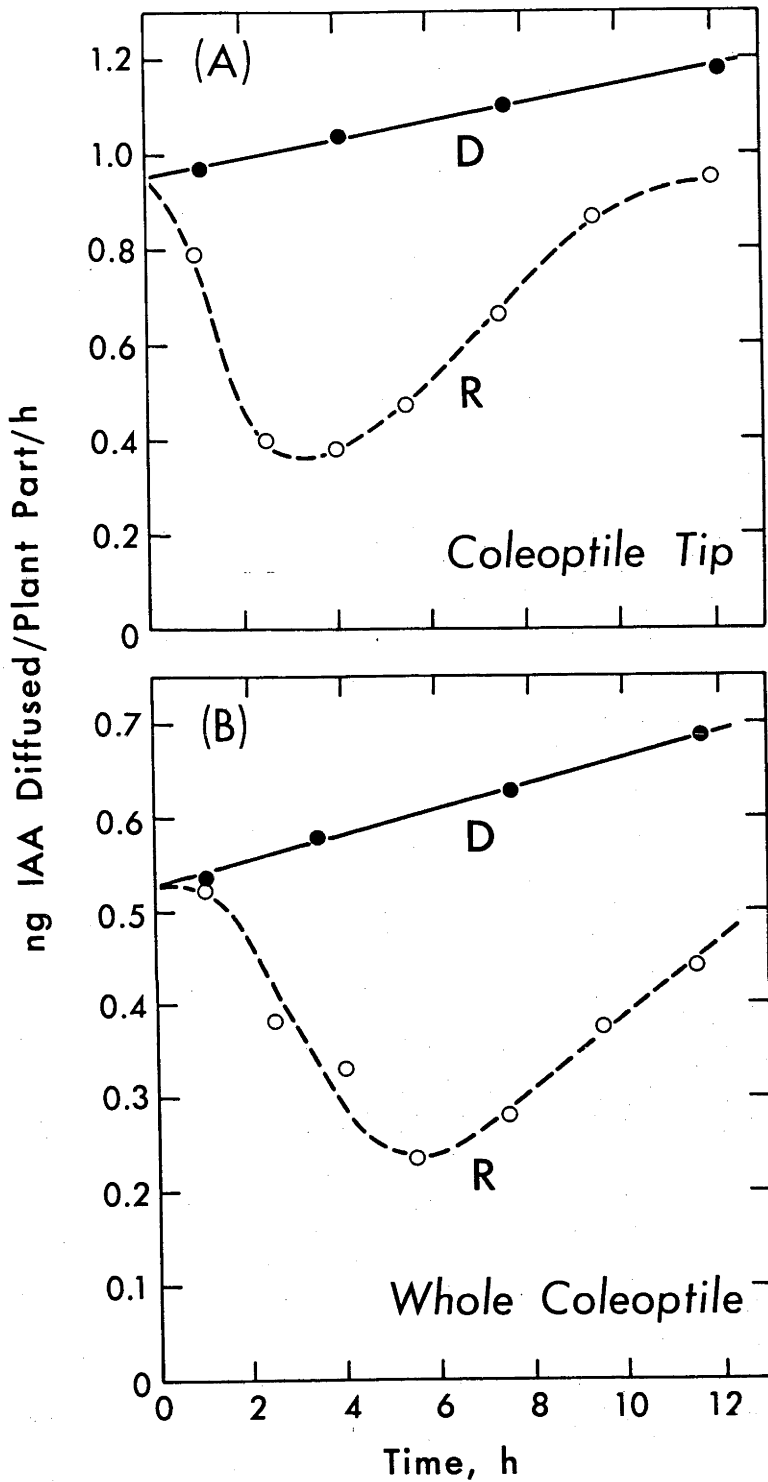


Fig. 2. Time course of the effect of red light on the yield of diffusible IAA. Intact seedlings were irradiated with red light (18 mWs/cm^2 , in 10 min). After a given dark interval, diffusible IAA was collected for 1 h from 3mm-long 20 coleoptile tips (A), or the bases of 24 whole coleoptiles obtained by cutting at about 1 mm below the node (B). The estimated diffusible IAA yield is plotted in the figure at the middle of a 1 h diffusion period. D: dark control, R: red irradiated.

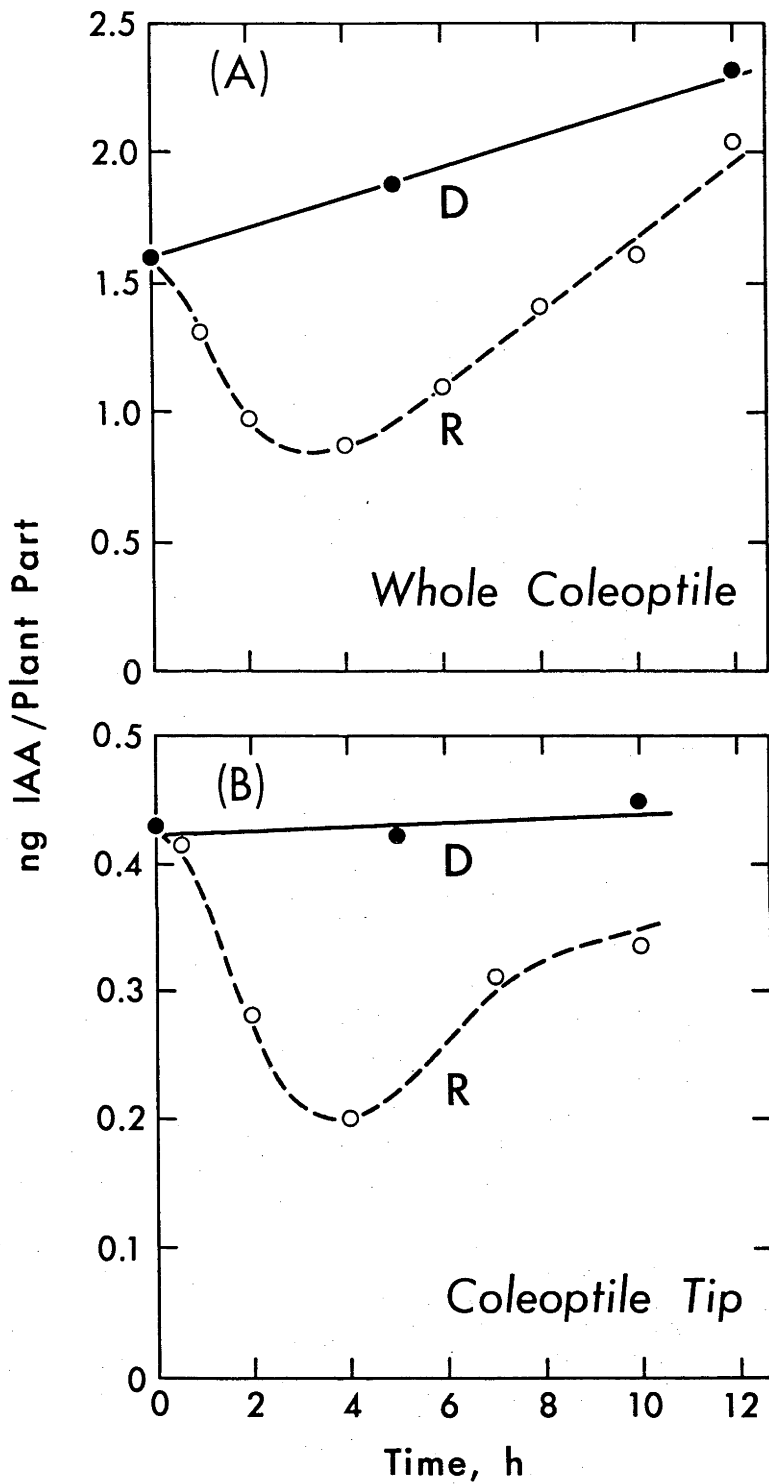


Fig. 3. Time course of the effect of red light on the free IAA content of the coleoptile. Intact seedlings were irradiated with red light, and after a given dark interval the free IAA content of whole coleoptiles (A) or 5 mm-long coleoptile tips (B) was estimated. For each estimate, 30 plant parts were used. Other conditions as in Fig. 2.

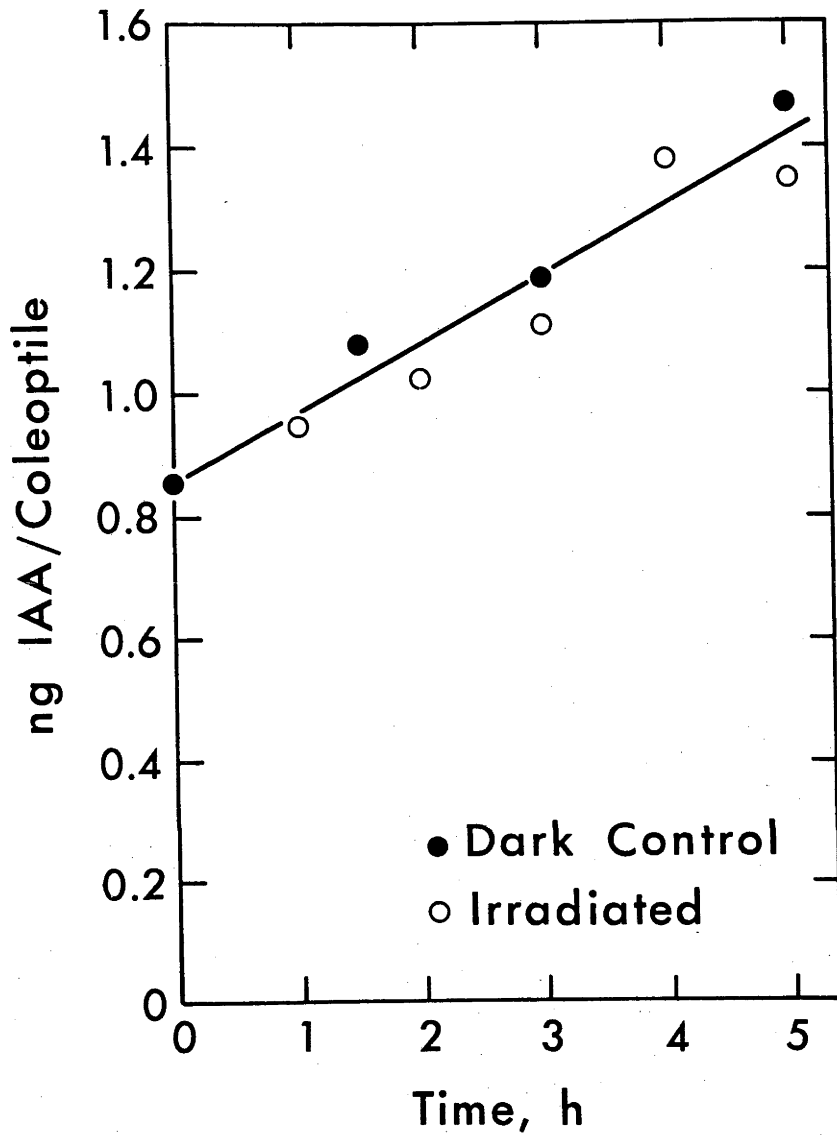


Fig. 4. Time course of the effect of red light on the conjugated IAA content of the coleoptile. Intact seedlings were irradiated with red light, and after a dark interval the conjugated IAA content of the whole coleoptiles was estimated. For each estimate, 30 seedlings were used. Other conditions as in Fig. 2. The measurements of free and free-plus-conjugated IAA, used to estimate the conjugated IAA content, are given in Table 1.

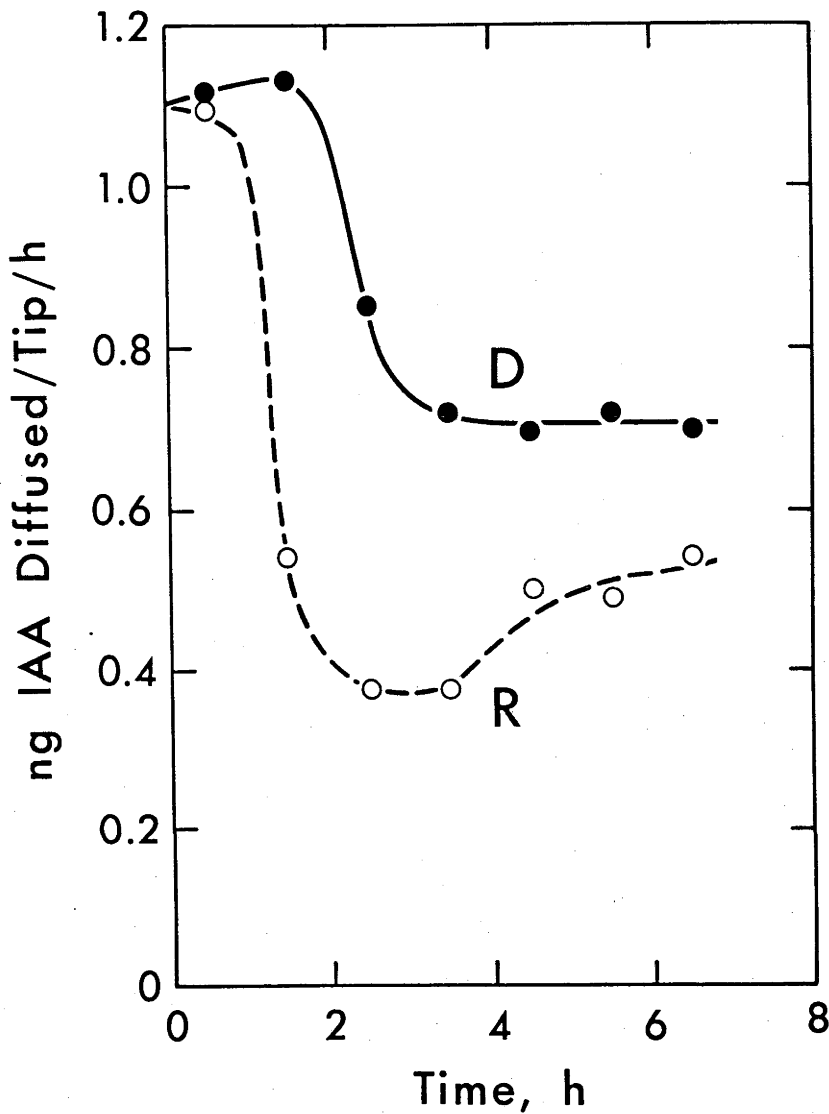


Fig. 5. Effect of red light irradiation of isolated coleoptile tips on the yield of diffusible IAA. Coleoptile tips, 3 mm long, were excised and pooled in water. The tips were taken from the pool alternately into two vials containing water, 30 tips per vial. Tips in one vial were then irradiated with red light (18 mWs/cm^2 , in 10 min) (R); those in the other vial were kept in the dark (D). They were then incubated in the dark, replacing the incubation water every hour with a fresh aliquot. Diffusible IAA obtained in each collection was estimated. The yield of IAA is plotted at the middle of the 1 h diffusion period.

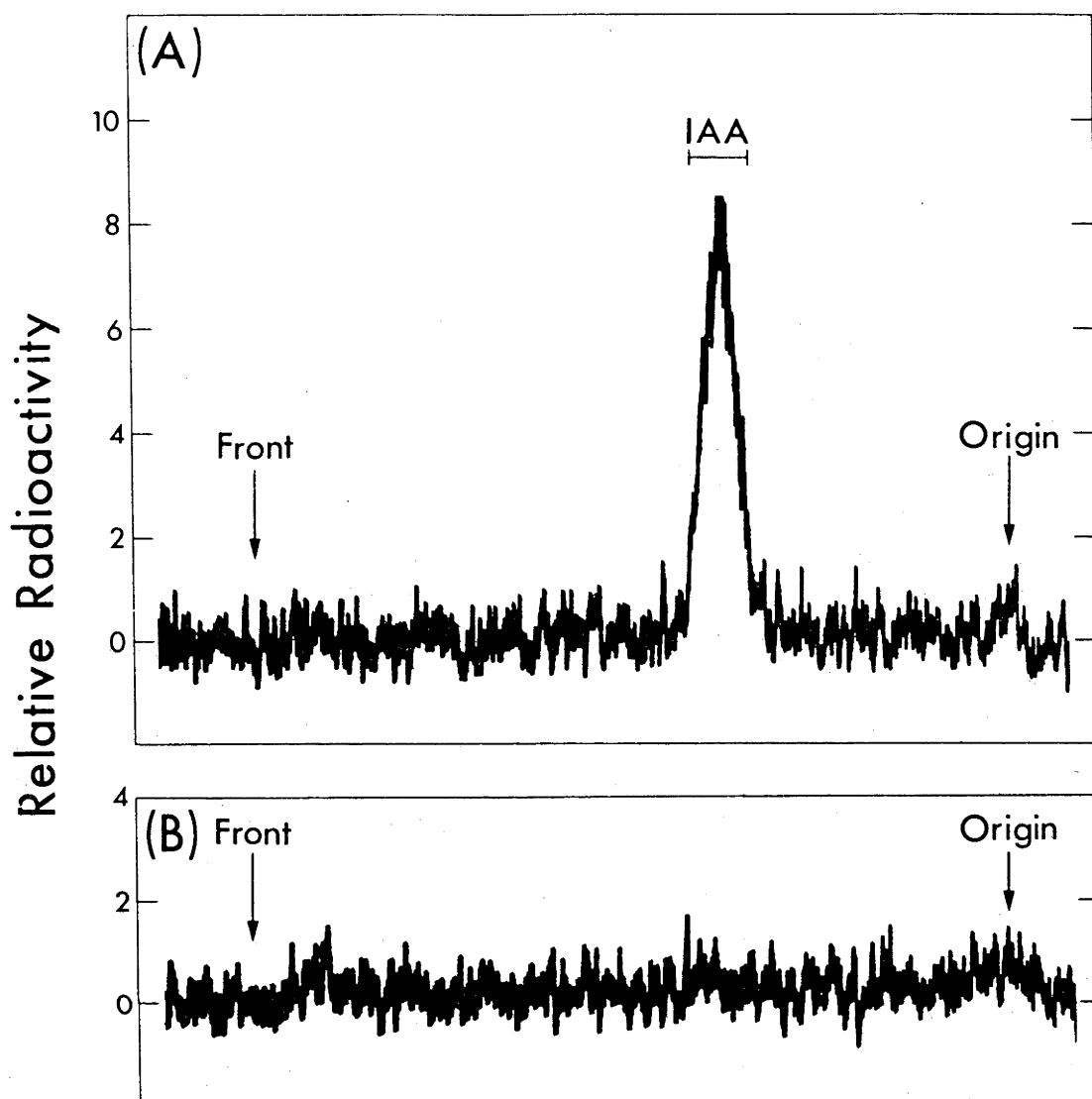


Fig. 6. Identification of $[^3\text{H}]$ IAA produced from $[^3\text{H}]$ tryptophan by coleoptile tips. A: Incubation of tissue in $[^3\text{H}]$ tryptophan medium. Excised 30 coleoptile tips, 3 mm long, were added to 1 ml of 10 mM phosphate buffer (pH 6.5) containing 10^7 dpm $[^3\text{H}]$ tryptophan and 0.1 mg streptomycin. Three lots were prepared successively. After an initial 30 min incubation, the medium was discarded and a fresh aliquot added. After a further 2h incubation, the medium from each lot was combined and 50 μg carrier IAA added. The medium, adjusted to pH 3.0, was shaken 3 times against half its volume of ether. After washing twice with a 1/3 volume of 10 mM KH_2PO_4 , the ether phase was dried by passing through a column of anhydrous Na_2SO_4 and evaporated to dryness. The residue was loaded on a polyamide TLC plate (DC-Alufolien Polyamid 11 F254, Merck) and developed using the solvent system, benzene:ethyl acetate:acetic acid (70:25:5). Radioactivity on the plate was scanned using a Berthold Thin-Layer Scanner. B: $[^3\text{H}]$ tryptophan medium incubated without tissue. An ether extract, obtained from parallel aliquots (3 x 1 ml) of the incubation medium without tissue, was developed on TLC and scanned.

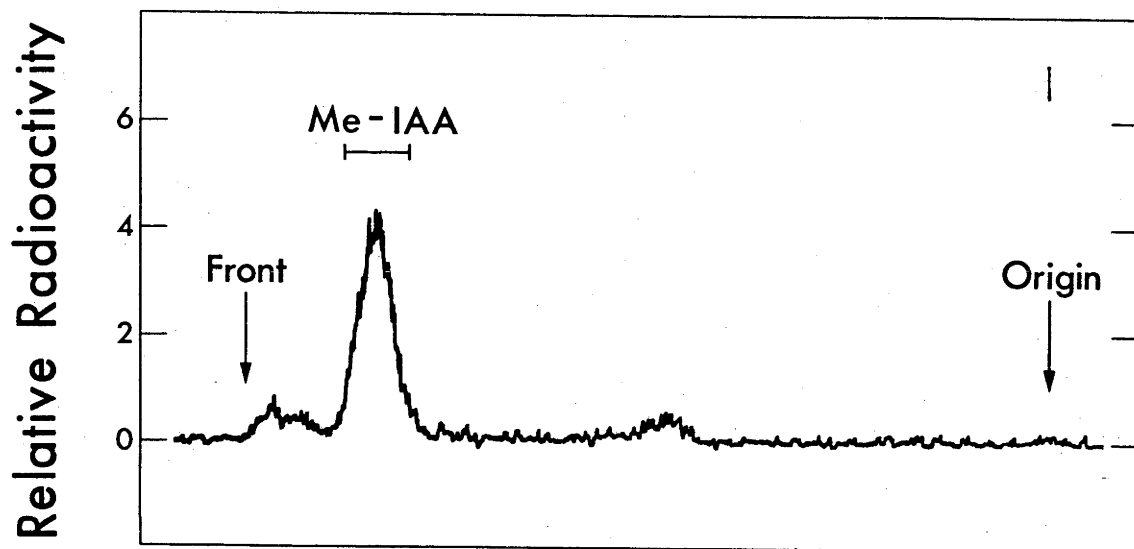


Fig. 7. Identification of [^3H]IAA produced from [^3H]tryptophan by coleoptile tips: methyl ester. An ether extract obtained using the method described in Fig. 6 (double the number of tips), was methylated with diazo-methane. The extract was then developed on polyamide TLC and radioactivity was scanned.

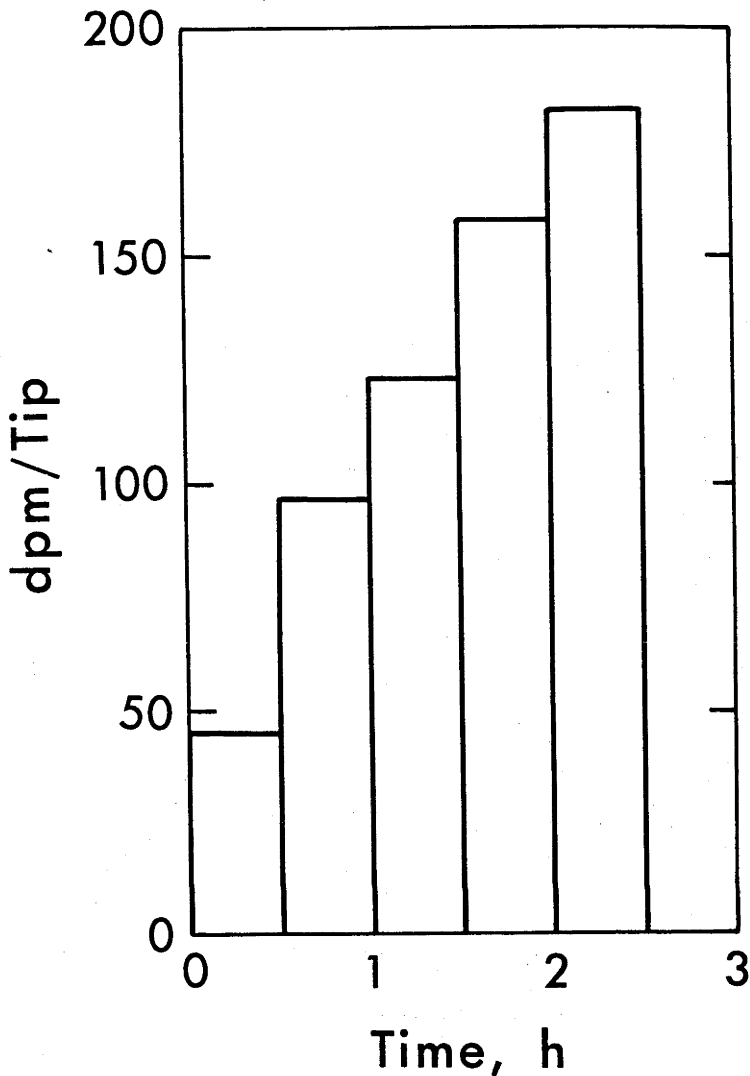


Fig. 8. Rates of [^3H]IAA production from [^3H]tryptophan during tissue incubation. Excised 3 mm-long coleoptile tips, 30 in number, were incubated in 1 ml incubation medium (10 mM phosphate buffer) containing 10^7 dpm [^3H]tryptophan and 0.1 mg streptomycin. The incubation medium was replaced with a fresh aliquot every 30 min and, from each collection, IAA was extracted into ether and purified by polyamide TLC. Radioactivity of the IAA fraction was measured by scintillation counting. Parallel aliquots of incubation medium, without tissue, were taken and radioactivity obtained from the IAA fraction of these aliquots was used to correct for background counts. Correction for losses of [^3H]IAA during its extraction and purification were made in terms of the UV absorbance of carrier IAA added to the incubation medium. Counts were corrected for counting efficiency and quenching.

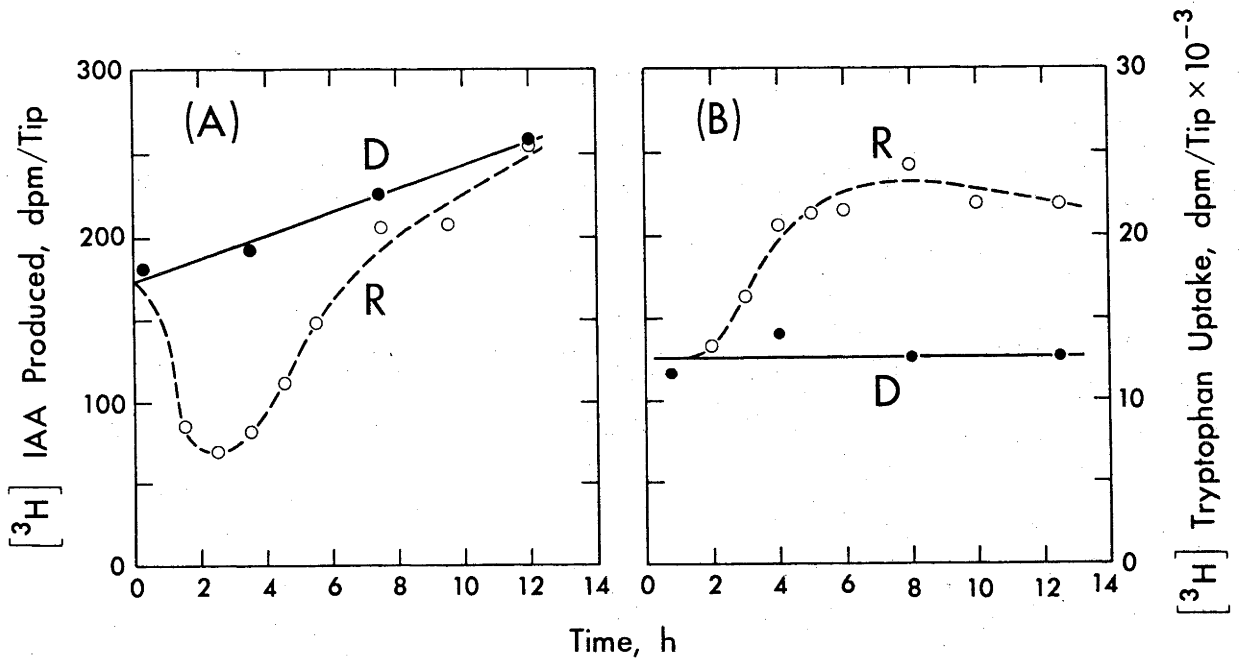
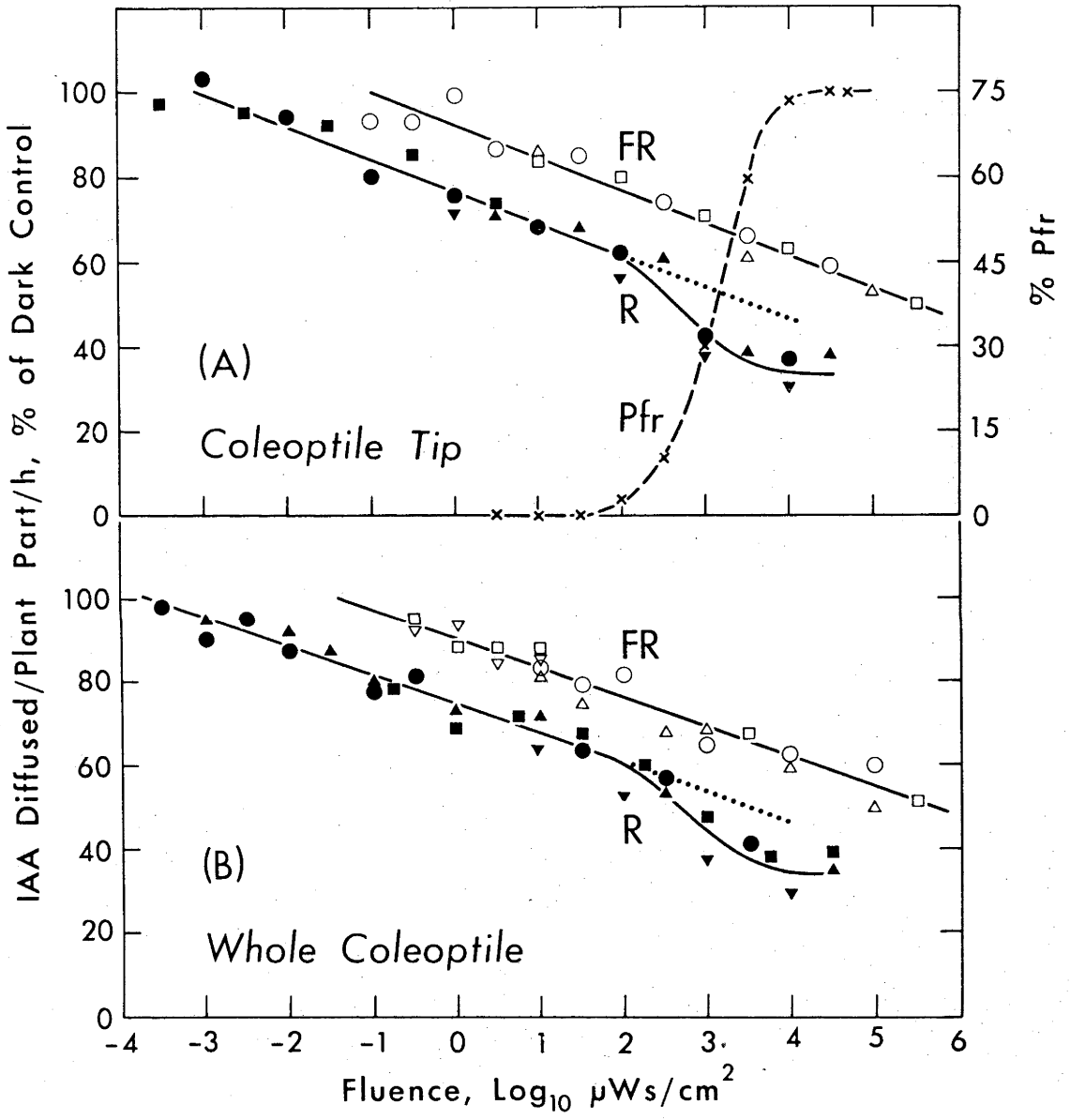


Fig. 9. Time course of effects of red light on $[^3\text{H}]$ IAA formation and $[^3\text{H}]$ tryptophan uptake by the coleoptile tip. Intact seedlings were irradiated with red light for 10 min and after a dark interval, 25-30 coleoptile tips were excised and incubated in 1 ml medium containing $[^3\text{H}]$ tryptophan. The medium was discarded after the initial 30 min incubation, and a fresh aliquot was added. The tissue was incubated a further 1 h, and the radioactivity of IAA, extracted from the medium, was estimated. On the termination of incubation, the remaining coleoptile tips were washed by shaking for 2 min on a stainless steel mesh in an excess of ice-cold water, and added directly to scintillation fluid for estimation of $[^3\text{H}]$ tryptophan uptake. The radioactivity of $[^3\text{H}]$ IAA produced is plotted at the middle of 1 h incubation (A), that of $[^3\text{H}]$ tryptophan incorporated in the tissue at the end of incubation (B). Other details as in Figs. 2 and 8.

Fig. 10. Fluence-response relationships. Intact seedlings were irradiated with various fluences of red or far-red light, which were obtained by means of a number of light sources, neutral-density filters and varying the duration of irradiation. Neutral-density filters used were Kodak No. 96 Wratten gelatin filters (for red light), and Oriel G-66 quartz filters (for far-red light). Duration of irradiation was varied between 30 sec and 6 min, except for the highest fluences of red (32 mWs/cm^2) and far-red (320 mWs/cm^2), which were about 18 and 12 min respectively.

A: Reduction in diffusible IAA yield from the coleoptile tip, and in vivo transformation of Pr to Pfr. Coleoptile tips, 3 mm long, were excised 3 h after the onset of irradiation and diffusible IAA, collected for 1 h, was estimated. Different symbols indicate results obtained on separate occasions. Red ($x \leq 2$): Slope=-7.69, $r=-0.962$, $n=15$; Far-red: Slope=-7.58, $r=-0.973$, $n=16$. Pfr level in the coleoptile tip was estimated spectrophotometrically on termination of irradiation of intact seedlings. The broken line in the figure shows Pfr level established under red light. Means of estimates of 2 separate experiments are given. Far-red light did not cause detectable Pfr formation at any fluence.

B: Reduction in diffusible IAA yield from the base of the whole coleoptile. Whole coleoptiles were excised 5 h after the onset of irradiation, and diffusible IAA, collected for 1 h, was estimated. Red ($x \leq 2$): Slope=-7.17, $r=-0.960$, $n=19$; Far-red: Slope=-7.20, $r=-0.973$, $n=22$.



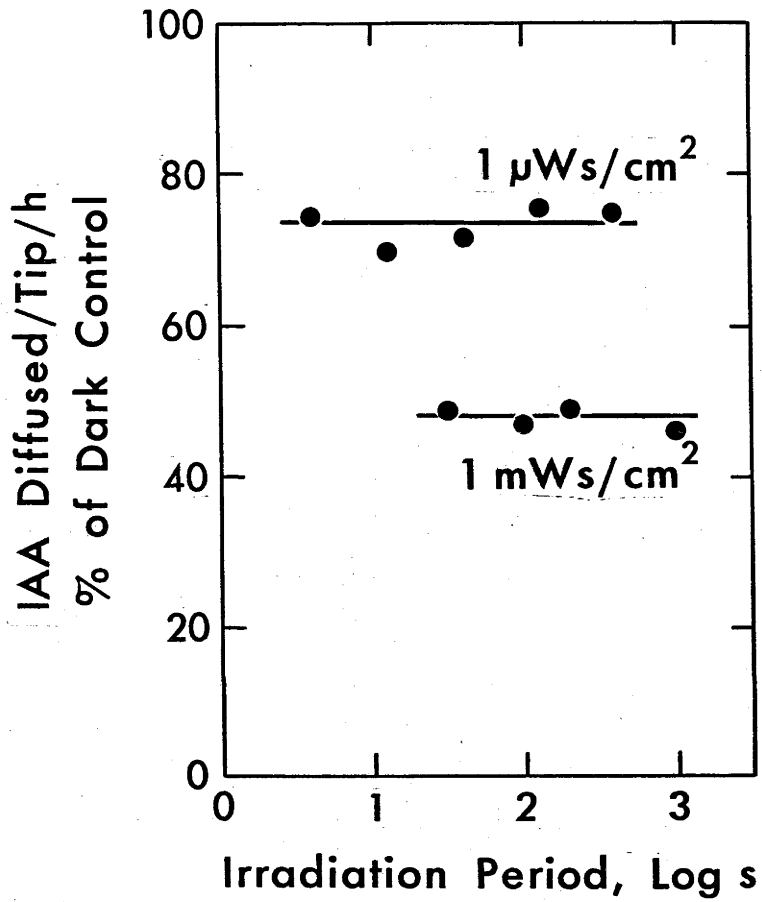


Fig. 11. Reciprocity of the red light effect on diffusible IAA yield. Intact seedlings were irradiated with red light of $1 \mu\text{Ws}/\text{cm}^2$ or $1 \text{mWs}/\text{cm}^2$ for various periods. Diffusible IAA was collected from coleoptile tips as in Fig. 10-A.

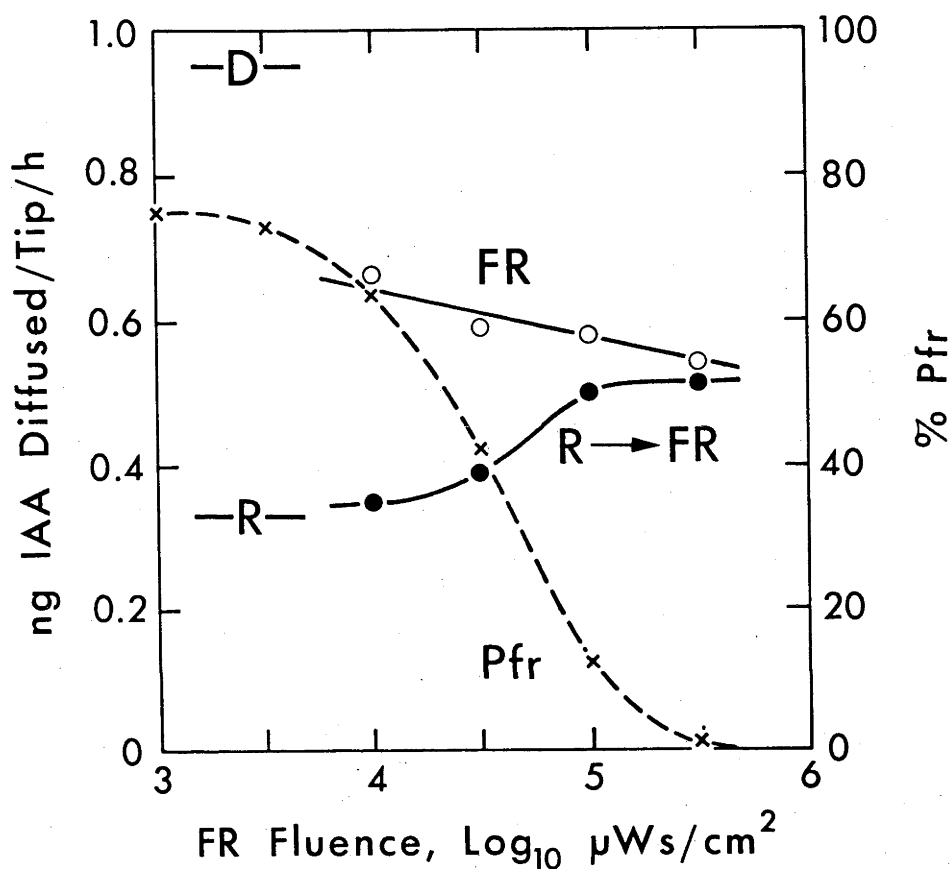


Fig. 12. Reversibility of red light effects by far-red light. Intact seedlings were irradiated with red light ($10 \text{ mWs}/\text{cm}^2$, given in about 5 min) and then immediately afterwards with far-red light of a given fluence. The diffusible IAA yield from the coleoptile tips and their Pfr level were estimated as described in Fig. 10. D: diffusible IAA yield from non-irradiated seedlings. R: that from seedlings irradiated with red light only.

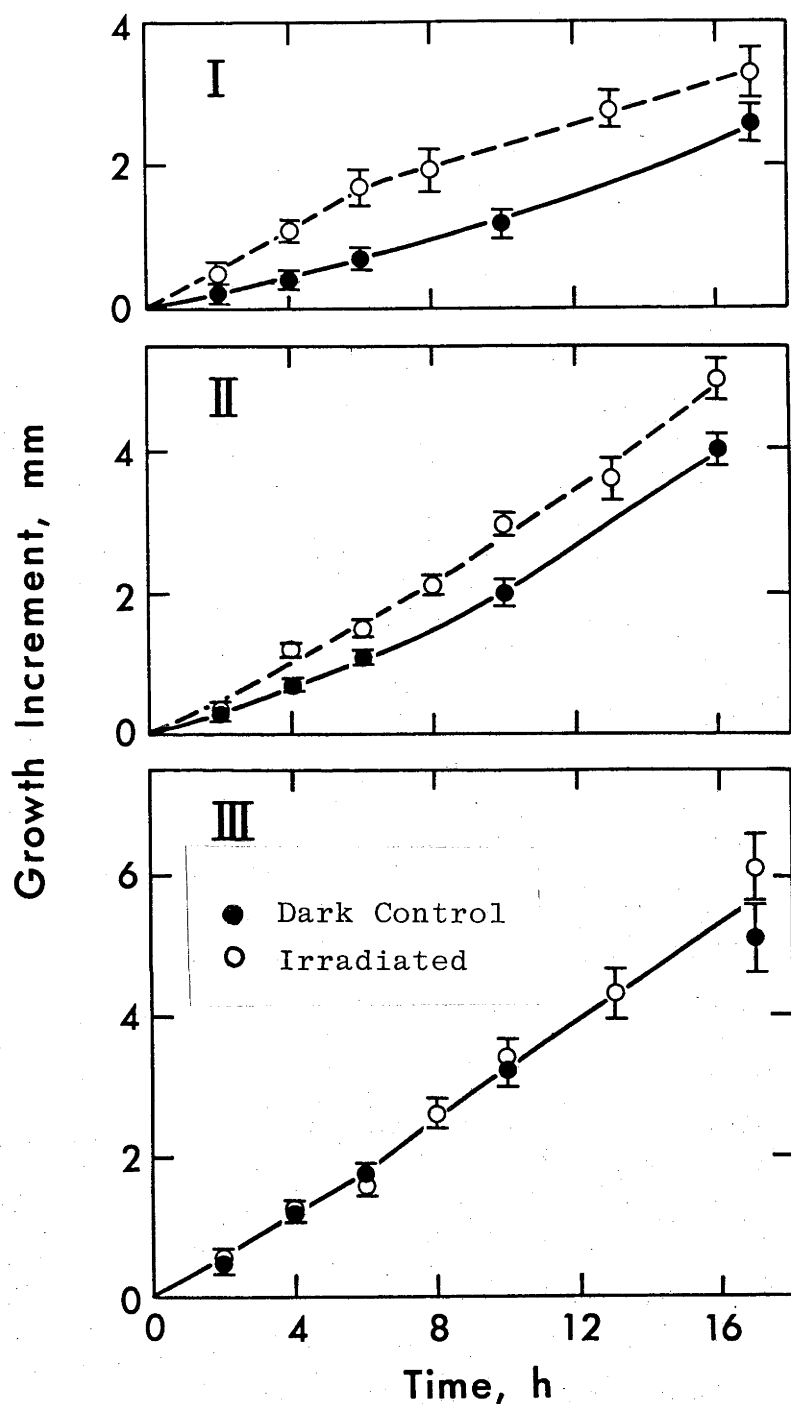


Fig. 13. Effects of red light on the growth of different zones of the coleoptile. The coleoptiles of intact seedlings were marked every 5 mm from the tip (threads fixed at 5 mm intervals in a frame were used to apply Indian ink to the coleoptiles), and then irradiated with red light (18 mWs/cm^2 , given in 10 min). After a dark interval, the coleoptiles were cut at the marks and the length of each segment measured (photographic image, tenfold enlargement). Only seedlings having the coleoptilar node in the zone 15 mm to 20 mm from the tip were used for measurement, and the number of these seedlings was 10 to 15 per treatment. Zones are numbered I to III from the top. Vertical bars indicate SE.

CHAPTER 6

SOURCES OF FREE IAA IN THE MESOCOTYL OF
ETIOLATED MAIZE SEEDLINGS

IINO, M, CARR, D.J. (1982)

PLANT PHYSIOL., ACCEPTED FOR PUBLICATION.

SUMMARY

Sources of free IAA for the mesocotyl of intact etiolated maize (Zea may L.) seedlings are evaluated. The coleoptile unit, which includes the primary leaves and the coleoptilar node, is the main source of free IAA for the mesocotyl. The seed and the roots are not immediate sources of IAA supply. Dependence of the apical growing region of the mesocotyl on the coleoptile unit as a source of free IAA is almost total. Half or more of the supply of IAA comes from the coleoptile tip, the rest mainly from the primary leaves. Removal of the coleoptile tip results in inhibition of mesocotyl elongation. The hypothesis that growth of the mesocotyl is regulated by auxin supplied by the coleoptile is supported. Conjugated forms of IAA appear to play little part in regulating the levels of free IAA in the shoot.

INTRODUCTION

In 1928 Went expressed the opinion (Went 1928, p 76) that the growth of the mesocotyl is determined by a growth substance (later called auxin) which reached it from the tip of the coleoptile. This hypothesis was experimentally supported by van Overbeek, who showed that inhibition of mesocotyl elongation due to a genetical factor (dwarfism) or external treatments such as heat and light is accompanied by a lower yield of diffusible auxin from the tip or the base of the coleoptile (van Overbeek 1935, 1936, 1938). He was also able to reverse the heat-induced inhibition by applying hetero-auxin (IAA) to the tip of the coleoptile (van Overbeek 1936). He (1936) and Went (1928) cited Beyer's

finding (1927), that decapitation of the oat coleoptile tip inhibits the mesocotyl elongation, in support of the hypothesis. With maize a similar effect of decapitation was found by Inge and Loomis (1937). Light-induced inhibition has recently been shown to be reversed by applied IAA (Vanderhoef and Briggs 1979)

On the other hand, the auxin hypothesis has not always received support. Schneider (1941), working on inhibition by light, concluded that "if there is an indirect mechanism involving the mediation of another part of the plant, the mediation can hardly be by way of auxin". Mer also marshalled evidence from his many experiments to contradict the auxin hypothesis (1951). The most striking evidence was the claim that decapitation of the coleoptile tip exerted no appreciable effect on the elongation of the oat mesocotyl even though successive decapitations of the coleoptile were carried out to reduce the possibility of auxin production at the coleoptile stump, known as "regeneration of the physiological tip" (see Went and Thimann 1937). Dattary and Mer (1964) further claimed to show that the effect of light or heat treatment on mesocotyl elongation is not accompanied by a fall in the auxin content of the mesocotyl. Thus, the experimental evidence that mesocotyl growth is controlled by auxin originating in the coleoptile is contradictory and further research is needed.

The results of Iino and Carr (1982, Chapter 4) provide a reaffirmation of the classical concept of IAA production at the coleoptile tip. The mesocotyl, however, is unlikely to depend totally for its IAA on the coleoptile tip.

The primary leaves, for instance, appear to be able to supply relatively large amounts of IAA (Iino and Carr 1982, Chapter 4). There is also a possibility that the mesocotyl receives some IAA in free (Pohl 1936, Shelldrake 1973, Hall and Bandurski 1978) or conjugated (Epstein et al 1980, Nowacki and Bandurski 1980) form from the seed. If regulation of mesocotyl growth by IAA is to continue to be regarded as an example of hormone-mediated growth correlations, it is necessary at least to show that the IAA reaches it from elsewhere in the seedling. The present study investigates the extent of the dependence of the maize mesocotyl on other parts of the seedling as sources of IAA.

MATERIALS AND METHODS

Etiolated seedlings of maize (Zea mays L. cv. GH 390) were raised in seedling boxes as previously reported (Iino and Carr 1981, Chapter 2), and used when three days old. The box was opened only when seedlings were treated. Surgical treatments to seedlings were carried out in the dark using an IR-scope and physiologically safe IR radiation (Iino and Carr 1981, Chapter 2). Dim green light (Iino and Carr 1981, Chapter 2) was used when plant materials were harvested and frozen in liquid N₂ for estimation of their free IAA content. All these procedures were carried out in a dark room at 25°C and 80% RH. The humidity was raised to about 90% when the seedling box was opened for surgical treatments. Free, conjugated and diffusible IAA were

estimated essentially as previously reported (Iino and Carr 1982, Chapter 4; see also Iino et al 1980, Chapter 3).

RESULTS AND DISCUSSION

1. Origin of Free IAA in the Mesocotyl

In 3-day-old seedlings, the yield of diffusible IAA from the base of the whole coleoptile (coleoptile plus primary leaves and nodal region, obtained by cutting at about 1 mm below the node) was 0.6 ng/h (Iino and Carr 1982, Chapter 4). Knowing that a mesocotyl (about 3 cm long at this age) contains only 1.5 ng of free IAA (Iino and Carr 1982, Chapter 4), the yield of diffusible IAA suggests that IAA in the mesocotyl originates largely in the coleoptile unit. To evaluate its dependency on the coleoptile unit, the free IAA content of the mesocotyl was monitored after removal of the whole coleoptile (Fig. 1). The result, however, did not permit a clear-cut evaluation, which can be made only when the IAA content reaches a steady state following removal of the source. A decrease was followed by an increase. At the lowest level, 3 h after decapitation, the content was more than half that of the undecapitated control. However, when the free IAA content of the apical 5 mm of the mesocotyl stump was monitored following removal of the whole coleoptile, the content fell almost to zero at 1 h after cutting, despite the following increase (Fig. 2). The result clearly indicates that the free IAA in the apical part of the mesocotyl is almost entirely derived from the coleoptile unit.

It has been suggested that the seed of cereals supplies IAA to the shoot (Pohl 1936, Sheldrake 1973, Hall and Bandurski 1980). The results indicate that there is no substantial acropetal supply of IAA at least for the apical region of the mesocotyl, but there may be some supply to the basal region. To investigate the supply of IAA to the mesocotyl from the parts below it, i.e. the seed and the roots, the whole shoot was excised from the seedling, and changes in the free IAA content of the mesocotyl then monitored. The excised shoots were incubated in vials, their bases in contact with water. The free IAA content of the mesocotyl was unaffected (Fig. 3); therefore, the seed and the roots are unlikely to supply free IAA to the mesocotyl in appreciable amounts.

The rise in IAA content of the mesocotyl following the initial fall is probably due to production of IAA in the mesocotyl itself, induced by removal of the coleoptile unit, as has been shown in the coleoptile after its decapitation (see below). In the apical 5 mm of the mesocotyl, the increase is apparent from 1 h after cutting (Fig. 2). The induced IAA production appears to start very soon after cutting.

It can be concluded that the external source of free IAA in the mesocotyl of the intact seedling is the coleoptile unit, and the dependence of the apical region of the mesocotyl on the coleoptile unit as the source is almost total. Considering that direct measurement (see Chapter 7) shows that the growth of the mesocotyl is located in its

apical region, the important source of free IAA for mesocotyl growth must be the coleoptile unit.

2. Supply of IAA from the Coleoptile Unit

The most active site of IAA production in the coleoptile unit is demonstrably the coleoptile tip (Iino and Carr 1982, Chapter 4). However, the primary leaves may also be such sites. Skoog (1937) could detect some auxin diffusing out of oat primary leaves using his sensitive "deseeded Avena test". Iino and Carr (1982, Chapter 4) estimated the yield of diffusible IAA from the base of a single set of primary leaves as about 0.2 ng/h, a substantial part of the yield (0.6 ng/h) from the base of the whole coleoptile. In order to evaluate what fraction of the IAA supplied by the coleoptile unit to the mesocotyl originates in the coleoptile tip, the yield of diffusible IAA from the base of the coleoptile unit was monitored after removal of the tip. The result is shown in Fig. 4. The yield fell rapidly after decapitation. The fall was followed by a gradual rise, due probably to development of IAA production in the coleoptile stump induced by decapitation (Went and Thimann 1937, van Oberbeek 1941, see also below). The minimum yield obtained, at around 3 h after decapitation, was about half that of the undecapitated control. From this it can be concluded that at least half of the diffusible IAA at the base originates in the coleoptile tip. The amount, however, could be greater since IAA production induced by decapitation probably begins earlier than the measured rise. A large part of the

diffusible IAA not derived from the coleoptile tip is probably supplied by the primary leaves (see above). However, the data do not exclude the possibility that parts of the coleoptile unit other than the coleoptile tip and the primary leaves contribute to some small extent.

Production of IAA in the coleoptile induced by removal of its tip was demonstrated by monitoring diffusible IAA from the apical region of the decapitated coleoptile. Each hour, apical 5 mm stumps of coleoptiles, without included leaves, were excised from seedlings decapitated at time zero (3 mm tip removed). The yield of diffusible IAA in an hour from these excised coleoptile cylinders was estimated. The yield fell to a minimum during the first 2 hours, then increased until at least 8 h from the time of decapitation (e.g. 0-1 h: 0.26 ng, 2-3 h: 0.09 ng, 8-9 h: 0.28 ng; diffusion time after decapitation: IAA yield per segment). A similar increase, following an initial decrease, was induced even in coleoptile cylinders (5 mm long) excised from intact seedlings and incubated in water (not shown, see also Fig.5), and these isolated segments were found to give off diffusible IAA in amounts far exceeding their initial free IAA content. These results are consistent with those obtained by van Overbeek using a bioassay (1941). It is, however, not clear whether this increased IAA production is due to newly-generated or merely enhanced production, since the results do not exclude the possibility that the non-tip tissues of the intact coleoptile produce IAA.

The yield of diffusible IAA from the base of the whole coleoptile showed a gradual increase with seedling age

(see undecapitated control, Fig. 4). This probably reflects a normal increase of IAA production in the intact coleoptile, since the yield of diffusible IAA from the coleoptile tip also showed an increase with age (see Fig. 2 in Chapter 5). An age-dependent increase in diffusible auxin yield from the maize coleoptile tip has already been reported by van Overbeek (1938) and Briggs (1963).

3. Roles of Conjugated IAA

Conjugated IAA is known to occur in seeds (Ueda and Bandurski 1969, Percival and Bandurski 1976, Bandurski and Schelze 1977) and seedling shoots (Bandurski and Schulze 1974; Iino and Carr 1982, Chapter 4) of cereals. Bandurski and co-workers have developed a hypothesis, based on the transport and metabolism of radioactive indole-3-acetyl-myo-inositol applied to the cut surface of the maize endosperm, that this substance, a major component of IAA conjugates in the maize seed, is transported into the shoot and serves as the source of free IAA (Epstein et al 1980, Nowacki and Bandurski 1980). Nevertheless, the most active IAA production at the coleoptile tip has been shown to be able to proceed without a supply of conjugated IAA (Iino and Carr 1982, Chapter 4). Conjugated IAA might be converted to free IAA in the mesocotyl, since the free IAA content of the whole mesocotyl did not fall greatly after removal of the coleoptile unit (Fig. 1). However, such supplies of IAA are probably not important for growth in the intact seedling, because, as shown above, the apical growing portion of the

mesocotyl depends largely on the coleoptile unit for its supplies of free IAA.

Interconversion of free IAA and conjugated IAA shown by Bandurski and co-workers (Epstein et al 1980, Nowacki and Bandurski 1980) also may have little importance in the control of free IAA levels in the etiolated seedling. To investigate this problem, coleoptile segments (1 cm long) excised 3 mm below the tip were used. In such a segment, incubated in water, the free IAA content decreases after excision owing to removal of the supply of IAA from the coleoptile tip and diffusion of IAA from the segment into water. This decrease is then followed by an increase due probably to IAA production induced by removal of the coleoptile tip. In spite of these changes, however, the conjugated IAA content remained constant (Fig. 5). Thus the changes in the content of free IAA were not reflected in that of conjugated IAA. This suggests that free IAA and conjugated IAA are not freely interconvertible, at least in the coleoptile tissue used.

The IAA supplied from the coleoptile unit to the mesocotyl is probably produced from precursors such as tryptophan or tryptamine, not by release of IAA from its conjugates. Some of the IAA produced in the coleoptile unit appeared to be lost during its downward transport (Iino and Carr 1982, Chapter 4). This loss is more likely to be due to decomposition than to conjugation (see also Iino and Carr 1982, Chapter 4).

4. IAA and Mesocotyl Growth

Contrary to the finding of Beyer (1927), Mer has reported (1951) that decapitation of the coleoptile tip of oats does not inhibit elongation of the mesocotyl. If mesocotyl elongation is regulated by IAA, the result shown in Fig. 4 leads to the expectation that coleoptile decapitation should inhibit mesocotyl growth. This is demonstrated in Table 1. During 8 h incubation following decapitation, the increment of elongation was reduced to 64% of the undecapitated control. Mer's claim cannot be sustained at least for maize.

The hypothesis that mesocotyl elongation is regulated by auxin produced at the coleoptile tip is supported, with the proviso that parts other than the coleoptile tip, particularly primary leaves, are also sources of auxin. The concept of auxin as a correlation carrier, which extends back to the time of its discovery (see Went and Thimann 1937), found support in parallel effects of environmental factors, e.g. light, on the growth and on the auxin status of plants. However, the supporting experimental evidence was later contradicted or questioned (Schneider 1941, Mer 1951, Mer 1969, Firn and Digby 1980). The dependence of the apical growing region of the mesocotyl on supplies of IAA from the coleoptile unit is shown in this study. Mesocotyl elongation will be an excellent subject for the further study of growth correlations mediated by auxin.

Table 1. Effect of removal of the coleoptile tip on elongation of the mesocotyl. Seedlings in two boxes were uncovered at the same time under safe IR radiation. From the seedlings in one box, coleoptile tips (3 mm) were removed; the seedlings in the other box were kept as undecapitated control. The seedlings in both boxes were covered again. Immediately afterwards, a third box was transferred to a cold room (4°C) for an estimate of the initial mesocotyl length. These procedures, which took c. 10 min to perform, were repeated 3 times. After another 8 h incubation, the decapitated and control seedlings were transferred to the cold room. Mesocotyl length was measured to the nearest 1 mm. Mean length ($\pm$ SE) of 30 to 33 seedlings in each box is presented.

	Mesocotyl Length (mm)		
	Initial	After 8 h	
		Control	Decapitated
	36.7 $\pm$ 0.9	50.5 $\pm$ 1.8 ^a	45.3 $\pm$ 1.2 ^a
	36.8 $\pm$ 1.2	51.3 $\pm$ 1.6 ^b	46.5 $\pm$ 1.2 ^b
	37.4 $\pm$ 1.0	52.9 $\pm$ 1.9 ^c	47.3 $\pm$ 1.4 ^c
Mean	37.0	51.6	46.4
Increment	—	14.6	9.4

a,b,c Means (control, decapitated) are significantly different (a and c: $0.01 < p < 0.02$, b: $0.02 < p < 0.05$).

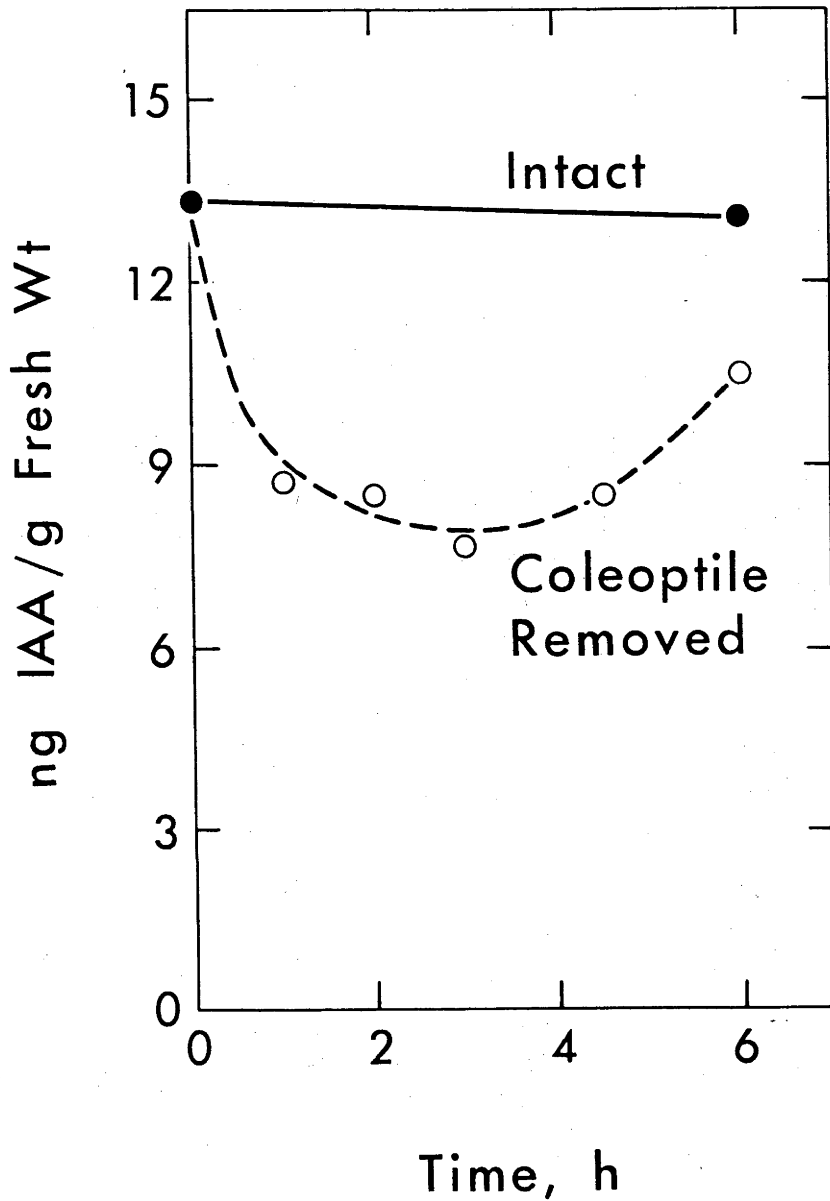


Fig. 1. Effect of removal of the whole coleoptile on the free IAA content of the mesocotyl. The coleoptiles were removed from seedlings, at time zero, by cutting at about 1 mm below the node. Following incubation of the seedlings for a given time, mesocotyls were excised and their free IAA content estimated. For each estimate, 30 mesocotyls were used. Controls were mesocotyls freshly excised from intact seedlings.

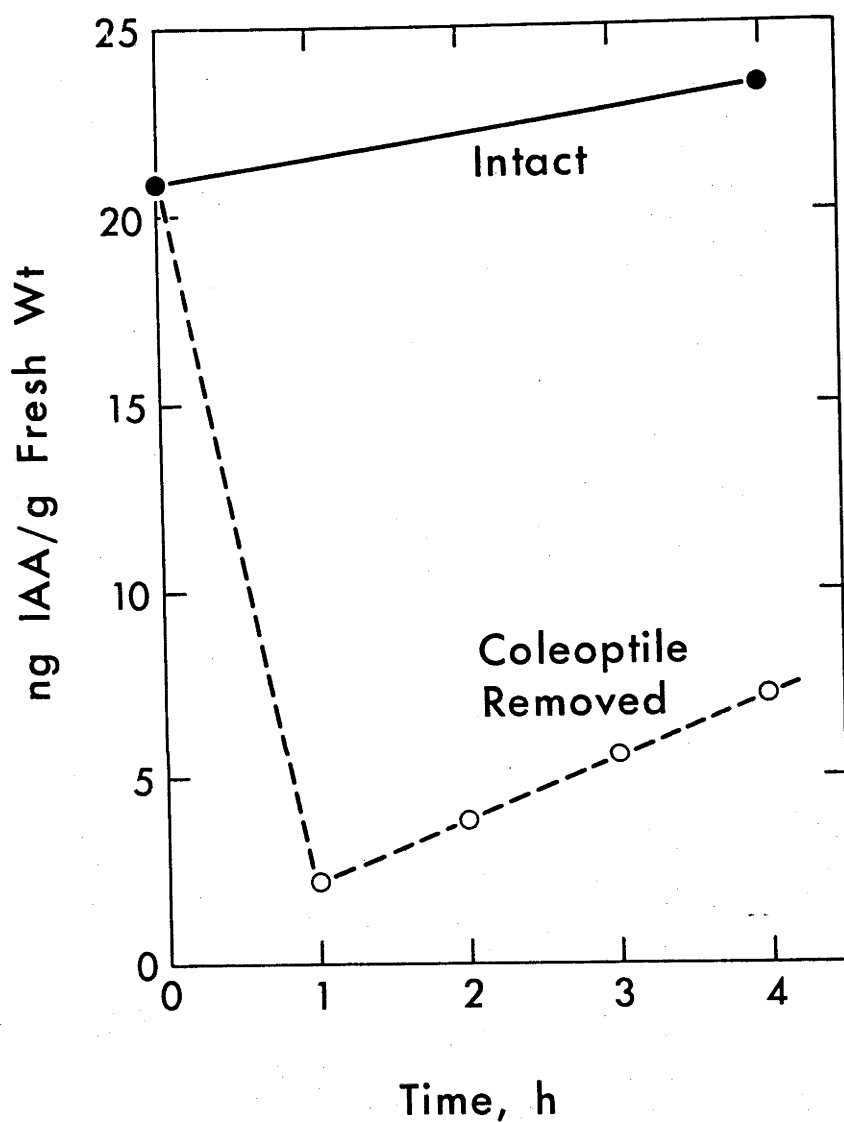


Fig. 2. Effect of removal of the whole coleoptile on the free IAA content of the apical 5 mm of the mesocotyl. Experimental treatments were as given in the legend to Fig. 1, but only the apical 5 mm of the mesocotyl was subjected to estimation of free IAA. For each estimate, 30 mesocotyls were used.

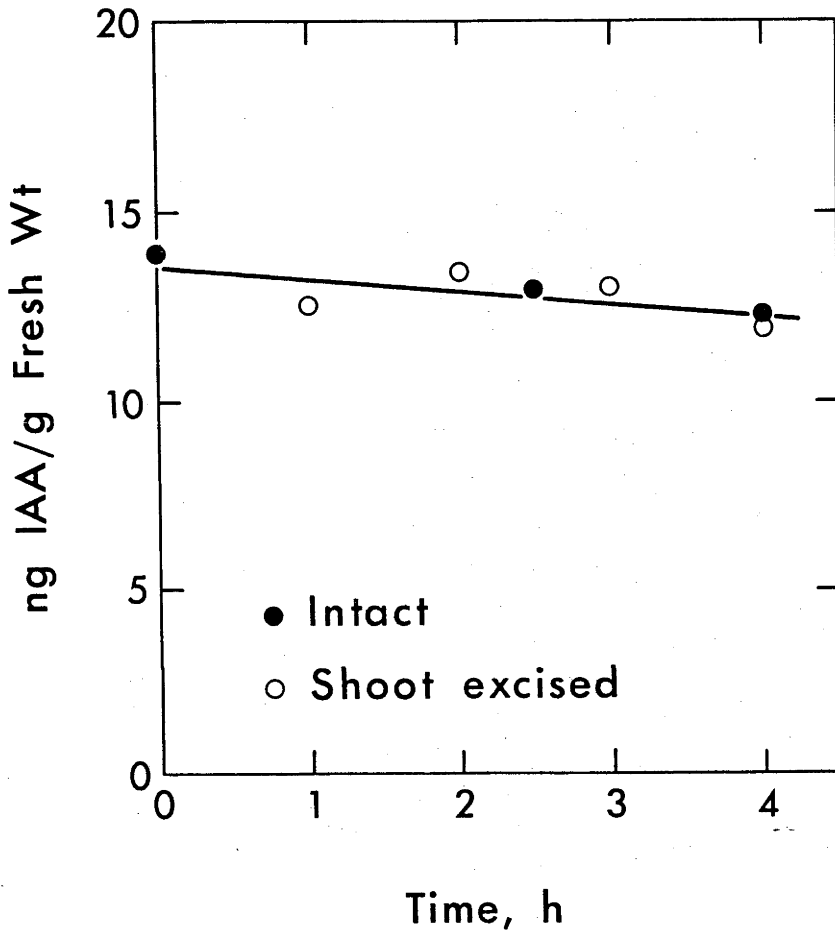


Fig. 3. Effect of removal of the seed and the roots on the free IAA content of the mesocotyl. Ten whole shoots, excised from seedlings, were stood in a vial, cut surfaces in contact with water (3 ml). Following incubation for a given period, the mesocotyls were excised and their free IAA content estimated. For each estimate, mesocotyls from three vials were used.

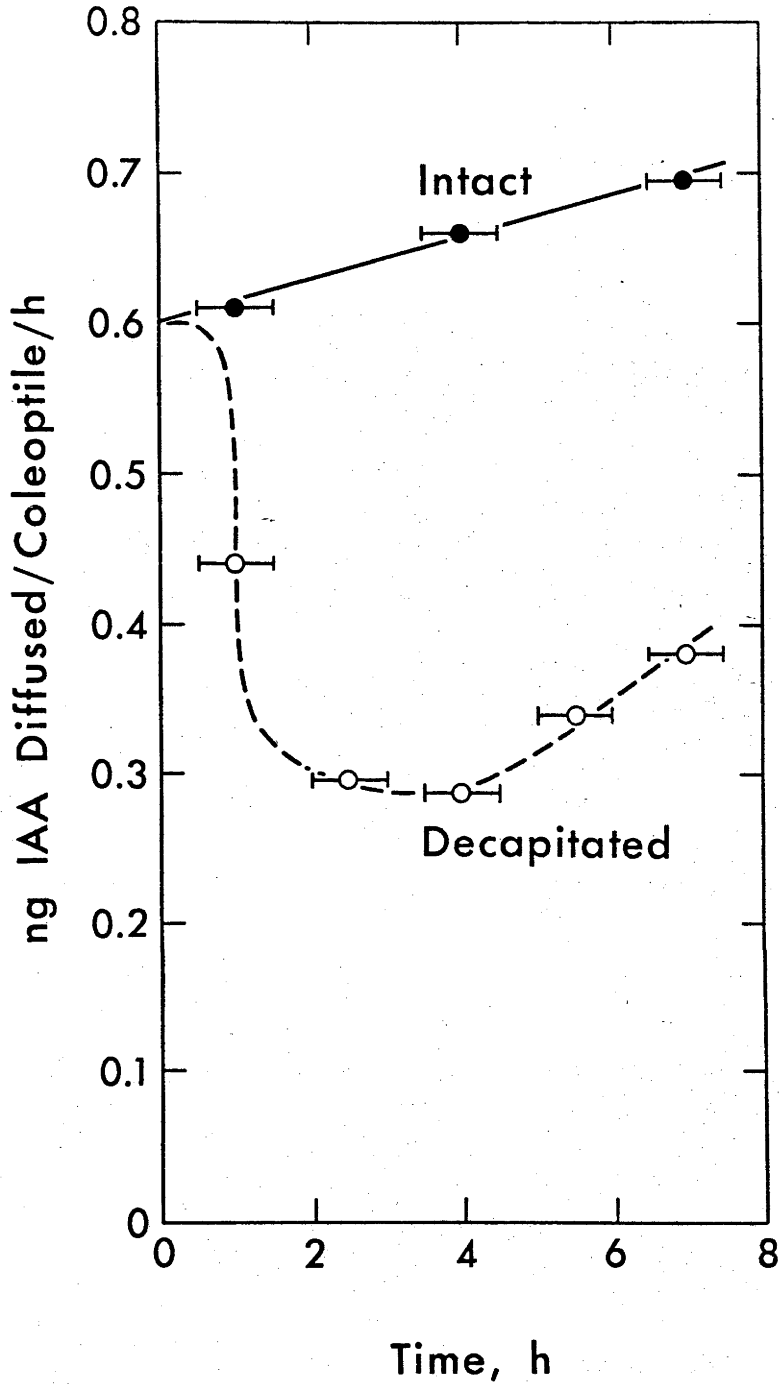


Fig. 4. Effect of removal of the coleoptile tip on diffusible IAA yield from the base of the whole coleoptile. The coleoptile tips (3 mm) were removed from seedlings at time zero. Following incubation of the seedlings for a given time, the coleoptiles were excised from the seedlings by cutting at about 1 mm below the node, and the yield of diffusible IAA, collected from the basal cut surfaces for 1 h, was estimated. For each estimate, 24 coleoptiles were used. Controls were prepared from non-decapitated coleoptiles. Each estimate was plotted at the centre of the diffusion time of 1 h, indicated by a horizontal bar.

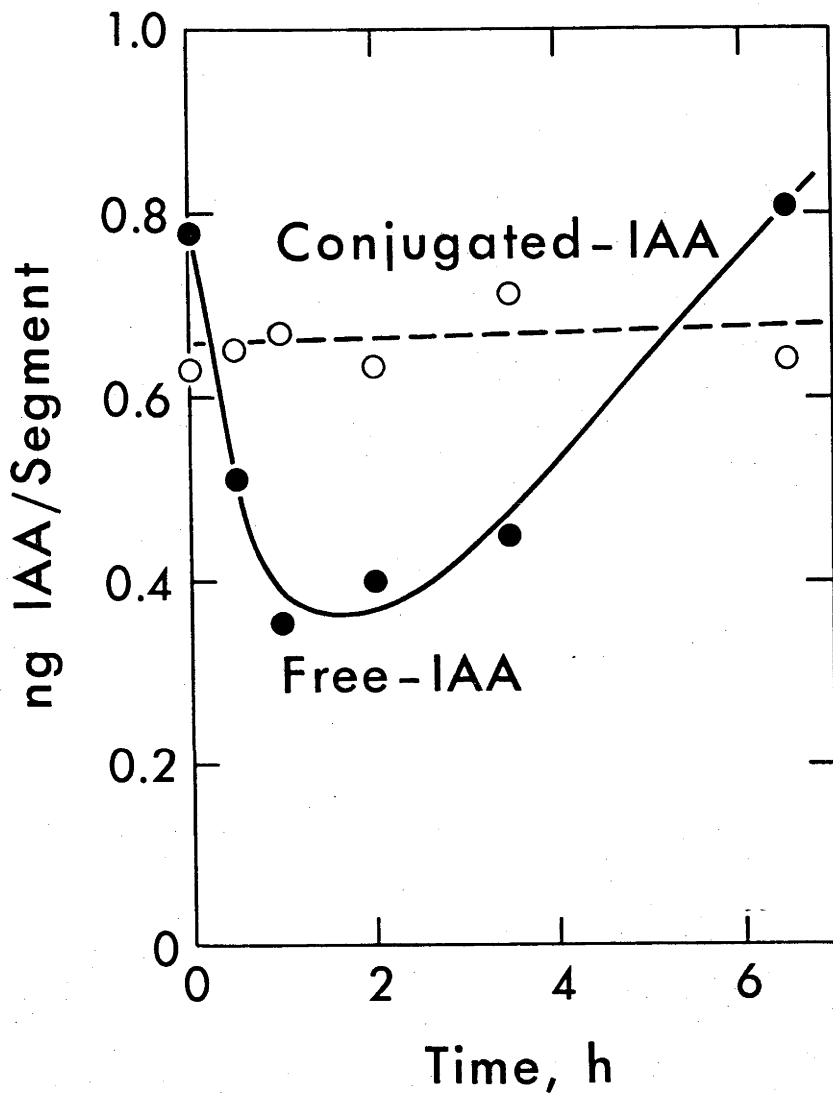


Fig. 5. Changes in free and conjugated IAA content of coleoptile segments following excision. A coleoptile segment, 1 cm long, was excised at 3 mm below the tip, and leaves removed. Twenty such segments were incubated for a given time floating on 10 ml water in a shaken vial. The segments were then subjected to estimation of free and conjugated IAA content.

CHAPTER 7

INHIBITORY ACTION OF RED LIGHT ON GROWTH OF MAIZE MESOCOTYL:
EVALUATION OF THE AUXIN HYPOTHESIS

IINO, M (1982) PLANTA, SUBMITTED FOR PUBLICATION.

SUMMARY

Brief irradiation of 3-day-old maize (Zea mays L.) seedlings with red light (18 mWs/cm²) inhibits elongation of the mesocotyl (70-80% inhibition in 8 h) and reduces its IAA content. The reduction in IAA content, apparent within a few hours, is due to reduction in the supply of IAA from the coleoptile unit (which includes the shoot apex and primary leaves). The fluence-response relationships for the inhibition of the mesocotyl growth by red and far-red light closely resemble those for the reduction of the IAA supply from the coleoptile. The relationship between the concentration of IAA (1-10 μ M) supplied to the cut surface of the mesocotyl of seedlings with their coleoptile removed and the growth increment of the mesocotyl in short term (4 h) is linear. The hypothesis that red light inhibits mesocotyl growth mainly by reducing the IAA supply from the coleoptile is supported. However, mesocotyl growth in seedlings from which the coleoptiles have been removed is also inhibited by red light (about 25% inhibition in 8 h). This inhibition is not related to changes in the IAA level, and is not relieved by applied IAA. In intact seedlings, this more direct effect may also participate in the red light inhibition of mesocotyl growth. Inhibition of cell division by red light, whose mechanism is not known, will also result in reduced mesocotyl elongation especially in the long term (e.g. 24 h).

INTRODUCTION

Growth of the mesocotyl of etiolated cereal seedlings is inhibited upon exposure to light, especially red light (e.g. Johnston 1937, Goodwin 1941, Weintraub and McAlister 1942). It was later shown that phytochrome is a photo-

receptor (Loercher 1966, Duke et al 1977). More detailed studies, however, have revealed complexities: (1) three steps have been distinguished in the fluence-response curves, only one of which is under photoreversible phytochrome control (Blaauw et al 1968, Mandoli and Briggs 1981); (2) the time course of growth under continuous exposure to red light showed two steps of inhibition (Vanderhoef et al 1979); (3) an action spectrum showed two peaks in the red region (Vanderhoef et al 1979). It has also been shown that light inhibits both elongation and division of mesocotyl cells (Avery et al 1937, Araki 1939, Goodwin 1941, Thomson 1954, Mer and Causton 1967). Inhibition of cell division was apparent with brief red light irradiation (Goodwin 1941, Mer and Causton 1967). Light inhibition of mesocotyl growth is thus complex, and probably involves a variety of mechanisms.

Went (1928) and van Overbeek (1936) held that light inhibits mesocotyl growth by reducing its auxin supply from the coleoptile tip, believed to be the site of auxin production in the shoot. The latter showed that illumination of oat seedlings reduces the yield of diffusible auxin from the excised coleoptile tip. This was confirmed by various investigators specifically using red light (see Chapter 5 for references). Kondo et al (1969) were able to detect a decrease in diffusible auxin from the base of the coleoptile of oat seedlings following red irradiation. These effects of red light on diffusible auxin yields have recently been confirmed by Iino (1982, Chapter 5) using maize seedlings and specific physicochemical

determinations of IAA. Iino and Carr (1982, Chapter 6) have further shown that the IAA in the mesocotyl comes mainly from the coleoptile, and the apical, rapidly growing region of the mesocotyl depends almost entirely on the coleoptile for its IAA. The fluence-response relationship between red or far-red light and the diffusible IAA yield (Iino 1982, Chapter 5) showed a resemblance to that for oat mesocotyl growth (Blaauw et al 1968, Mandoli and Briggs 1981). Thus, reduction in IAA supply from the coleoptile is consistent with inhibition of mesocotyl elongation by red light. Applied IAA has also been shown to counteract the inhibitory effect of red light on mesocotyl elongation (Vanderhoef and Briggs 1978).

The actual effect of red light on IAA content of the mesocotyl remains, however, to be shown. Dattaray and Mer (1964), measuring the content daily following a single brief irradiation, claimed to show that the auxin content of oat mesocotyls is not reduced by red light. However, the effect of such a brief red light treatment, reducing the diffusible IAA yield from the base of the coleoptile, is transient, the lowest yield being at around 5 to 6 h after the irradiation (Iino 1982, Chapter 5). It is therefore necessary to investigate this red light effect over much shorter periods than a whole day.

Other data have also been presented which appear to be inconsistent with the auxin hypothesis. Schneider (1941) concluded that inhibition of mesocotyl growth is direct and not through modulation of the auxin supply from the coleoptile, by showing that red light inhibits elongation of

excised mesocotyl segments and that this inhibition is not relieved by applied IAA. Other authors (Huisinga 1964, Vanderhoef and Briggs 1978) have observed a similar inhibition of the elongation of excised mesocotyl segments by red light. Although they concluded that the main mechanism for the inhibition of mesocotyl growth is nevertheless that involving auxin, there appears to be some evidence for a growth-inhibiting photomorphogenetic system which does not directly involve auxin. Also, there is no evidence that light inhibition of cell division in the mesocotyl is mediated by auxin.

The present contribution further characterizes the inhibition of maize mesocotyl growth by red light, and re-examines the validity of the auxin hypothesis.

MATERIALS AND METHODS

Etiolated seedlings of maize (Zea mays L. cv. GH 390) 3 days old, were raised on moist paper towels in seedling boxes (Iino and Carr 1981, Chapter 2). During experimental treatments to seedlings, objects were visualized, when required, in the dark using infrared radiation and an infrared-scope (Iino and Carr 1981, Chapter 2; Iino 1982, Chapter 5). Dim green light was used only in strictly limited instances (for the use of green light, see Iino and Carr 1981, Chapter 2, and Iino 1982, Chapter 5). Red and far-red light were obtained as previously reported (Iino 1982, Chapter 5). The free IAA content was estimated as previously reported (Iino and Carr 1982, Chapter 4; see also

Iino et al 1980, Chapter 3).

Measurement of mesocotyl elongation. For measurements of zonal elongation, the mesocotyl was marked with Indian ink, applied using threads fixed at intervals on a frame. Following incubation for a given period, the marked zones were excised and their length was recorded (10-fold magnification, photographic image). Elongation of the whole mesocotyl was measured essentially as previously reported (Iino and Carr 1981, Chapter 2).

Counting of cell numbers. Mesocotyl segments were fixed in a mixture of formaldehyde(40%)-acetic acid-ethanol (70%) (5:5:90, v/v) (FAA), and embedded in paraffin. Median longitudinal sections (14 μ m) were stained with toluidine blue O. Cell numbers of the 5th row of cortical cells from the epidermis were counted. The mean cell number of each mesocotyl segment was obtained from counts made on each side of each of 3 sections.

RESULTS

1. Mesocotyl Growth in Intact Seedlings;

Effect of Red Light.

The mesocotyls of etiolated maize seedlings, raised under the conditions chosen, elongated at a constant rate (1.8 mm/h) from 3 days after sowing for at least a day (the mesocotyl length of the 3-day-old seedlings was about 35 mm). When the seedlings, 3 days old, were irradiated with 18 mWs/cm² red light (10 min), which saturates spectrophotometrically measurable in vivo Pfr formation at the

coleoptile tip (Iino 1982, Chapter 5) and in the apical 5 mm portion of the mesocotyl (Fig. 1), the elongation rate of the mesocotyl was reduced within a few hours to about 20% of that of the dark control. The rate of elongation remained depressed from 4 to 10 h after the irradiation, but then started to increase over a few hours. The mesocotyl then elongated at an approximately constant rate, 50-60% of that of the dark control, for at least another 12 h.

Fig. 2 shows time courses of elongation of mesocotyl zones. In non-irradiated seedlings, the apical 6 mm zone continued to elongate actively becoming five times as long in 18 h. The elongation rate of this zone showed an increase during the first few hours. The elongation rate of the second 6 mm zone (6-12 mm) decreased gradually over a period of 18 h. The initial elongation rate of this zone, however, was about the same as that of the apical zone. When marking took place, the third 6 mm zone (12-18 mm) was about to cease elongation. Thus, the high elongation rate is confined to the apical c. 12 mm, and below this zone, the rate falls basipetally to zero. This zone-dependent distribution of elongation appears to be kept up for 18 h, since the top zone showed an increase in its elongation rate at least until it become about 12 mm long and its final steady rate is about the sum of the elongation rates of zones 1 to 3 at the time of marking.

Red light inhibited elongation of all three zones. Inhibition of the apical zone was apparent within a few hours after irradiation. Inhibition of the second zone was almost complete after 4 h, and no further elongation took

place over at least 14 hours. Red light also terminated elongation of the third zone before its length reached that of non-irradiated seedlings.

Longitudinal cell numbers (the 5th row of cortical cells) of each zone were also obtained from the mesocotyls used in the experiment described above (Fig. 2). The mean cell numbers of the three zones at the time of marking were, from the top zone downward, 129, 40 and 27. Thus, the mean cell lengths in these zones were 47, 150 and 220 μm , respectively. The cell number of the top zone increased about 50% in 18 h, indicating that cell division is taking place in this zone. Red light appeared to inhibit this cell division within 6 h after irradiation, supporting earlier findings (Goodwin 1941, Mer and Causton 1967). There was no increase in the cell numbers of the second and the third zones, indicating that at this stage in development no cell division is taking place in them.

In the apical zone, although cell division appeared to be inhibited by red light, the rapid inhibition of elongation must be attributed to an effect on the elongation of its initial complement of cells: the mean cell length in the top zone is one third of that of the second zone, which is still elongating at a high rate (see above); the rate of cell division in the apical zone (about 50% increase in 18 h) is far less than the rate of elongation (about 400% increase in 18 h). However, inhibition of cell division must in the long term cause inhibition of mesocotyl elongation or reduction in the finally attainable mesocotyl length. In the second and the third zones, it is apparent

that the effect of red light is to inhibit cell elongation. It must be stressed that in these zones, red light terminated elongation before the component cells reached their potential final length. It thus appears that red light terminates cell elongation in parts composed of cells already relatively elongated.

2. Mesocotyl Growth in Isolated Segments or Seedlings Without Coleoptiles; Effect of Red Light.

The reported inhibition of the elongation of excised mesocotyl segments by red light (see Introduction) is reproducible. Apical 1 cm-long mesocotyl segments were floated on water, irradiated with red light (18 mWs/cm^2), and incubated for 8 h. Non-irradiated segments elongated 1.8 mm. Elongation of irradiated segments was about 75% of the dark control (not shown).

This direct red light effect was further characterized using seedlings from which the coleoptiles had been removed. A positive response of such seedlings does not necessarily imply a direct effect of red light on the mesocotyl in view of the presence of the seed and roots, but the response would at least be closer to the intact situation. The coleoptile was removed by cutting the seedling shoot at about 1 mm below the node. The mesocotyl was then marked at 10 mm, or 5 and 10 mm from its apical end, and elongation of only these zones was measured.

The removal of the coleoptile strongly inhibited elongation of the mesocotyl. For example, during 8 h after cutting, elongation of the apical 1 cm zone was reduced to

about 20% of that of the equivalent zone in the intact seedling. Red light inhibited still further the elongation of the mesocotyl. For example, red light irradiation (18 mWs/cm^2) immediately following removal of the coleoptile reduced elongation of the apical 1 cm zone during 8 h to 70 to 75% of the dark control, a reduction which corresponds well with that of excised mesocotyl segments.

Fig. 3 shows time courses of zonal mesocotyl elongation following removal of the coleoptile. Elongation of the apical and the next 5 mm-zones was measured. In either zone (non-irradiated seedlings), a reduction in the high elongation rate of the intact seedling was evident as early as 2 h after cutting (these two zones roughly correspond to the two apical zones of intact seedlings shown in Fig. 2). The elongation rate of the apical 5 mm-zone increased gradually over a period of about 10 h, and then decreased. In the next 5 mm zone, the elongation rate rapidly declined at around 4 h after the removal of the coleoptile and then fell almost to zero. Following irradiation of the seedlings, elongation of the apical 5 mm zone was inhibited gradually over about 8 h. In the subjacent 5 mm zone, elongation was inhibited within a few hours after irradiation, and was terminated before the cell length reached that of non-irradiated seedlings.

In the above experiments, seedlings were irradiated after removal of the coleoptile. However, irradiation applied just before its removal produced essentially similar overall effects (Fig. 4).

3. Mesocotyl Growth in Seedlings Without Coleoptiles and Supplied with IAA; Effect of Red Light.

After removal of the coleoptile, the mesocotyl was marked at 10 mm from the cut end and agar containing various concentrations of IAA was applied to the cut surface. Zonal elongation, measured after 4 h, was stimulated in a narrow range of IAA concentrations, 1 to 20 μ M; elongation at higher concentrations was less than the maximum reached at 10-20 μ M. The elongation induced by applied IAA closely approached that of the equivalent zone in intact seedlings, but never exceeded it. At concentrations between 1 and 10 μ M, the growth increment produced by applied IAA was linear to concentration (see Fig. 5). A linear relationship between growth increment of decapitated oat coleoptiles and concentration of auxin (Rhizopus suinus culture extract) applied in agar to the cut surface had earlier been shown by Thimann and Bonner (1933). It should be noted, in contrast, that the effects of IAA applied in solution on elongation of isolated coleoptile or mesocotyl segments are linear to log of concentration (e.g. Nitsch and Nitsch 1956).

Fig. 5 shows the effects of red light on mesocotyl elongation in seedlings following removal of the coleoptile and application of IAA. Within the range of IAA concentrations in which the growth response is linear, the inhibitory effect of red light was still manifest.

4. Effect of Red Light on IAA Content of the Mesocotyl.

IAA content of the mesocotyl was monitored following red irradiation of intact seedlings (Fig. 6). In the

non-irradiated control, IAA content per mesocotyl increased with time, whereas IAA content per g fresh weight remained almost constant. Red light stopped the increase in IAA content per mesocotyl. After about 6 h, the content started to increase again. Since the reduction in tissue weight (relative to controls) following irradiation becomes considerable with time, a better parameter for comparison was sought in the content per g fresh weight. Evidently red light reduced the IAA content per g fresh weight (Fig. 6). The lowest level was reached at about 6 h after irradiation, followed by a recovery towards the level of the control during a further 6 h. This time course is similar to that obtained for diffusible IAA yield from the base of the coleoptile (Iino 1982, Chapter 5), but the actual decrease observed here is much less. Nevertheless, it has already been shown that there is a fraction of IAA, particularly in the basal non-elongating region of the mesocotyl, the amount of which is not affected immediately by the removal of the coleoptile (Iino and Carr 1982, Chapter 6). In fact, the reduction was more pronounced when the IAA content of only the apical elongating region (1 cm) of the mesocotyl was monitored following irradiation (Fig. 7).

The effect of red light on the IAA content of the mesocotyl was also investigated in seedlings irradiated with red light immediately following removal of the coleoptiles. The changes in IAA content of the mesocotyl due to removal of the coleoptile (see Chapter 6) were not affected by red light (Fig. 8). The changes in IAA content of the apical 5 mm region were also unaffected by red light (Fig. 9).

5. Fluence-response Relationships.

Fig. 10 shows fluence-response curves obtained for the elongation of the whole mesocotyl in intact seedlings. Although some difficulty arises through variability in measurements, the threshold of the response to red light can be seen to be at around 1-10 nWs/cm², which is 4-5 orders of magnitude less than the threshold of spectrophotometrically detectable Pfr formation (see Iino 1982, Chapter 5) and corresponds well to that obtained for the inhibition of oat mesocotyl elongation (Blaauw et al 1968, Mandoli and Briggs 1981). Thus, the very low irradiance response, which has so far been shown with oat seedlings is also to be found in maize seedlings.

Mesocotyl elongation was also inhibited by far-red light at relatively low fluences, as has already been shown for the oat mesocotyl (Blaauw et al 1968, Mandoli and Briggs 1981). Although the response to far-red light appears to be about linear for 6 orders of magnitude, that to red light changes abruptly at above 0.1 mWs/cm², in good correspondence with the threshold for the formation of spectrophotometrically detectable Pfr (see Iino 1982, Chapter 5). The inhibition induced by 10 mWs/cm² red light, which almost saturates Pfr formation (Iino 1982, Chapter 5) was reversed by 0.3 to 1 Ws/cm² of far-red light, which reverses most of the spectrophotometrically measurable Pfr to Pr (Iino 1982, Chapter 5), up to about the level induced by far-red alone (data not shown). The response above 0.1 mWs/cm² red light, which is apparent as a fall in the fluence-response curve, appears to be

controlled by the photoreversible phytochrome system. The photoreversibility data are consistent with those obtained with oat seedlings (Blaauw et al 1968, Mandoli and Briggs 1981). The saturation phase of the very sensitive response shown in the work using oat seedlings, however, was not apparent in the present study, although it is possible that this saturation was concealed within the variability experienced with maize.

Fig. 11 shows the effects of red light at two different fluences, 18×10^{-3} and 18 mWs/cm^2 , and of far-red light at 320 mWs/cm^2 , on mesocotyl elongation in seedlings with their coleoptiles removed. The inhibition of mesocotyl elongation by 18 mWs/cm^2 red light, saturating in vivo Pfr formation, was already induced and almost saturated by $18 \times 10^{-3} \text{ mWs/cm}^2$ red light, which causes no detectable Pfr formation (Iino 1982, Chapter 5). Far-red also inhibited elongation to about the level induced by red light, and red followed by far-red irradiation produced no detectable difference. The fluence of far-red light used was that sufficient to reverse most Pfr to Pr (Chapter 5). The results make it clear that the inhibition of mesocotyl elongation in seedlings with the coleoptiles removed is at least considerably induced with red or far-red light which produces no spectrophotometrically detectable Pfr. These sensitive responses to red and far-red light are highly consistent with those observed by Huisinga (1964) using oat mesocotyl segments. Similar results have also been obtained in an investigation of the stimulatory effect of red and far-red light on maize coleoptile elongation (Chapter 5).

DISCUSSION

The IAA content of the mesocotyl, especially of its actively growing apical region, was found to be reduced by red light, following a time course (Figs. 6 and 7) in good accord with that of the diffusible IAA yield from the base of the coleoptile (Iino 1982, Chapter 5). The observed decrease in IAA content can be attributed to a reduced IAA supply from the coleoptile, which in turn is probably due to inhibition of IAA biosynthesis in the coleoptile. Inhibition by red light of IAA biosynthesis in the coleoptile tip has been shown (Iino 1982, Chapter 5). Although the coleoptile tip is not the only potential source of IAA supply to the mesocotyl (Iino and Carr 1982, Chapter 6) and at least the primary leaves can produce IAA (Iino and Carr 1982, Chapter 4), it is likely that IAA biosynthesis in parts other than the coleoptile tip is also inhibited by red light.

The relationship between the concentration of IAA applied to the cut surface of the mesocotyls of seedlings from which the coleoptiles were removed and the growth increment of the mesocotyl measured after a brief incubation (4 h) is linear (Fig. 5). IAA (in 0.1% Tween 20 solution) applied with a brush to the surface around the nodal and the apical mesocotyl regions (about 1.5 cm) of intact seedlings appeared to be able to stimulate slightly the elongation of the mesocotyl; although the results were variable, repeated experiments showed a maximal increase of about 10-15% at

around 30 μ M IAA, elongation being measured 6 h after the application (not shown). This suggests that the endogenous IAA level of the mesocotyl is nearly saturating for growth, but is nevertheless within the linear stimulatory range. Thus, any reduction in IAA content of the mesocotyl may result in reduced elongation.

In seedlings with coleoptiles removed, the elongation of the second 5 mm-zone of the mesocotyl declined rapidly at about 4 h after cutting and almost ceased before the zone reached the final length attained in intact seedlings (Fig. 3). At around this time, 4 h after the removal of the coleoptile, the mesocotyl contains a small but increasing amount of IAA (see Fig. 9). It is suggested that reduction in the IAA content, short of its complete removal, results in termination of elongation of the subapical portion. Thus, the termination of elongation induced by red irradiation in intact seedlings (second 6 mm-zone, Fig. 2) may also be attributed to the reduced supply of IAA.

In irradiated seedlings, the elongation rate of the mesocotyl was restored to some extent following initial strong inhibition. This restoration was associated only with the apical 6 mm-zone (Fig. 2). This may indicate that the young cells in this zone whose elongation has not been totally terminated, resume elongation in response to the rise in IAA content following its initial fall (Fig. 6, see also Iino 1982, Chapter 5).

It was confirmed that red light can inhibit mesocotyl elongation directly without intervention of the coleoptile. As part of this direct effect, the attainable cell length in

the subapical portion is further reduced (second 5 mm-zone, Fig. 3). A possible interpretation is, as suggested by Vanderhoef and Briggs (1978), that red light inhibits the regeneration of IAA production in the cut stump of the mesocotyl following removal of the coleoptile (see Iino and Carr 1982, Chapter 6). This view is not supported, since the eventual increase in IAA content of the mesocotyl, following removal of the coleoptile, was not affected by red light (Figs. 8, 9). Although regeneration of IAA production may be inhibited under continuous illumination as practised by Schneider (1941) and Vanderhoef and Briggs (1978), it does not appear to be so following brief irradiation. The direct effect of red light on mesocotyl growth was not removed by applied IAA (Fig. 5). This observation further negates the possible involvement of an inhibitory effect on IAA production as an effective system. Most likely, the direct effect of red light on mesocotyl growth is due to some mechanism not involving the control of IAA level. It is suggested that, in intact seedlings, this direct effect of red light also has a role in the inhibition of mesocotyl growth in addition to the IAA-mediated indirect one.

Not much is known about the inhibition of cell division by red light. It has long been known that applied IAA can stimulate cell division in the cambium (Snow 1935), and it is possible that red light inhibition of cell division in the mesocotyl might also involve IAA. A direct effect of light on the mechanism of mitosis is also possible. This intriguing aspect of red light action is largely open for future studies.

The fluence-response relationships for the inhibition of mesocotyl growth by red or far-red light (Fig. 10) are similar to those for reduction in diffusible IAA yield (Iino 1982, Chapter 5), rather than to that of the direct effect (Fig. 11). The results support the significance of the IAA-mediated mechanism in the inhibition of mesocotyl growth. The fluence-response curves obtained by Blaauw et al (1968) and Mandoli and Briggs (1981) for the inhibition of oat mesocotyl growth are essentially similar, but they observed a saturation phase of the very low irradiance response at fluences insufficient to induce the photoreversible one. This might be due to the direct effect, which appears almost saturated with a fluence of red light with which no detectable Pfr is formed. The longer incubation period (24 h) used by these authors might have made easier the detection of the direct effect: the data of Fig. 3 suggest that the direct effect is retained over a long incubation period. Inhibition of cell division is also likely to result in considerable inhibition of elongation over long incubation times. Detailed fluence-response relationships for this response are needed before further discussion can be entertained.

Time courses of mesocotyl elongation of maize seedlings induced by red light have already been reported by Vanderhoef et al (1979). Although not specified in that report, it can be assumed that green safelights, which induce the very sensitive response (Blaauw et al 1968, Mandoli and Briggs 1981; Iino and Carr 1981, Chapter 2), were used in various steps of the experiments. The reported

fluence-response relationships suggest that the study was carried out in the absence of the very sensitive response. Direct comparison with the present study is therefore not feasible. Nevertheless, the minute-by-minute observations of the initial stages of inhibition hold considerable significance. Their data show a lag of about 30 min in the appearance of the growth inhibition. From Fig. 7, it may be seen that reduction of the IAA content is also induced with a similar lag. However, a detailed comparison of time courses, including that of the direct effect, must await further study.

The red light action investigated in the present study occurs at or below a fluence (18 mWs/cm^2) which saturates in vivo Pfr formation. However, long or continuous irradiation with red light exceeding this fluence has been shown to have an additional inhibitory effect on mesocotyl growth. This is indicated in the fluence-response curve (Blaauw et al 1968, Vanderhoef et al 1979) and in the time course (Vanderhoef et al 1979). Schneider (1941) observed a growth inhibition of excised mesocotyl segments irradiated with continuous red light in the presence of applied IAA, even when excision was carried out under dim red light, sufficient to saturate the sensitive direct effect shown in the present study. The growth inhibition by high fluence might be another kind of direct effect. The result of Wolton and Ray (1980) is suggestive of such an effect. They found that the number of specific binding sites for IAA is decreased in the mesocotyl of continuously-illuminated maize seedlings.

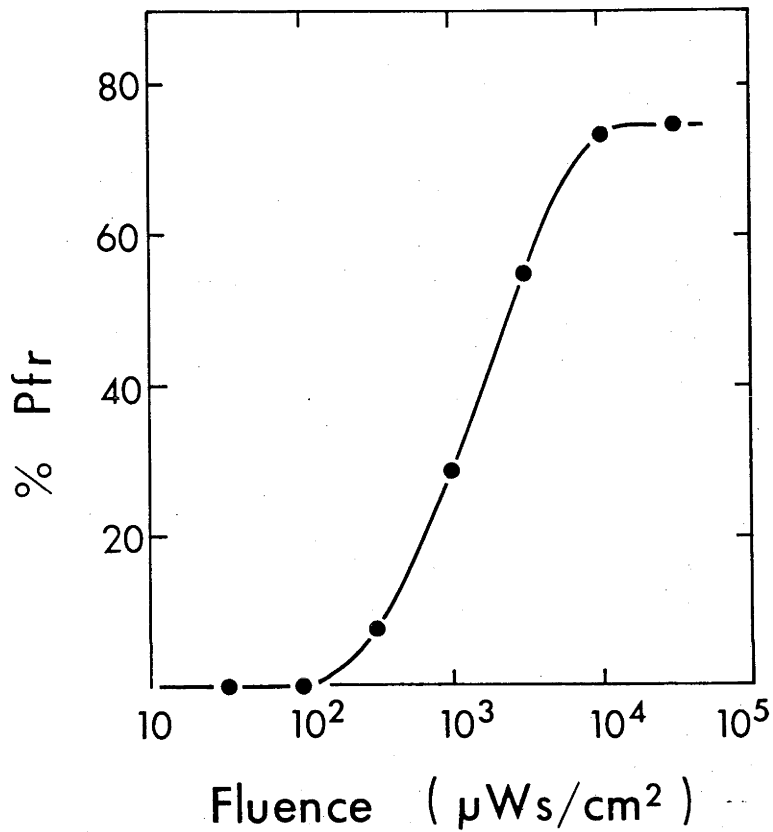


Fig. 1. In vivo transformation of Pr to Pfr in the apical portion of the mesocotyl. Apical 5 mm segments of mesocotyls were excised immediately following irradiation of intact seedlings with red light, and their Pfr level was estimated spectrophotometrically (see Methods in Chapter 5). For each determination, 32 segments were used.

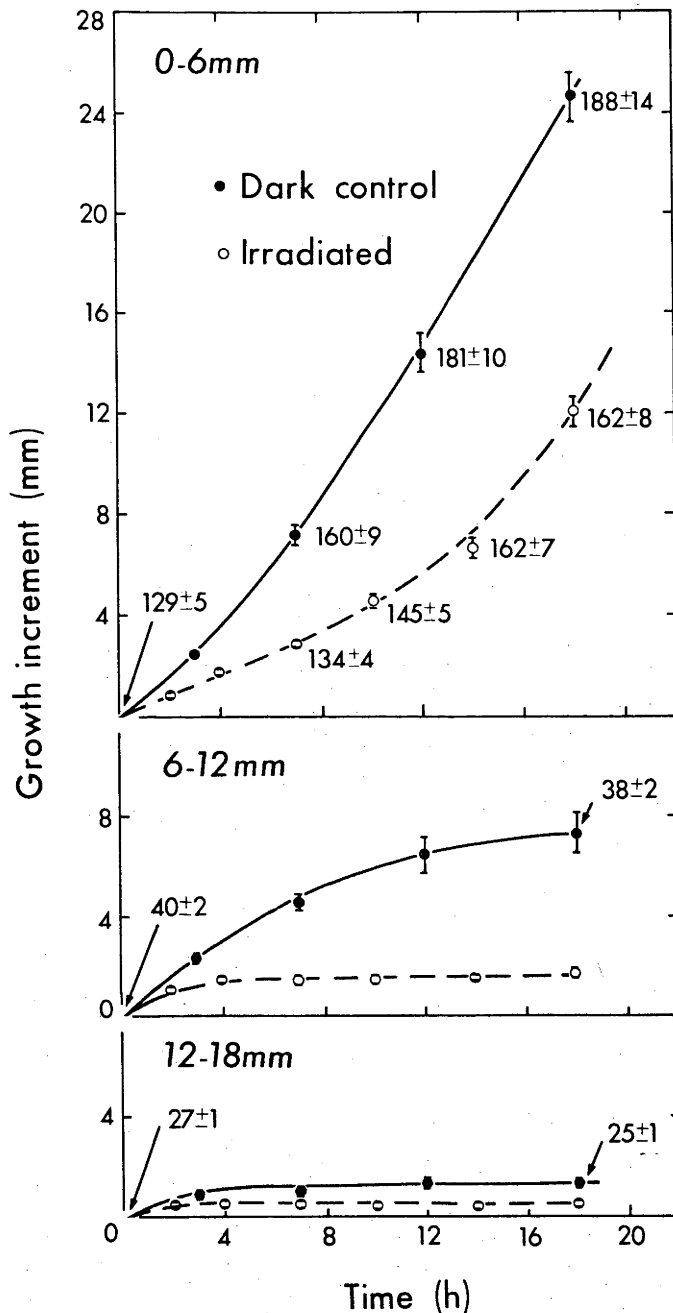


Fig. 2. Time courses of zone elongation and cell division in the mesocotyl: effects of red light. The shoots of etiolated seedlings were each marked at intervals of 2 mm from the basal region of the coleoptile down the mesocotyl (time 0). The seedlings were then irradiated with red light (18mWs/cm^2 in 10 min) or kept in the dark (control). Following incubation for a given period, three zones of the mesocotyl were excised from the node: each zone was initially 6 mm-long and the node was located within the top 2 mm-interval of the top zone. The length of the segments was promptly recorded, and the segments fixed in FAA (see Methods) for counting of cell numbers. For each treatment, 16 to 20 seedlings were used. Mean increment in zone length is presented with SE (vertical bars). The numbers in the figure are mean cell numbers $\pm$ SE.

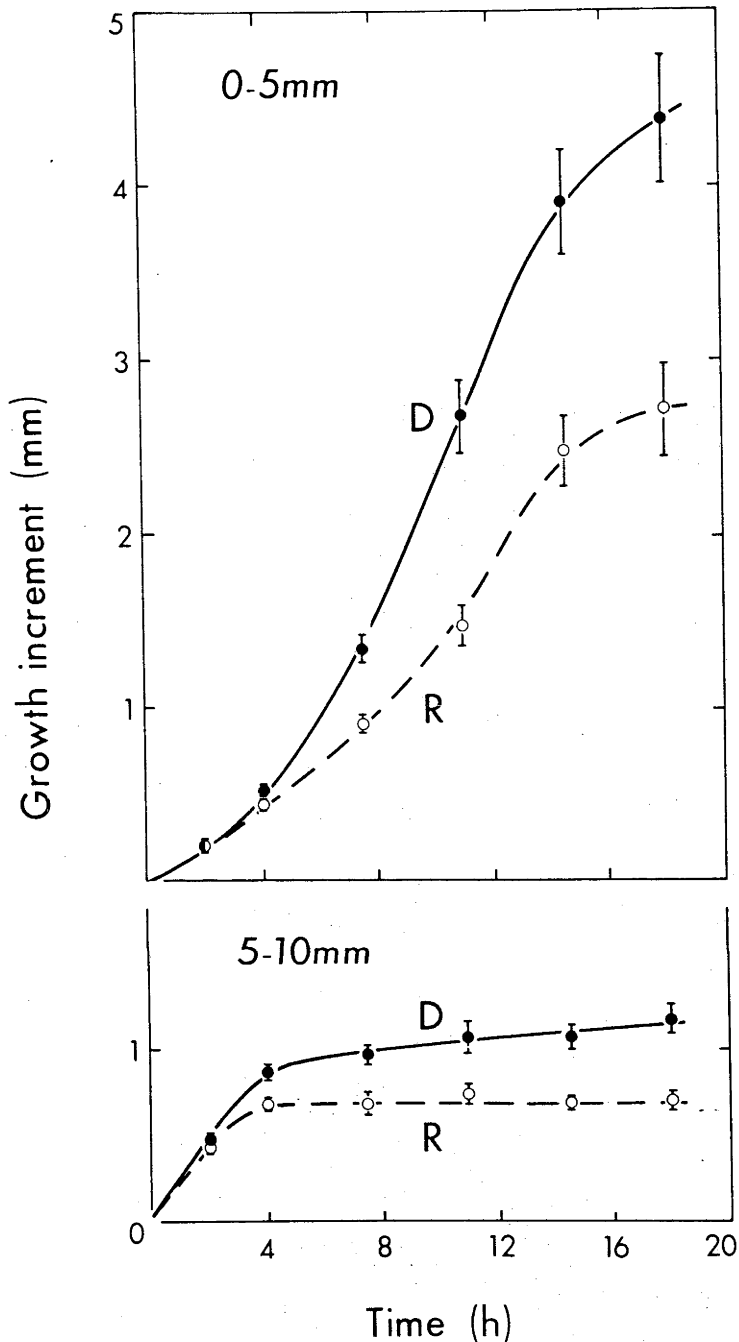


Fig. 3. Time courses of zonal mesocotyl elongation following removal of the coleoptile: effect of red light. Coleoptiles were removed from seedlings, by cutting at about 1 mm below the node, and the mesocotyls were each marked at 5 and 10 mm from the cut end (time 0); these treatments were completed within 10 min. The seedlings were then irradiated with red light (18 mWs/cm² in 10 min) or kept in the dark (control), and incubated for a given period. For each treatment, 20-24 seedlings were used. Mean increment in length of two zones (initially 0-5 mm and 5-10 mm) is presented with SE.

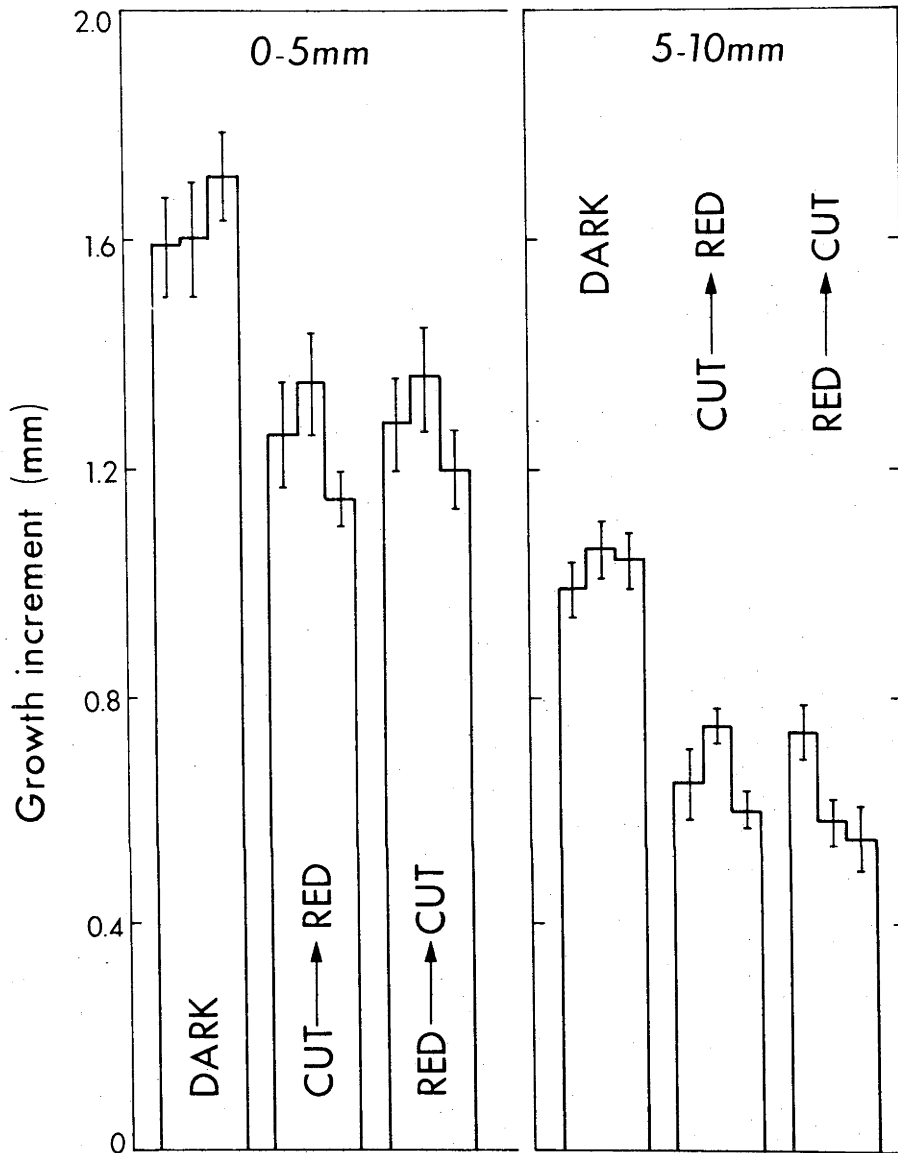


Fig. 4. Zonal mesocotyl elongation in seedlings with coleoptiles removed: irradiation before or after cutting. Removal of coleoptiles and marking of mesocotyls were carried out as for Fig. 3. Immediately before or after these procedures the seedlings were irradiated with red light (18 mWs/cm²). For each treatment, 20-24 seedlings were used. Results, mean increment in zone length following 8 h incubation (from the time of marking) of three replicates, are presented with SE.

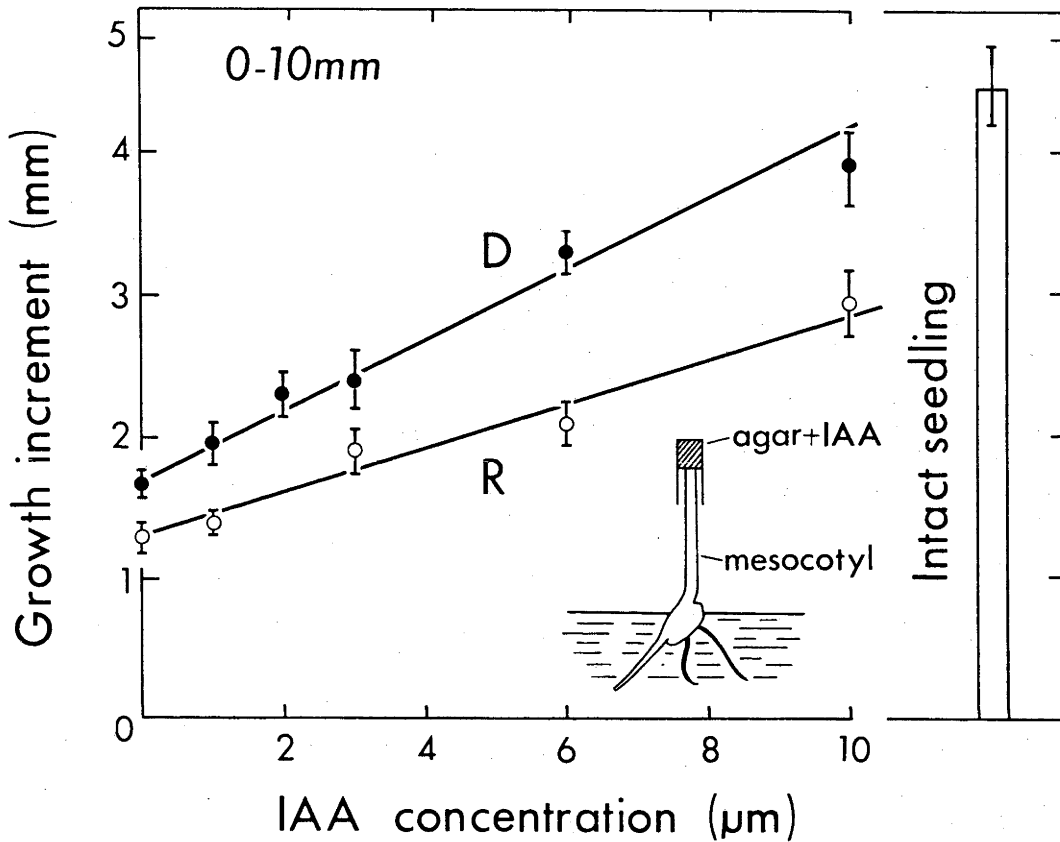


Fig. 5. IAA-induced zonal mesocotyl elongation: effect of red light. Seedlings selected for uniformity on the second day of sowing were grown subsequently on 1% plain agar. The coleoptiles were removed from the seedlings as for Fig. 3, and the mesocotyls each marked at 10 mm from the cut end. Agar (1.5%, 50 μl) contained in a tube (i.d. 3 mm), which had been equilibrated in a given concentration of IAA solution for a day, was then placed on the cut surface of the mesocotyl. For each treatment, 10 seedlings were used. Mean increment in zone length following 4 h incubation was obtained. R: seedlings were irradiated with red light (18 mWs/cm²) just before removal of the coleoptiles. D: dark control. Vertical bars indicate SE.

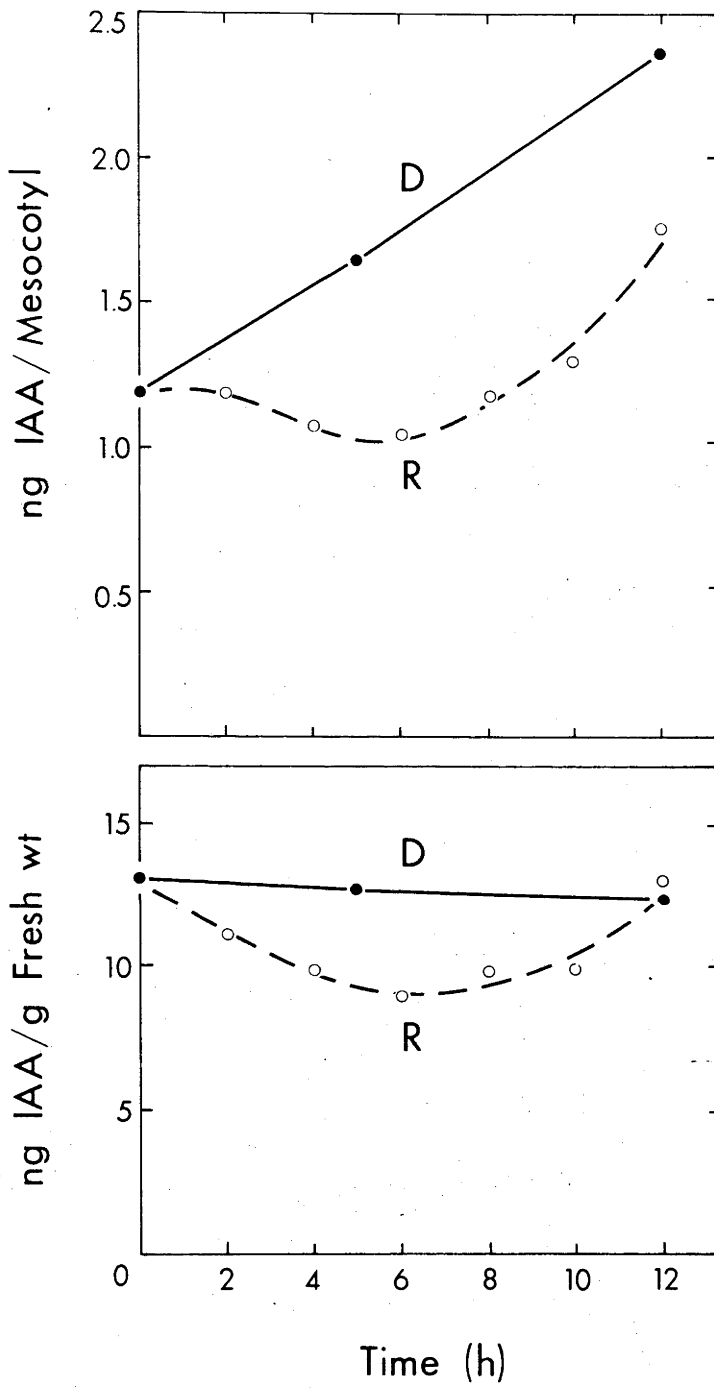


Fig. 6. Effect of red light on IAA content of the whole mesocotyl. Intact seedlings were irradiated with red light (18 mWs/cm^2). Following incubation for a given period, mesocotyls (30 for each estimate) were excised and their free IAA content estimated. R: red irradiated. D: dark control.

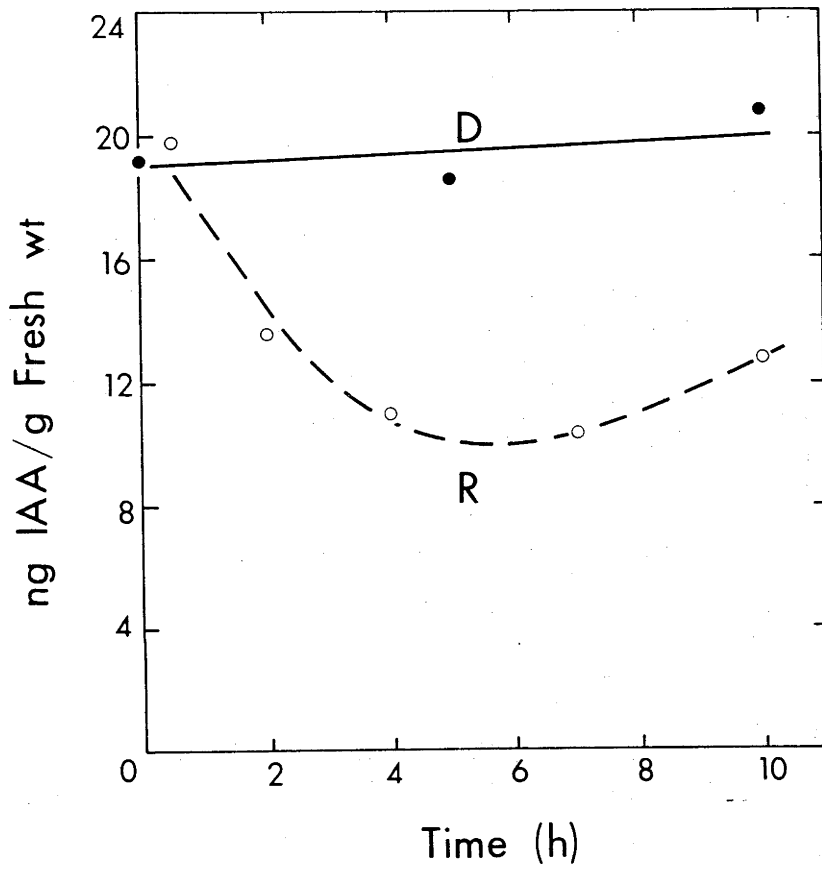


Fig. 7. Effect of red light on IAA content of apical portion of the mesocotyl. Experimental treatments as for Fig. 6, but only the apical 10 mm-portion of the mesocotyl was subjected to estimation of free IAA content. For each estimate, 30 mesocotyls were used.

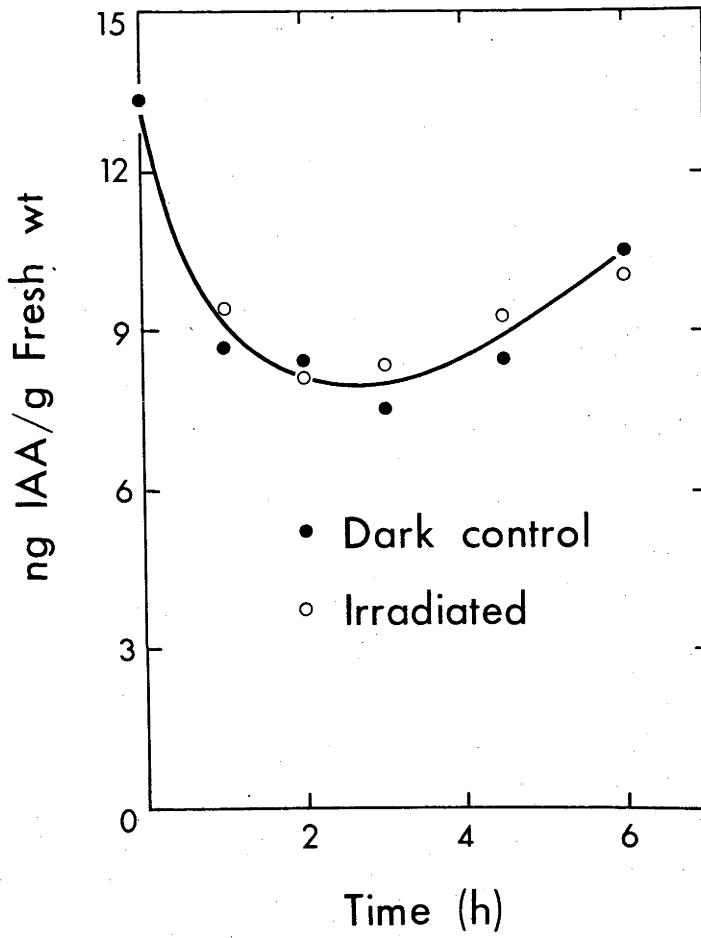


Fig. 8. IAA content of the mesocotyl of seedlings irradiated with red light (18 mWs/cm²) immediately following removal (time 0) of coleoptiles. For each treatment, 30 mesocotyls were used.

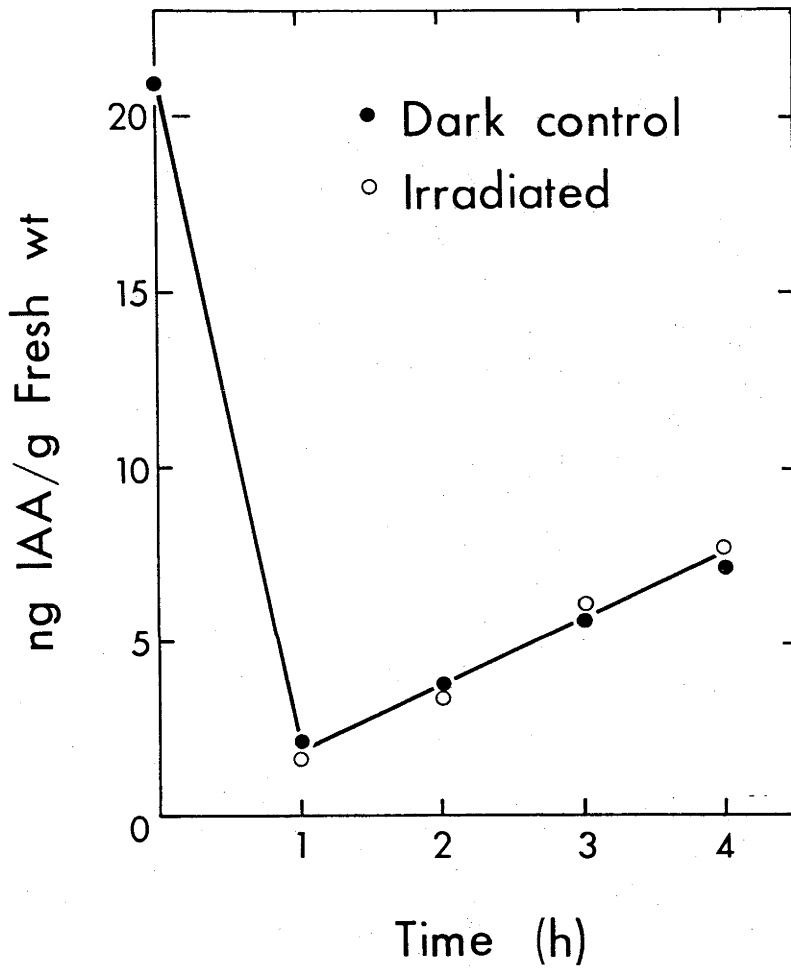


Fig. 9. IAA content of apical portion of the mesocotyl of seedlings irradiated with red light, immediately following removal (time 0) of coleoptiles. After a given period following irradiation (18 mWs/cm^2), the IAA content of the apical 5 mm portion was estimated. For each treatment, 30 mesocotyls were used.

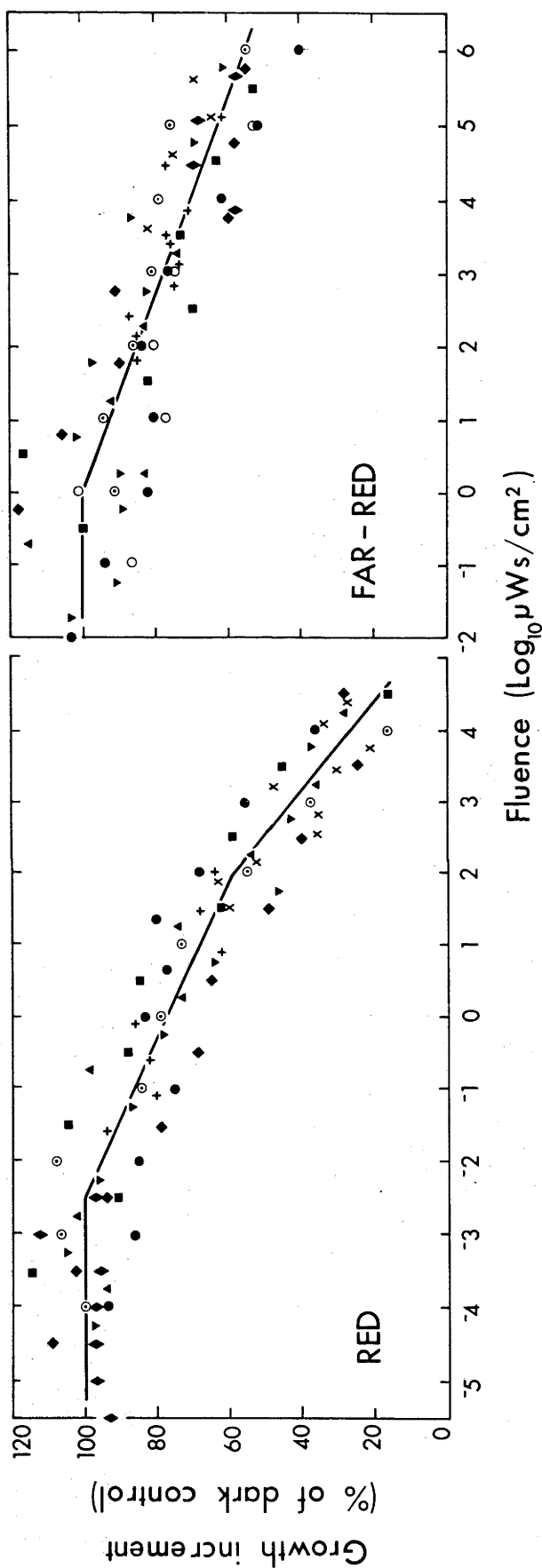


Fig. 10. Fluence-response relationships for mesocotyl elongation in intact seedlings. Seedlings were irradiated with red or far-red light of various fluences. The highest fluence rates used were 30 (red) and 470 (far-red) $\mu\text{W}/\text{cm}^2$. Fluences lower than 3.2×10^3 (red) and 5.7×10^4 (far-red) $\mu\text{Ws}/\text{cm}^2$ were given within 2 min. Increment in mesocotyl length was measured following 8 h incubation. Each point is the mean of 32 to 36 seedlings. Different symbols indicate the results of separate experiments. Red ($-2 \leq x \leq 2$): Slope = -8.9, $r = -0.796$, $n = 35$; Red ($2 \leq x \leq 4$): Slope = -16.4, $r = -0.787$, $n = 21$; Far-red ($0 \leq x$): Slope = -6.9, $r = -0.828$, $n = 60$.

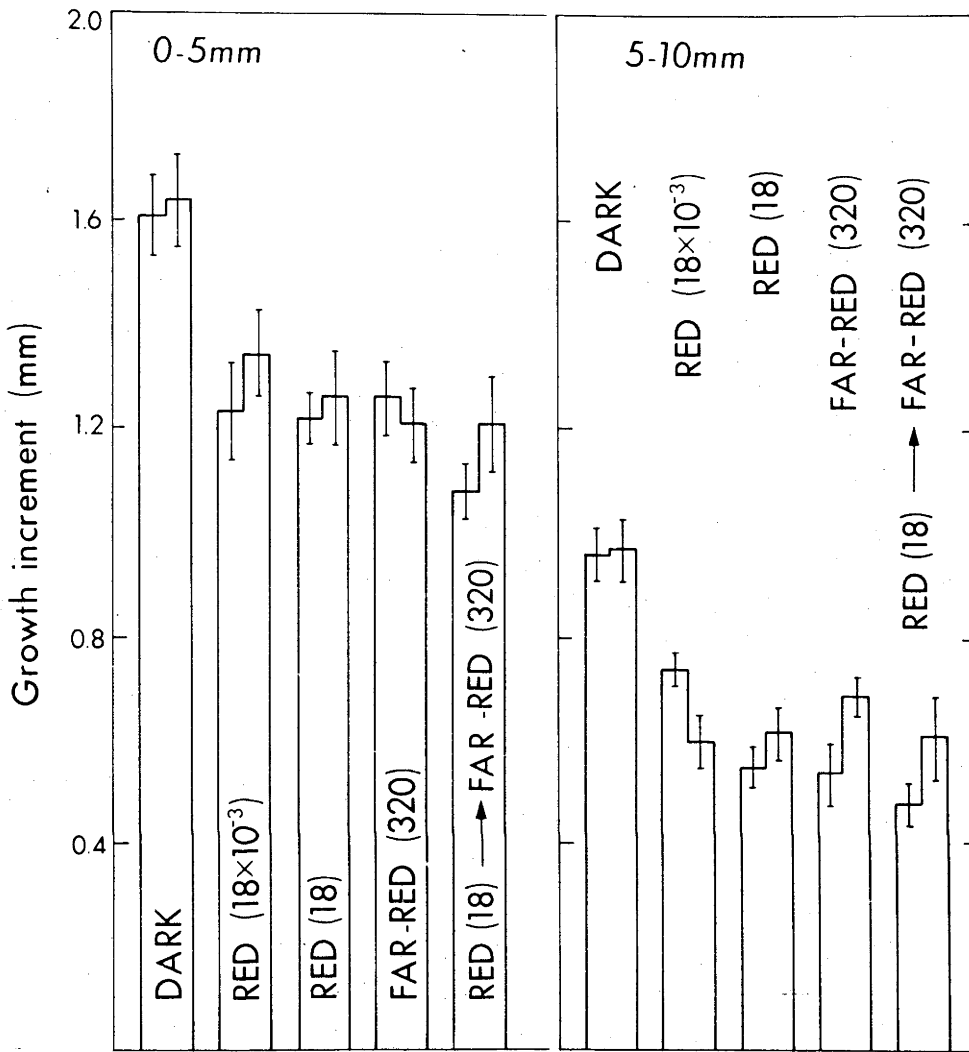


Fig. 11. Effects of red and far-red light on mesocotyl elongation in seedlings with coleoptiles removed. Following removal of coleoptiles and marking of mesocotyls (see Fig. 3), the seedlings were irradiated with red light (18×10^{-3} mWs/cm² in 70 sec, or 18 mWs/cm² in 10 min), or far-red light (320 mWs/cm² in 12 min), or far-red of the same fluence immediately following red irradiation (18mWs/cm²). For each treatment, 20 to 24 seedlings were used. Results, mean increment in zone length following 8 h incubation of two replicates, are presented with SE.

CHAPTER 8

GENERAL DISCUSSION

1. Technical Problems

Prior to this investigation of the physiological roles of IAA and light in the growth of maize seedling shoots, two major technical problems required attention: the development of a method of handling dark-grown seedlings in safelights which induce no growth responses (i.e. are absolutely safe), and improvements to procedures of extracting and quantifying endogenous IAA from small samples of tissue such as may conveniently be irradiated with monochromatic light or subjected to other physiological treatments over a short duration.

Safelight. In the earliest studies on hormonal physiology and photomorphogenesis of dark grown seedlings, phototropically inactive orange or red light was used as a supposed "safelight". Following the discovery of red-sensitive growth responses such as the inhibition of mesocotyl growth and later, the red/far-red reversible responses mediated by phytochrome, green safelights were introduced on the grounds that absorption by that pigment was minimum in the green. Despite their widespread use, green safelights have long been suspect since it became recognized that even at fluences merely conferring visibility, green radiation could induce growth responses (briefly summarized in the Introduction to Chapter 2). Over the last two decades reports have accumulated demonstrating green light effects on plant and animal cell growth (Klein 1979). However, the green-specific effects on plant cell

growth require high fluence and are apparently not induced by conventional dim green safelights. Highly sensitive responses of etiolated seedlings to green light can be attributed to extremely red-sensitive photomorphogenetic systems which react also to relatively low fluences of green light (Blaauw et al 1968, Mandoli and Briggs 1981).

The work of Goodwin (1941) and Mer (1954) suggested the use of invisible infra-red (IR) radiation, in which objects can be visualized by the use of an IR-scope, as an alternative to visible safelights. Commercially-available monocular IR-scopes are adequate for visualising dark-grown plants in IR radiation which appears not to affect their growth (Chapter 2). The technique is inconvenient but nevertheless proves its worth by making it possible to excise plant parts and mark growth zones without activating the very sensitive light responses. A binocular IR-scope would be advantageous in improving stereoscopic imaging and reducing fatigue, and eventual improvements to the sensitivity of the detectors may even do away with the necessity for a source of IR radiation other than the background heat of the room.

Estimation of endogenous IAA. A variety of new physicochemical methods for quantitative determination of IAA has been introduced in the last decade, replacing older bioassay methods. Of the new methods, GC-MS, especially its selected ion monitoring variant, is the most highly developed and reliable (e.g. Magnus et al 1980). With adequate internal standards, it is at present the least

equivocal method for quantification of IAA. Sensitive and specific IAA determination procedures based on high performance liquid chromatography (Sweetser and Swartzfager 1978) high performance GC (Hofinger 1980, Crozier et al 1980) or GC using a nitrogen-phosphorus detector (Martin et al 1980) have recently been proposed. The use of radioimmunoassay in plant hormones has begun (e.g. Pengelly and Meins 1977, Weiler and Ziegler 1981). The method still suffers from problems arising from the degree of specificity and batch variation of antibodies. The technique is likely to be advanced greatly by the introduction of monoclonal antibodies with unique specificities and greater uniformity, and would then be most useful for routine work.

The indolo- α -pyrone fluorescence method, first introduced by Stoessl and Venis in 1970, has been improved by various workers (references in Chapter 3, also Mousdale 1980). Following the lead of Kamisaka and Larsen (1980), further improvements are described in Chapter 3, which include the use of the antioxidant, BHT, and the introduction of polyamide TLC which allows the separation from IAA of compounds likely to interfere with the fluorescence method. The reliability of the improved method was checked in parallel determinations of endogenous IAA extracts using GC-MS in the selected ion monitoring mode (Chapter 4). The fluorometric assay is particularly useful as a routine procedure for IAA determination in physiological experiments with small amounts of tissue owing to its high sensitivity and specificity and its relative simplicity and speed.

IAA is particularly susceptible to decomposition during extraction and purification and this must be minimized. Isotopically-labelled IAA is useful as an internal standard to correct for losses during extraction, but some caution is due since the labelled decomposition products may not be totally separated from IAA itself (McDougall and Hillman 1978). Large and irregular losses of IAA during extraction and purification make it difficult to design experiments with relatively small samples of tissue. The spontaneous conversion of IPyA to IAA during extraction (Atsumi et al 1976, 1979) must also be minimized. The improvements and precautions described in Chapter 3, including the use of polyamide TLC, various treatments to solvents and working in low light, all help to minimize or avoid the problems described above. The improvements will also be useful whatever assay method is used.

2. IAA Status in Cereal Seedling Shoots.

Site and mode of IAA production. The tip of the coleoptile is a source of IAA supply for the maize seedling shoot (Chapter 4). The pool of IAA in the tip is kept very small: as rapidly as IAA forms, it is released from the tip. IAA is not merely liberated from conjugates but is produced by active synthesis from precursors (see Discussion in Chapter 4). Excised oat and maize coleoptiles are able to convert applied tryptophan to IAA (references in Chapter 5). Tryptophan is probably a primary precursor of IAA biosynthesis. This view was especially supported in

experiments which showed that red light not only caused a reduction in diffusible IAA yield from the coleoptile tip but affected in the same way the biosynthesis of IAA from labelled tryptophan (Chapter 5).

According to the generally accepted scheme of IAA synthesis from tryptophan in higher plants (see Schneider and Wightmann 1978), the immediate precursor is indole acetaldehyde which is produced from tryptophan via either indolepyruvic acid or tryptamine. Applied tryptophan had little effect on growth of the coleoptile but tryptamine stimulated it considerably (Winter 1966, Thimann and Grochowska 1968). Tryptamine may be the major intermediate between tryptophan and indole acetaldehyde in the coleoptile, and the concentration of endogenous tryptophan may be saturating for tryptamine synthesis. However, contradictory results have been reported by Muir and Chang (1974), who showed that diffusible IAA yield from the coleoptile tip is enhanced by applied tryptophan but not by tryptamine. Effects of applied tryptophan and other possible intermediates on IAA production in, and growth of, the coleoptile should be investigated in more detail.

The site of IAA production is not restricted to the coleoptile tip, and other parts of the coleoptile unit (which includes primary leaves and the node) can produce IAA (Chapter 6). The mode of IAA production in parts other than coleoptile tip has not been studied, but may be expected to be similar to that in the coleoptile tip. IAA production in the mesocotyl of intact seedlings is not evident. At least the apical growing portion of the mesocotyl appears to

produce little IAA and to depend almost entirely on the coleoptile unit as its source of IAA (Chapter 6).

The maize coleoptile tip releases diffusible IAA for at least several hours after excision, at a rate which is constant for the first few hours. IAA is not merely released slowly and steadily from a large pool of IAA or conjugated IAA in the tip (see above). Prolonged IAA release from the excised coleoptile tip must be due to IAA biosynthesis. Thus, the results suggest that the maize coleoptile tip contains sufficient metabolites for IAA biosynthesis to enable it to maintain a steady production for a few hours and to continue production over a considerable period. Van Overbeek (1941) also reported long-lasting diffusion of auxin from excised coleoptile tips of maize as well as of oat seedlings.

Removal of the seed of an oat seedling was found to reduce the diffusible auxin yield from the coleoptile tip, and to inhibit growth of the coleoptile (Skoog 1937). Since auxin was not obtained from the cut surface of seedlings from which the coleoptile had been removed, Skoog concluded that the seed supplies specific auxin precursors to the coleoptile tip. However, the effect of removing the seed is immediate: a reduction in diffusible IAA yield was apparent within 4 h and the extrapolated time course suggests an instantaneous effect. Taking into account the considerable amount of metabolites for IAA production in the tip (see above) and also the probable time required for the transport of hypothetical precursor(s) from the seed to the coleoptile tip, the fall in IAA production is too sudden to be

accounted for in terms of removal of a supply of precursor(s). The explanation of the rapid effect of deseeding may lie in effects on water relations. Light effects arising from the safelights used (the type of which is not specified) also cannot be excluded. The experiments need repetition under highly controlled conditions.

Translocation and degradation of IAA. Transport of endogenous IAA in the shoot is basipetal, and acropetal transport is unlikely. Three points can be raised on this account: (1) IAA released from the coleoptile tip is produced there, and is not dependent on IAA which might be supplied from the parts below the tip (see above); (2) IAA in the apical region of the mesocotyl originates in the coleoptile unit, not in the parts below it (Chapter 6); (3) removal of the seed and the roots from the seedling does not, at least for several hours, reduce the IAA content of the mesocotyl (Chapter 6). Probably IAA produced in the coleoptile unit is translocated only basipetally from the sites of production. Recent reinvestigation of the polarity of IAA transport in the root, including that of maize (Wilkins and Scott 1968, Scott and Wilkins 1968, Elliott 1977), has shown that it is acropetal, i.e. from base to tip of the root (see Schneider and Wightmann 1978). IAA translocated downwards in the shoot may eventually enter the root and be transported toward its tip.

Some of the IAA produced in the coleoptile tip appears to be lost during its downward transport in the coleoptile (Chapter 4), possibly due to conjugation or decomposition.

It was concluded that decomposition is the major process, since increase in the conjugated IAA content of the coleoptile is too small to account for the loss of IAA (see Discussion to Chapter 4).

IAA obtained by diffusion reflects, perhaps, the fraction of IAA which is transportable in the tissue. Comparison of solvent extractable IAA (here it is called "free IAA") and diffusible IAA suggests that at least in some tissues free IAA is mostly transportable. A 3 mm-long coleoptile tip contains 0.2 ng IAA, a very small amount compared to that obtainable by diffusion from the same tissue (1 ng/h). This suggests that IAA is produced at the tip in a transportable form. Nearly all of the free IAA of the apical portion of the mesocotyl disappears rapidly following removal of the coleoptile (Chapter 6). Thus, it appears that most of the free IAA in this part of the mesocotyl is transportable. In contrast, the free IAA in the more basal region of the mesocotyl may not be transported as rapidly as that in the apical tissue. This follows from the smaller effect of removal of the coleoptile on the free IAA content of whole mesocotyl (Chapter 6). Polarity of IAA transport may weaken in tissue composed of already elongated cells. It would be interesting to re-investigate the capacity for polar IAA transport in relation to the growth capacity of tissue.

Effect of light on IAA status. Red light has been shown not to affect transport or conjugation of IAA, but to inhibit its biosynthesis (Chapter 5). Further evidence for this conclusion is that IAA biosynthesis from applied ^3H -tryptophan by isolated coleoptile tips is inhibited by pretreatment of seedlings with red light (Chapter 5). Although stimulation or induction of IAA decomposition cannot be totally ignored as a possibility, it is considered improbable (see Chapter 5 for discussion). Further studies should be made to characterize the metabolic step inhibited by red light. The mode of action is also not known. Two major possibilities can be raised: (1) inhibition of enzyme action, and (2) inhibition of precursor supply to the IAA biosynthetic site in cells. The effect on IAA metabolism is almost instantaneous and mechanisms involving gene expression are not likely.

Photoreversible phytochrome is at least partially involved in the inhibition of IAA production by red light, and the Pfr level of spectrophotometrically detectable bulk phytochrome reflects the extent of the effect (Chapter 5). Relationships between the phytochrome system and IAA metabolism would form a project worthy of further research. The photoinhibition of IAA production, however, cannot be explained solely in terms of phytochrome action (Chapter 5). The nature of the photoinhibitory system which has its threshold at very low fluences of red light and reacts also to far-red light, needs to be investigated, not least in terms of its action spectrum.

In contrast to the red light effect on IAA biosynthesis, blue light has been shown to inhibit the transport of applied IAA (see Chapter 1). This blue light effect has still to be shown on the transport of endogenous IAA. Measurements of diffusible and solvent extractable IAA might provide information required to elucidate the action of blue light. If transport of endogenous IAA is inhibited by blue light, different regions of the spectrum affect the status of endogenous IAA through two distinct mechanisms, i.e. inhibition of production and transport.

3. Red Light and Cereal Seedling Growth.

Growth of the coleoptile. Red light irradiation of intact etiolated seedlings of maize and oat stimulates elongation of their coleoptiles (Blauuw et al 1968, Duke et al 1977, Mandoli and Briggs 1978). This stimulation is more apparent in the apical portion of the coleoptile than in its basal, more rapidly-elongating zone (Huisinga 1967, Chapter 5). Stimulation of maize coleoptile elongation is saturated with fluences insufficient to induce any detectable Pfr formation and far-red light also saturates the response (Chapter 5). This very red-sensitive response has also been shown with oat seedlings (Blauuw et al 1968, Mandoli and Briggs 1981), although photoreversible phytochrome control was also evident at higher fluences.

The stimulatory action of red light on coleoptile elongation is not explicable in terms of IAA level, which is

reduced by red irradiation (Chapter 5). If IAA is used up in growth, the decrease in the amount of IAA in the coleoptile could be due to red light enhancement of IAA action in growth. Although such a possibility is not to be totally rejected, it lacks convincing evidence. Recent work on acid-induced cell elongation provides information which might account for the enhancement of coleoptile elongation by red light. It was shown that red light stimulates proton excretion in oat coleoptile segments which include the tip (Pike and Richardson 1977). Acidification of the cell wall is known to promote cell elongation by loosening of the complexes of the cell wall (see Tepfer and Cleland 1979). It is thus possible that elongation of coleoptile cells is stimulated following proton excretion induced by red light.

Uptake of potassium ions has also been shown to be stimulated by red light (Pike and Richardson 1979). Excretion of protons and uptake of potassium ions are likely, at least in part, to be mediated by the antiport carrier system (Haschke and Lüttge 1973, 1975, Marre et al 1974, Cleland 1976). This suggests that the intercellular content of potassium ions could be rate-limiting for proton excretion under certain conditions. It is possible that lack of photoreversible phytochrome control with maize (see above) is due to insufficient availability of intercellular potassium ions, i.e. uptake of the ions present is already maximally stimulated by red light at fluences insufficient to induce photoreversible phytochrome action.

The observed extent of red light effects on proton and potassium fluxes is small (Pike and Richardson 1977, 1979). However, since the experiments were carried out using green safelights, the actual red light effects could well be greater. It is of interest to reinvestigate this red light effect using safe IR radiation and an IR-scope. Detailed investigation of the fluence-response relationships of proton excretion (in the presence or absence of added potassium) and on potassium uptake might provide valuable information in the control of coleoptile growth by light.

Growth of the mesocotyl. The supply of IAA from the coleoptile unit to the mesocotyl is inhibited by red light (Chapters 5). This is perhaps a major mechanism involved in the inhibition of mesocotyl elongation by red light (Chapter 7), but it is not the whole explanation. One apparent effect of red light, which does not involve control of IAA level, is that exerted directly on the mesocotyl (Chapter 7). This effect also may depend on the proton-potassium exchange mechanism. Work using bean hypocotyl sections showed that the effect of red light on the uptake of potassium ions can be stimulatory or inhibitory according to their morphological position within the hypocotyl (Brownlee and Kendrick 1977). The effect of red light on proton excretion in the mesocotyl may be opposite to that in apical coleoptile segments. Inhibition of cell division by red light (Chapter 7), a topic which clearly requires further study, may also be independent of auxin level.

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