PHYTOCHROME REGULATION OF PEROXIDASE ACTIVITY IN *ŻEA MAYS*

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CONTENTS

PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full for any other degree or diploma of any university.

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ABBREVIATIONS

ABA - Abscisic acid

ACH - Acetylcholine chloride

ACT-D - Actinomycin-D

AMO-1618 - 2-Isopropyl-4-dimethylamino-5-methylphenylpiperidine carboxylate methylchloride

ANT-A - Antimycin-A

AT - Atropine

ATP - Adenosine triphosphate

c-AMP - Adenosine 3',5'-(hydrogen phosphate), Na_2-sodium salt, DB_2 - N602 - dibutryl salt.

CAP - D-threo Chloramphenicol

CCC - 2-chloroethyl trimethylammonium chloride

CCH - Chlorocholine chloride

CHI - Cycloheximide

Chl - Chlorophyll

D - Dark

DCMU - 3-(3,4-Dichlorophenyl)-1, 1-dimethylurea

DCPIP - 2,6 dichlorophenol indophenol

DNA - Deoxyribonucleic acid

DNP - 2,4-Dinitrophenol

EDTA - Ethylenediaminetetracetic acid tetrasodium salt

ES - Eserine sulphate

FR -Far-red light

FW - Fresh weight

GA - Gibberellic acid

GLU - L-Glutamine

GTP - Guanosine triphosphate

HIR - High irradiance reaction

h or hr - hour

IAA - Indole 3-acetic acid

KN - Kinetin, 6-furfurylaminopurine

M - Molarity

MAK - Methylated abumin on kieselgur

NAD - Nicotinamide adenine dinucleotide

NADP - Nicotinamide adenine dinucleotide phosphate

P - mg protein basis

PAL - Pherylalanine ammonia lyase

PCA - Perchloric acid

Pfr - Far-red absorbing form of of phytochrome, phy, physiologically active form

Pfr^{*} - Excited state of Pfr form

POPOP - 1,4-bis 2-5-(Phenoxyoxazolyl) benzene

PP - Photophosphorylation

PPO - 2,5-Diphenyloxazole

Pr - Red absorbing form of phytochrome

PRB-8 - α-Chloro-β-(3chloro-o-totyl) propionitrile

PSI - Photosystem-I

PSII - Photosystem-II

Ptot - Total phytochrome (Pr + Pfr)

PUR - Puromycin dehydrochloride

PVP - Insoluble polyvinylpolypyrrolidone

R - Red light

RA - Relative activity

RNA - Ribonucleic acid, r-ribosomal, t-transfer and m-messenger

TCA - Trichloroacetic acid

TH - Theophylline

U - Units/mg protein

UV - Ultra violet

WL - White light

INTRODUCTION

While the development of an organism is programmed by its genetic information and on its environment, plants are particularly sensitive to environmental factor of light. The foremost importance of light as the ultimate source of energy for living organisms is well recognised. Green plants besides harbouring the photosynthetic process, have evolved mechanisms dependent on seasonal variations in duration of daylength, and alternation of light and dark period as a time signal for onset of various morphogenetic events such as germination, dormancy, reproduction and synchronization of metabolic activity. All light signals for such developmental processes are percieved by a pigment, termed phytochrome. The molecular mechanism by which Pfr (active form of phytochrome) translates the physical stimuli into physiological function or into a morphological event is still unresolved.

Since the development of a plant is primarily the consequence of orderly changes in the enzyme complement, the studies on Pfr control of enzymatic machinery may lead to an answer to the understanding of photomorphogenesis and with this view Pfr regulation of many enzymes have been investigated (see Schopfer, 1977). However, in most of cases, work has been carried out only with a cause and effect relationship, only in case of phenylalanine ammonia-lyase intensive research was done to decipher the control at the molecular level.

The objective in the present investigation was to study the mechanism of action of phytochrome in controlling the activity of the enzyme peroxidase. The work was carried out to decipher the level of control by phytochrome and possible intermediate factors whose level or modulation could affect peroxidase activity. The investigation of regulation of peroxidase activity can also shed light on how phytochrome may participate in photomorphogenetic events by regulating an enzyme which has been shown to be associated with differentiation processes by controlling hormonal level in plants.

PREVIOUS WORK

Brief History

The importance of duration of daylength as clocksetter for morphogenetic events was first recognised by Garner and Allard in 1920 by their elegantly designed experiments discovering the phenomenon of photoperiodism. Their work was followed by a group led by Borthwick and Hendricks at Beltsville, U.S.A. These workers studied the effect of monochromatic light on flowering and compared action spectras for many plants (see Borthwick, 1972). These action spectras were almost identical showing maximum effect for induction in red region between 600 and 700 nm. The action spectra for other light dependent growth responses, like lettuce seed germination and inhibition of stem growth were also found to be essentially similar to those for flowering. Beltsville group repeated an earlier discovery made by Flint and McAlister (1935) where they found that light dependent germination of lettuce seed was promoted by red light and inhibited by far-red light. They confirmed their results and, in addition found that, on alternately exposing the seeds with red and far-red light, the physiological response always depended on the last exposure given (Borthwick et al., 1952). This red/far-red reversibility was subsequently found for flowering and for many other photoresponses.

The similarities in action spectra for different photoresponses and their reversibility by red and far-red light

indicated existence of a common photoreceptor pigment, subsequently named as phytochrome. The discovery of various biochemical and physiological responses triggered by phytochrome is much more recent. The last two decades have witnessed an elaborate study of molecular properties and function of this pigment, but still its mode of action is debatable and controversial.

Localization and Molecular Properties

The *in vivo* detection of phytochrome was done by Butler *et al.* (1959) in a sample of etiolated maize seedlings by detecting absorption characteristics at 660 and 730 nm. It is distributed ubiquitously in the green plants. The maximum concentration of phytochrome usually occurs in meristematic tissues e.g. coleoptile apex, root tips and at nodes, while its amount gradually decreases along the axis (Pratt and Coleman, 1974; Briggs and Siegelman, 1965; Corell *et al.*, 1968). At the subcellular level, it has been detected in plasmalemma (Haupt, 1970; Marme *et al.*, 1974), nucleus (Galston, 1968), plastids (Cooke *et al.*, 1975; Evans and Smith, 1976), mitochondria (Manabe and Furuya, 1974) and endoplasmic reticulum (Williamson *et al.*, 1975). Coleman and Pratt (1974) studied phytochrome localization by immunocytochemistry and electron microscopy. In their study phytochrome is found to be uniformly distributed throughout the cytoplasm and associated with both amyloplasts and mitochondria but not inside nuclei. The electron micrographs

also suggest for a possible association of phytochrome with membranes such as plasma membrane, endoplasmic reticulum and nuclear membrane.

Phytochrome has been extracted and purified from a number of species by several groups (see Briggs and Rice, 1972). It is a biliprotein, which exists in two spectrally different forms, the Pr form absorbs light maximally in red region at 660 nm, and the Pfr form absorbs light maximally in the far-red region at 730 nm. These two forms are interconvertible by absorbing light of appropiate wavelengths, which may be represented as follows:

$$\text{Pr or } P_{660} \underset{730\ \text{nm}}{\overset{660\ \text{nm}}{\rightleftharpoons}} \text{Pfr or } P_{730}$$

Out of these two, Pfr is regarded as physiologically active form of phytochrome. Phytochrome has a molecular weight of approximately 2,40,000 *in vivo*, however, during purification high molecular weight species is generally broken down by a endogenous protease to a smaller species of molecular weight of 60,000 (Gardner *et al.*, 1971). The protein moiety of phytochrome occurs primarily in β-chain configuration (Anderson *et al.*, 1970) having an open chain tetrapyrrole chromophore (Grombein *et al.*, 1975). The phototransformation of Pr to Pfr and vice versa follows kinetics of first order reaction and occurs via several parallel pathways involving number of

20,000 x g for 30 minutes to obtain plastid and mitochondrial pellets respectively. Supernatant was then centrifuged in an Ultra-centrifuge (Janetzki Vac 601) at 150,000 x g for 1 hour to obtain ribosomal pellet and final supernatant. The pellets were washed twice with isolation buffer and gently resuspended in 1 ml of isolation buffer. Samples from respective fractions were assayed for enzyme activity and total protein.

Enzyme Assay - Peroxidase activity (E.C. 1.11.1.7) was assayed by recorded change in absorbance at 610 nm at 15 second intervals after adding 50 µl of sample supernatant to 5 ml of assay mixture at 25°C. The assay mixture contained benzidine, 2.4×10^{-3} M; hydrogen peroxide, 3×10^{-2}M; acetic acid, 7×10^{-2}M, pH 3.8. Solution of benzidine was prepared in acetic acid according to Scandalios (1964). One unit of enzyme activity was defined as change in absorbance of 0.1 at 610 nm per 15 seconds (1 unit = $0.1A_{610\ nm}$/15 seconds).

Protein Estimation - Protein was estimated after dissolving the trichloroacetic acid (5% final conc.) precipitate in 1 N NaOH by procedure of Lowry et al. (1951). The standard curve was made by using bovine serum albumin fraction V.

Electrophoresis - Isozymes of peroxidase were separated by polyacrylamide gel electrophoresis at 4°C. Samples

(Kendrick and Hillman, 1971). The process of dark reversion follows kinetics of first order reaction and its rate is temperature dependent (Mumford, 1966; Pike and Briggs, 1972). Decay of Pfr, which is independent of light, follows first order kinetics in dicots and zero order kinetics in monocots (Pike and Briggs, 1972; Schafer et al., 1975) and its rate depends on temperature and is sensitive to metabolic inhibitors (Manabe and Furuya, 1971). In certain imbibing seeds, generation of Pfr from Pr form has been detected and named as 'inverse dark reversion' (Boisard and Malcoste, 1970). Such a reaction is implausible on thermodynamical ground, since Pr is more stable form than Pfr. It has been suggested that such a conversion may occur from an intermediate between Pr and Pfr due to its slow transformation to Pfr (Kendrick and Spruit, 1974).

High Irradiance Reaction (HIR)

It has been proposed that phytochrome can control photomorphogenesis by two reactions:

1. The 'low irradiance reaction' of phytochrome is one which is saturated by low energies and an effect induced by brief irradiation with red light can be fully reversed by a subsequent pulse of far-red light. In addition, for short induction times (within minutes), reciprocity (intensity x time = constant) relationship is valid indicating that no cooperative effects are occurring (Mohr, 1972). In this

reaction photoresponse, either increases with Pfr concentration (graded response) or requires a threshold concentration of Pfr (Mohr, 1972). This threshold mechanism has been attributed to a matrix capable of cooperatively changing its property after the ligand Pfr has reached the threshold level (Mohr and Oelze-Karow, 1976).

2. In action spectral studies of many photoresponses with prolonged irradiation period, the maximum action is, however, localised in blue and far-red region of the spectrum and none or very less effect in the red light region e.g. hypocotyl elongation in Lactuca sativa seedlings (Hartmann, 1966). Such a response has been termed as 'high irradiance reaction' and characterized by the magnitude of response being function of irradiance, lack of photoreversibility and reciprocity failures. There has been much controversy over the nature of HIR photoreceptor, particularly for far-red light (λ>700 nm). Far-red mediated HIR has been explained by two hypotheses, a) phytochrome is only photoreceptor involved, b) it arise by an interaction between Pfr and photosynthesis, particularly PSI.

a) Hartmann (1966) proposed that HIR responses are brought about by phytochrome, by maintaining a low but relatively constant level of Pfr, the maximum effect achieved close to 720 nm when Pfr/Ptot equilibrium is about 3%. The Pfr/Ptot equilibrium is a function of wavelength but not of irradiance. Since HIR

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of this complex may be much more than that under 'low irradiance reaction'. Mancinelli and Rabino (1975) studied / effect of cyclic far-red treatments on anthocyanin synthesis of cabbage and mustard seedlings. They observed that under this condition, the magnitude of photoresponse depends on the total dose, and cyclic treatments are as effective as continuous, if total radiation dose is equal and applied over equal period of time. The reciprocity relationship is valid under cyclic far-red treatment. Their model, however, does not explain irradiance dependence of HIR, on the basis of data presently available for phytochrome binding. Recently Rabino et al. (1977) have extended these observations for blue light mediated HIR too, however, failure of reciprocity under continuous far-red irradiation is yet to be explained.

b) The possibilities of a second photoreceptor beside phytochrome in HIR has been raised by Grill and Vince (1970). The possible contribution of photosynthesis to HIR response has been considered by many workers (Downs et al., 1965; Creasy, 1968; Schneider and Stimson, 1971, 1972). The evidences in favour of such participation are mostly based on inhibitor studies. Phytochrome-mediated anthocyanin biosynthesis is inhibited by inhibitors of cyclic photophosphorylation (PP) such as NH_4^+, DNP and antimycin-A (Schneider and Stimson, 1971, 1972; Margulies, 1962).

Inhibition of chlorophyll biosynthesis by streptomycin (Margulies, 1962) and levulinic acid (Giudici de Nicola et al., 1974; Schneider and Stimson, 1972) also reduced photo-induced flavonoid formation. Photo-induced synthesis of anthocyanin (Creasy, 1968; Giudici de Nicola et al., 1972a; Downs et al., 1965) and phenylalanine ammonia-lyase (Zucker, 1969) is effectively inhibited by DCMU. The PSI inhibitor salicylaldoxime has been shown to inhibit light-induced amaranthin formation (Giudici de Nicola et al., 1972a). Further support for photosynthetic participation in HIR is based on operation of cyclic PP under far-red light (Arnon et al., 1967) and development of photosynthesis under continuous FR (Schneider and Stimson, 1971, 1972; Oelze-Karow and Butler, 1971; De Greff et al., 1971).

Evidences for non-involvement of photosynthesis also come from inhibitor studies, as greening inhibitors like chloramphenicol and streptomycin enhance anthocyanin formation (Mancinelli et al., 1974; 1975). The PSII inhibitor DCMU (Amhrein and Zenk, 1971; Mancinelli et al., 1974) and cyclic PP inhibitor NH_4^+ (Eddy and Mapson, 1951) have no effect on photo-induced anthocyanin synthesis. Normal anthocyanin (Bertsch and Mohr, 1965) and betacyanin (Kohler, 1973) synthesis occurred in absence of CO_2. A negative correlation between greening and anthocyanin synthesis in maize seedlings is demonstrated by Duke et al.,

(1976). Furthermore, under FR light chlorophyll synthesis is minimal (Masoner et al., 1972; Mohr, 1972) and anthocyanin synthesis can be correlated to phytochrome level (Drumm et al., 1975).

Membranes - A Possible Site of Primary Action of Phytochrome

Some of the phytochrome controlled responses have a lag period of only 1-5 minutes such as nyctinastic movement of Mimosa leaflets (Fondeville et al., 1966) or attachment of excised mung bean root tips to negatively charged glass surface (Tanada, 1968). To explain such responses, Hendricks and Borthwick (1967) suggested that primary action of phytochrome may take place via some changes in functional properties of membranes. Smith (1970, 1975, 1976a) has proposed a model which envisage that phytochrome is localized in membranes, and acts by regulating transport of some metabolite, perhaps a second messenger, which may subsequently elicit photoresponse (Fig. 1). Haupt and Weisenseel (1976) speculated Pfr may function as Ca^{2+} carrier in membranes.

It has been established by recent studies that one of primary act of phytochrome on phototransformation to Pfr is its association to a particulate fraction (Quail, 1975; Marme, 1977). Using immunocytochemical techniques for localization of phytochrome, Mackenzie et al. (1975) demonstrated that in oat and rice, within 8 minutes of phototransformation to Pfr, most of phytochrome from a homogenous distribution orients into discrete regions of cell. These discrete regions do not appear to be nuclei, plastids or mitochondria.

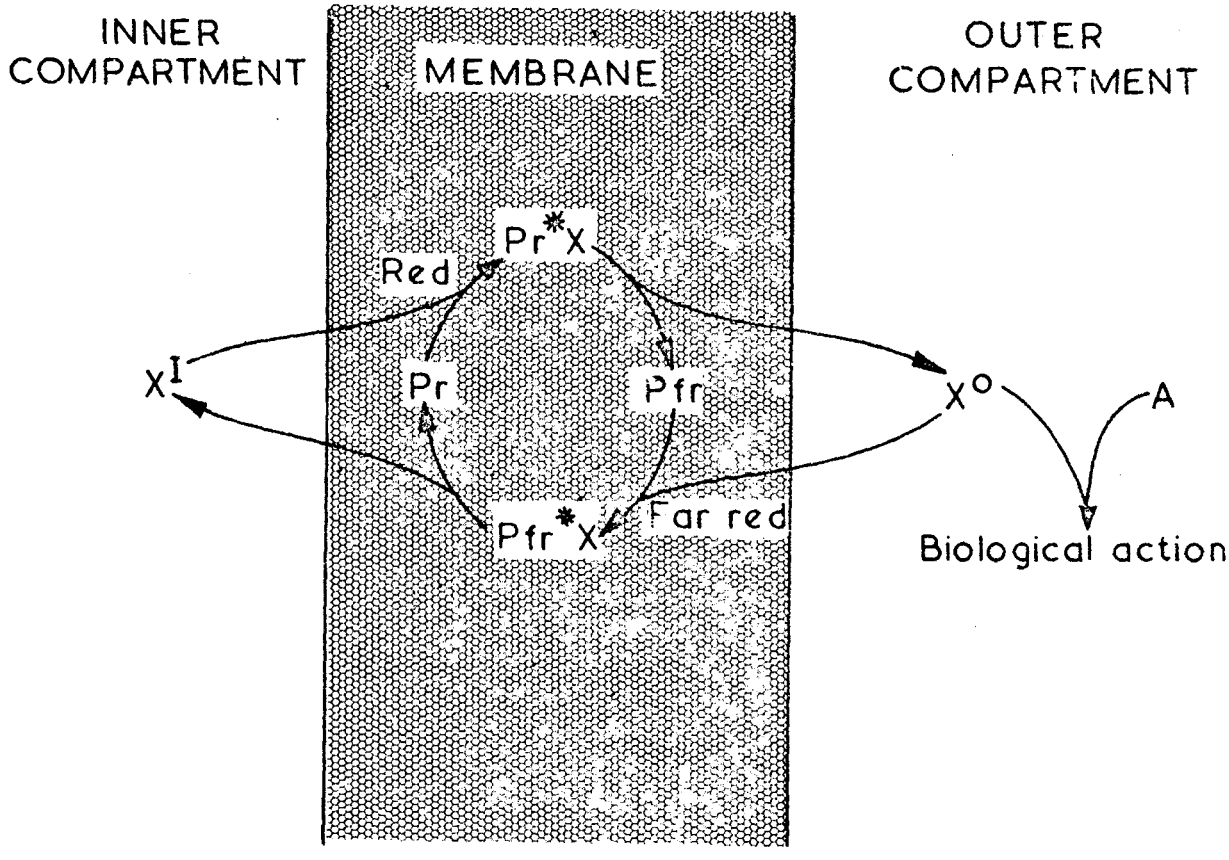


Fig. 1. The transport factor hypothesis for phytochrome action. Active phytochrome is considered to be located in one or more critical membranes in which it acts to control transport of a second messenger, X, from one side to other; X is then able to interact with many processes.

The red irradiation given to intact plants (Quail et al. 1973a; Yu et al., 1976) or to crude homogenate (Marme et al., 1974) enhances phytochrome pelletibility on subsequent centrifugation to a particulate fraction. Such binding of phytochrome to a particulate fractions has been reported for number of species (Pratt and Marme, 1976). It has been argued that irradiation of intact plants leads to phytochrome binding to a membrane fraction (Pratt and Marme, 1976; Marme et al., 1976) or to mitochondria (Manabe and Furuya, 1975), while in vitro irradiation may lead to electrostatic adsorption to degraded ribosomal particles (Quail and Gressel, 1976). It has been speculated that this phytochrome binding represents initial events in the chain of reaction that ultimately lead to photoresponses. Phytochorme interaction to membrane may lead to change in membrane properties, which can be any one of following:

Bioelectrical Potentials - Red/far-red reversible change in bioelectrical potential of order of 1 mV was found by Jaffe (1968) in excised mung bean root tip and this effect occurred within less than a minute of irradiation. Racusen and Etherton (1975) demonstrated that these changes are associated with root cap cells and localised on plasma membranes. Phytochrome mediated bioelectrical potential changes have also been demonstrated in oat coleoptiles (Newman and Briggs, 1972) and in nyctinastic movements of

Samanea pulvini (Racusen and Satter, 1975). The electrical conductance change by red/far-red light is also detected in vitro for black lipid membrane incorporating purified phytochrome (Roux and Yugerabide, 1973).

Ionic fluxes - Some of the phytochrome controlled responses could eventually result from ionic fluxes. The nyctinastic closure of Albizzia julibrissin leaflets is brought through phytochrome-mediated change in volume of motor cell of pulvinule (Satter et al., 1970). During closure of leaflets, subepidermal ventral cells of pulvinule shrink, and dorsal cells swell as a result of K^+ influx in dorsal cell and efflux from ventral cells (Satter et al., 1970). Red light initiates, efflux of H^+ ion (Yunghans and Jaffe, 1972; Pike and Richardson, 1977), and uptake of Na-acetate in mung bean root tips (Jaffe and Thoma, 1973), and ^{14}C sucrose in pea plumules (Goren and Galston, 1966). While red light inhibits uptake of phosphate in Phaseolus aureus hypocotyl (Tezuka and Yamamoto, 1975), and K^+ uptake in pea seedlings (Kohler, 1969).

Energy change - The membrane specific association of phytochrome has suggested that it may act by bringing a change in the energetics of cell. Phytochrome mediated change in level of ATP has been reported for mung bean root tips (Yunghans and Jaffe, 1972), bean buds (White and Pike, 1974), bean hooks (De Greff et al., 1976), Avena

mesocotyl tissue (Sandmeier and Ivart, 1972) and in maize leaves (Michel and Thibault, 1973). In all these cases the effect is very rapid, taking place within 5-15 minutes and the concentration of ATP and ADP return to original values within 30 minutes. There was no change in activities of ATPase in mung bean root tips (Yunghans and Jaffe, 1972). Freiderich and Mohr (1975), however, could not detect any difference in total ATP level in dark-grown and far-red grown mustard seedlings.

The change in level of NADP under the influence of phytochrome was found in Pharbitis nil (Tezuka and Yamamoto, 1969). Furthermore, a partially purified phytochrome preparation had NAD kinase activity which could be modulated in vitro by red/far-red light. Nevertheless, pure phytochrome does not have NAD kinase activity as evident by the fact that these could be separated by calcium phosphate gel chromatography (Tezuka and Yamamoto, 1972, 1974). Manabe and Furuya (1974) have also shown phytochrome mediated reduction of exogenous NADP by isolated mitochondria by changing membrane properties. Frosch et al. (1974) also reported control of nicotinamide nucleotides level by phytochrome, but excluded them to act as a link between phytochrome and photoresponse.

Pfr-mediation through Hormone, Acetylcholine and c-AMP

Hormones - The probability of phytochrome action being carried by hormones has been considered recently (Black and Vlitos, 1972; Kandeler, 1974; Wareing and Thompson, 1976; Evans and Smith, 1976). This view primarily rests on the observations that phytochrome effects in certain cases can be mimicked by exogenous supply of hormones. In addition, many physiological processes, which require endogenous hormones are affected by phytochrome. Phytochrome control of endogenous hormonal level has been reported in many cases. Changes in the concentration of auxin-like substances have been found following red and far-red light treatments (Blaauw-Jansen, 1959; Briggs, 1963; Fletcher and Zalik, 1964). A good correlation has been reported between phytochrome dependent decrease in ethylene production and increased plumular expansion in etiolated pea seedlings (Goeschel et al., 1967). Many workers have suggested that phytochrome control of ethylene biosynthesis can intervene as a regulator in hook opening (Kang and Ray, 1969), elongation of coleoptiles (Ku et al., 1969), carotenoid synthesis and geotropism (Kang and Burg, 1972a,b). Phytochrome control of cytokinins have also been observed in light sensitive seeds of Rumex obtusifolia (van Staden and Wareing, 1972) and in green leaves of Populus X robusta (Hewett and Wareing, 1973). The effect of phytochrome on ABA level could not be detected in pea shoots (Barnes, 1972).

Of different hormones, phytochrome control of endogenous gibberellin level has been given most attention. A rapid surge of gibberellin activity occurs in cereal leaves following a brief red irradiation (Reid et al., 1968; Reeves et al., 1970; Loveys and Wareing, 1971). This increase occurred between 10-20 minutes and is thought to be, either due to release of active GA from bound form (Loveys and Wareing, 1971), or due to de novo synthesis (Reid et al., 1968). Irradiation of homogenates of etiolated barley leaves has similarly been shown to increase GA level and furthermore, such a homogenate converted exogenously added GA_9 to other gibberellins (Reid et al., 1972). Recently, it has been shown that some phytochrome is located in etioplasts, where it rapidly controls an in vitro efflux of GA across the etioplast envelope (Cooke et al., 1975; Evans and Smith, 1976). Taking this fact into account, Evans and Smith (1976) reiterated support to their earlier hypothesis (see Smith, 1970, 1975) that phytochrome is located in intimate association with specific critical membrane over which it exerts a regulatory function perhaps by acting as permease (Fig. 1).

Occasionally phytochrome-controlled responses can be mimicked by exogenous hormones e.g., root tip attachment by IAA and ABA in mung bean and barley (Tanada, 1973a,b). IAA is required for the initiation of measurable response to phytochrome in some systems (Furuya et al., 1969;

Furuya and Torrey, 1964; Tanada, 1968). Similarly, in amaranthin synthesis, phytochrome action depends on supply of exogenous cytokinins (Kohler, 1972). Exogenous GA mimicks the action of phytochrome in lettuce seed germination (Kahn, 1960) and unrolling of cereal leaves (Beevers et al., 1970). Also there are reports of blocking of certain phytochrome controlled responses by application of inhibitors of GA biosynthesis (Black, 1969; Smolenska and Lewak, 1971).

The action of phytochrome and hormones therefore seems to be linked apparently. However, this relationship is purely circumstantial in many cases and it is possible that phytochrome and hormone act as independent factors on the same response (Mohr, 1972; Drumm et al., 1971). Bajracharya et al. (1975) could not find any specific role of ABA in photomorphogenesis. Giudici de Nicola et al., (1972a,b) concluded that in amaranthin synthesis phytochrome and kinetin actions are located at different sites, former acts at the translation level, while the latter at the transcription level. Even the participation of GA could not be detected in phytochrome-mediated amylase production (Drumm et al., 1971) and in lettuce seed germination (Bewley et al., 1968). Kende and Lang (1964) could not detect difference between GA level in red light grown and dark grown, dwarf and tall peas. Schiebe and Lang (1965) brought forward evidence that, in lettuce seed, phytochrome

and gibberellin promotes germination through different mechanisms.

Acetylcholine - Since the phenomena of ion flux and bioelectrical potential in animal nervous systems are known to be mediated by neurohumors such as acetylcholine, similar search for such a role for acetylcholine has also been made in plants. Jaffe (1970) demonstrated phytochrome-mediated concentration change of acetylcholine in mung bean root tips and such change has also been detected in moss callus (Hartmann and Kilbinger, 1974). Acetylcholine mimicked the phenomenon of red light mediated root tip attachment to negatively charged surface, and the red light effect could be effectively blocked by acetylcholine inhibitors (Jaffe, 1970; Jaffe and Thoma, 1973). Jaffe (1970) proposed that acetylcholine may act as local hormone regulating phytochrome-mediated phenomenon at the membrane level.

Jaffe's hypothesis has aroused much scepticism and many other workers could not find any involvement of acetylcholine in other photoresponses, e.g., nyctinastic leaflet movement in Albizzia (Satter et al., 1972), induction of phenylalanine ammonia-lyase (Kasemir and Mohr, 1972), flavonoids level (Saunders and McClure, 1972) and ATP level (Kirshner et al., 1975). Tanada (1972) suggested that acetylcholine may function by its cation interfering capaci-

ties, while Kasemir and Mohr (1972) proposed that acetylcholine action may be restricted to plants or organs in touch with water.

Cyclic AMP - The role of c-AMP as a second messenger for certain hormone mediated responses in animals has evoked a similar search in plants. Phytochrome controlled concentration of c-AMP was detected in mustard (Janistyn and Drumm, 1975). Rast et al. (1973) reported that exogenous c-AMP could substitute for red light mediated betacyanin synthesis and theophylline, a putative inhibitor of c-AMP phosphodiesterase enhanced this effect. Similar photomimitic action of c-AMP was also reported on cell elongation growth (Weintraub and Lawson, 1972). Janystin and Drumm (1975), however, could not find any effect of c-AMP on photoresponses, such as hypocotyl elongation and anthocyanin synthesis in mustard seedlings. Kirshner et al., (1975) and Giudici de Nicola et al. (1975) ruled out the possibility of any participation of c-AMP as a second messenger in phytochrome controlled responses. The very existence of c-AMP in plants is infact doubtful (see Amhrein, 1974).

Phytochrome Control of Development-Mechanism of Enzyme Regulation

Although it is fairly well established that primary action of phytochrome may result after its association to

certain matrix perhaps a membrane, still the knowledge of signal chain to final photomorphogenetic response is far from complete. The action of phytochrome in development has been recently envisaged as a simple trigger molecule, however, initiation of photoresponse would depend on specific state of differentiation of an organism (Mohr, 1972; Schopfer, 1977). This specific state of differentiation is dynamic and changes with time, and has two levels of control:

a) primary differentiation - where genetic information of an organism determines spatial pattern and temporal progress of development independently of environment,

b) secondary differentiation - where external factor may modify developmental process by their action on epigenetic processes.

In essense this concept implies that, primary pattern of differentiation preprogramm the morphogenetic responses in time and space, and phytochrome simply acts as a nonspecific trigger molecule leading to secondary differentiation (Fig. 2).

The spatial and temporal pattern of primary differentiation determine the competence of a cell to respond to Pfr (Competence Pfr). The existance of such spatial pattern of differentiation has been shown at organ level e.g. phytochrome increases peroxidase activity in mustard cotyledon and tap root but causes inhibition in hypocotyl (Schopfer and Plachy.

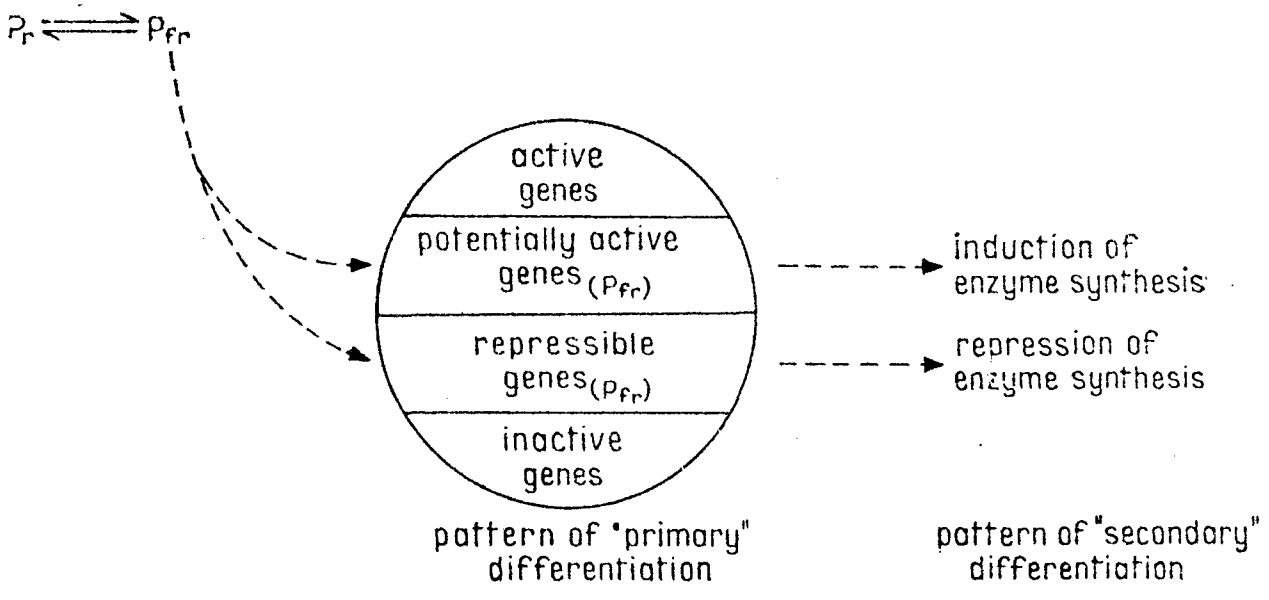


Fig. 2. A general model illustrating the hypothesis of differential gene activation and repression as the mode of action in differential induction and repression of enzyme synthesis by P_{fr}. (After Schopfer, 1977)

1973), on the other hand ascorbate oxidase (Drumm et al., 1972) and amylase (Drumm et al., 1971) can be induced in whole seedlings. The temporal pattern of competence-Pfr has been shown most elegantly in regulation of lipoxygenase activity, where phytochrome mediated repression become/ evident only in between 33.3 to 48 h after sowing (25°C) (Mohr and Oelze-Karow, 1976). This response has been particularly taken as evidence for hypothesis that time course of primary differentiation is independent of Pfr as starting and termination point of photoresponse are fixed, irrespective of irradiance programme. The age dependence of onset of photoresponse has also been shown for anthocyani synthesis in mustard seedlings, however, in this case light signal recieved before onset of competence can be stored, until the starting point of competence is reached (Steinitz et al., 1976).

The mechanism of regulation of enzymes can be decipher by the kinetic analysis of competence, as identical kinetics of competence provides best evidence for coordinate regulati of a group of enzymes, in addition to coincident induction kinetics. The kinetics of enzyme activity in presence and in absence of phytochrome can also provide clue to the mode of action of phytochrome (Schopfer, 1973). Schopfer (1973) distinguished two kinds of control mechanisms regulating enzyme synthesis in presence or after removal of Pfr in syste

viz. photomodulation and photodetermination. In photomodulation, the manifestation of photoresponse intimately depends upon continuous presence of Pfr in the system, whereas in photodetermination the response once triggered by Pfr can continue even in its absence. Schopfer and Plachy (1973) noted kinetic pattern of photodetermination for far-red mediated increase in peroxidase activity in mustard seedlings. In this case Pfr is effective only when it is formed before 96 hours after sowing, however, peroxidase activity increases only after 96 hours of sowing. The formation of Pfr in the cotyledon leads to enzyme synthesis in a period during which competence to respond to Pfr is already lost. The inhibitor and density labelling data has provided evidence that in this case Pfr induces an inactive enzyme, which is however, activated only after 96 hours of sowing (Schopfer, 1977). The kinetics, characteristic of photomodulation, has been observed for many photoresponses e.g. ascorbic acid accumulation (Bienger and Schopfer, 1970), amylase (Drumm et al., 1971), phenylalanine ammonia-lyase (Dittes et al., 1971) etc.

The characteristic differences in induction kinetics and dose response behaviour with respect to Pfr has been observed for Pfr-controlled enzymes in same organ (Mohr, 1972 and even in the same organelle (Bajracharya et al., 1975; Bruning et al., 1975). Phytochrome does not affect activity of all enzymes but specifically enhance or repress the

activity of relatively few enzymes. This specificity and multiplicity in the action of phytochrome has been taken as a proof for hypothesis that phytochrome may have different initial actions in controlling different photoresponses - multiple switch concept (Mohr, 1972). The existance of graded responses where phytochrome action depends on level of Pfr e.g. phenylalanine ammonia-lyase (Schopfer and Mohr, 1972; Mohr, 1972) and threshold response where phytochrome act in all or none fashion e.g. lipoxygenase (Oelze-Karow et al., 1970) points to differences even in quality of primary action of phytochrome (Schopfer, 1977).

While the molecular mechanisms underlying specificity and competence are yet to be deciphered, speculations have been advanced for mechanism of regulation of enzyme (see Schopfer, 1977; Mohr, 1972; Smith, 1976a,b). Though initially it was proposed that phytochrome action should be at transcriptional level (Mohr, 1966, 1972), it is now accepted that Pfr action is probably also at the level of translation, activation, inactivation or degradation of enzyme (Schopfer, 1977; Smith, 1976a,b). Mohr (1966, 1972) proposed, after an elaborate study of various phytochrome controlled photoresponses, that it acts by regulating gene expression in a differential manner i.e. by repressing or derepressing certain genes, thus resulting in either positive or negative photoresponses (Fig. 2). Some positive responses are, increase in the level of anthocyanin (Lange et al., 1971), phenylalanine

ammonia-lyase (Dittes et al., 1971; Schopfer and Mohr, 1972), ascrobate oxidase (Drumm et al., 1972), while a negative photoresponse is repression in the activity of lipoxygenase (Oelze-Karow et al., 1970). This hypothesis is further substantiated by blocking expression of genetic information by inhibitors of nucleic acid and protein synthesis (Carr and Reid, 1966; Mohr, 1966, 1972), which in turn also blocked the manifestation of the photoresponse. The experiments with these inhibitors are only suggestive, but do not exclusively prove, that phytochrome action is localised on genome. Therefore, the effect of phytochrome was also investigated at the level of protein and nucleic acid synthesis.

Although phytochrome control of specific mRNA for enzymes has not been reported, the regulation of RNA level has been repoted for many plants (Koller and Smith, 1972; Weidner and Mohr, 1967; Jaffe, 1969). The characterization of RNA has revealed a specific increase in rRNA of cytoplasm (Thien and Schopfer, 1975; Koller and Smith, 1972) and plastids (Scott et al., 1971; Thien and Schopfer, 1975), and in tRNA (Okoloko et al., 1970). In some cases phytochrome mediated decrease in RNA level, e.g., in hypocotyl of Sinapis alba (Weidner and Mohr, 1967) and pea epicotyl (Koller and Smith, 1972), and in the level of tenaciously bound RNA on MAK column (Okoloko et al., 1970), has been reported. In most of these cases lag before increase in RNA

level is always longer than the photoresponse. In their earlier studies, Dittes and Mohr (1970), who double labelled the RNA of mustard cotyledon for 20 minutes and fractionated on MAK column, could not resolve any difference between dark grown and light treated plants. They concluded that since phytochrome controls a relatively small number of enzymes, the change in the level of mRNA may be very less, whose detection was limited by existing procedures. Recently however, Ragy et al. (1977) and Schroder (1977) have shown light induced increase in the amount of translatable mRNA for phenylalanine ammonia-lyase in reticulocyte cell free system. Although phytochrome enhanced plastid and nuclear RNA polymerases activity in pea buds, yet it also had a longer lag phase than many other photoresponses (Bottomley, 1970).

Red light mediated enhancement in the rate of leucine incorporation in wheat leaves, could be effectively blocked by translation inhibitors (Kang, 1971), suggesting an active involvement of protein synthesis machinery during Pfr-mediated responses. These observations have been supported by experiments of Travis et al. (1974) who isolated ribosomes from red light irradiated leaves and found them to be more active in in vitro incorporation of phenylalanine, demonstrating phytochrome-mediated activation of ribosomes. Similarly, an increase in the level of polysomes in bean leaves has been shown in response to red

light irradiation (Pine and Klein, 1972; Klein and Pine, 1977; Smith, 1976b). Since polysome formation in bean starts before the onset of RNA synthesis and blocking of mRNA synthesis by cordycepin does not affect the formation of polysome, Smith (1976b) proposed that phytochrome may control enzyme synthesis at the translation level. Klein and Pine (1977) however, studied the effect of cycloheximide, actinomycin-D and rifampicin on polysome formation, and found resultant decrease in polysome level. They concluded that RNA synthesis is involved in phytochrome control of polysome formation, on contrary to conclusions of Smith (1976b).

Phytochrome controlled de novo synthesis has been established for ribonuclease (Acton and Schopfer, 1974) and ascorbate oxidase (Attridge, 1974; Acton et al., 1974). Phytochrome mediated increase in phenylalanine ammonia-lyase (PAL), whether represents de novo synthesis or activation from preexisting inactive enzyme molecule has been extensively debated. The activation of enzyme has been inferred from cycloheximide insensitive rise in enzyme activity under light treatments in gherkin hypocotyls (Attridge and Smith, 1975) and radish cotyledons (Blondel et al., 1973; Klein-Eude et al., 1974), and increase in enzyme activity in mustard seedlings on transfer from low (5°C) to high (25°C) temperature

(Attridge and Johnson, 1976). This was substantiated by existence of specific PAL inactivator in gherkin hypocotyl (French and Smith, 1975). The density labelling experiments demonstrated that under light treatment buyoant density of PAL is lesser than dark grown control, favouring concept of activation in mustard (Attridge et al., 1974) and gherkin (Attridge and Smith, 1974). Another group (Acton and Schopfer, 1975; Tong and Schopfer, 1976) has however, pointed out flaws in the techniques of Attridge et al. (1974) on the grounds that no suitable density marker enzyme was used, labelling period was too long for short lived enzyme PAL (3-4 h half-life), use of 100% D_2O increased lag phase to 24 hours under Pfr making tissue unrepresentative to water control and determination of density shift was not supplemented with band width measurement. Using ^{15}N as nitrogen source Wellman and Schopfer (1975) demonstrated Pfr-mediated de novo synthesis of PAL in parsley cell suspension cultures. Tong and Schopfer (1976) employed preirradiated mustard seedlings to reduce the lag in the induction of PAL and demonstrated de novo synthesis on the contrary to results obtained by Attridge et al. (1974).

Peroxidase

Peroxidases (E.C. 1.11.1.7) are enzymes that can utilize hydrogen peroxide to oxidize range of hydrogen donor such as phenolic substance, cytochrome C, nitrite, leuco-dyes, ascorbic acid, indole amino and certain inorganic ions

(Saunders et al., 1964; Burris, 1960). Peroxidase is ubiquitously distributed among higher plants, comprising many electrophoretically distinguishable isozymes (Scandalios, 1974).

Peroxidase activity in cell is usually soluble (Penon et al., 1970; Plesnicar et al., 1967; Liu and Lamport, 1974), but it may also be associated with cell walls and various organelles. The presence of peroxidase in cell wall has been detected histochemically (Nougarede, 1971) and by extraction (Stafford and Bravinder-Bree, 1972; Ridge and Osborne, 1970; Liu and Lamport, 1974). In these studies, isozyme composition of wall bound peroxidases is found to be different from cytoplasm. The presence of peroxidase in membranes has also been reported, e.g., in plasmalemma, tonoplasts, and peroxisomes gave strong reaction (Nougarede, 1971; Penel et al., 1976). This is further evident by the fact that membrane disruption increases peroxidase solubility (De Jong, 1972; Plesnicar et al., 1967). The association of distinct peroxidase isozymes to ribosomes has been reported in wheat (Lanzani and Galante, 1964), lens (Penon et al., 1970) and cabbage (Raa, 1973). It therefore, appears that peroxidase may be associated with many subcellular components and since some of the isozymes are site-specific, it may be suggested that the enzyme carries out different functions at different locations.

The activity of peroxidase in plants has been reported to be regulated by an interplay of hormonal, environmental and genetic factofs (Galston and Davies, 1969). The application of GA to promote growth in the genetic dwarfs brings about reduction in peroxidase activity (McCune and Galston, 1959), while stunting growth of normal plant by GA inhibitors increases peroxidase activity (Gasper and Lacoppe, 1969). In maize there are 10 electrophoretically detectable zones of peroxidase activity variably expressed in tissues throughout the life cycle of plants. Polymorphism of these 10 loci are determined by allellic variations of each of 10 distinct gene loci (Brewbaker and Hasegawa, 1975).

Peroxidases are known to be temporally and spatially determined. It is well documented that peroxidase undergoes dramatic shifts in course of development and differentiation, e.g., increase in activity of peroxidase in senescent pea tissues (Birecka and Galston, 1970), and it is associated with appearance of a new isozyme, which fails to appear in the presence of auxin (Ockerse et al., 1966). Similarly, changes in pattern of peroxidases are reported during rooting of hypocotyl cuttings (Gurumurti and Nanda, 1974) and seed germination (Gasper et al., 1973). Ku et al. (1970) have reported that during tomato ripening three fold increase in peroxidase activity is associated with loss of one and appearance of three new isozymes.

Hormonal regulation of peroxidase activity has been extensively studied. Nearly all hormones and many other compounds have been shown to affect its activity or isozyme pattern. Only selected examples are cited here. In tobacco pith culture, auxin represses 2nd cathodic isozyme but induces a 3rd cathodic isozyme (Galston et al., 1968), while in Pelargonium pith culture initially it inhibits but latter promotes the peroxidase activity (Lavee and Galston, 1968). Similarly, GA inhibits peroxidase activity in dwarf pea internodes (Birecka and Galston, 1970), while in tobacco pith culture it promotes the activity (Galston et al., 1968). Ethylene triggers peroxidase synthesis (Gahagan et al., 1969), and kinetin increases peroxidase activity in lens roots (Gasper and Xhaufflaire, 1967) but represses in tobacco callus (Lee, 1974). A complex interaction between hormones controlling peroxidase activity and isozyme pattern is reported for lentil embryonic axis (Gasper et al., 1973). Besides growth regulators, other compounds may also play a role in regulating peroxidase activity. Dezsi et al. (1970) reported amino acids mediated enhancement in peroxidase activity during leaf infection and senescence, furthermore exogenous amino acids like glutamine glycine, theronine and asparagine enhanced peroxidase activi Similarly, phenolic-compounds also play an important role in determining peroxidase activity by acting as an inhibitor or activator (Russell and Galston, 1968; Kosuage, 1969).

The role of environmental factor, such as light, has not been investigated in detail in regulating peroxidase activity. Light-mediated enhancement in peroxidase activity has been reported in detached maize leaves (Graham et al., 1970). In detached barley leaves both light and kinetin arrested the decline in peroxidase level (Sharma and Biswal, 1976). Red light is found to enhance peroxidase activity in pea seedlings (Anstis et al., 1975). Russell and Galston (1969) speculated that red-light mediated modulation of phenolics level may control the activity of peroxidase. In spinach red/far-red reversible effect on peroxidase activity is shown by Penel and Greppin (1973), DCMU abolish this response, while acetylcholine promote it. The alteration is peroxidase isozyme composition takes place in spinach with shift from continuous light to short day or vice versa (Penel and Greppin, 1975) and this effect can also be evoked by exogenous GA. In Cucurbita pepo instant modulation of peroxidase activity is found in membrane vesicles under the influence of phytochrome (Penel et al., 1976). Light represses some anionic peroxidases in tobacco leaves (De Jong, 1973), while in pea there is no difference in isozymes in light grown and dark grown plants (Siegel and Galston, 1967). In Sinapis alba, peroxidase activity increased in cotyledon under phytochrome influence (Schopfer and Plachy, 1973). Light-mediated increase in peroxidase activity is reported in maize seedlings

(Jain, 1973) which is subsequently shown to be mediated by phytochrome (Sharma, 1974).

The *in vivo* function of peroxidase is a matter of controversy. Peroxidase may play a role in IAA oxidation, since it has been reported that IAA-oxidase and peroxidase are same enzyme by isozymic similarities on polyacrylamide gels (Gove and Hoyle, 1975) and on gel filteration (Derbyshire, 1973; Hoyle, 1972). It may also play a role in healing wounds and resisting infections (Kosuge, 1969), by participating in lignin formation (Herkin and Obst, 1973).

MATERIALS AND METHODS

Fresh seeds of maize (Zea mays var shakti) were obtained every year in July from Indian Agricultural Research Institute, New Delhi, and stored dessicated at 4°C. Seeds were germinated on moist absorbent paper in petri dishes at 27 ± 1°C in complete darkness in BOD incubator. Seedlings were watered once a day with distilled water. A daily screening of petri dishes was done and ungerminated and infected seeds, if any were removed. Seedlings of uniform height and morphology were selected for experiments. The watering and subsequent treatments to seedlings were performed under dim green safe light.

Treatments - Seedlings were selected for uniform height and morphology for experiments. They were transferred to fresh petri dishes supplied with respective solutions or distilled water. All solutions were supplied only to roots.

Light Sources - The green safe light was obtained through 8 layers of green cellophane papers from a 40 watt tungsten lamp or a coolwhite fluorescent tubelight. The intensity of light at the plant level was never more than 1 $\mu W\ cm^{-2}$. Red light was obtained, either from two 100 watt tungsten lamps wrapped with two layers of red cellophane papers (emission maxima, 650 nm), or from a single 300 watt reflector lamp (Westinghouse, U.S.A.) filtered through a CBS-650 (Carolina Biological Supply Company) filter (emission maxima,

650 nm). The intensity of red-light at the height of seedlings was 500 $\mu W\ cm^{-2}$. The light from a 300 watt reflector lamp was filtered through 8 cm of continuously flowing tap water and a CBS-750 filter (emission maxima-750 nm) to obtain far-red light. The intensity of far-red light was 140 $\mu W\ cm^{-2}$ at the plant level. White light of an intensity of 1200 $\mu W\ cm^{-2}$ was obtained by two cool white fluorescent tubelights. Temperature during all these treatments was maintained at 27 $\pm$ 1°C.

Enzyme Extraction - Apical leaves or roots were homogenized in Na-phosphate buffer, 0.1 M; pH 7.0 (10 ml buffer/gm fresh weight) with insoluble PVP (2.5% W/V) in mortar and pestle at 4°C in cold room. The resulting brei was centrifuged at 26,000 x g for 30 minutes at 2°C and the supernatant obtained used for enzyme assay, protein estimation and gel electrophoresis.

For subcellular localization studies, apical leaves (7 ml buffer/gm fresh weight) were homogenized in tris-HCl buffer, 0.05 M; pH 7.4; sorbitol, 0.5 M; Ficoll, 2.5% W/V; dithiothreitol, 1 mM; in Wareing blendor (10 x 3 secs at top speed). Homogenate was strained through 8 layers of cheese cloth to get rid of cellular debris. The separation of organelles was achieved by differential centrifugation, the homogenate was first centrifuged at 500 x g for 10 minutes to obtain nuclear pellet and later at 2,000 x g and then at

20,000 x g for 30 minutes to obtain plastid and mitochondrial pellets respectively. Supernatant was then centrifuged in an Ultra-centrifuge (Janetzki Vac 601) at 150,000 x g for 1 hour to obtain ribosomal pellet and final supernatant. The pellets were washed twice with isolation buffer and gently resuspended in 1 ml of isolation buffer. Samples from respective fractions were assayed for enzyme activity and total protein.

Enzyme Assay - Peroxidase activity (E.C. 1.11.1.7) was assayed by recorded change in absorbance at 610 nm at 15 second intervals after adding 50 µl of sample supernatant to 5 ml of assay mixture at 25°C. The assay mixture contained benzidine, 2.4×10^{-3} M; hydrogen peroxide, 3×10^{-2}M; acetic acid, 7×10^{-2}M, pH 3.8. Solution of benzidine was prepared in acetic acid according to Scandalios (1964). One unit of enzyme activity was defined as change in absorbance of 0.1 at 610 nm per 15 seconds (1 unit = $0.1A_{610\ nm}$/15 seconds).

Protein Estimation - Protein was estimated after dissolving the trichloroacetic acid (5% final conc.) precipitate in 1 N NaOH by procedure of Lowry et al. (1951). The standard curve was made by using bovine serum albumin fraction V.

Electrophoresis - Isozymes of peroxidase were separated by polyacrylamide gel electrophoresis at 4°C. Samples

(100 µl), prepared by mixing supernatant and glycerol (2:1 ratio), were layered on the top of the gel. The anionic and cationic isozymes were resolved by the methods of Davis (1964) and Reisfield et al. (1962) respectively. A current of 4 mA per tube was employed. After completion of the run, gels were taken out and stained by first immersing in benzidine-acetic acid mixture (2.4×10^{-4}M and 7×10^{-3}M, respectively) for 10 minutes and then in hydrogen peroxide (3×10^{-2}M) for 2 minutes. Intense blue bands which latter turned brown on storage, were obtained. Gels were immediately washed and stored in 7% acetic acid.

Nucleic Acid Estimation - The procedure followed was essentially that of Cherry (1973). Apical leaves (0.5 gm) were homogenized in 6 ml of methanol and centrifuged at 5,000 x g for 10 minutes. Pellet was extracted twice in 4 ml of methanol, twice with 4 ml of 0.2 M perchloric acid and then twice with 4 ml of ethanol. All these steps were carried out in cold room at 0-2°C. Pellet was then extracted with 5 ml of ethanol-ether (2:1) at 50°C for 30 minutes, suspended in 0.5 N KOH, and incubated at 37°C for 16 hours. Tubes were then chilled at 0°C, acidified (to 0.5 N) with perchloric acid and incubated at 0°C for 10 minutes. These were again centrifuged and the pellet and the supernatant (Sup-A) were retained. Pellet was rewashed with perchloric acid and the supernatant was added to Sup-A.

Pellet was extracted at 80°C in 5% $HClO_4$, to hydrolyze DNA, for 50 minutes. It was then centrifuged and the supernatant was retained (Sup-B). Pellet was rewashed with 5% $HClO_4$ and supernatant added to Sup-B. Both supernatants containing RNA (Sup-A) and DNA (Sup-B) were neutralized with KOH to pH 7.0 and centrifuged in cold to remove $KClO_4$. Nucleic acid content was measured spectrophotometrically by recording absorbance in UV at 260 and 290 nm following formula, $\Delta A(A_{260} - A_{290}) \times 57 = \mu g$ nucleic acid/ml).

Radioactivity Incorporation Studies - ^3H L-leucine (1400 mci/mmoles) 1 µci/ml were supplied to roots to measure incorporation into proteins. The leaves were weighed and homogenized in 5 ml of 10% TCA, the homogenate was incubated at room temperature for 10 minutes and centrifuged at 20,000 x g for 20 minutes and supernatant retained for TCA soluble counts. TCA insoluble pellet was extracted twice with 5 ml of 5% TCA and then with 5 ml of ethanol, and washed with 4 ml of cold ethanol-ether (3:1), dried, and dissolved by leaving overnight in 4.0 ml of 1 N NaOH. Aliquots were taken and counted in 8 ml of Bray's Scintillation liquid (Napthelene, 60 gm; Methanol, 100 ml; Ethylene glycol, 20 ml; 1-4-dioxane to 1 litre; PPO 4 gm and POPOP, 0.2 gm) of TCA soluble and insoluble portions.

For incorporation into RNA, 2.5 µci/ml ^3H uridine (7500 mci/mmoles) was supplied to roots. Leaves were weighed

and homogenized in 7 ml of 0.3 M perchloric acid at 0°C. Homogenate was centrifuged at 20,000 x g at 0°C and supernatant retained for perchloric acid (PCA) soluble counts. The PCA insoluble pellet was extracted twice with 0.3 M perchloric acid and then with 5 ml of 80% ethanol, finally extracted with 5 ml of ethanol-ether (3:1). The resulting pellet was dried and resuspended in 0.3 N KOH and incubated at 37°C for 16 hrs. Aliquots were neutralized with HCl and counted in 8.0 ml of Bray's Scintillation liquid of PCA soluble and insoluble portions.

Chloroplast Isolation - Apical leaves of maize were weighed, chopped in small pieces and homogenized in buffer containing Na-phosphate, 0.1 M; pH 7.8; EDTA, 0.01 M; sucrose, 0.4 M; in Wareing blendor at top speed (15 x 2 sec). For photophosphorylation studies, chloroplasts were isolated in EDTA free buffer. The homogenate was strained through 8 layers of cheese cloth and first centrifuged at 200 x g for 2 minutes and then at 2,000 x g for 10 minutes. Pellet was washed and suspended in a minimal amount of buffer. Chloroplasts obtained were of C types as classified by Hall (1972).

The spectra of chloroplasts were taken at 25°C in medium comprising 50% glycerol and 50% isolation buffer. A constant amount of chloroplasts scattering at 750 nm was

taken as a reference. The procedures of isolation and studies on chloroplast function were carried under dim green safe light. Light source for measurement of photochemical activity was a 300 watt projector lamp, the incident beam was passed through 10 cm path of water layer and CS-3-69 (Cornings) cut off filter to eliminate infra-red light. The intensity of light was 1.5×10^5 ergs cm^{-2}.

Estimation of Pigments - Apical leaves were homogenized in 80% acetone with a pinch of $MgCO_3$ in mortar and pestle, in cold room under dim green safe light. The homogenate was centrifuged at 3,000 x g for 10 minutes and pellet was re-extracted with 80% acetone and the supernatants were combined. The content of chlorophyll was estimated according to Vernon (1960) by formula as follows:

$$\text{Chla} = 11.63\ A_{665} - 2.39\ A_{649} = \mu g\ \text{Chla/ml}$$

$$\text{Chlb} = 20.11\ A_{649} - 5.18\ A_{665} = \mu g\ \text{Chlb/ml}$$

$$\text{Total Chl} = 6.45\ A_{665} + 17.72\ A_{649} = \mu g\ \text{Chl/ml}$$

Carotenoids were estimated according to Liaaen-Jensen and Jensen (1971) by following formula :

$$\frac{A_{473} \times \text{dilution of sample} \times 4}{\text{fresh weight (gm)}} = \mu g\ \text{carotenoids/gm FW}$$

The amount of chlorophyll in isolated chloroplast was measured by lysing them in 80% acetone and centrifuging at 3,000 x g for 10 minutes according to Bruinsma (1961) by

following formula:

$$\frac{1000 \times A_{652}}{36} = \mu g \text{ Chl/ml}$$

Photochemical Activity Measurement - PSII-mediated DCPIP reduction was measured spectrophotometrically in reaction mixture (final volume 3 ml) containing DCPIP, 15 μM; KCl, 100 mM; $MgSO_4$, 0.1 mM, Na-phosphate buffer, 10 mM; pH 6.8 at 25°C, and chloroplasts equivalent to 10 μg of chlorophyll. The reduction of DCPIP was measured at 600 nm using extinction coefficient as reported by Armstrong (1964).

PSI activity was assayed polarographically in a reaction mixture (final volume 3 ml) containing tricine-NaOH, 50 mM, pH 8.0; NaCl, 35 mM; $MgCl_2$, 5 mM; DCMU, 10 μM; Na_2-ascorbate, 4 mM; NaN_3, 2 mM; ethanol, 0.5%; DCPIP, 20 μM; methyl viologen, 150 μM; and chloroplast equivalent to 60 μg chlorophyll. In case of far-red grown plants the chloroplasts equivalent to 20 μg chlorophyll were used. PSI activity was measured as O_2 uptake by methyl viologen at 25°C in YSI-Kettering oxygen monitor having clark type oxygen electrode connected with Riken-Denshi recorder. The electrode was calibrated against air saturated distilled water and oxygen content was calculated from standard tables.

The co-operation between PSII-PSI was assayed polarographically as O_2 uptake by methyl viologen in a reaction mixture (final volume, 3 ml) containing tricine-NaOH, 50 mM, pH 8.0;

NaCl, 35 mM; $MgCl_2$, 5 mM; NaN_3, 2 mM; ethanol, 0.5%; methyl viologen, 50 µM; and chloroplasts equivalent to 60 µg chlorophyll at 25°C. In case of FR grown plants chloroplasts equivalent to 20 µg chlorophyll was added.

Photophosphorylation - The reaction was run at 25°C in 1 ml of assay mixture containing tricine-NaOH, 50 mM, pH 8.0; ADP, 1 mM; K_2HPO_4, 2 mM; $MgCl_2$, 5 mM; NaCl, 10 mM; Bovine serum albumin, 0.5%; ^{32}Pi, 1 µci (carrier free) and chloroplasts amounting to 10 µg of chlorophyll. In case of cyclic photophosphorylation assay mixture was supplemented by 50 µM phenazine methosulfate, while in noncyclic photophosphorylation 1 mM K_3FeCN_6 was added. Samples were illuminated for 4 minutes in air and the reaction was terminated by adding 0.2 ml of 10% TCA. After centrifugation at 3,000 x g for 10 minutes, to the supernatant 150 mg of activated charcoal was added to absorb all phosphorylated compounds. The mixture was then filtered through Whatman no 1 filter paper on a Millipore filtration assembly and repeatedly washed with distilled water. The filter papers containing charcoal were dried at 50°C and counted in a GM counter (ECIL, India). In all cases a zero time control was also taken and the amount of counts added to reaction mixture was also determined.

Commercially obtained activated charcoal was purified for use by the following procedure. It was washed by suspending in 10 volumes of 1 N HCl at 70-80°C for 30 minutes, then with

distilled water on a sintered glass funnel till it become chloride free and finally with double distilled water. The charcoal was taken out, dried and packed in a bottle.

Pyrophosphate contamination of ^{32}Pi was overcome by first treating it to 100°C in 1 N HCl for 1 hr followed by addition of acid washed charcoal, centrifugation and filteration. Supernatant was suitably diluted with distilled water.

Oxygen Evolution and Uptake Studies - Photosynthetic activity in intact leaves was assayed by oxygen monitor. Sample chamber was filled with equal parts of CO_2 buffer (7 parts 0.1 M $NaHCO_3$ + 3 parts 0.1 M Na_2CO_3) and Avrons medium (tris-HCl, 15 mM, pH 7.8; NaCl, 2 mM; $MgCl_2$, 4 mM and K-phosphate buffer, 4 mM). Leaves weighing 150 mg were chopped to small pieces (2 mm^2) and used as sample. A constant rate of O_2 uptake due to respiration was established as basal rate before and after irradiation, from which rate of oxygen evolution was calculated. The reaction was carried out at 30°C and [redacted] electrode was calibrated with air saturated distilled water.

In case of O_2 uptake due to respiration the reaction was carried out in same buffer but under dim green safe light.

Polyribosomes Isolation - The method was followed from Travis et al. (1970) with a few modifications. Three gms of leaves were chopped to 1 cm^2 sections and frozen on dry ice immediately. Leaves were homogenized in mortar and pestle in 15 ml of tris buffer (tris-HCl, pH 7.5, 200 mM; $MgCl_2$, 20 mM; KCl, 50 mM; sucrose, 250 mM; Triton X-100, 1%; diethylpyrocarbonate, 0.1%). The RNAase free sucrose was used for all preparations and RNAase activity was inhibited by including diethylpyrocarbonate to homogenization buffer immediately before homogenization. The homogenate was centrifuged at 1,000 x g for 5 minutes and twice at 20,000 x g for 30 minutes. The polyribosomes were purified by layering the supernatant (5 ml) on 2.5 ml of a sucrose pad in tris-buffer (tris-HCl, pH 7.5, 50 mM; $MgCl_2$, 5 mM; KCl, 15 mM; sucrose 1.5 M) and centrifuging at 160,000 x g for 2 hours. Polyribosomal pellet was washed in same buffer but lacking sucrose and suspended gently by a glass rod in buffer to a final concentration of 10 A_{260}/ml. A 0.6 to 0.9 ml sample was layered on 27 ml of 10% to 34% linear sucrose gradient and centrifuged in (3 x 35 ml) swing-out rotor at 60,000 x g for 2 hours. The gradients were fractionated from top and monitored by passing through a quartz flow cell at 260 nm in Carl-Zeiss-PMQII spectrophotometer connected with Riken-Denshi recorder. Gradient linearty was checked by Abbe-refractometer at 20°C. All extraction and centrifugation steps were carried out at 0-2°C.

The polyribosome fraction was taken to be area of polyribosome region of recorded absorbance pattern divided by total area represented by monomer + polyribosomes. Monomer area was determined by multiplying peak height by its half the distance from base of apex.

In Vitro Translation - Polyribosome pellets were stored in deep freeze at 20°C for amino acid incorporation studies. The reaction mixture in 1 ml contained tris-HCl pH 7.5, 100 mM; $MgCl_2$, 10 mM; KCl, 16 mM; ATP, 1 mM; GTP, 0.3 mM; phosphoenol-pyruvate, 7 mM; pyruvic kinase, 7.0 e.u./ml, ^{14}C-lysine (120 mci/mmole) 1 µci, 19 unlabeled amino acids, 0.5 µ moles each, enzyme protein, 1.0 mg, polyribosomes equivalent to 3 A_{260} units. The reaction was carried out at 37°C for 30 minutes and terminated by adding TCA (final conc. 5%). The precipitate was heated at 90°C for 30 minutes and collected on millipore filters, (0.45 u/25 mm od), filters were repeatedly washed with 5% TCA and dried at 70°C. To the vials containing the dried filters, 8 ml of Toluene based scintillation cocktail (Toluene, 1000 ml; Methanol, 100 ml; PPO, 6 gm; POPOP, 0.5 gm) was added and counted in Packard-Tricarb Liquid Scintillation counter.

The enzyme protein was prepared from plumules excised from 2-day-old maize seedlings (Ramgopal and Hsiao, 1973). About 500 plumules were homogenized in 10 ml of buffer (tris-HCl, pH 7.8, 50 mM; $MgCl_2$, 0.5 mM; sucrose, 0.45 M)

and centrifuged at 160,000 x g for 1 hr. Supernatant was divided in 1 ml aliquots and frozen immediately and stored at -20°C.

RESULTS

In our earlier investigations (Sharma, 1974) it was found that peroxidase activity in 6-day-old etiolated seedlings of maize is controlled by phytochrome. In present investigation experiments were performed to characterize this response in detail and also to elucidate possible mechanism(s) for phytochrome mediated response.

Characterization of Photoresponse

Experiments were performed to study the development of photoresponse at different ages, its kinetics and the localization of photoresponse.

I. Effect of age on photoresponse - This experiment was carried out to see at what stage the effects of phytochrome can be seen during seed germination. Etiolated seedlings of different ages were irradiated with red and far-red light for a brief duration and transferred back to darkness for the next 24 h after which the enzyme activity was measured in leaves (Table 1). A brief irradiation with red light brought an enhancement in enzyme activity in 4-6-day-old seedlings, but was ineffective in 3-day-old seedlings. Irradiation with far-red light was, however, ineffective in evoking a significant photoresponse. Furthermore, red light-mediated enhancement in all cases was totally nullified when followed by far-red light.

TABLE 1

EFFECT OF BRIEF IRRADIATION OF RED AND FAR-RED LIGHT ON PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Treatments were given to seedlings at the age indicated in table and enzyme activity was measured 24 h later. Values are mean of four separate experiments done in duplicate. The activities are expressed as percentage of control which is taken as 100%.

Treatment	Age of seedlings (days)			
	3	4	5	6
D (control)	100	100	100	100
R	100	135	153	162
FR	89	100	110	121
R + FR	98	110	115	123

R-5 minutes, FR - 5 minutes.

Red light mediated enhancement increased with the age of seedlings.

Similar experiments were also conducted with roots (Table 2). Irradiation with red and far-red light did not have any effect on peroxidase activity in 3-5-day-old seedlings. In 6-day-old seedlings red light depressed the activity of peroxidase, while far-red relieved it, however, magnitude of difference was very little. All subsequent experiments therefore were done with leaves of 6-day-old seedlings.

II. Subcellular localisation - Phytochrome-mediated enhancement in peroxidase activity was studied in different subcellular fractions which were separated by differential centrifugation. Though peroxidase activity was detectable in all subcellular fractions, but most of the activity was present in the final supernatant (Table 3). The irradiation with far-red light did not bring about any significant change in recovery pattern of peroxidase, as amount of recovery in both, in dark grown and far-red irradiated seedlings was identical. On comparing peroxidase activity in different subcellular fractions in far-red irradiated plants, it was apparent that far-red increased the enzyme activity, both on fresh weight and protein basis (Table 4). The increase in enzyme activity was maximum in final supernatant after 24 hours of far-red treatment. The enzyme activity also increased in nuclear and ribosomal fractions but no significant

TABLE 2

EFFECT OF BRIEF IRRADIATION OF RED AND FAR-RED LIGHT ON PEROXIDASE ACTIVITY OF ROOTS

Treatments were given to seedlings at the age indicated in table and the enzyme activity was measured 24 h later. Values are mean of four experiments done in duplicate. The activities in table are expressed as percentage to control which is taken as 100%.

Treatment	Age of seedlings (days)			
	3	4	5	6
D (control)	100	100	100	100
R	100	118	107	80
FR	106	113	110	112
R + FR	119	107	103	100

R - 5 minutes, FR - 5 minutes.

TABLE 3

RECOVERY PATTERN OF PEROXIDASE ACTIVITY DURING SUBCELLULAR FRACTIONATION BY DIFFERENTIAL CENTRIFUGATION

Etiolated 6-day-old seedlings (0 time control) were transferred to continuous far-red irradiation and enzyme activity were measured at time intervals mentioned below. Values are presented as percentage of total activity originally present in crude homogenates.

Fractions	Relative peroxidase activity						
	0 h	8 h		16 h		24 h	
	D	D	FR	D	FR	D	FR
Homogenate	100	100	100	100	100	100	100
500 x g Pellet	1*	1	1	1.2	1.8	1	1
2000 x g Pellet	1	1	1	1	1	1	1
20000 x g Pellet	1	1	1	1	1	1	1
150000 x g Pellet	1	1	1	1	1	1.3	1.5
Supernatant	82	86	90	95	95	73	75

*One percent or less than it.

TABLE 4

INTRACELLULAR LOCALISATION OF PEROXIDASE ACTIVITY. EFFECT OF CONTINUOUS FAR-RED LIGHT ON PEROXIDASE ACTIVITY IN DIFFERENT SUBCELLULAR FRACTIONS

Etiolated seedlings (6-day-old) were transferred to continuous far-red irradiation and enzyme activities measured at time intervals indicated below. Values for peroxidase activity of far-red grown plants are presented in table as percentage to respective dark control for each fraction, which is taken as 100%. Values are represented on fresh weight or on protein basis.

Fractions	Relative peroxidase activity					
	8 h		16 h		24 h	
	P	FW	P	FW	P	FW
Homogenate	100	106	118	125	134	137
600 x g Pellet	105	137	154	177	125	133
2000 x g Pellet	87	80	125	86	112	100
20000 x g Pellet	48	52	111	100	100	131
150000 x g Pellet	108	130	170	100	161	136
Supernatant	129	110	142	135	176	144

P - mg protein basis, FW - gm fresh weight basis.

change were detected in plastid or mitochondrial fractions.

III. Kinetics of photoresponse - The kinetics of the enhancement in peroxidase activity was studied in seedlings irradiated with continuous far-red light (Fig.3). After a lag period, the increase in peroxidase activity took place steadily for 20 h following which it levelled off. On turning off the far-red light at the 12 h, the rate of increase in peroxidase activity declined but was still maintained at a higher rate than the dark control. Irradiation of 6.5-day-old seedlings with far-red light increased peroxidase activity but only after a lag of 2 h. In dark control, the peroxidase activity increased with age at an almost constant rate.

Interaction with Hormones, Acetylcholine and c-AMP

Experiments were conducted with an aim to study the possible role of hormones, acetylcholine and c-AMP in phytochrome-mediated enhancement in peroxidase activity. These experiments were done under three different conditions. Firstly, the effects of hormones, acetylcholine and c-AMP were seen in dark grown seedlings. Secondly, in seedlings exposed to red light and thirdly, under continuous far-red irradiation for 24 hours. Under these three conditions, the effects of inhibitors of hormones, and acetylcholine and c-AMP metabolism on peroxidase activity were also studied.

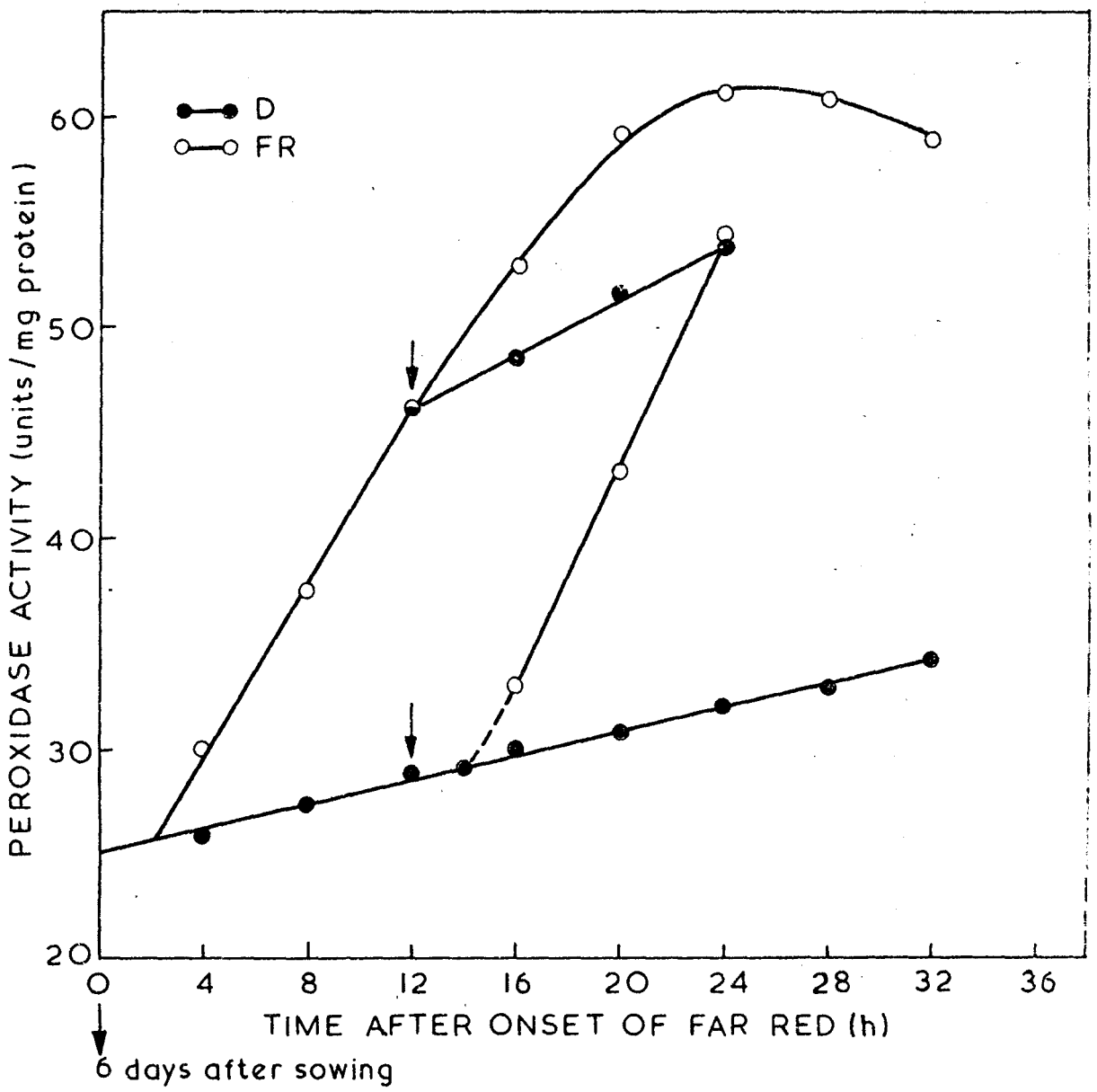


Fig. 3. Effect of continuous FR irradiation on the peroxidase activity in maize. The arrows indicate the time when seedlings were transfered from dark to FR (o——o) and from FR to darkness (●——●).

I. Effect of c-AMP - No stimulation in peroxidase activity was noticed in seedlings supplied with Na_2 c-AMP from 10^{-8}M to 10^{-5}M (Table 5). Since it had been reported that uptake of Na_2 c-AMP is not efficient (Robinson et al., 1971) we also tried Dibutryl-c-AMP from 10^{-5} to 10^{-8}M concentrations. However, it also had no effect on the peroxidase activity.

II. Effect of acetylcholine - Exogenous acetylcholine brought about an enhancement in peroxidase activity in dark at concentration of 10^{-5}M (Table 6). This effect of acetylcholine was specific since choline chloride, a compound closely resembling acetylcholine structurally had no effect on the peroxidase activity at the same concentration.

III. Effect of GA, IAA and kinetin - The effect of these three hormones were also seen at concentrations ranging from 10^{-4} to 10^{-7}M (Table 7). As is seen in Table 7, in the presence of GA and kinetin the peroxidase activity increased after 24 h, the optimum concentrations were 10^{-6} and 10^{-5}M, respectively. IAA had no effect on peroxidase activity at all concentrations tested. In further experiments, only optimal concentrations of the effective hormones and acetylcholine were employed, while IAA and DB_2-c-AMP were used at 10^{-4} and 10^{-5}M, respectively.

IV. Interaction of hormones, ACH and DB_2 c-AMP with red and continuous far-red light - The exposure of seedlings to red light for 5 minutes resulted in enhanced peroxidase

TABLE 5

EFFECT OF c-AMP ON PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE IN DARK

Etiolated seedlings (6-day-old) were supplied with respective solutions. Controls were incubated in distilled water. Enzyme activities were measured after 24 h. The results are mean values of four experiments done in duplicate.

Conc.(M)	Peroxidase activity			
	Na_2c-AMP		DB_2c-AMP	
	U	RA	U	RA
0	44.0	100	35.0	100
10^{-5}	52.0	118	33.5	96
10^{-6}	48.0	109	41.0	116
10^{-7}	50.0	114	38.0	109
10^{-8}	47.0	107	38.0	109

TABLE 6

EFFECT OF ACETYLCHOLINE AND CHOLINE CHLORIDE ON PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE IN DARK

Etiolated seedlings (6-day-old) were transferred to respective solutions. Controls were incubated in distilled water. Enzyme activity was measured after 24 hours of treatment. The results are mean values of 4 experiments done in duplicate.

Conc.(M)	Peroxidase activity			
	ACH		CCH	
	U	RA	U	RA
0	43.0	100	44.5	100
10^{-4}	55.0	128	47.0	105
10^{-5}	62.0	146	44.5	100

TABLE 7

EFFECT OF DIFFERENT HORMONES ON PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE IN DARK

Etiolated seedlings (6-day-old) were supplied with respective hormone solutions. Controls were incubated in distilled water only. Enzyme activity was measured after 24 h. The results are mean value of 4 separate experiments done in duplicate.

Conc.(M)	Peroxidase activity					
	GA		Kinetin		IAA	
	U	RA	U	RA	U	RA
0	32.0	100	36.0	100	49.0	100
10^{-4}	43.0	134	50.8	140	49.0	100
10^{-5}	43.0	134	52.5	146	50.0	102
10^{-6}	48.0	147	48.0	131	48.0	98
10^{-7}	40.0	125	48.0	131	52.0	107

activity. Supply of hormones and acetylcholine during the red light treatment increased peroxidase activity over the red light control (Table 8). The magnitude of enhancement, however, was marginal and in none of these cases it was found to be additive. DB_2 c-AMP which was ineffective in dark, decreased the peroxidase activity in red light irradiated seedlings. In presence of continuous far-red light (24 hours), the hormone, DB_2 c-AMP and acetylcholine showed lesser enhancement than the control. The repression was more with IAA and acetylcholine and least with GA.

V. Kinetics of peroxidase activity in presence of hormones, acetylcholine, c-AMP - Seedlings irradiated with far-red light showed an enhanced activity of peroxidase after a lag phase of 2 h, reaching to a maximum between 20 to 24 h (see Fig. 3). The presence of IAA and DB_2 c-AMP had no effect on peroxidase activity in dark, while under far-red light they repressed the enhancement after following a similar kinetics upto 8 h, (Fig. 4). GA enhanced the peroxidase activity in dark after a lag phase of 2hh, but the rate of enhancement was slower than far-red light alone. It repressed the far-red mediated enhancement after 8 h but at 24 h repression was marginal (Fig. 5). Kinetin steadily enhanced the peroxidase activity in dark after a lag period of 4 h but under, far-red light the increase was repressed after 8 h (Fig. 6) and after 24 h it was almost equal in far-red light irradiated and dark grown seedlings. Acetyl-

TABLE 8

INTERACTION OF HORMONE, ACETYLCHOLINE AND c-AMP WITH RED LIGHT AND CONTINUOUS FAR-RED LIGHT IN REGULATING PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Treatments were given to 6-day-old etiolated seedlings and enzyme activity was measured after 24 h. Controls were incubated in distilled water. Dark control is taken as 100% for relative activity measurement. Results are expressed as mean value of four experiments done in duplicate.

Treatment	Peroxidase activity					
	D		R		FR	
	U	RA	U	RA	U	RA
Control	43.0	100	73.0	170	82.0	190
DB_2c-AMP(10^{-5}M)	43.0	100	65.0	151	70.0	162
GA(10^{-6}M)	64.0	148	78.0	181	78.0	181
IAA(10^{-4}M)	43.0	100	76.0	176	66.0	153
KN(10^{-5}M)	67.0	155	79.0	183	75.0	174
ACH(10^{-5}M)	64.0	148	77.0	179	67.0	155

R - 5 minutes, FR - 24 h.

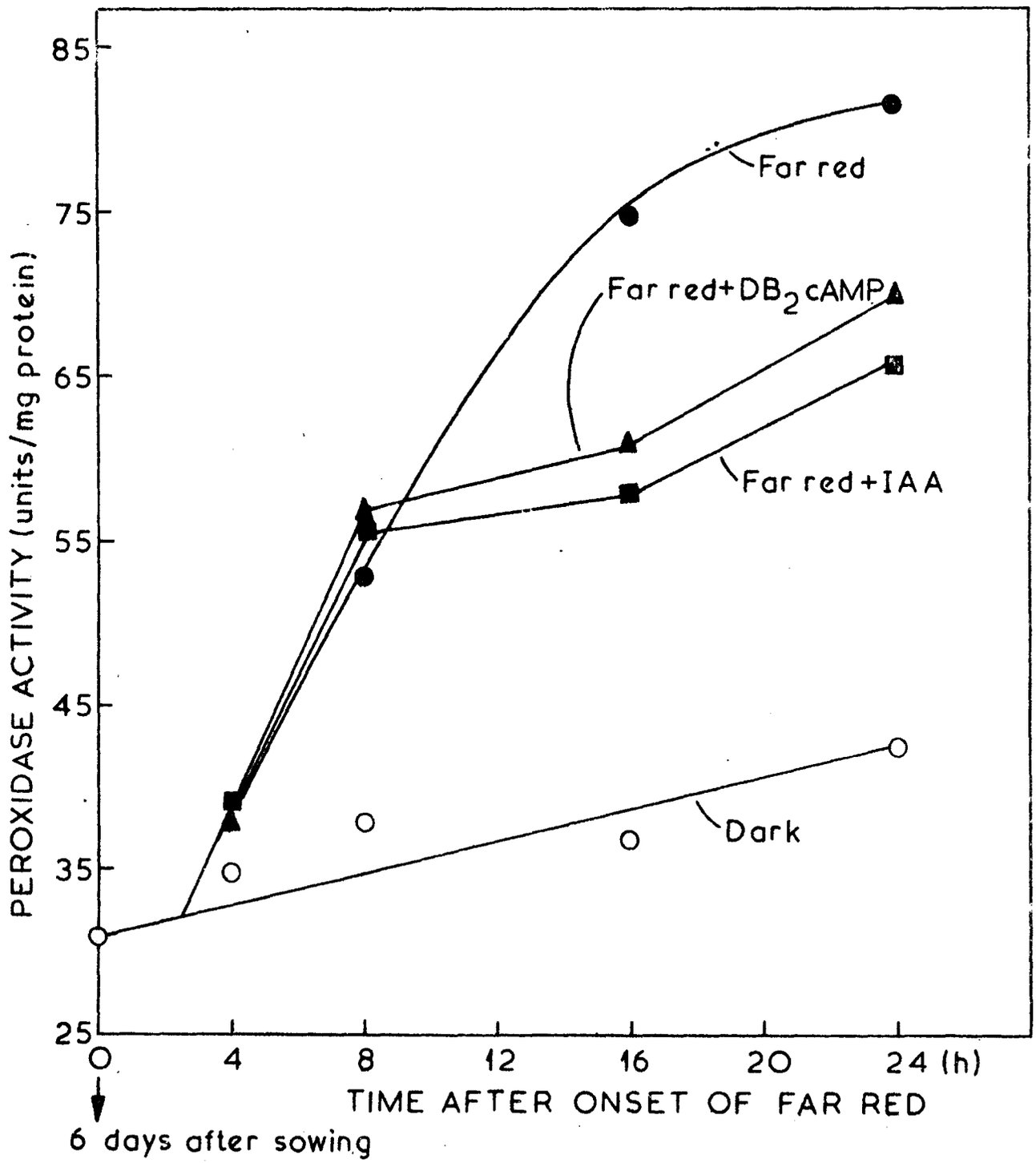


Fig. 4. The kinetics of peroxidase activity in apical leaves of maize under continuous FR light (●—●) and darkness (○—○) also in presence of DB_2cAMP (10^{-5}M) (▲—▲) and IAA (10^{-4}M) (■—■) under continuous FR light.

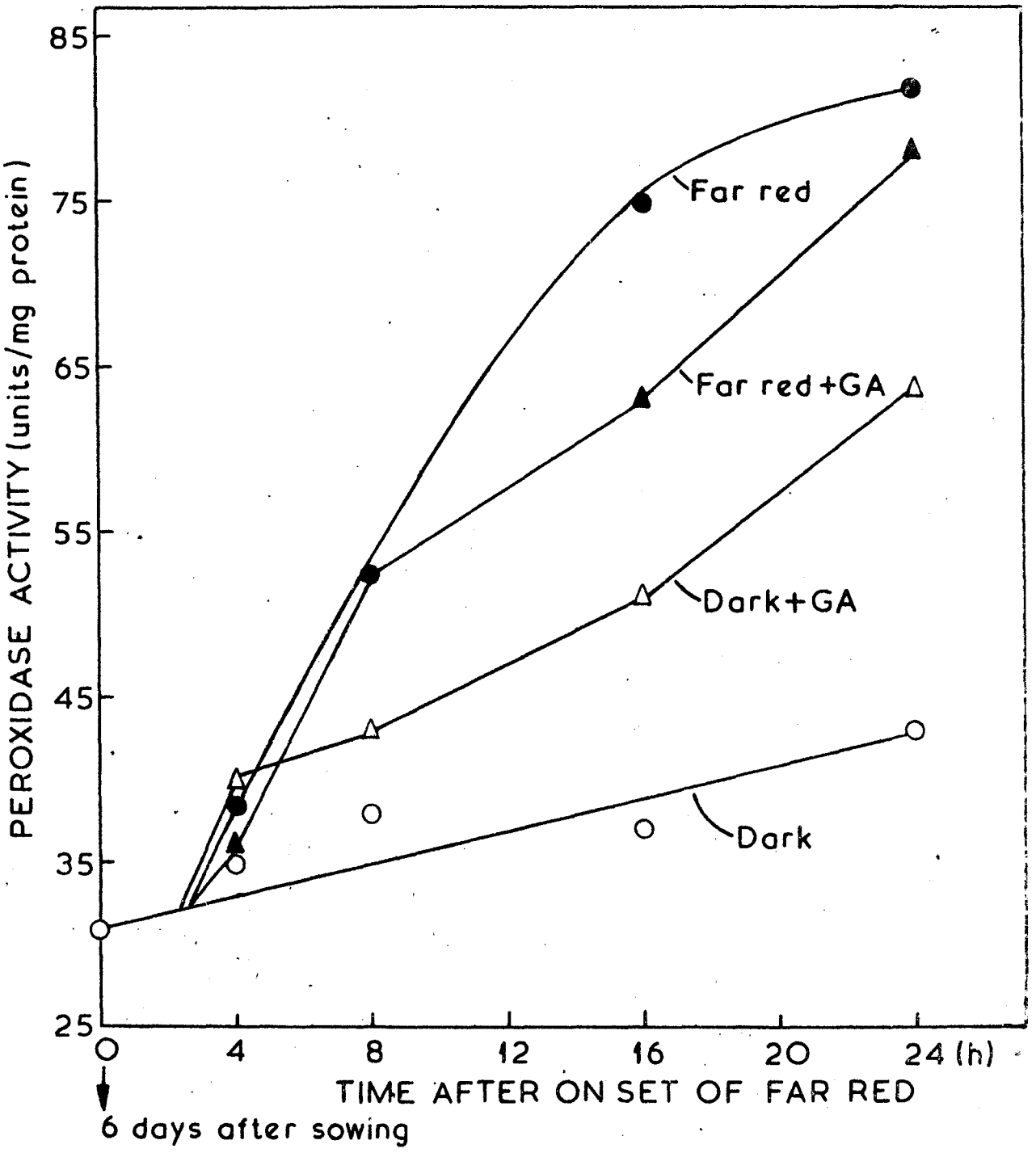


Fig. 5. The kinetics of peroxidase activity in apical leaves of maize in presence of GA(10^{-6}M) under continuous FR light (▲—▲) and darkness (Δ—Δ).

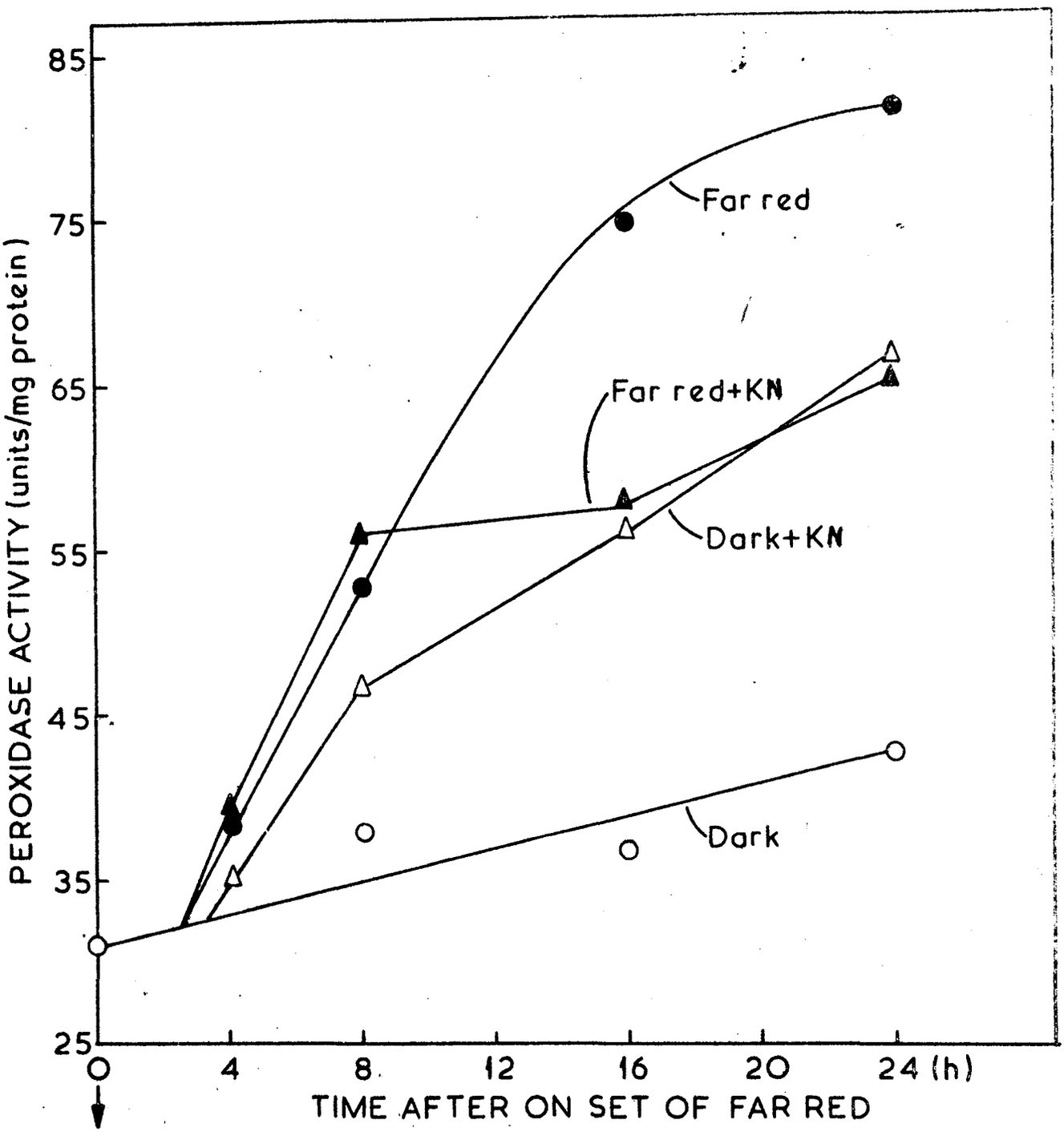


Fig. 6. The kinetics of peroxidase activity in apical leaves of maize in presence of kinetin (10^{-5} M) under continuous FR light (▲—▲) and darkness (△—△).

choline-mediated enhancement in peroxidase activity also had a lag phase of 4 h in dark. In far-red irradiated seedlings, acetylcholine repressed the far-red mediated enhancement in peroxidase activity after 4 h (Fig. 7).

VI. Interactions with antagonists of hormone, ACH and and c-AMP - The data presented in Table 9 show that most of the antagonists tried did not exert any significant effect on peroxidase activity in dark. The irradiation with red light which resulted in enhanced peroxidase activity, was however, suppressed by 20% by CCC. Under these conditions, ABA, atropine and eserine sulphate had no effect whereas theophylline potentiated the enhancement. Under continuous far-red light, all these compounds inhibited the peroxidase activity by 20-30%, atropine being the most effective. However, none of these could totally repress the enzyme activity.

VII. Effect on isozymes of peroxidase - There was no qualitative difference in isoperoxidase pattern in seedlings supplied with hormones, acetylcholine, c-AMP and their inhibitors, 5 minutes treatment of red light or under continuous far-red light. There were 3 bands of cationic isozymes and 8 bands of anonoic isozymes (Fig. 8). There was no change either in number of bands or in their Rf values.

TABLE 9

INTERACTIONS OF HORMONE ANTAGONISTS, INHIBITORS OF ACETYLCHOLINE AND c-AMP METABOLISM WITH RED LIGHT AND CONTINUOUS FAR-RED LIGHT, IN REGULATING PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Treatments were given to 6-day-old etiolated seedlings and enzyme activity was measured after 24 h. Controls were incubated in distilled water. Dark control is taken as 100% for relative activity measurement. Results are expressed as mean values of four experiments done in duplicate.

Treatments	Peroxidase activity					
	D		R		FR	
	U	RA	U	RA	U	RA
Control	43.0	100	71.0	166	82.0	190
ABA(10^{-4}M)	43.0	100	71.0	166	69.0	160
CCC(10^{-4}M)	43.0	100	63.0	147	72.0	167
ES(10^{-3}M)	49.5	115	69.0	160	73.0	170
AT(10^{-4}M)	46.0	107	73.0	170	66.5	155
TH(10^{-3}M)	40.0	93	76.5	178	72.0	167

R- 5 minutes, FR - 24 h.

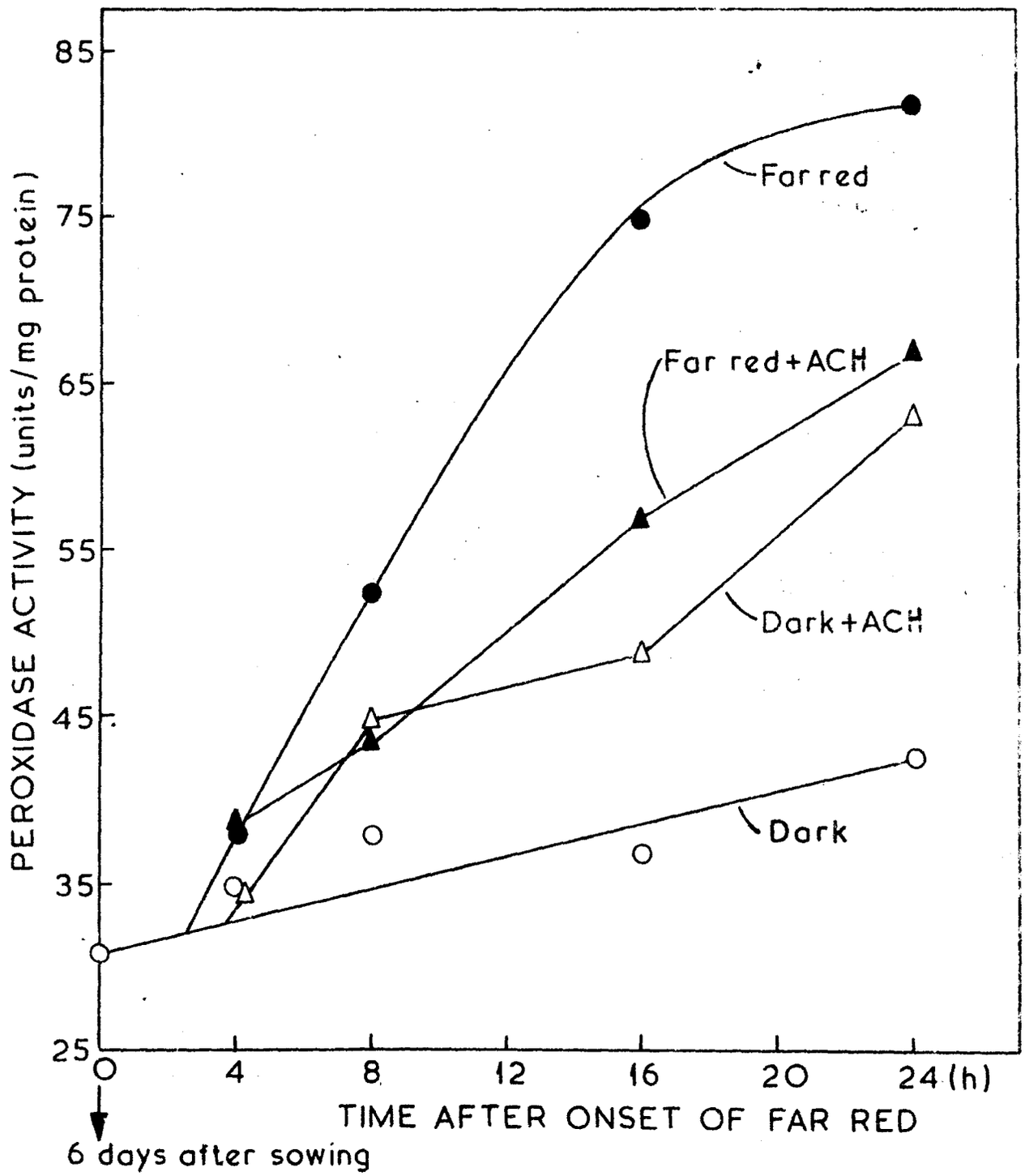
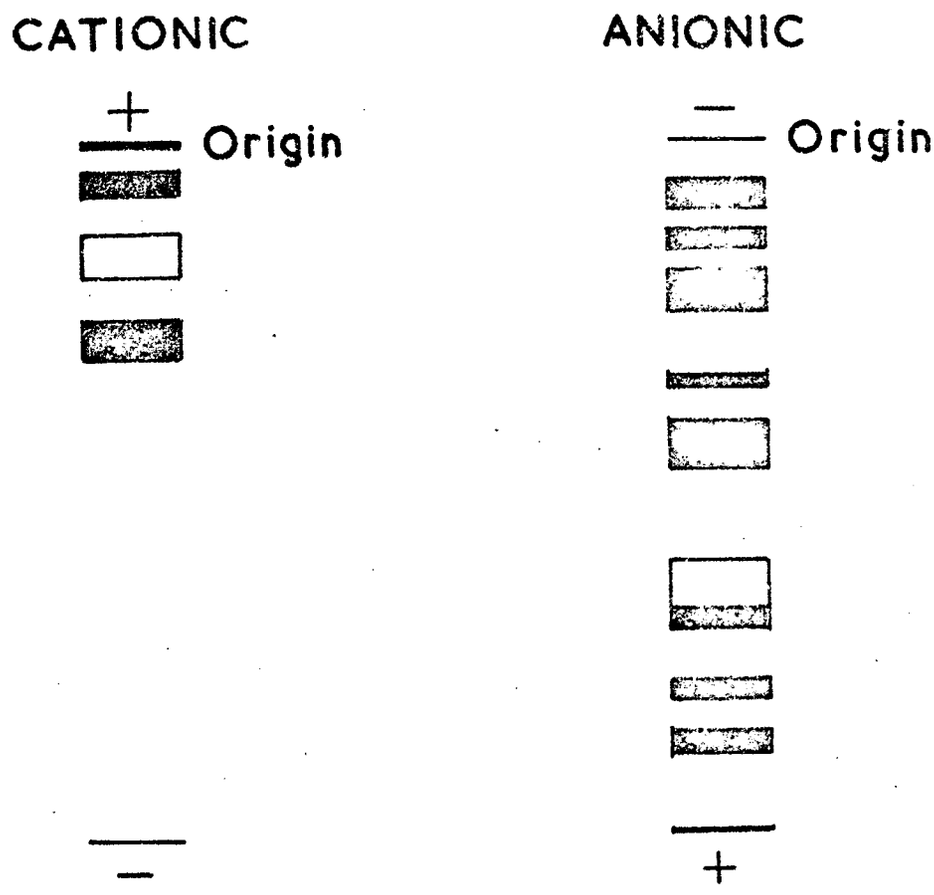


Fig. 2. The kinetics of peroxidase activity in apical leaves of maize in presence of (10^{-5}M) ACH under continuous FR light (▲—▲) and darkness (△—△).



8. Isozyme pattern of peroxidase on polyacrylamide gel.

Role of Photosynthesis in HIR Response

Studies were conducted to decipher possible contribution of photosynthesis to far-red-mediated HIR response in enhancing peroxidase activity in maize seedlings. Experiments were done to see the effect of photosynthetic inhibitors on FR-mediated enhancement in peroxidase activity and development of photosynthesis under continuous far-red light.

I. Effect of inhibitors on peroxidase activity - The increase in peroxidase activity under far-red light was affected by photosynthetic inhibitors. DCMU slightly stimulated the peroxidase activity at concentrations of 10^{-4} and 10^{-5}M, but under far-red light it repressed the enhancement in peroxidase activity by 20-25%, (Table 10). Ammonium sulphate did not have much effect on peroxidase activity in dark grown plants, while under far-red light it repressed the enhancement by 51% and 31% at concentrations of 10^{-2}M and 10^{-3}M, respectively (Table 10). Peroxidase activity in dark grown plants was slightly stimulated in the presence of DNP but under far-red light it repressed the enhancement by 40-60% (Table 11). Antimycin A, likewise, inhibited the peroxidase activity under far-red light, repression was about 65% at concentration of 10^{-6}M, while in dark same concentration was slightly stimulatory (Table 11).

TABLE 10

EFFECT OF DCMU AND $(NH_4)_2SO_4$ ON PHYTOCHROME MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were transferred to respective solutions 2 h prior to onset of far-red irradiation and enzyme activity was measured after 24 h. Controls were incubated in distilled water. Values are mean of three separate experiments done in duplicate. Dark control is taken as 100% for relative activity measurement.

Conc. (M)	Peroxidase activity DCMU				$(NH_4)_2SO_4$			
	D		FR		D		FR	
	U	RA	U	RA	U	RA	U	RA
0	50.0	100	93.0	185	49.0	100	88.0	180
10^{-2}	-	-	-	-	47.0	96	68.0	139
10^{-3}	-	-	-	-	54.0	110	78.0	155
10^{-4}	58.0	116	85.0	169	-	-	-	-
10^{-5}	57.0	114	83.0	166	-	-	-	-

FR - 24 h.

TABLE 11

EFFECT OF DNP AND ANTIMYCIN A ON PHYTOCHROME MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were tranferred to respective solutions 2 h prior to onset of far-red irradiation and enzyme activity was measured after 24 h. Controls were incubated in distilled water. Values are mean of three separate experiments done in duplicate.

Conc. (M)	Peroxidase activity							
	DNP				ANT-A			
	D		FR		D		FR	
	U	RA	U	RA	U	RA	U	RA
0	44.0	100	81.0	185	45.0	100	83.0	183
10^{-4}	50.0	114	66.0	150	-	-	-	-
10^{-5}	47.0	108	61.0	139	48.0	106	69.0	153
10^{-6}	-	-	-	-	52.0	115	58.0	129

FR - 24 h.

II. Effect of inhibitors on O_2 uptake - Excised leaves were studied for O_2 uptake. There was no significant difference in rate of oxygen uptake in dark and far-red grown plants (Table 12). The difference between different treatments and inhibitors were not very significant. Mostly, the respiration rate was slightly stimulated in plants incubated with inhibitors. Maximum enhancement was obtained in case of FR irradiated plants supplied with DNP (10^{-4}M), which increased O_2 uptake by 22%.

III. Effect of far-red and white light on pigment content- On irradiating maize seedlings with far-red or white light, amount of chlorophyll increased during the irradiation period from a basal level of zero. In plants irradiated with white light the rate of increase in total chlorophyll content was faster than the far-red irradiated plants and at 24 h it was 8.5 fold more (Fig. 9 and 10). Both chlorophyll a and b developed in far-red grown and white light grown plants, the Chl. a/b ratio was greater in far-red grown plants at all time intervals, it was 3.35 and 2.24 in far-red grown and light grown plants respectively at 24 h. The spectra of pigments in 80% acetone were also taken to ascertain the nature of pigments (Fig. 11). The spectra of pigments extracted with 80% acetone from seedlings kept under far-red and white light showed a single peak at 663 nm, while in dark grown plants there was no

TABLE 12

EFFECT OF PHOTOSYNTHETIC INHIBITORS ON RESPIRATION RATE IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were supplied with respective solutions and irradiated with far-red light for 24 h. Controls were supplied with distilled water. Respiration was measured in leaf slices in oxygen moniter under dim green safe light. Values for dark and far-red irradiated controls were 23.11 µmoles O_2 uptake/gm fresh weight.h. and 23.32 µmoles O_2 uptake/gm fresh weight.h. respectively. The results are expressed as percentage to dark dontrol which is taken as 100% and are mean of three independent experiments.

Conc. (M)	Relative Respiration rate							
	DCMU		$(NH_4)_2SO_4$		DNP		ANT-A	
	D	FR	D	FR	D	FR	D	FR
10^{-2}	-	-	109	105	-	-	-	-
10^{-3}	-	-	111	116	-	-	-	-
10^{-4}	94	97	-	-	105	122	-	-
10^{-5}	103	111	-	-	96	109	118	109
10^{-6}	-	-	-	-	-	-	108	109

FR - 24 h.

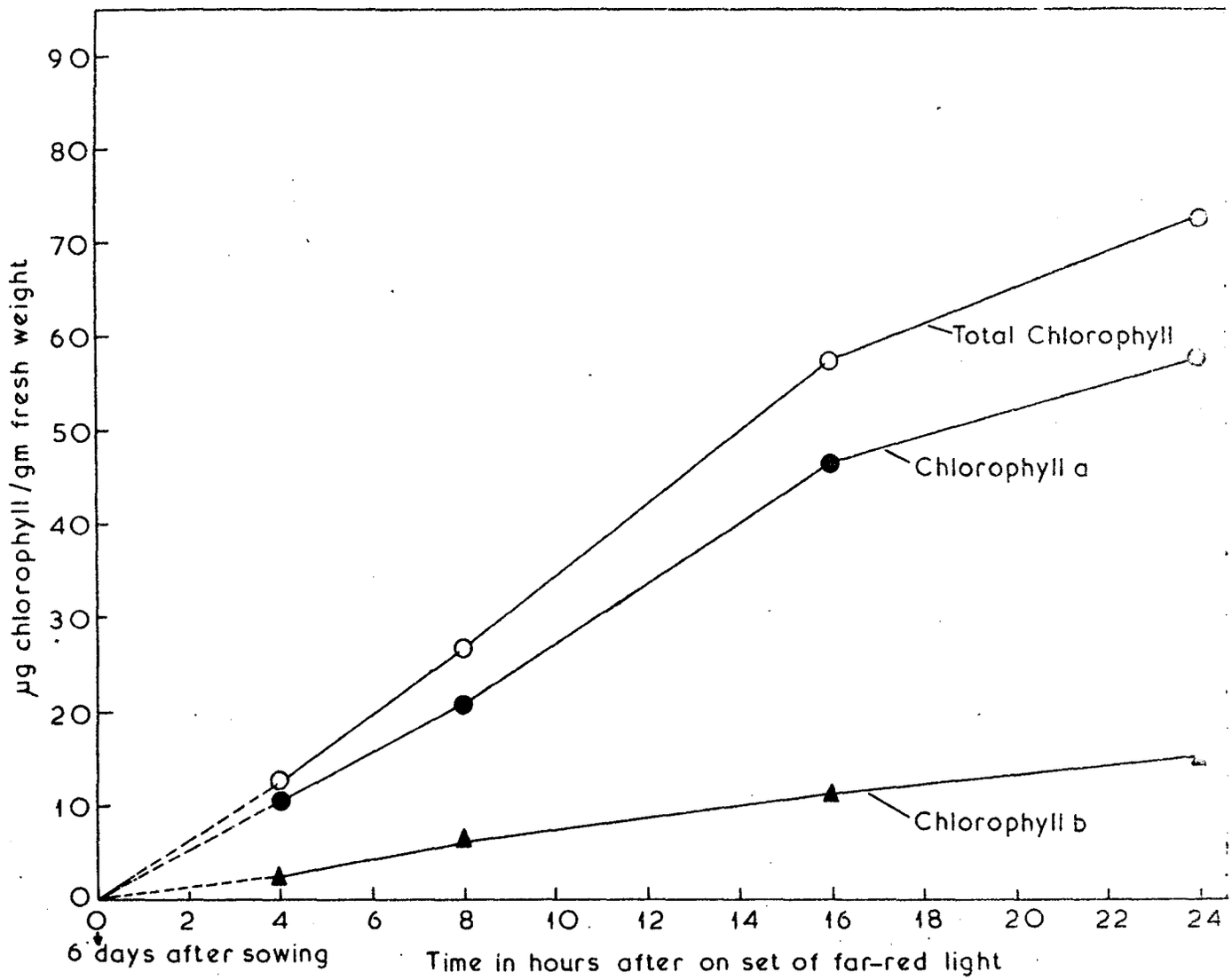


Fig. 9. Development of chlorophyll in apical leaves of maize under far-red light.

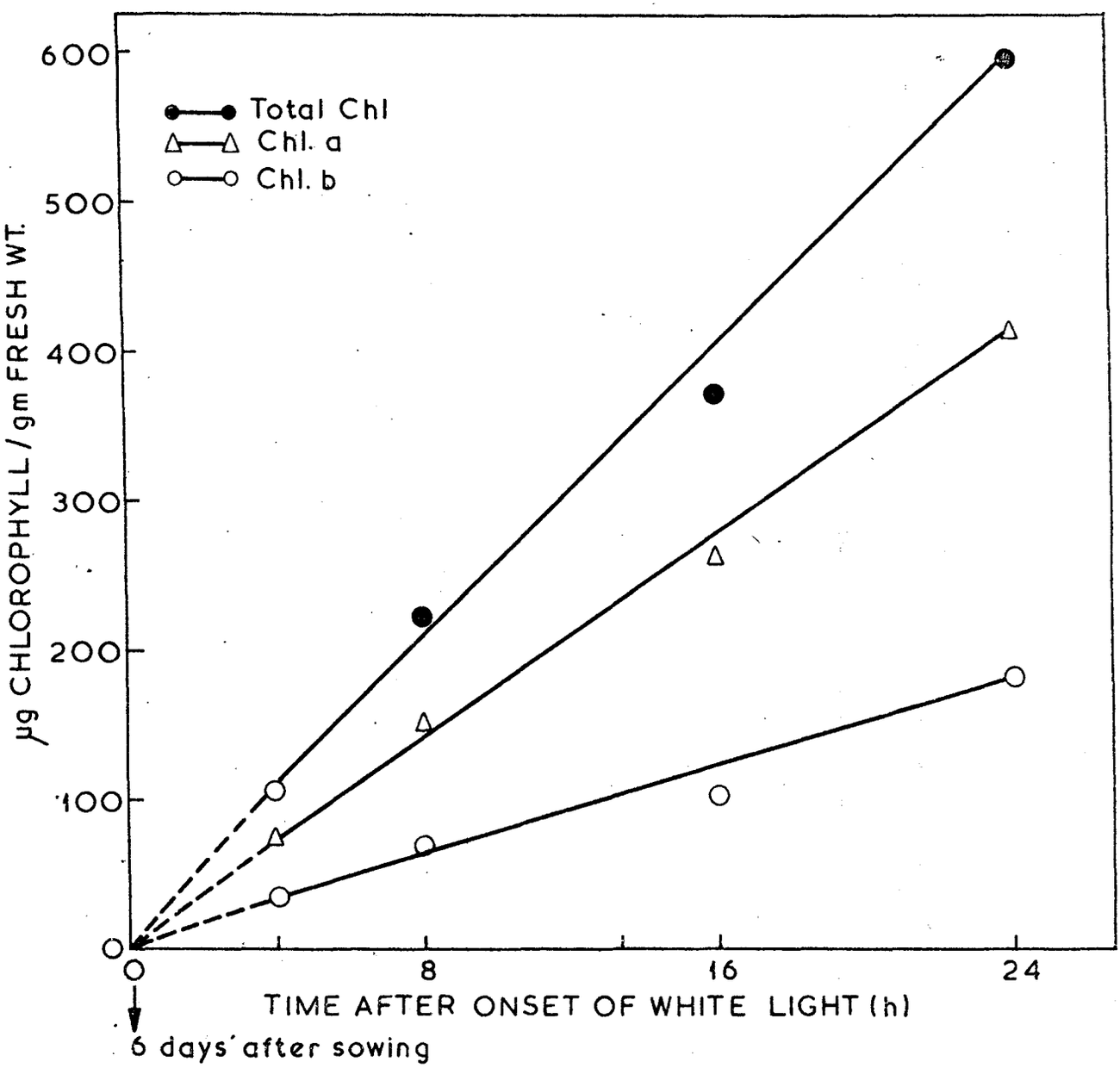


Fig. 10. Development of chlorophyll in apical leaves of maize under white light.

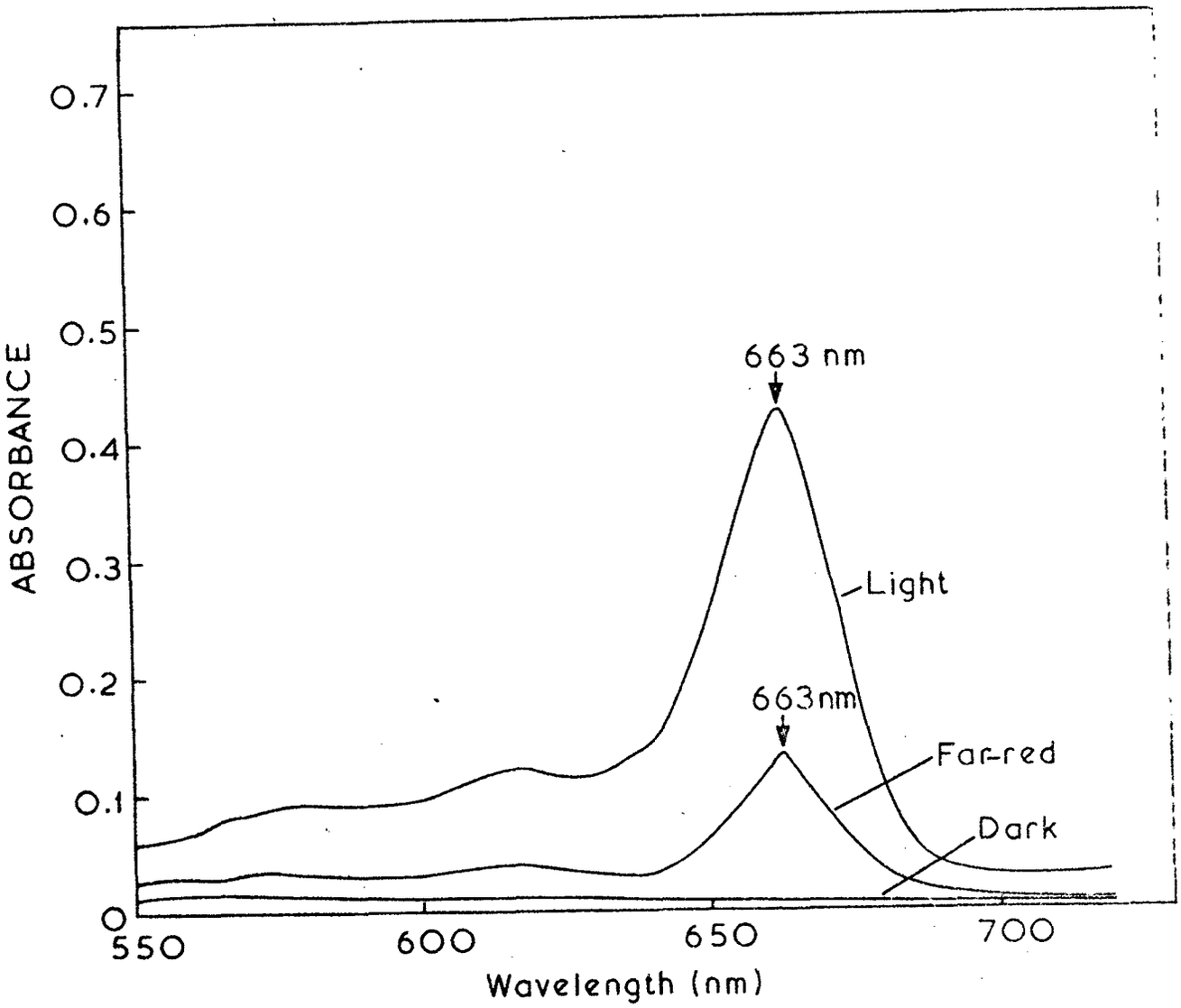


Fig. 11. Absorption spectra of chlorophylls in 80% acetone, extracted from apical leaves greened under far-red or white light for 24 hours, or kept in darkness.

measurable peak as no chlorophyll was present. The amount of carotenoids also increased along with chlorophyll under continuous far-red and white light (Fig. 12), the increase was however greater in case of white light grown plants. Chloramphenicol (250 µg/ml) repressed the chlorophyll development by 55% and increased the Chl a/b ratio in far-red grown plants (Table 13).

IV. Effect of far-red and white light on absorption spectra of chloroplasts - The isolated plastid from maize seedlings subjected to far-red and white light irradiation were analyzed for their spectral properties (Fig. 13). Plastids isolated from white light grown-plants presented a spectral profile indicative of mature chloroplasts, showed a peak at 678 nm in red region (Biswal and Mohanty, 1976), and characteristic soret band in blue region. On the other hand, plastids isolated from totally dark grown plants did not show any feature of a differentiated plastids. Plastids isolated from far-red grown plants showed a peak at 673 nm in contrast to light grown plants and had no organized soret peak in blue region. The absence of soret peak may, however, result due to huge scattering change, which could not be eliminated in method employed.

V. Development of O_2 evolution - Excised leaves from far-red and white light grown plants were studied for time course of development of CO_2-dependent O_2 evolution (Fig. 14).

TABLE 13

EFFECT OF CHLORAMPHENICOL ON DEVELOPMENT OF CHLOROPHYLL IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6½day-old) were transferred to inhibitor solution 2 h prior to far-red irradiation. Controls were incubated in distilled water. The chlorophyll content was measured after 24 h of far-red irradiation.

Treatment	Chlorophyll amount		
	µg/gm fresh wt.	Relative amount	Chl.a/b ratio
FR (H_2O)	68.59	100	3.42
FR(CAP 250 µg/ml)	31.06	45.2	4.91

FR - 24 h.

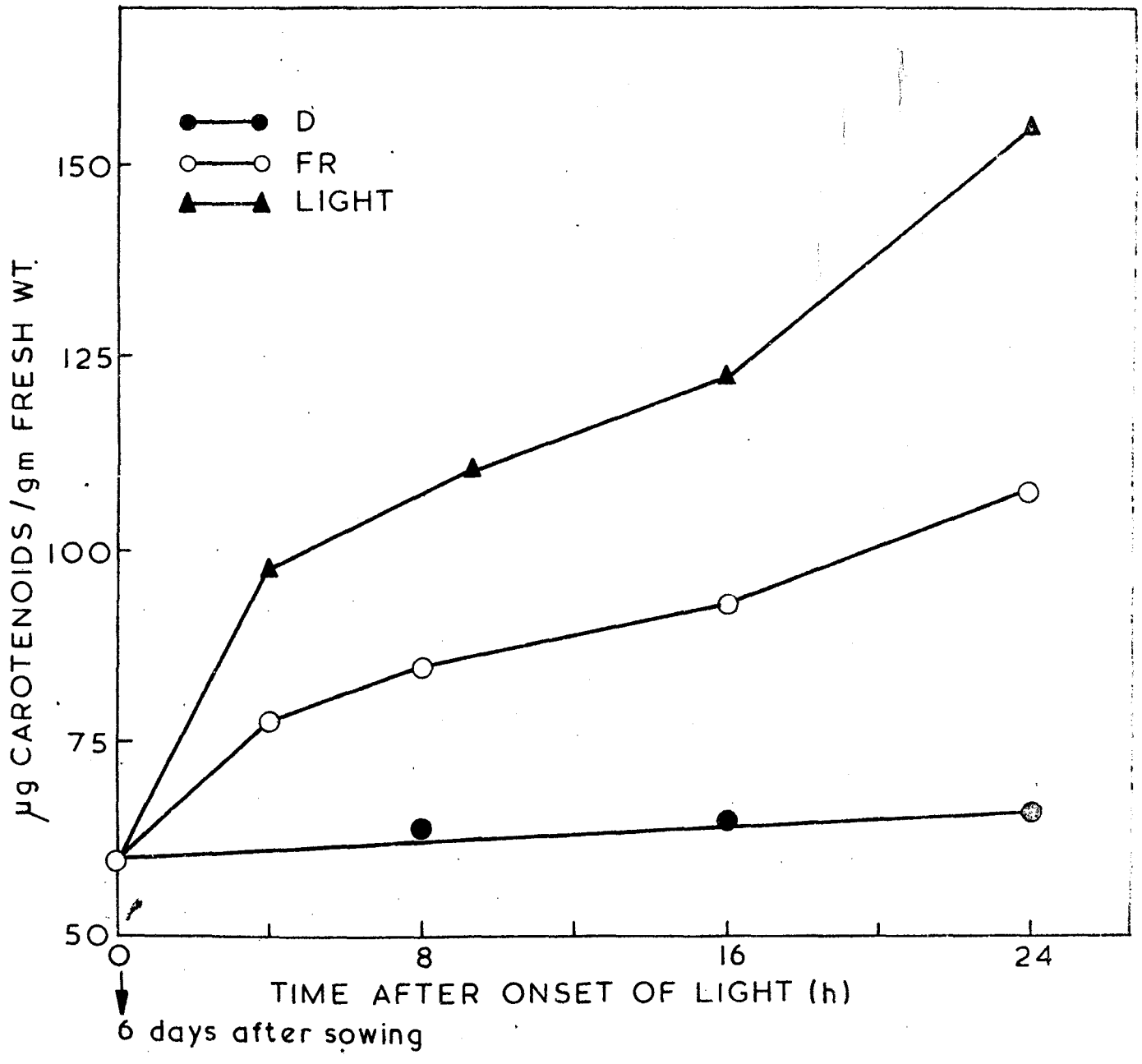


Fig. 12. Development of carotenoids in apical leaves of seedlings irradiated with far-red or white light, or kept in darkness.

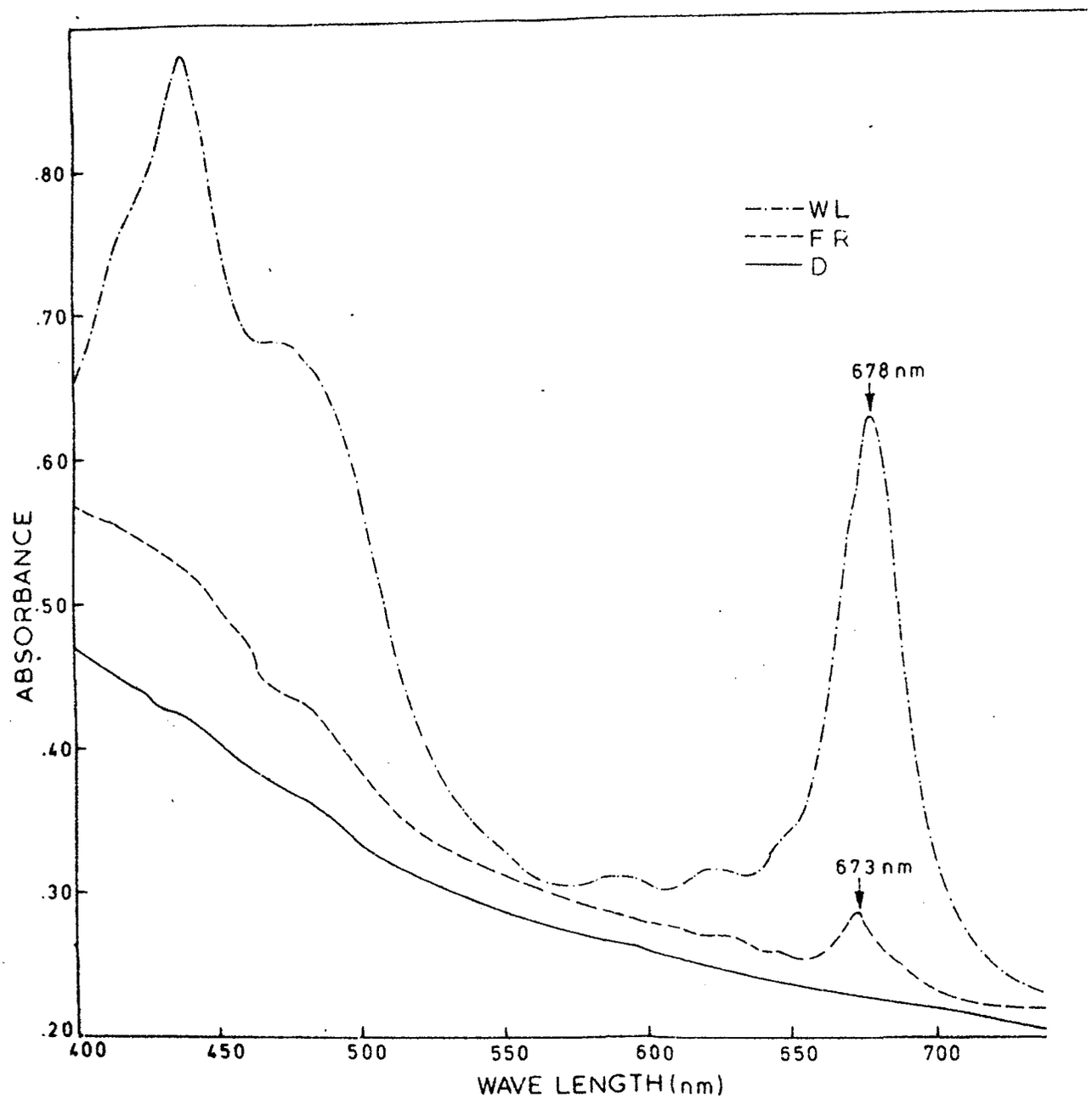


Fig. 13. Absorption spectrum of plastids isolated from dark grown seedlings irradiated with white or far-red light for 24 hours.

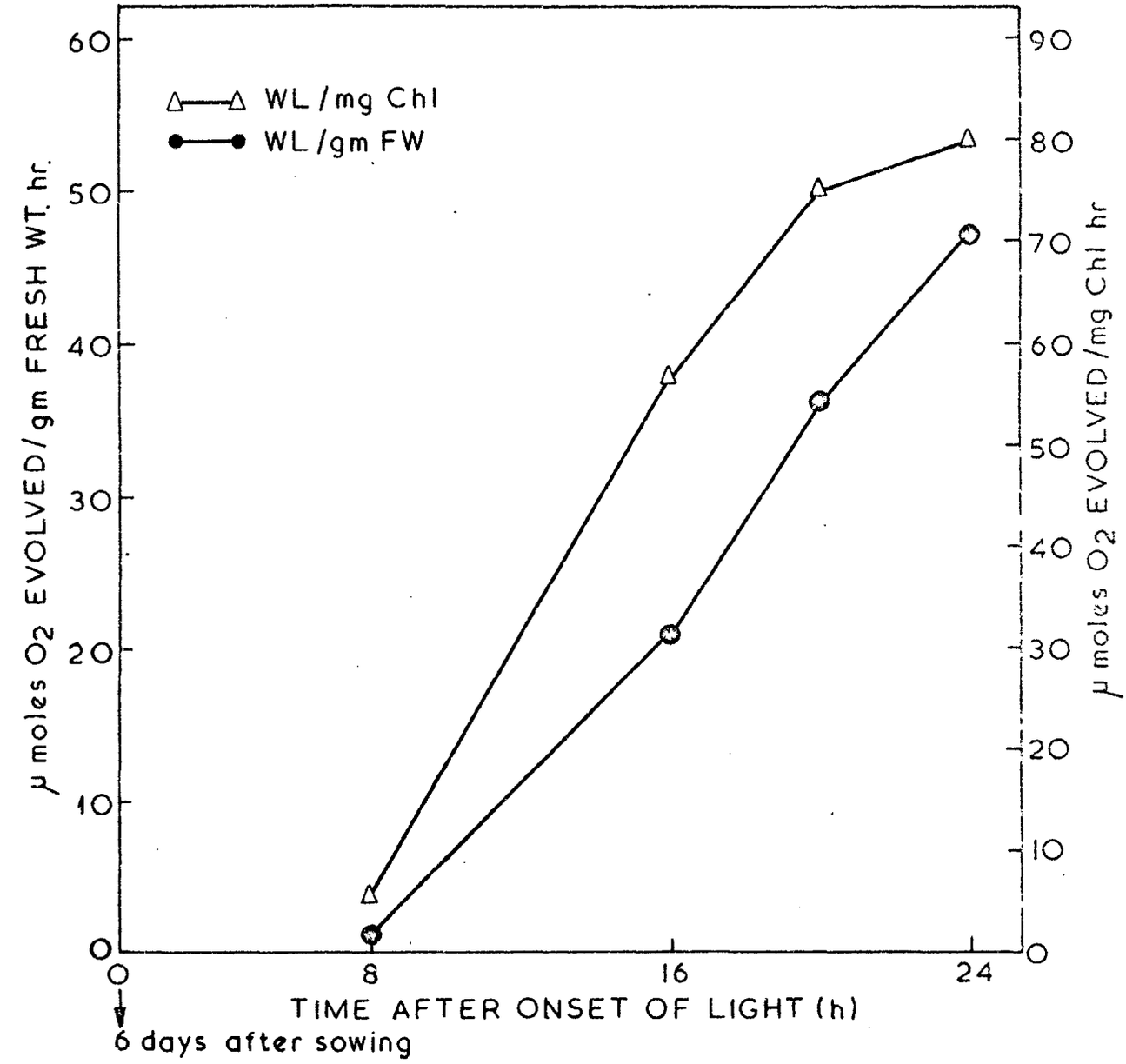


Fig. 14. Development of CO_2-dependent-O_2 evolution in maize leaves greened under white light. In FR grown plants no 02 evolution could be detected till 24 hours.

The oxygen evolution could not be detected in far-red grown plants till 24 h. In case of white light grown plants O_2 evolution was detected after 8 h of irradiation and it increased steadily till 24 h.

VI. Development of electron transport chain - The development of ability of DCPIP - mediated Hill reaction was studied in isolated plastids of far-red and white light grown plants (Fig. 15). Hill activity could not be detected upto 2 h, at 4 h of irradiation it could be detected both in case of far-red and white light grown plants. The PSII activity in light on chlorophyll basis reached a maximum at 8 h and declined later, while under far-red light it increased steadily but, it was lesser than white light. At 24 h both far-red grown and white light grown plants had almost equal Hill activity when compared on chlorophyll basis. However, the difference between the two treatments was about 8 folds when the Hill activity was expressed on fresh weight basis. The activity in far-red light increased steadily, while in white light it reached to a peak at 8 h, and then declined at 16 h, and again increased at 24 h.

The activity of PSI could be detected at 2 h in case of white light grown plants, and at 4 h in far-red light grown plants (Fig. 16). The activity of PSI on the basis of chlorophyll was higher in far-red light grown plants and declined steadily. In white light grown plants, PSI activity reached a maximum at 8 h and thereafter declined. On fresh

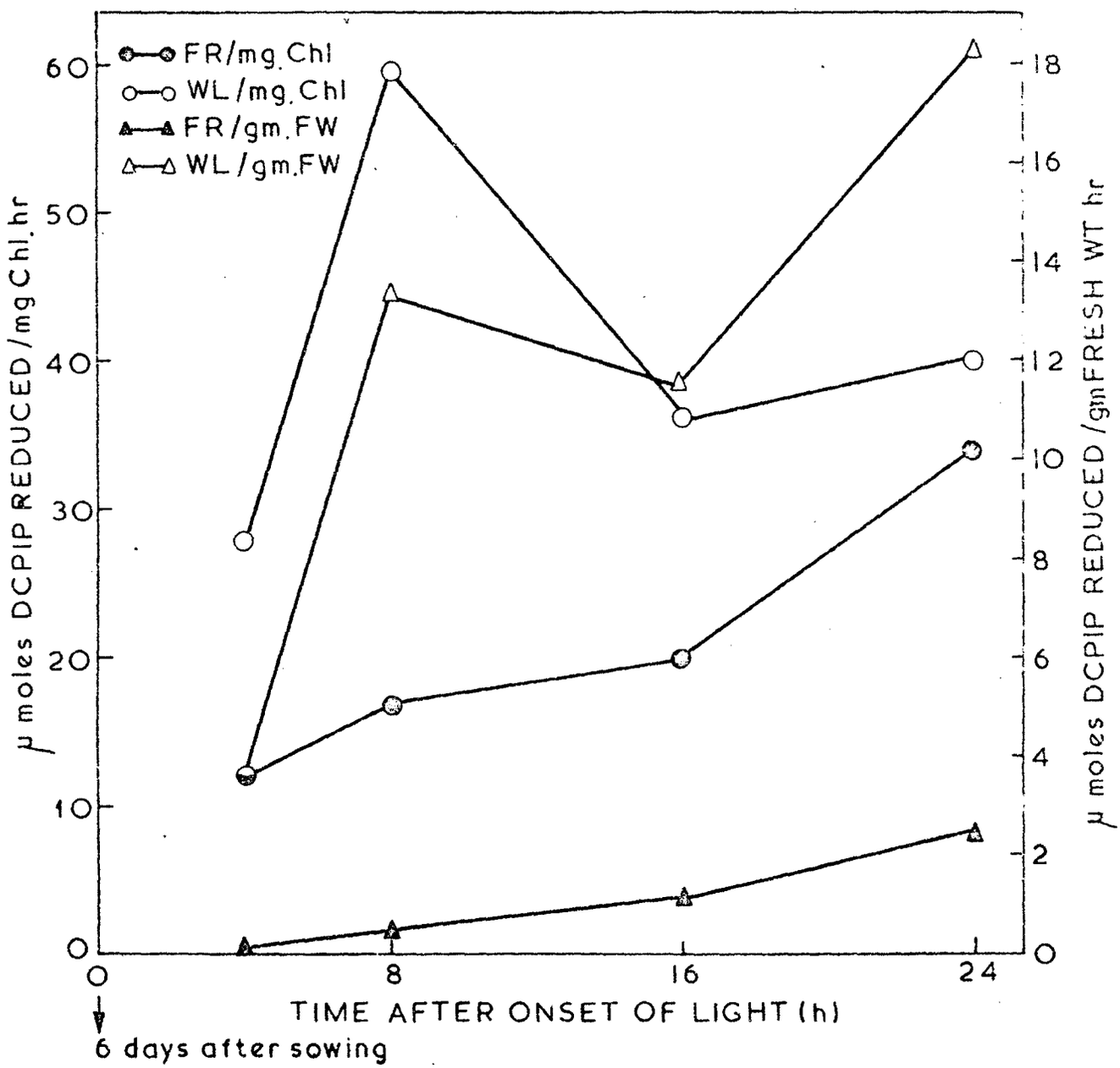


Fig. 15. Development of Hill activity (PSII) in isolated plastids from apical leaves of maize under white or far-red light.

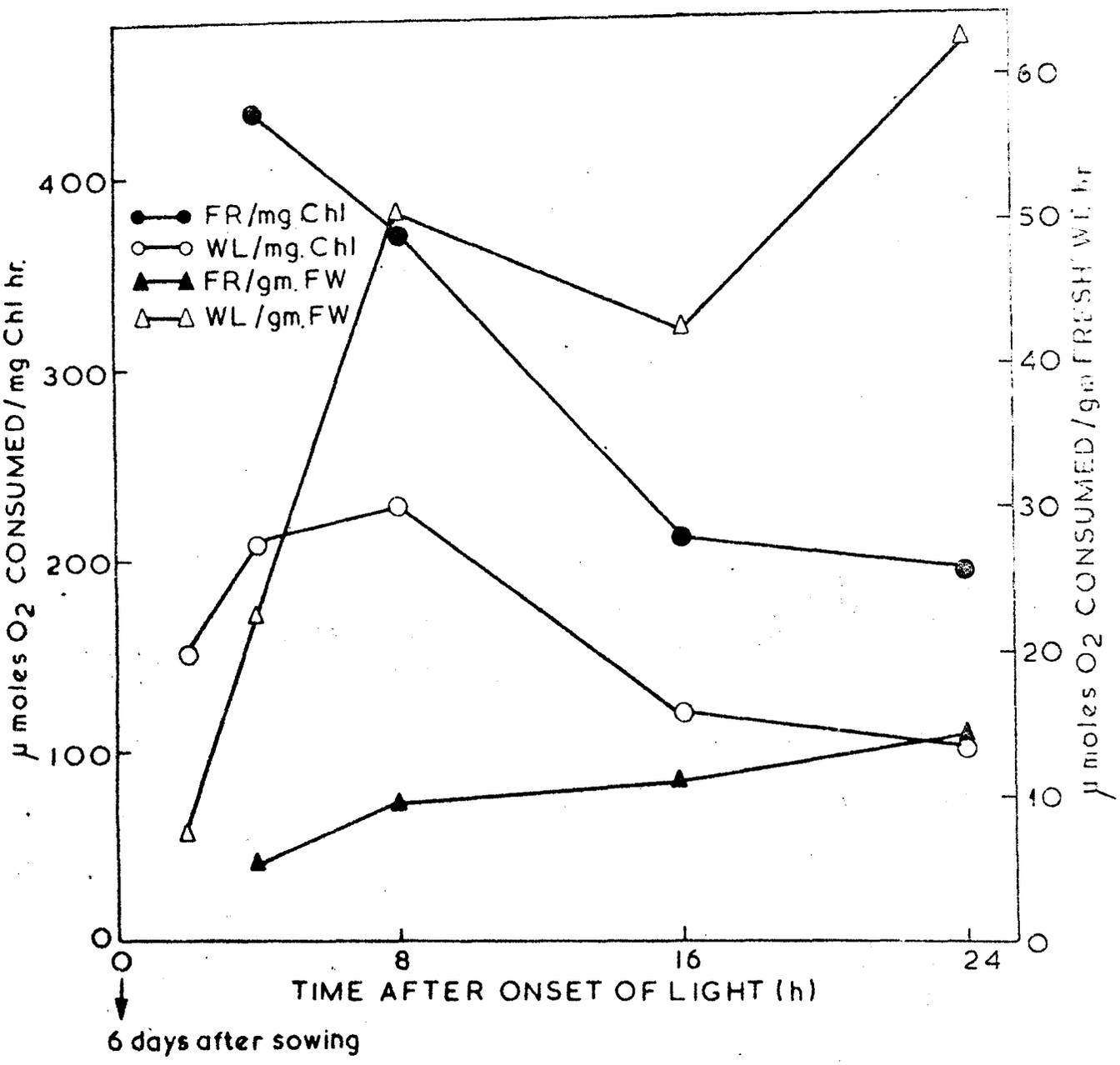


Fig. 16. Development of PSI activity in isolated plastids from apical leaves of maize under white or far-red light.

weight basis, however, the activity of PSI in white light was 4 fold more than far-red light. It increased with time in far-red treatments, while in white light it increased steadily till 8 h, declined till 16 h, again increased at 24 h.

The cooperation between two photosystem followed similar kinetics as noticed for PSI and PSII (Fig. 17). At 2 h since no PSII activity was present in white light grown plants, no cooperation between PSII and PSI could be detected both in far-red and white light grown plants before 4 h. On chlorophyll basis it reached a maximum at 8 h in white light grown plants and thereafter declined. In far-red grown plants it declined steadily. On fresh weight basis it remained at constant level in far-red grown plants, while in light grown plants it reached maximum at 8 h, declined and again increased at 24 h.

VII. Development of photophosphorylation - Unlike other photochemical activities photophosphorylation developed much latter. Cyclic PP developed earlier in case of far-red grown plants, while noncyclic PP could be detected only at 24 h. (Fig. 18, 19). In case of white light grown plants both cyclic and noncyclic PP could be detected at 8 h of irradiation and activity of these increased steadily. In case of white light grown plants, cyclic PP developed much faster than noncyclic PP. On the chlorophyll basis cyclic

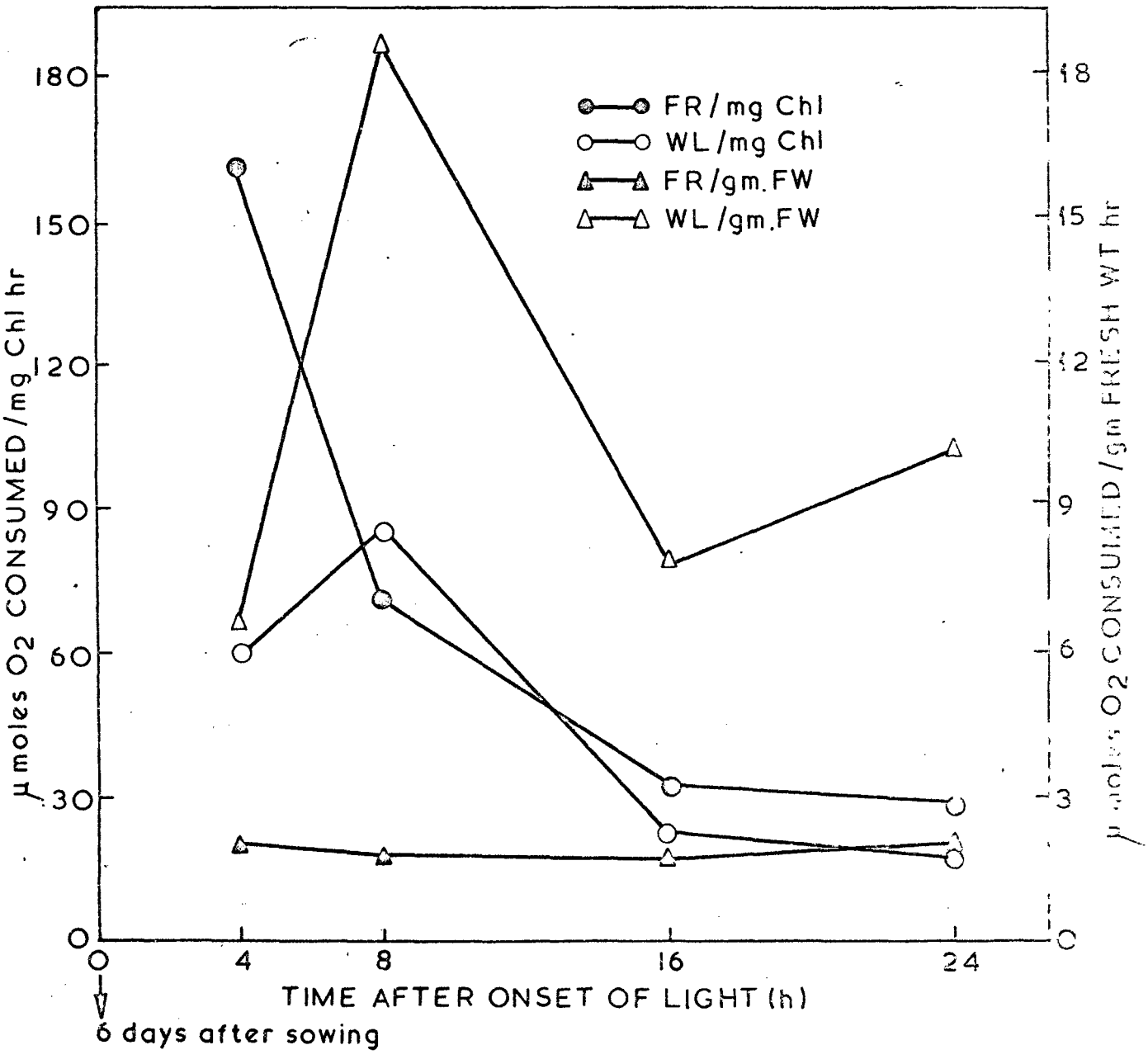


Fig. 17. Development of PSII→PSI cooperation in isolated plastids from apical leaves of maize under white or far-red light.

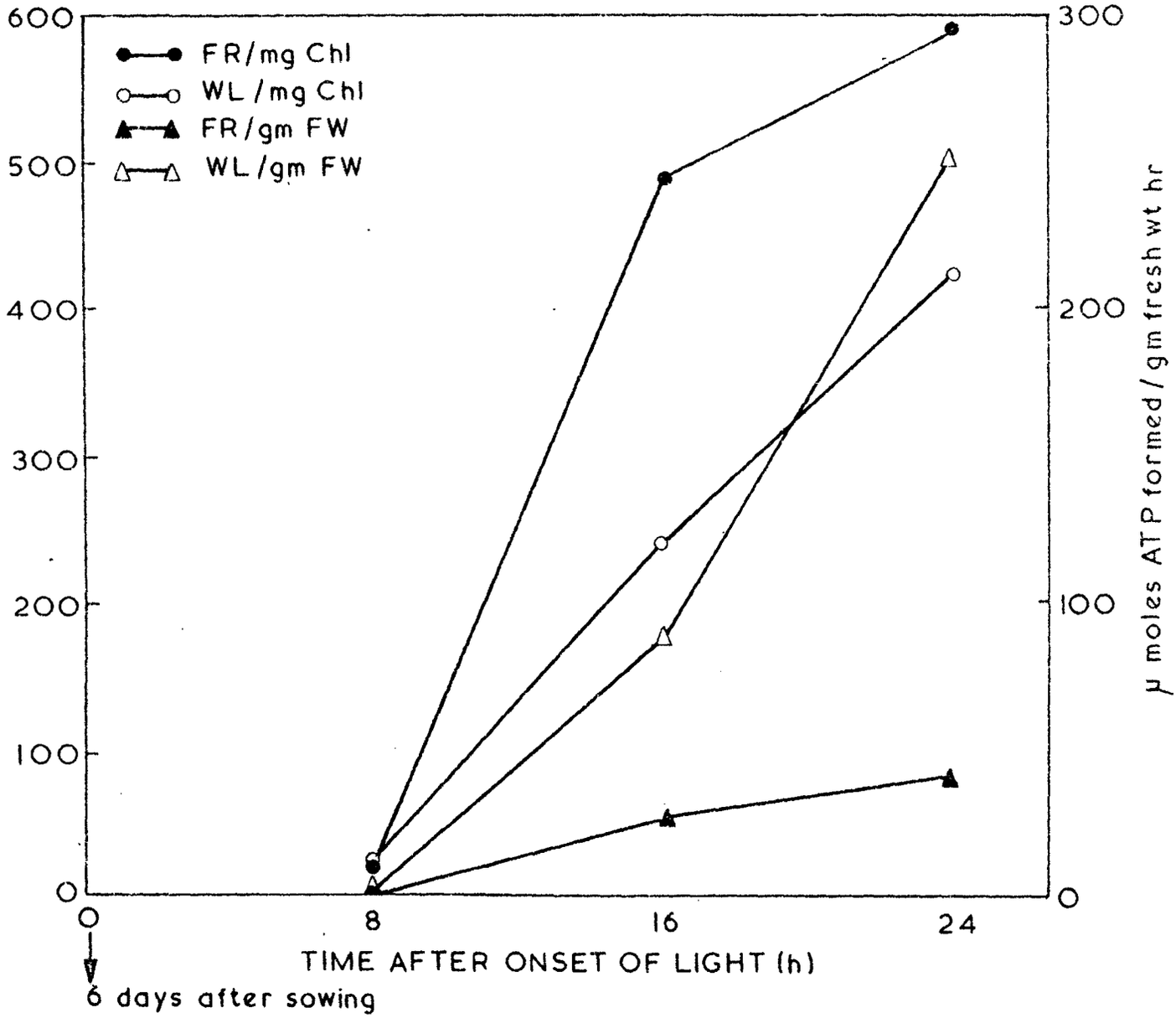


Fig. 18. Development of cyclic photophosphorylation in isolated plastids from apical leaves of maize under white or far-red light.

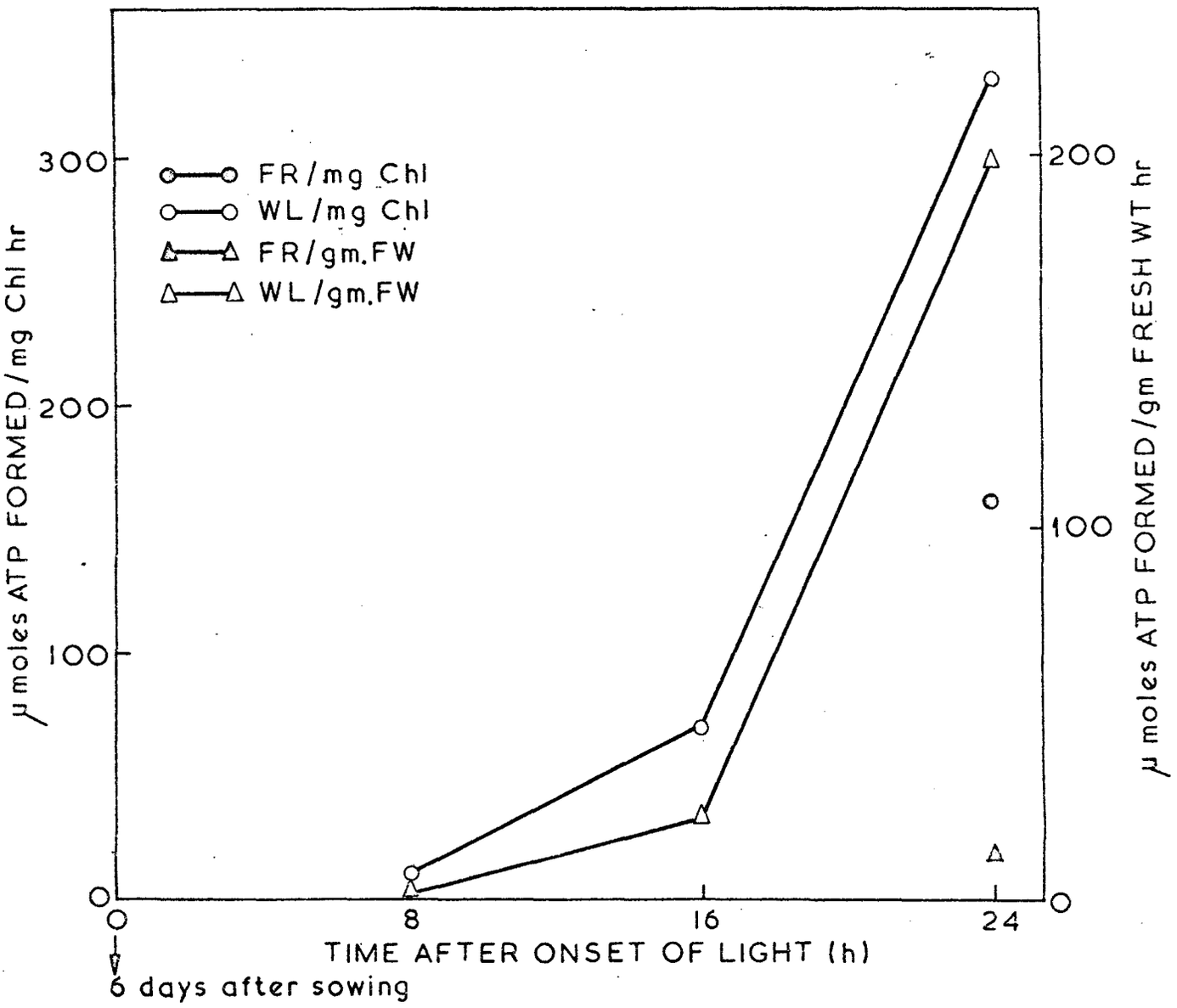


Fig. 19. Development of noncyclic photophosphorylation in isolated plastids from apical leaves of maize under white or far-red light.

PP was higher in far-red grown plants but on fresh weight basis it was 6 fold lesser than white light grown plants. Noncyclic PP in far-red grown plants was 2 fold lesser on chlorophyll basis and 18 fold lesser on fresh weight basis than white light grown plants.

Role of Transcription and Translation

These experiments were carried out to decipher the state of transcriptional and translational machinery during far-red irradiation period, and to see if there is any positive correlation to the enzyme increase.

I. Effect of actinomycin D and chloramphenicol - To elucidate the possible role of RNA synthesis and protein synthesis in this photoresponse, seedlings were supplied with actinomycin-D and D-threo chloramphenicol. These inhibitors did not exert any significant effect on peroxidase activity of etiolated seedlings (Table 14) as compared to control. In case of FR irradiated plants the presence of chloramphenicol did not hamper the enhancement in peroxidase activity, while actinomycin-D marginally repressed peroxidase activity. These inhibitors were effective in repressing RNA and protein synthesis at the concentrations employed (see below).

II. Effect of puromycin - Puromycin was also employed to ascertain the role of protein synthesis in photoresponse. As is seen in Table 15, puromycin (100 µg/ml) did not repress

TABLE 14

EFFECT OF ACTINOMYCIN D AND CHLORAMPHENICOL ON PHYTOCHROME-MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were transferred to respective solutions in petri dishes 2 h prior to onset of far-red irradiation. Controls were incubated in distilled water. Enzyme activity was measured after 24 h of irradiation. Values are mean of four separate experiments done in duplicate.

Treatment	Peroxidase activity	
	U	RA
D(H_2O)	33.0	100
D(ACT-D 8 µg/ml)	31.0	94
D(CAP 50 µg/ml)	36.0	109
D(CAP 250 µg/ml)	36.0	109
FR(H_2O)	61.0	183
FR(ACT-D 8 µg/ml)	57.0	170
FR(CAP 50 µg/ml)	59.5	179
FR(CAP 250 µg/ml)	59.0	177

FR - 24 h.

TABLE 15

EFFECT OF PUROMYCIN ON PHYTOCHROME-MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were transferred to respective solutions 2 h prior to onset of irradiation. Controls were incubated in distilled water. Enzyme activity was measured after 24 h of irradiation. Values are mean of three separate experiments done in duplicate.

Treatment	Peroxidase activity	
	U	RA
D(H_2O)	33.0	100
D(PURO 50 µg/ml)	31.0	94
D(PURO 100 µg/ml)	34.0	103
FR(H_2O)	61.0	183
FR(PURO 50 µg/ml)	43.5	132
FR(PURO 100 µg/ml)	32.0	97

FR - 24 h.

the peroxidase activity in dark control but brought a total repression of phytochrome-mediated rise in peroxidase activity. At concentration of 50 µg/ml it repressed the enhancement by 60%. It also effectively blocked *in vivo* protein synthesis (see below).

III. Effect of cycloheximide - Cycloheximide, an inhibitor of protein synthesis, displayed an entirely different response. It brought about an increase in enzyme activity in dark control and, this increase, was more pronounced at low concentration (5 µg/ml) than at high concentration (20 µg/ml) (Table 16). In its presence (5 µg/ml), even the FR-mediated increase in peroxidase activity was further enhanced, but at 20 ug/ml, the increase in dark grown and far-red grown plants was almost equal and was lesser than that obtained at low concentration (5 µg/ml). If 20 µg/ml cycloheximide was applied at the time when FR-light was turned off at 12 h, it again enhanced the increase in acitivity by 36%.

Since cycloheximide lead to an increase in peroxidase activity a kinetical study was made (Fig. 20). In this experiment application of cycloheximide (20 µg/ml) was done just before the begining of far-red irradiation. As can be seen, cycloheximide-mediated increase had a lag of 2 h and reached a maximum at 8 h, thereafter, no significant

TABLE 16

EFFECT OF CYCLOHEXIMIDE ON PHYTOCHROME-MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were transferred to respective solutions 2 h prior to irradiation. One set of seedlings, irradiated with far-red light for 12 h in water, was supplied with cyclochximide at the time they were transferred to darkness. Controls were incubated in distilled water. Enzyme activity was measured after 24 h of irradiation Values are mean of four experiments done in duplicate.

Treatment	Peroxidase activity	
	U	RA
D(H_2O)	33.0	100
D(CHI 5 µg/ml)	56.2	170
D(CHI 20 µg/ml)	44.6	135
FR(H_2O)	61.0	183
FR(CHI 5 µg/ml)	67.8	205
FR(CHI 20 µg/ml)	44.0	133
12 h FR (H_2O) + 12 h D(H_2O)	53.0	161
12 h FR (H_2O) + 12 h D (CHI 20 µg/ml)	65.0	197

FR - 24 h except in last two treatments where time has been indicated.

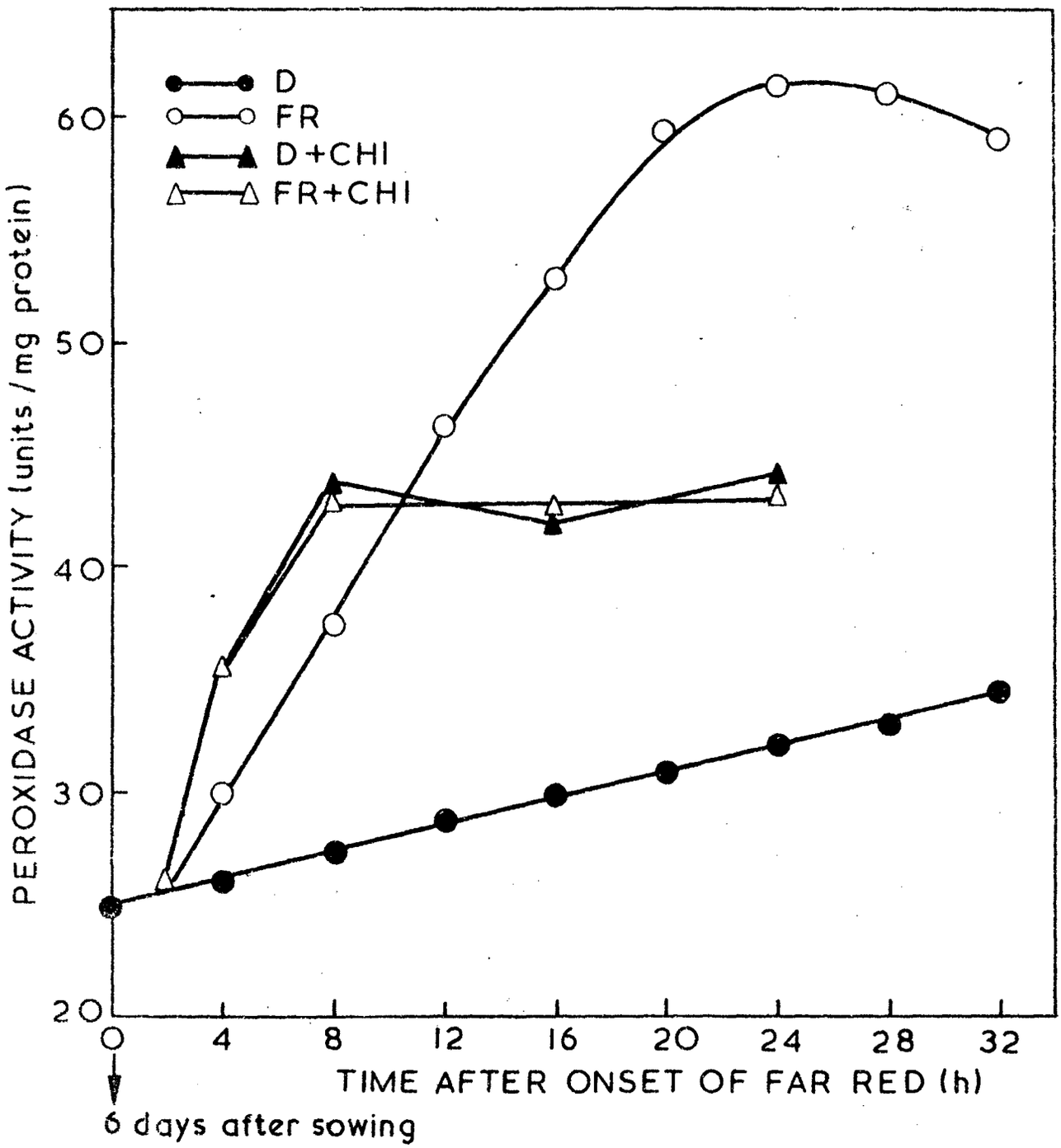


Fig. 20. Kinetics of peroxidase activity in dark and in continuous FR irradiation in presence (Δ—Δ) or absence of (0—0) of cycloheximide (20 µg/ml) in apical leaves of maize.

increase took place. The kinetics of cycloheximide-mediated enhancement was same in dark grown seedlings and in those irradiated with far-red light. The increase in activity was in fact lesser in magnitude than the FR-mediated increase and followed a different kinetic pattern.

IV. Effect of glutamine - Exogenous supply of L-glutamine lead to an enhancement in peroxidase activity, the optimal concentration was 5 mM, the stimulation at lower and higher concentration was not significant (Table 17). In far-red light irradiated seedlings supplied with 5 mM glutamine, the magnitude of enhancement was greater than control (Table 18). On applying both cycloheximide and glutamine at equimolar concentrations (0.1 mM) the cycloheximide increased the enzyme activity but not glutamine. Furthermore, glutamine did not affect cycloheximide mediated enhancement when both were applied together (Table 19).

V. Effect of inhibitors on protein and RNA synthesis - In previous experiments, it was observed that puromycin (100 µg/ml) and cycloheximide (20 µg/ml) blocked the phytochrome-mediated enhancement in peroxidase activity but chloramphenicol (250 µg/ml) had no effect. We also measured the incorporation of exogenously supplied, ^{3}H L-leucine into total proteins in dark and far-red light to determine the degree of inhibition of protein synthesis in presence of respective inhibitors (Table 20). Since both inhibitors and far-red light affected the rate of

TABLE 17

EFFECT OF L-GLUTAMINE ON PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE.

Etiolated seedlings (6-day-old) were transferred to respective solutions. Controls were incubated in distilled water. Enzyme activity was measured 24 h later. Values are mean of three experiments done in duplicate.

Treatment	Peroxidase activity	
	U	RA
D(H_2O)	41.0	100
D(1 mM GLU)	46.0	112
D(5 mM GLU)	55.0	134
D(25 mM GLU)	47.5	116

TABLE 18

EFFECT OF L-GLUTAMINE ON PHYTOCHROME-MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were transferred to glutamine solution 2 h prior to onset of irradiation. Controls were incubated in distilled water. Enzyme activity was measured 24 h after the irradiation. Values are mean of three experiments done in duplicate.

Treatment	Peroxidase activity	
	U	RA
D(H_2O)	33.0	100
D(5 mM GLU)	46.5	140
FR(H_2O)	59.0	179
FR(5 mM GLU)	63.0	191

FR - 24 h.

TABLE 19

EFFECT OF EQUIMOLAR CONCENTRATION (0.1 mM) OF CYCLOHEXIMIDE AND GLUTAMINE ON PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were transferred to respective solutions. Controls ware incubated in distilled water. Enzyme activity was measured after 24 h of treatment. Values are mean of two experiments done in duplicate.

Treatment	Peroxidase activity	
	U	RA
D(H_2O)	37.0	100
D(GLU)	39.0	105
D(CHI)	52.5	142
D(CHI + GLU)	54.0	146

TABLE 20

INCORPORATION OF ^{3}H LEUCINE INTO PROTEINS IN APICAL LEAVES OF MAIZE, IN PRESENCE OF INHIBITORS OF PROTEIN SYNTHESIS

Etiolated seedlings (6-day-old) were transferred to respective inhibitor solutions also containing ^{3}H leucine (1 µci/ml) 2 h prior to onset of far-red irradiation. Controls were incubated in ^{3}H leucine only. Values are expressed as ratio of total incorporation by total uptake on the basis of fresh weight.

Treatment	Incorporation/Uptake	
	D	FR
Control	0.448	0.210
CHI (20 µg/ml)	0.377	0.056
CHI (5 µg/ml)	0.361	0.109
CAP (250 µg/ml)	0.235	0.188
PURO (100 µg/ml)	0.242	0.171

FR - 24 h.

uptake, the results are expressed as ratio of total incorporation by total uptake, on fresh weight basis. It can be seen from the data that in the presence of inhibitors the incorporation of leucine in protein was less as evident by decrease in ratio. All inhibitors affected the incorporation, but effect of cycloheximide was most pronounced in FR irradiated seedlings. Effect of actinomycin D on incorporation of uridine into total RNA was also studied. In presence of actinomycin D the ratio of incorporation/uptake is lowered both in dark and far-red light (Table 21).

VI. Effect of nucleic acid level - There was no difference in DNA level of seedlings irradiated with far-red light and those kept in darkness (Fig. 21). It declined slightly during the experimental period. The level of RNA increased in seedlings kept under continuous far-red light after 2 h of irradiation and reached a maximum at 8 h of irradiation then declined (Fig. 22). In dark controls, there was a continuous decline in RNA during the experimental period. The rate of decline in RNA was higher in dark controls than in far-red grown plants. At 24 h, RNA in far-red grown plants was 36% higher. On irradiating maize seedlings with brief red and far-red light no significant change in RNA level was observed which could be ascribed to phytochrome (Table 22).

TABLE 21

INCORPORATION OF ^{3}H URIDINE INTO RNA OF APICAL LEAVES OF MAIZE IN PRESENCE OF ACTINOMYCIN-D (10 µg/ml)

Etiolated seedlings (6-day-old) were transferred to actinomycin D solution also containing ^{3}H uridine (2.5 µci/ml) two h prior to onset of far-red irradiation. Controls were incubated in ^{3}H uridine only. Values are expressed as ratio of total incorporation by uptake on the basis of fresh weight.

Treatment	Incorporation/Uptake	
	D	FR
Control	0.036	0.096
ACT-D	0.026	0.067

FR - 24 h.

TABLE 22

EFFECT OF BRIEF IRRADIATION OF RED AND FAR-RED LIGHT ON RNA LEVEL IN APICAL LEAVES OF MAIZE

Etiolated seedlings (6-day-old) were irradiated with red and/or far-red light and RNA level was measured after 24 h of dark treatment.

Treatment	RNA amount	
	ug RNA/gm FW	Relative amount
D (control)	1240	100
R	1160	93.6
FR	1160	93.6
R + FR	1137	91.7

R- 5 minutes; FR - 5 minutes.

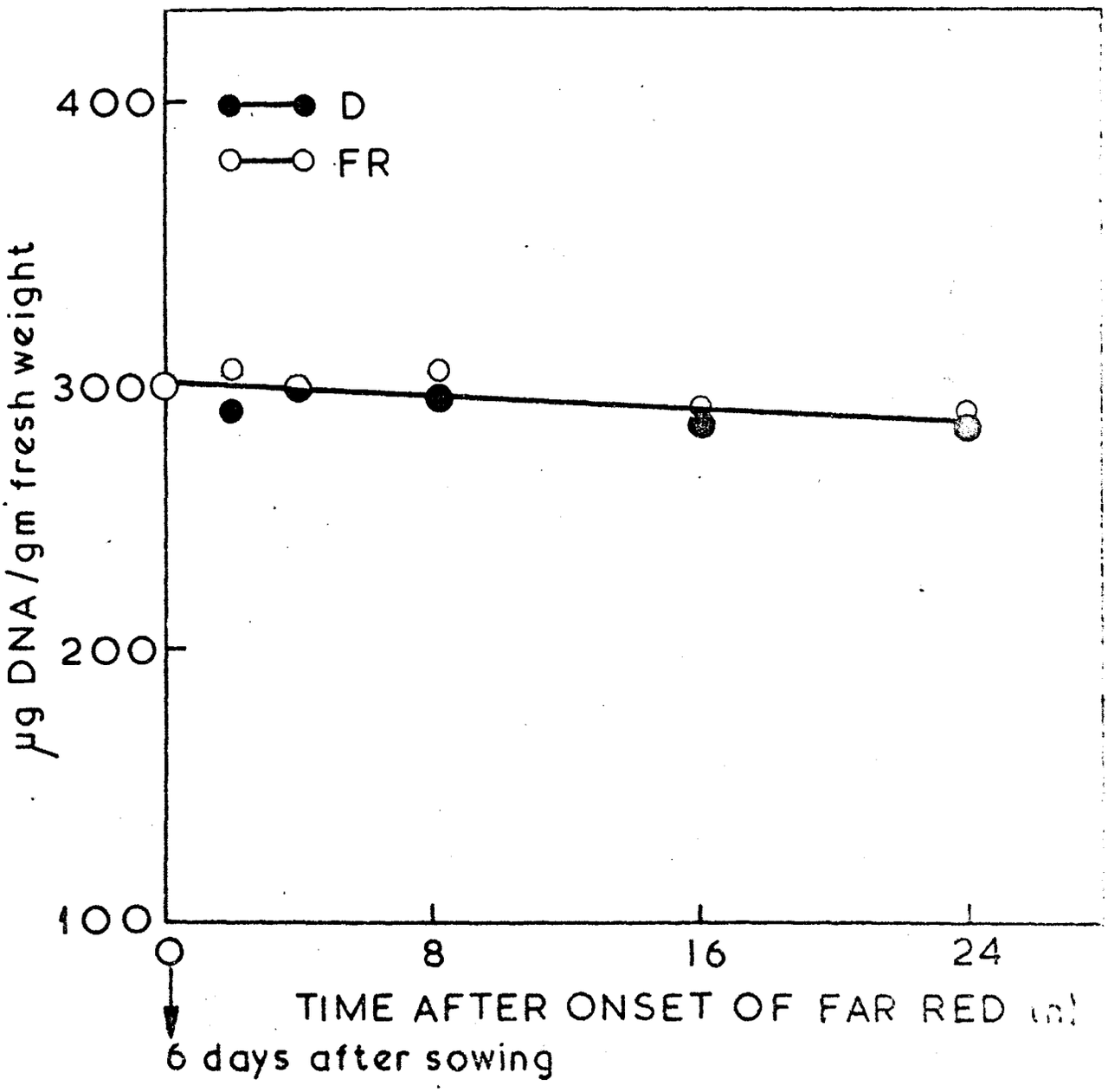


Fig. 21. DNA level of apical leaves of maize under continuous far-red light.

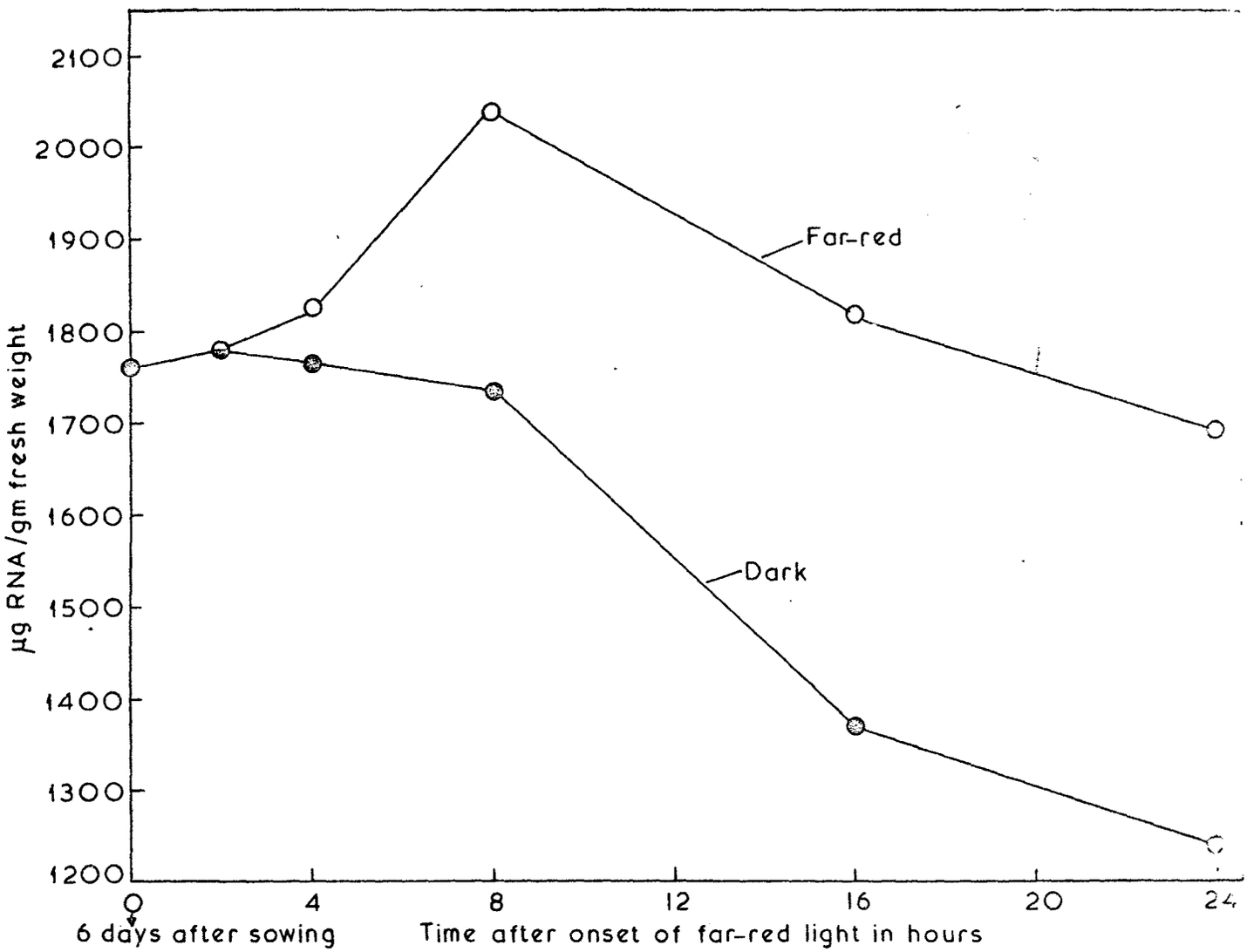


Fig. 22. RNA level in apical leaves of maize under continuous far-red light.

VII. Effect on polyribosomes - The polyribosomal level was about 54% in 6-day old etiolated seedlings which declined to 50% during the next 8 h (Fig. 23, 24). When plants were irradiated with continuous far-red light the level of polyribosome increased after about 2 h of lag phase and by 8 h it was 18% more than the dark control. Polyribosomes isolated from far-red grown seedlings were more active in in vitro incorporation of ^{14}C-lysine into proteins than dark control (Table 23).

TABLE 23

THE INCORPORATION OF ^{14}C-LYSINE INTO PROTEINS BY POLYRIBOSOMES ISOLATED FROM DARK GROWN AND FAR-RED IRRADIATED SEEDLINGS

The values are mean of three different samples.

Treatment	cpm/10$A_{260\ nm}$	RA
D (0 h)	18,650	100
FR (8 h)	25,000	134

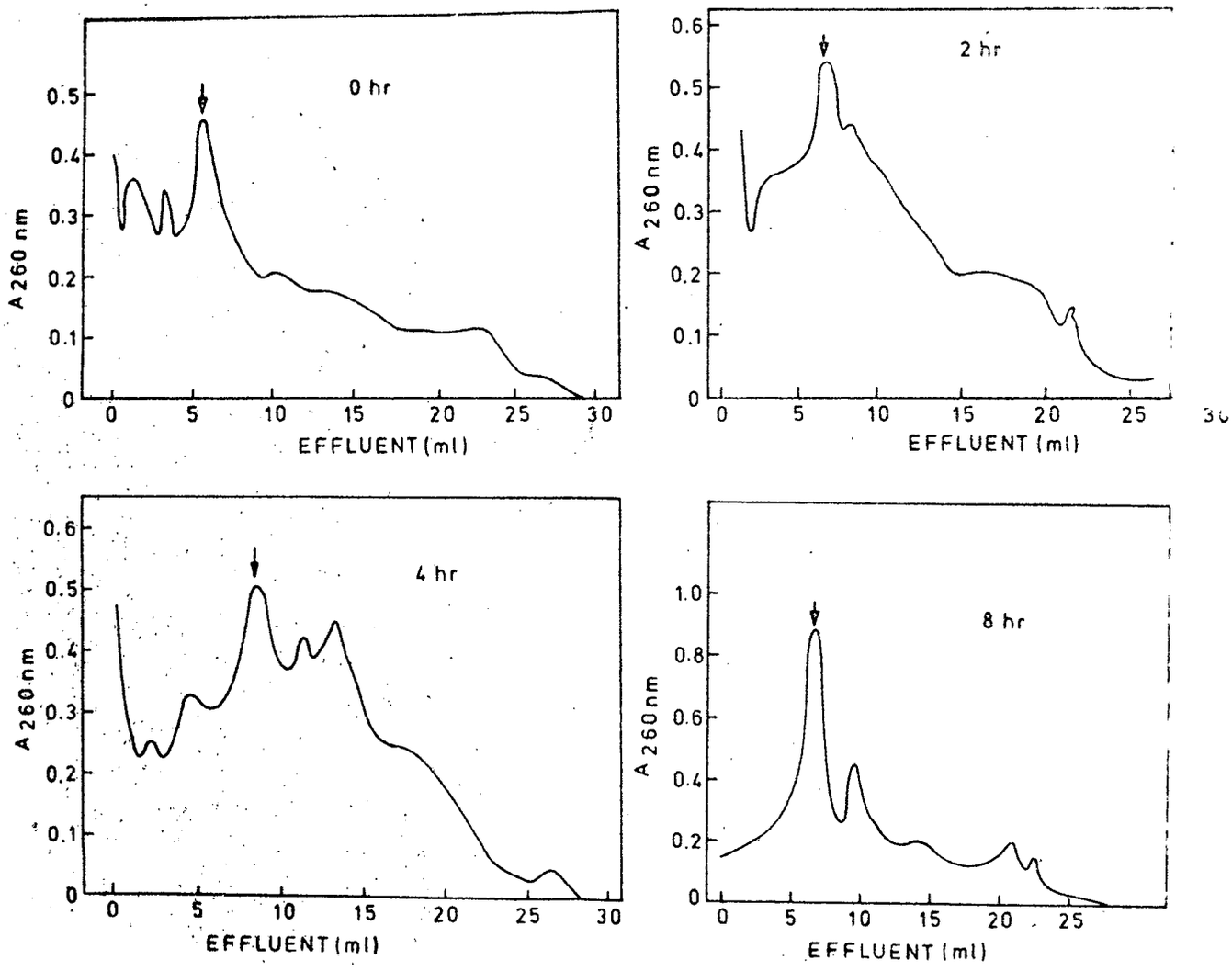


Fig. 23. Polyribosome prolile in apical leaves of maize seedlings at different time of far-red irradiation.

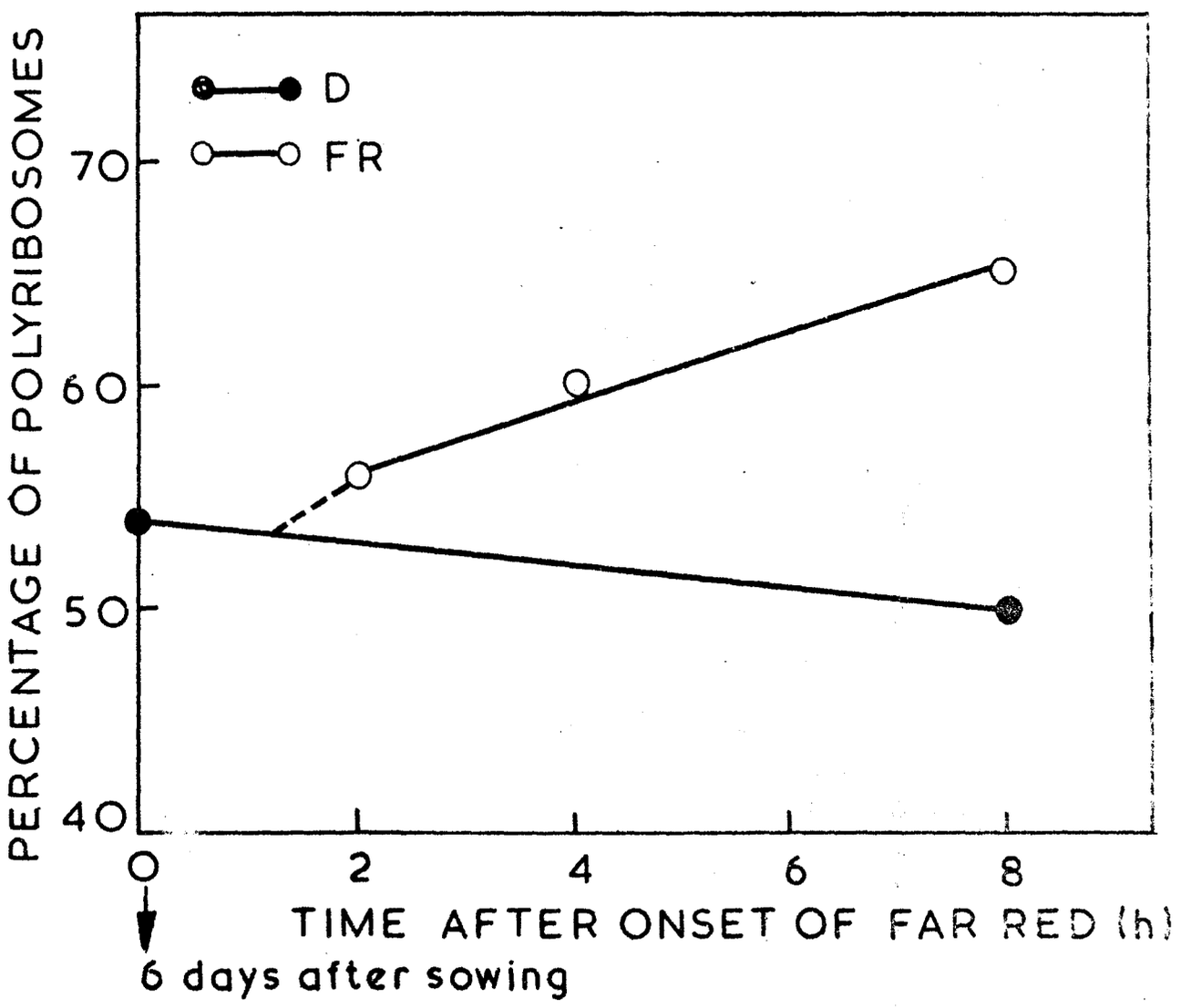


Fig. 24. Time course of total polyribosome level in dark grown and far-red irradiated seedlings . Polyribosome profiles are given in Figure 23.

DISCUSSION

The mechanism by which phytochrome displays its action on plant development is yet unknown. Since development in biochemical terms is an orderly sequence of change in enzyme complement and modulation of activities of certain key enzymes, the role of phytochrome in enzyme regulation has been much investigated. The mechanism by which phytochrome controls activity of enzymes is currently under intense debate (Mohr, 1972; Smith, 1975; Schopfer, 1977). In present investigation, we have endeavoured to decipher mechanism of action of phytochrome in controlling peroxidase activity in plants.

Dependence of Photoresponse on Organ and its Age

Phytochrome enhances peroxidase activity in apical leaves of 6-day-old etiolated maize seedlings (Sharma, 1974). In present investigation, this response was characterized in detail. Phytochrome regulation of peroxidase activity is found to be age dependent (Table 1). There is no enhancement in enzyme activity in 4-day-old seedlings. The photoresponse becomes conspicous in 5-day-old seedlings and increased in magnitude with age. The enhancement in enzyme activity is brought about by a brief irradiation of red light, while a far-red light irradiation of similar duration is ineffective. The reversal of red light mediated enhancement by subsequent far-red light fulfills the

operational criterion for involvement of phytochrome in this response (Mohr, 1972).

The effect of red/far-red light on roots demonstrated a greater degree of variability and modulation of enzyme activity is not significant (Table 2). It shows lack of effective phytochrome involvement in this organ, and points that the photoresponse is organ specific. The age dependence and specificity of photoresponse supports the hypothesis advanced by Mohr (1972) that expression of Pfr-triggered photoresponse depends on 'specific state of differentiation' of particular tissue or cell. In Sinapis alba, phytochrome mediated increase in peroxidase activity is specifically confined to cotyledons and tap root, while in hypocotyl there is an inhibition (Schopfer and Plachy, 1973).

Pfr-mediates through Activators or Inactivation-a Comment

Phenolics are one of the factors which may regulate peroxidase activity in vivo. Russell and Galston (1969) reported that red light mediated modulation of level of phenolics could account for change in the level of IAA-oxidase activity in vivo. They proposed that phytochrome regulates growth by controlling synthesis of cofactors of oxidizing enzyme which catalyzes destruction of auxins. Phenolics also create nuisance during extraction of enzymes, as on oxidation they inactivate many enzymes irreversibly

(Loomis, 1974). This problem is overcome in our experiments by use of insoluble PVP in extraction buffer which adsorbs these phenolics (Loomis, 1974). The presence of such phenolic inhibitors and/or activators were also checked by additive experiments (data not shown). On mixing extracts from dark-grown and light grown plants, enzyme activity was always additive, thereby ruling out the possibility that enhancement in enzyme activity results due to change in level of activators or inactivators.

Localization of Peroxidase

Phytochrome mediated enhancement in peroxidase activity may result from either due to transfer of enzyme in different subcellular components or increase may be confined to some sub-cellular components. Peroxidase in apical leaves is mainly soluble enzyme, the other subcellular fractions had very little activity as compared to the final supernatant, obtained at 150,000 x g (Table 3). This observation is similar to Plesnicar et al. (1967) who reported that about 90% of peroxidase activity is soluble and 6% is associated with microsomes in mungbean seeds. In horse radish roots too, 80% of peroxidase activity is found to be soluble in phase, while 20% is associated with cell wall (Liu and Lamport, 1974). Since the recovery pattern of enzyme activity in dark and far-red light grown seedlings are similar (Table 3), it rules out the possibility

of intracellular transfer of enzyme under influence of phytochrome. In radish, it has been reported, that β-fructosidase transfers from cytoplasm to cell wall in roots and hypocotyl under the influence of phytochrome, and cycloheximide inhibits the Pfr-mediated increase in enzyme activity, but does not affect the transfer (Zouaghi and Rollin, 1976). FR-light mediated increase in peroxidase activity is mainly confined to supernatant (Table 4), this also justify our use of supernatant to study the change in enzyme activity under influence of phytochrome.

Control of Peroxidase Activity by Phytochrome - A Case of Photomodulation -

The enhancement in enzyme activity in seedlings irradiated with continuous far-red light (Fig. 3), demonstrates the operation of 'High Irradiance Reaction' presumably to be operating through phytochrome (Hartmann, 1966; Mohr, 1972; Schopfer and Mohr, 1972; Schafer, 1976; Rabino et al., 1977). Under continuous far-red light the peroxidase activity increases after a lag period of 2 h and reaches a steady state level at 20 h (Fig. 3). The presence of lag phase before the increase in enzyme activity points that the effect of phytochrome is not instantaneous, but requires build up of a potential to initiate the photoresponse. The existence of lag also rules out the possibility of direct photoactivation of enzyme as reported for nitrate reductase in mustard (Johnson, 1976). On transferring 6.5-day-old

seedlings to far-red light, peroxidase activity increase after a lag of 2 h. This observation is contrary to the results of Penel and Greppin (1973), who noted an immediate modulation in peroxidase activity after red/far-red irradiation in spinach and also in membrane vesicles isolated from Cucurbita pepo (Penel et al., 1976). Most of phytochrome mediated enhancement in enzyme activity is preceded by a lag phase e.g., 2 h for inorganic pyrophosphatase in maize (Butler and Benett, 1969) and 3 h for ascorbic oxidase (Drumm et al., 1972) etc. Mohr (1972) assumes that the lag phase can be explained by the time necessary to make genes acessible to far-red irradiation, therefore, no lag phase can be detected in case of secondary irradiation. Huault (1974) has shown activation of inactive phenylalanine ammonia-lyase in cotyledons of Raphanus sativus, and suggests that in this case lag phase is related to the critical level of Ptot, which is about 27 ± 3% of maximum content of Ptot and the presence of lag phase is encountered only when Ptot content is below the critical level.

The termination of far-red light after 12 h of irradiation brings a drop in rate of increase in peroxidase activity, albeit it is maintained at a higher rate than the dark control. Since the termination of far-red irradiation also leads to decline in Pfr level due to Pfr-decay

(Butler et al., 1963), one can correlate enhancement in peroxidase activity and presence of Pfr in the system. In other words, enhancement in peroxidase activity requires continuous presence of Pfr in system. It is evident from kinetics of enhancement that Pfr-regulates peroxidase activity in maize leaves in a manner characteristic of photomodulation, while it is a case of photodetermination in Sinapis alba, (Schopfer and Planchy, 1973). A similar case of photomodulation has been reported for Pfr-mediated ascorbic acid accumulation in mustard seedlings (Bienger and Schopfer, 1970).

The continuous far-red light may not establish a stationary level of Pfr as predicted by Hartmann (1966) and Mohr (1972), because in maize the Pfr-decay is very rapid and get saturated at low energies (Schafer et al., 1975; Butler et al., 1963). Pfr-decay also depends on wavelength and at 750 nm it is slower than at 720 nm, however, level of Pfr is quite low at 750 nm (Schafer et al., 1975). Nevertheless, this low Pfr level controlled peroxidase activity to a significant extent. Since we have employed a filter emitting maximally at 750 nm, the Pfr-decay would have proceded at a slow rate enabling us to detect HIR in controlling peroxidase activity.

The peroxidase activity remains stable in leaves as evident by kinetics of its activity (Fig. 3), once it is enhanced to a high level it does not decline back to the

level of dark control. Such stability of enzyme has earlier been observed for ascorbate oxidase (Drumm et al., 1972) and amylase (Drumm et al., 1971) while in case of PAL its level drops when Pfr is removed from the system indicating a rapid turnover (Dittes et al., 1971). The termination of far-red light could not completely stop increase in peroxidase activity, but the rate of enhancement is slowed down (Fig. 3). It appears that either peroxidase mRNA is stable to maintain peroxidase synthesis or rate of peroxidase turnover is slow.

Does Pfr controls through Hormones, Acetylcholine and c-AMP?

Hormones and phytochrome influence a variety of similar developmental processes in plants, still the participation of hormones in phytochrome mediated responses is controversial (Mohr, 1972; Smith, 1975; Wareing and Thompson, 1976). Hormonal regulation of enzyme activity has been studied in detail, for example, in case of GA induced amylase formation in barley aleurone layers (Mann, 1975). The involvement of hormones in phytochrome regulated enzyme activity, however, has not been investigated in detail. In the present study we have made an attempt to study the role of hormones, ACH and c-AMP in phytochrome metidated regulation of peroxidase activity in maize. Any particular hormone, in order to qualify as a mediator in phytochrome action, should satisfy the following criteria:

1. The *in vivo* titer of hormone should be either controlled by phytochrome and/or phytochrome should modify its distribution, transport or change the affinity of target of action.
2. Exogenously supplied hormone should mimick the action of phytochrome and kinetics of response induced by exogenous hormone should be similar to the kinetics of photoresponse.
3. In case phytochrome does not alter *in vivo* titer of hormone but change the affinity of target of action then in presence of exogenous hormone phytochrome action should either be enhanced or repressed.
4. The inhibitors which are known to affect the hormonal level or counter their action should nullify the phytochrome response.

In the present investigation, we have not studied the first criterion. Phytochrome control of hormonal level, transport and distribution are, however, well known (Black and Vlitos, 1972; Kandeler, 1974; Wareing and Thompson, 1976). In cereals, phytochrome lead to an increase in extractable gibberellin activity (Loveys and Wareing, 1971; Beevers, 1970; Reid *et al.*, 1968), and its transport out of etioplast (Evans and Smith, 1976; Cooke *et al.*, 1975). The control of level of other hormones has not been investigated in cereals, but such changes have been reported for other systems.

Regarding phytochrome mediated change in the affinity of target of action, it can be speculated on the basis of its association to particulate fraction in maize after red light irradiation (Quail et al., 1973).

When the other three criteria were studied, it was found that none of the hormones tested could absolutely be considered as a mediator in phytochrome controlled peroxidase activity in apical leaves of maize. The exogenous supply of GA, kinetin and ACH enhance the peroxidase activity in dark whereas c-AMP and IAA are ineffective (Table 5-7). The magnitude of enhancement is, however, always less than that obtained by 5 minutes of red light or continuous far-red light alone (Table 8). Moreover, the kinetics of enhancement obtained by these hormones are different and usually had a longer lag phase of 4 h, except GA, where it is of 2 h (Fig. 4-7) and the rate of enhancement is much slower than continuous far-red light, which also has a shorter lag time of 2 h (cf Fig. 3) The results with ACH are different from those reported in spinach, where the enhancement in peroxidase activity after ACH or red and far-red light treatment is apparent after only a few minutes (Penel and Greppin, 1973). Gibberellin induces peroxidase in wheat half seeds (Pollard, 1969) and α-amylase in maize (Katsumi, 1970). Kinetin also enhances peroxidase activity in lens roots (Gasper and Xhaufflaire,

1967), while protects in barley leaves (Sharma and Biswal, 1976).

In presence of 5 minutes of red light or continuous far-red light the effect of hormones, ACH and c-AMP was different than in dark (Table 8, Fig. 4-7). Except DB_2 c-AMP, in 5 minutes red light, all others have a slight enhancing effect on peroxidase activity. Under continuous far-red irradiation, the enhancement is inhibited by all these regulators except with GA, which has only a marginal effect. In most cases, the repression in rate of enhancement became apparent only in latter phase of far-red irradiation. In DB_2c-AMP, IAA and kinetin it is apparent after 8 h, whereas with ACH it starts after 4 h.

Hormone mediated repression of photoresponses has been reported earlier also in a few cases e.g. in pea, where both auxins and GA negated phytochrome mediated responses (Russell and Galston, 1969; Purves and Hillman, 1958). Similarly, phytochrome mediated enhancement of phenylalanine ammonia lyase, anthocyanin and carotenoid synthesis is shown to be blocked by exogenous supply of RRB-8, a synthetic growth regulator (Dierickx and Vendring, 1973). In mustard seedlings, GA repressed, when supplied exogenously, the far-red controlled amylase activity (Drumm et al., 1971). With ACH too, a complex interaction with light treatment has been reported in moss callus (Hartmann, 1974). On the basis of

kinetic studies, Giudici de Nicola et al. (1975) also ruled out possibility of kinetin and c-AMP acting as intermediates in FR light-mediated amaranthin synthesis. These compounds enhance the far-red mediated amaranthin synthesis in Amaranthus caudatus, while repress it in A. tricolor. Red light mediated lycopene synthesis in tomato is reversed by GA and kinetin but ABA potentiated this effect (Khudairi and Arboleda, 1971). In the present studies also, since hormone, irrespective of their stimulatory effect on peroxidase activity in darkness, are repressing the enzyme activity under continuous far-red light, it seems that in latter case some secondarly processes are triggered by hormone application which compete with the far-red induced photoresponse, thereby limiting the rate of enhancement.

The application of chemicals, which oppose or interact with the action of hormones, also did not give any results in favour of participation of ACH, c-AMP or hormones in phytochrome control of peroxidase activity (Table 9). Eserine, an inhibitor of ACH esterase (Fluck and Jaffe, 1975) does not affect red light mediated enhancement. We have not tested the effect of AMO-1618, another inhibitor of ACH esterase, which is shown to stimulate the red light mediated uptake of ^{14}C-acetate in mung bean root tip (Jaffe and Thoma, 1973). However, another inhibitor atropine, which blocks ACH receptors (Goodman and Gilman, 1967), also failed to affect the red light mediated enhancement of perxidase

activity, though in Avena coleoptiles it negated the action of ACH (Evans, 1972). Under continuous far-red light, both eserine and atropine repress the enhancement in enzyme activity. Theophylline, an inhibitor of phosphodiesterase (Robinson et al., 1971), is ineffective in dark but inhibited far-red mediated enhancement.

Similarly, the inhibitors of hormones also had no definite effect. Red light enhancement of peroxidase activity is not found to be sensitive to ABA, at a concentration where it blocks the action of most hormones (Milborrow, 1974). CCC, an antagonist of GA biosynthesis (Denis et al., 1965) has little effect under red light, which may be due to its inhibitory action on protein synthesis (Kinsman et al., 1975). Under continuous far-red light the repression by ABA and CCC is never more than 20 to 30%. In mustard seedlings too, Bajracharya et al. (1975) could not find any specific role of ABA in phytochrome mediated photomorphogenesis. Inhibitors employed in present investigation do affect hormonal level in cereal leaves, e.g., the pretreatment with CCC strongly inhibited light mediated increase in gibberellins in cereal leaves (Reid et al., 1968), similar results were also obtained with ABA pretreatment (Beevers et al., 1970).

Light has been shown to promote the formation of catalase isozymes in Sinapis (Drumm and Schopfer, 1974),

and hormones can alter the activity of peroxidases by a qualitative and quantitative change in isozyme pattern (Gasper et al., 1973). In our experiments, no qualitative difference is discernible with respects to different treatments in anionic or cationic isozymes of peroxidase on polyacrylamide gels (Fig. 8).

The involvement of hormones in phytochrome mediated photoresponse can be unequivocally considered only when a particular hormone satisfies all the criteria mentioned before. In the present investigation, although some of the compounds enhanced the peroxidase activity in dark, they can not be considered as a mediator in phytochrome response because, 1) the enhancement in dark is always less than that obtained by 5 minutes of red light or under continuous far-red light alone; 2) the kinetics of enhancement are different with longer lag phase; 3) the inhibitors tested could not bring down the far-red mediated enhancement more than 20-30%. It is very likely, therefore, that the enhancement induced by hormones, and their interactions with phytochrome mediated photoresponse, result from their independent action on a similar process. For instance Gibberellic acid (Wasilewiska and Kleczkowski, 1976) and c-AMP (Tarantowicz-Marek and Kleczkowski, 1975) enhance RNA synthesis in maize seedlings. On the other hand, ABA inhibits nucleic acid metabolism and RNA polymerase activity in maize (Bex, 1972a,b; Tarantowicz-Marek and Kleczkowski, 1975), ABA also

affects membrane properties as it inhibits ion accumulation in maize (Shaner et al., 1975). Any generalization on participation of hormones, ACH or c-AMP on phytochrome mediated photoresponse should be, therefore, carefully interpreted and based on rigorous analysis and not merely by showing the mimicking effect e.g., as has been done for c-AMP (Rast et al., 1973; Weintraub and Lawson, 1972) and ACH (Penel and Greppin, 1973).

Role of Photosynthesis in HIR

The nature of photoreceptor(s) controlling HIR response has been a matter of much discussion. Some people support the idea that phytochrome is the sole receptor involved in HIR responses (Borthwick et al., 1969; Hartmann, 1966; Mohr, 1972), whereas other argue for existance of a second photochemical system besides phytochrome (Grill and Vince, 1970; Schneider and Stimson, 1971, 1972; Downs et al., 1965). There is some evidence that this second photochemical system involved in HIR could be photosynthesis (Creasy, 1968; Downs, 1964; Downs et al., 1965; Schneider and Stimson, 1971, 1972).

I. Effect of Photosynthetic inhibitors - One of the principle argument in favour of participation of photosynthesis in HIR is effectiveness of photosynthetic inhibitors to block FR-mediated HIR (Schneider and Stimson, 1971, 1972). The inhibitors of cyclic PP like DNP and antimycin-A and

noncyclic PP like DCMU and NH_4^+ brought a reduction in enhancement in peroxidase activity mediated by far-red light (Table 10, 11). Schneider and Stimson (1971, 1972) proposed that in FR-mediated HIR besides phytochrome, cyclic PP also participates, on the basis of inhibition of FR-mediated anthocyanin synthesis by inhibitors of cyclic PP. Their proposal is, however, based on data obtained by Arnon et al., 1967) on effect of these inhibitors on PP in isolated chloroplasts. Therefore, the use of these inhibitors may not represent a true picture, as in vivo effect of these inhibitors may be considerably different from in vitro studies. In fact, these inhibitors stimulated respiration to some extent in treated seedlings in our studies (Table 12), though significant difference could not be seen. This may perhaps be due to use of excised leaves, as excision itself stimulates respiration, thereby making effect of inhibitors. It has been shown that these inhibitors are not specific in vivo and they do effect other cellular processes. In Chlorella for example, DNP at a concentrations which inhibits endogenous oxidative phosphorylation and increases the endogenous respiration maximally, do not inhibit photosynthesis (Kandler, 1958). Furthermore, both DNP and DCMU inhibit RNA and protein synthesis (Gruenhagen and Moreland, 1971; Moreland et al., 1969). Antimycin-A is a potent inhibitor of oxidative phosphorylation (Pomeroy, 1975; Storey, 1972) and NH_4^+ may affect respiration by blocking citric acid cycle

(Wakiuchi et al., 1971). The specificity of inhibitors is also questioned by Mancinelli et al. (1974), while studing far-red mediated increase in anthocyanin in cabbage and mustard seedlings.

II. Development of photosynthetic pigments in continuous far-red light - As the participation of photosynthesis in FR-mediated HIR can not be conclusively demonstrated by use of inhibitors, we have therefore alternatively studied the development of photosynthesis under continuous far-red light and compared it with the normal development under white light. Mohr and his associates (Mohr, 1972; Masoner et al., 1972) ruled out the possible contribution of photosynthesis in FR-mediated HIR in mustard seedlings, as there is no significant chlorophyll development under FR light. However, in maize as we can measure there is a substantial increase in chlorophyll level under FR light although it is much less in comparison to chlorophyll level under white light (Fig. 9, 10). The slow chlorophyll accumulation under far-red light is obvious as wavelengths only in the blue and red region are most effective, while far-red is least effective in mediating chlorophyll development (Koski et al., 1951). The characteristic peak at 663 nm of extract of pigments in 80% acetone confirmed their identity as chlorophylls (Fig. 11). There is no lag in chlorophyll development, both chlorophyll a and b developed simultaneously

(Fig. 9, 10). Chl. b development was slower in FR light then in white light as evident by high Chl a/b ratio under FR light. Shylk et al. (1970) have also obtain similar results in maize seedlings that Chl. b appeared within a few minutes of initial transformation of protochlorophyll. On the other hand, in bean (De Greff et al., 1971; Weistrop and Stern, 1977), no Chl b development take place upto 48 h of far-red irradiation. Beside chlorophyll, FR light also influenced development of photosynthetic accessory pigment carotenoids (Fig. 12). Red/far-red reversible control for carotenoid synthesis has been reported for maize seedlings (Cohen and Goodwin, 1962). Our observations confirm this by further demonstrating the operation of HIR in controlling carotenoid level.

Although there was substantial development of chlorophyll under continuous far-red light, there seemed to be no direct relation of this response with the increase in peroxidase activity by far-red light. In our experiments, the exogenous application of chloramphenicol in maize, though inhibits chlorophyll biosynthesis (Table 13) does not affect peroxidase activity under far-red light (Table 14). Similarly, another greening inhibitor, actinomycin D (Bogorad and Jacobson, 1965) also have no significant effect on peroxidase activity (Table 14). A similar conclusion is reached by Mancinelli et al. (1974, 1975) by using chloramphenicol and streptomycin which inhibit chlorophyll biosynthesis

but enhance FR light induced anthocyanin formation. Schneider and Stimsan (1972), however, have shown that inhibition of chlorophyll biosynthesis by levulinic acid also inhibited FR-mediated HIR in inducing anthocyanin synthesis.

Since there is a significant development of photosynthetic pigments under FR light, it seems quite possible that functional development of photosynthesis may also take place under FR light. Wellburn and Wellburn (1973) showed that FR light triggers development and differentiation in isolated plastids *in vitro*. The absorption spectra of isolated plastids from leaves greened under far-red light shows a peak in red region at 673 nm, while isolated plastids from leaves greened under white light shows a peak at 678 nm and characteristic soret band in blue region (Fig. 13). The position of peak at 673 nm indicates the organisation of plastid is not complete in far-red light. This is evident by the fact that the peak position is at 678 nm in plastids isolated from white light-grown seedlings. During development to mature organelle this peak in red region always shifts towards 678 nm (De Greff *et al.*, 1971), while treatments of mature chloroplasts to disorganization agents shifts peak toward the blue region (Biswal and Mohanty, 1976). In bean leaves greened under far-red light shows that although prolameller body disappears and thylakoids

are formed in plastids, there is no fusion of thylakoids to form grana unless plants are irradiated with white light (De Greff et al., 1971). It appears that though far-red light triggers pigment development, the plastids are not fully differentiated to be optimally active in photosynthesis. This is also evident by the fact that photochemical electron transport chain is not complete in far-red irradiated seedlings, as there is no light mediated O_2 evolution in vivo till 24 h of far-red irradiation, whereas this capacity develops at 8 h in light grown seedlings (Fig. 14). In bean leaves greened under far-red light, onset of photosynthetic O_2 evolution is detected after 24 h of irradiation (De Greff et al., 1971).

III. Effect of far-red light on development of photochemical activities - The onset and development of various photochemical activities under continuous far-red light were also studied by using isolated plastids. In all cases the development in photochemical activity is preceded by a lag phase. PSI develops after 2 h of irradiation under white light, while under far-red light it develops after 4 h (Fig. 16). Both under FR light and white light, PSII activities could be detected after 4 h (Fig. 16). The cooperation between two photosystems also develops along with the development of PSII (Fig. 17). The basis for expressing results of development in photochemical activities merit some discussion. The results in present

investigation are expressed both on the basis of chlorophyll and fresh weight of leaves. It is observed that whereas there is an enormous change in chlorophyll content with duration of irradiation, fresh weight does not change to the same magnitude. Furthermore, due to the difference in chlorophyll content in far-red light and white light grown seedlings, the activities can be better compared on fresh weight basis than on chlorophyll basis. When results are expressed on chlorophyll basis, the photochemical activities show a peak at 8 h, which decline at 16 h (Figs. 15-17) in white light-grown plants. Such a curve is not too evident when data are expressed on fresh weight basis. Under far-red light, however, such a characteristic peak at 8 h of irradiation is never noticed, however, activity of PSI and cooperation between PSII-PSI shows decrease with increase in amount of chlorophyll molecules. This occurrence of peak at 8 h in white light grown plants and declined of PSI and PSII-PSI activities in far-red grown plants may arise from different rate of synthesis of reaction center chlorophyll molecules and bulk chlorophyll molecules. It seems that reaction center molecules are synthesized predominantly in initial phase than the bulk chlorophyll molecules, thereby, resulting in high photochemical activity, when expressed on the basis of chlorophyll. However, during latter phase synthesis of bulk chlorophyll is faster than reaction center species, which lead to an apparent decline in photochemical activity. On

the basis of fresh weight, increase in activity is stationary in between 8-16 hours of white light irradiation. Similar curves in photochemical activity development on chlorophyll basis was also observed in barley, while it was absent when results were expressed on basis of fresh weight (Egneus et al., 1972). It is difficult to decide at present, whether this rise in photosynthetic activity reflect a higher rate of synthesis of reaction center molecule as compared to bulk chlorophyll or change in other properties, (e.g., change in enzyme properties) associated with photosynthetic unit. The evidence support the former view, since it has been observed by Faludi-Daniel et al. (1970) that between 3-12 h of greening of maize plastid, the synthesis of reaction center molecule P_{700} lagged behind synthesis of bulk chlorophyll. The cooperation in PSII-PSI remained at a constant level under far-red light on fresh weight basis, while PSI and PSII activity increased during same period (Fig. 15-17). It seems that some key component of electron transport chain is limiting in far-red grown plants, so cooperation remained at a steady state level. It is also supported by the fact that CO_2-dependent $-O_2$ evolution also could not be detected in vivo in far-red grown plants (Fig. 14), pointing to incomplete nature of electron transport chain.

In white light, both cyclic and noncyclic PP developed after 8 h of irradiation, while under far-red light cyclic

PP could be detected at 8 h, but noncyclic PP could be dectected only at 24 h (Fig.18,19). Our results are not in agreement with earlier reports where cyclic PP could be detected within 1.5 h of white light irradiation (Forger and Bogorad, 1971; Arntzen, 1970). Oelze-Karow and Butler (1971) detected DCMU insensitive ATP changes in bean leaves after 12 h of far-red irradiation presumbly representing cyclic PP *in vivo*, while DCMU sensitivity appeared at 24 h. However, such light induced change in ATP level *in vivo* may not necessarily be due to cyclic PP as light can also independently modulate ATP level as observed in bean (White and Pike, 1974; De Greff et al., 1976). The hypothesis elaborated by Schneider and Stimson (1971, 1972) is based on the fact that although spectrum of photosynthesis is localised in blue and red region, under FR light (> 700 nm) the cyclic PP mediated by PSI can operate. In *Chlorella*, it was shown that cyclic PP may provide energy for the induction of isocitrate lyase (Syrett, 1966). However, such participation of cyclic PP in enhancing peroxidase activity is not obvious, as under FR light cyclic PP develops much latter than the enhancement in peroxidase activity. The occurrence of cyclic PP, its capacity and physiological significance is also uncertain and controversial (Heber, 1969, 1973). In particular, the published rates of endogenous cyclic PP of isolated intact chloroplasts are extremely low, e.g., about 0.2-5 μ moles/mg Chl. h, whereas rate of CO_2 fixation may reach 200 μ moles/mg Chl.h (Kaiser and

Urbach, 1974). High rates of cyclic PP have only been observed *in vitro* in the presence of suitable cofactors such as phenazine methosulfate (Tanner *et al.*, 1969), *in vivo* cyclic PP furnishing ATP occurred under nitrogen, and Heber (1969) reported that in presence of oxygen it is suppressed and replaced by pseudocyclic electron transport. Heber (1973) further presented evidence that light dependent-increase of both chloroplasts NADPH and ATP is suppressed by DCMU at concentrations which are ineffective in suppressing PSI-dependent cyclic PP of broken chloroplasts. Simnois and Urbach (1973) considered that cyclic PP may be a relic of evolutionary past and exists today preferentially in lower plants. Mohanty *et al.* (1971) showed that in *Porphyridium* cyclic PP is absent in mature cells, but may be present during early developmental stages. Since enzyme enhancement occurs predominatly in cytoplasm (Table 4), the participation of cyclic PP in such response would also require transport of generated ATP to cytoplasm, but high energy compounds like ATP and NADP can not cross the plastid membrane barrier *in vivo* (Heber, 1974). Only compounds which can be exchanged are 3-phosphoglyceric acid and dihydroxy acetone phosphate, whose generation depends on active CO_2 fixation by plastids, which is absent in seedlings grown under far-red light, as there was no light mediated CO_2-dependent-O_2-evolution (Fig. 14).

Our results on development of various photochemical activities are in aggrement with earlier reports. The development of PSI activity preceded PSII activity, however both cyclic and noncyclic PP develop at the same time in white light. Butler (1965) using light induced fluorescence changes demonstrated that PSI was active before PSII, in greening bean leaves. In greening bean leaves, Hill reaction could be detected in 5-10 h but NADP reduction appeared only after 16 h (Gyldenholm and Whatley, 1968). In this case cyclic PP appeared at 8 h, while noncyclic PP appeared at 10 h. In another study on bean leaves both cyclic and noncyclic PP could be detected at 3-4 h, but efficient coupling to electron transport chain was achieved only after 15-18 h (Howes and Stern, 1973). In barley PSI activity appeared within five minutes of illumination, while PSII activity appeared after 3 h of illumination (Egneus et al., 1972). In isolated plastids from far-red grown bean plants, cyclic PP was detected by 8 h, while noncylic electron transport and PP could only be measured after 12 and 16 h of far-red irradiation respectively (Weistrop and Stern, 1977). It seems that there is quite a variation in different species in onset and subsequent development of photochemical activity, furthermore, age and cultivar difference may also affect sequence of development.

It's evident from the above discussion that photosynthesis plays no role in FR-mediated HIR regulating peroxidase activity in maize. The onset and development of all photochemical activities under far-red light lag behind

the FR-mediated enhancement in peroxidase activity. The participation of PSI-mediated cyclic photophosphorylation in HIR can not be favoured, as it develops much later than increase in peroxidase activity. It seems that photosynthesis has no role in FR-mediated HIR atleast in etiolated maize seedlings and the effect of phytochrome on peroxidase activity and photosynthetic activity development are quite independent of each other.

Pfr-regulation of Peroxidase Activity at Transcription or Translation level

I. Effect of inhibitors - One obvious prediction of differential gene activation hypothesis is de novo synthesis of enzyme molecules in positive photoresponse under influence of Pfr. Evidence for this has come mainly from use of the inhibitors of transcription (Carr and Reid, 1966; Mohr and Bienger, 1976; Dittes et al., 1971; Drumm et al., 1971) and translation (Drumm et al., 1971, 1972). These inhibitors either reduce or prevent the phytochrome effect. In present investigations chloramphenicol an inhibitor of organelle protein synthesis, do not affect Pfr-mediated enhancement in peroxidase activity (Table 14) thereby ruling out the possibility of participation of organelles ribosomes in this response. The ineffectiveness of actinomycin-D to repress the enhancement of peroxidase activity suggests that there is no major participation of transcription (Table 14). The phytochrome-mediated enhancement in peroxidase activity in

the present system probably results from its synthesis on cytoplasmic ribosomes, as puromycin completely prevented the enhancement (Table 15). In sweet potato, actinomycin-D inhibited RNA synthesis by 60-70%, but did not block increase in peroxidase activity (Matsushita and Uritani, 1975). These authors interpreted that either actinomycin-D fails to repress mRNA synthesis for peroxidase, or peroxidase is synthesized on preformed mRNA which has relatively longer half life. Similar suggestions were also made for isolated wheat embryo by Taneja and Sacher (1976), in this system, while actinomycin-D (100 µg/ml) failed to repress peroxidase activity, cycloheximide (10 µg/ml) repressed it by 82%, at this concentration actinomycin-D inhibited RNA synthesis by 70% while cycloheximide inhibited protein synthesis almost completely. In our investigations also while actinomycin-D blocks RNA synthesis significantly (Table 21), it had very less effect on peroxidase activity (Table 14), indicating for a possible existance of a stable mRNA of peroxidase.

The enhancement in peroxidase activity in presence of cycloheximide (Table 16) suggests the existence of proteinaceous inhibitor of peroxidase with rapid turn over rate, however, alternative interpretations are also possible. Cycloheximide probably inhibits the synthesis of this inhibitor thus resulting in an enhancement in peroxidase

activity due to reduction in its rate of inactivation. The differential response noted at different concentrations of cycloheximide may be due to its differential effect on synthesis of peroxidase and inhibitor protein. At low concentration (5 µg/ml), cycloheximide may stop inactivation but may not hinder the synthesis of peroxidase thereby resulting in enhanced activity. This may be the reason for the differential increase observed in peroxidase activity in plants kept in darkness and those treated with far-red light (Table 16). At higher concentration (20 µg/ml) of cycloheximide, enzyme synthesis may also be inhibited thereby leading to an overall reduction in magnitude of enhancement. An almost identical response was noted in sunflower discs by Creasy et al. (1974), where on comparing cycloheximide mediated inhibition at different concentrations they showed that inactivation was much more sensitive to cycloheximide than the phenylalanine ammonia-lyase formation. From the kinetics of enzyme activity in presence of cycloheximide and continuous far-red light it seems that they do not have a common mode of operation (Fig. 20). Cycloheximide probably increased peroxidase activity by inhibiting enzyme inactivation, while in far-red light fresh enzyme synthesis takes place. The stationary level of peroxidase after enhancement by cycloheximide further supports the contention that peroxidase is a stable enzyme and does not decay during the course of investigation (Fig. 20).

Suggestions for the presence of inactivators, regulating enzyme activity, have been made for other systems too. These inactivators are classified as binding inhibitors, specific proteinases and modifying enzymes. Binding inhibitors are known for potato invertase (Matsushita and Uritani, 1976; Pressy, 1967) and phenylalanine ammonia-lyase (French and Smith, 1975). Wallace (1973) showed the existence of specific proteinases inactivating nitrate reductase in maize. The results of Blondel et al. (1973) suggest the existence of some modifying enzymes for phenylalanine ammonia-lyase.

Oaks and Jonson (1973) found that cycloheximide causes an accumulation of glutamine in maize roots. Glutamine in certain cases has been reported to affect activity of certain enzymes, e.g., asparagine synthetase (Rognes, 1970) and peroxidase (Dezsi et al., 1970), and cycloheximide may act as an antagonist of glutamine (Ross, 1974; Jones, 1977; Oaks and Johnson, 1973). These observations prompted us to investigate a possible relationship between cycloheximide-mediated enhancement and glutamine. Exogenous glutamine enhances the enzyme activity in etiolated seedlings maximally at 5 mM (Table 17), however, Dezsi et al. (1969) got enhancement in peroxidase activity at 150 mM. The enhancement in peroxidase activity is greater in presence of glutamine in far-red irradiated seedlings, but this effect is neither synergistic nor additive (Table 18). Cycloheximide does not act as an antagonist to

glutamine since at equimolar concentrations cycloheximide mediated enhancement is not prevented by glutamine (Table 19). In maize roots cycloheximide mediated inhibition in asparagine formation was relieved by exogenous glutamine (Jones, 1977). The possibility of cycloheximide-mediated change in glutamine level affecting peroxidase activity requires further investigation.

The use of inhibitors although suggests possible involvement of nucleic acid and protein synthesis, but by no means provides a proof for such a mechanism as the in vivo specificity of these inhibitors is questionable. In fact cycloheximide shows too many side effects in vivo e.g., inhibition of ion uptake, respiration (Ellis and MacDonald, 1970) and many other metabolic processes (McMahon, 1975). Similarly, chloramphenicol also inhibits ion uptake and oxidative phosphorylation (Ellis, 1969) and energy transfer in chloroplasts (Wara-Aswapati and Bradbeer, 1974)

II. Effect on DNA and RNA synthesis - The effectiveness of inhibitors of protein synthesis in repressing Pfr-mediated enhancement in peroxidase activity suggests a possible role of protein and nucleic acid synthesis in phytochrome action. In seedlings irradiated with continuous far-red light, DNA content in leaves is not changed (Fig. 21), whereas RNA level

increased after a lag phase of 2 h, reached its maximum at 8 h, then gradually declined, at a slower rate than the control (Fig. 22). This decline may result from the change in the rate of RNA synthesis and/or degradation. Gyurjan (1974) reported that white light has no effect on DNA level in maize leaves while it increases RNA synthesis, particularly of plastid rRNA. Weidner (1967) also could not detect change in DNA content in mustard cotyledons under far-red light while rRNA level increased in both cytoplasm and plastids after a lag period of 6 h and 12 h respectively (Weidner and Mohr, 1967; Thien and Schopfer, 1976). In barley leaves, synthesis of rRNA of cytoplasm precedes that of plastid rRNA, in light (Poulson and Beevers, 1970). In the present investigation we have not characterized the nature of the RNA components influenced by far-red irradiation, nevertheless existing studies suggests that it is mainly localised in rRNA.

In contrast to continuous far-red light a brief red irradiation does not bring about significant change in the RNA level (Table 22). Such changes have been reported for mustard cotyledon (Weidner and Mohr, 1967) and pea buds (Jaffe, 1969). In pea buds after brief red irradiation, rRNA level increased after 2 h to a maximum at 6 h (Koller and Smith, 1972). The efforts pertaining to demonstrate change in mRNA has so far met with failures (Dittes and Mohr, 1970),

however, a change in tRNA was reported (Okoloko et al., 1970). In maize, Harel and Bogorad (1973) after 2.5 h of light treatment detected a new RNA species presumably mRNA. A clear cut demonstration of phytochrome control of mRNA is still awaited, however, light mediated change in level of PAL mRNA has been reported (Ragy et al., 1977; Schrader, 1977). Phytochrome mediated increase in RNA level may result from a change in RNA polymerase activity. Light mediated changes in RNA polymerase activity have been reported for barley (Poulson and Beevers, 1970) and pea (Ellis and Hartley, 1971; Bottomley, 1970) and plastid and nuclear polymerase of maize (Bogorad, 1967; Stout et al., 1967).

III. Effect of Pfr on polyribosome formation - The synthesis of proteins takes place on ribosomes, which aggregates on mRNA to form polyribosome complex. The amount of polyribosomes, in a cell or tissue, is, therefore an indirect indication of rate of operation of protein synthesizing system. In present study, far-red light induces polyribosome formation after 2 h lag and it increase by 18% over the control by 8 h (Fig. 23, 24). Furthermore, polyribosomes isolated from FR grown plants incorporated ^{14}C-L lysine into proteins more actively than those from the dark control (Table 23). Our results on polyribosome level though different from earlier ones (Travis et al., 1970),

can be easily explained since we isolated total polyribosomes, comprising of membrane bound, free and organelle polyribosomes by the inclusion of Triton X-100, a membrane disrupting agent in homogenizing medium, while Travis et al.(1970) isolated only free cytoplasmic polyribosomes.

Nevertheless, results on the effect of continuous far-red light on polyribosome level and activity are quite comparable to the ones reported for maize seedlings. William and Novelli (1964) reported that white light enhanced protein synthesis in isolated polyribosomes in maize after a lag of 1.5 h to a maximum at 3 h and remained unchanged till 11 h. In a further study, they reported that white light-mediated increase in the polysome level was accompanied with increased in vitro leucine incorporation (Williams and Novelli, 1968). A lag phase of 2 h preceded the white light effect on in vitro incorporation of ^{14}C-leucine into protein, and of different light treatments, red light was most effective. Travis et al. (1970) also reported light-mediated increase in polyribosome level in maize, which was correlated with increase in activity of nitrate reductase (Travis and Key, 1971). Red light also activated monoribosomes in maize by increasing the level of peptidyl tRNA associated with ribosomes (Travis et al., 1974). It is very evident from these studies that light plays an important role in regulating protein synthesis by controlling polyribosome level in

maize and that the action of far-red light is very similar to that of white light. Since in our studies too, the lag period for enzyme induction was roughly equivalent to the lag found for polyribosomes formation, it seems that phytochrome mediated increase in peroxidase activity may result from the increase in polyribosome level. It is also apparent from our earlier experiments, that peroxidase mRNA may be a stable one. The presence of stable mRNA in seed development is also well documented (Weeks and Marcus, 1971) and similar suggestion was advanced for stored peroxidase mRNA in wheat embryo (Taneja and Sacher, 1976). The enhancement in peroxidase activity may result from increased level of polyribosome leading to increased rate of translation of mRNA of peroxidase. Travis and Key (1971) also speculated same type of translational control for light-mediated induction of nitrate reductase in maize leaves.

Recently, Smith (1976) has advanced a model suggesting translational control by phytochrome of protein synthesis on the basis of studies conducted on bean and other plants. In bean, inhibition of mRNA synthesis by cordycepin did not affect the light-mediated development of polyribosomes, and the lag phase (1 h) of polyribosome development was shorter than that of RNA synthesis (2 h). In a recent publication (Giles et al., 1977), they have further shown

that light mediated enhancement in protein synthesis may be due to mobilization of stored mRNA into polyribosomes in bean leaves. Although the classicial operon concept of Jacob and Monod (1961) argued that all regulation of gene expression should be at the transcriptional level for bacterial system. Sufficient experimental evidences have now been accumulated that in eucaryotes, amount of enzyme in tissue may be a direct result of post transcriptional control. The results obtained in present investigations also suggest some post transcriptional control for Pfr-regulation of peroxidase level in maize leaves.

SUMMARY

The present investigation was carried out with an aim to decipher mechanism of regulation of peroxidase activity in apical leaves of etiolated maize seedlings by phytochrome.

A brief irradiation of red light to maize seedlings lead to enhancement in peroxidase activity in leaves, the onset and magnitude of photoresponse was age dependent. Phytochrome participation in this photoresponse was established by reversibility of red light effect by far-red light. The kinetics of peroxidase activity was followed under continuous far-red light, where phytochrome operates through 'high irradiance reaction'. The enhancement in peroxidase activity was preceded by 2 h lag and reached stady state level after 20 h of irradiation. The kinetics of enhancement in peroxidase activity was essentially that of photomodulations, as it depended on continuous presence of Pfr in system. Peroxidase in leaves was found to be a soluble enzyme and phytochrome mediated increase was confined to supernatant fraction.

The role of hormones, acetylcholine and c-AMP as intermediates in phytochrome action was also studied. When supplied exogenously kinetin, gibberellin and acetylcholine enhanced peroxidase activity in dark but c-AMP and indole acetic acid were ineffective. With

brief red light irradiation, peroxidase activity slightly enhanced in presence of hormones and acetylcholine but not with c-AMP. Under continuous far-red light in presence of hormones, acetylcholine and c-AMP enhancement in peroxidase activity was less in magnitude than control. Furthermore, kinetics of enhancement in presence of kinetin, gibberellin and acetylcholine in dark were different from those elicited by far-red light. The application of abscisic acid, CCC and inhibitors of c-AMP and acetylcholine metabolism did not significantly effect the peroxidase activity in dark or in red light (5 minutes). Under continuous far-red light these compounds reduced magnitude of enhancement by only 20-30%. The results did not favour any participation of hormones, acetylcholine and c-AMP as mediator of phytochrome action. There was no qualitative difference in isozyme complement of peroxidase during various light and chemical treatments.

The participation of photosynthesis in far-red light mediated 'high irradiance reaction' was also investigated. Continuous far-red mediated enhancement in peroxidase activity was repressed by inhibitors of cyclic and non-cyclic photophosphorylation. Under far-red light the chlorophyll development was minimal and plastids were only partially differentiated. Isolated plastids from far-red grown plants developed photosystem I and II activity after

a lag of 4 h, cyclic photophosphorylation after 8 h and noncyclic photophosphorylation at 24 h. Far-red grown plants however failed to show CO_2-dependent oxygen evolution uptill 24 h of irradiation. The magnitude of various photochemical activities developed under far-red light was considerably lower than that developed under white light. Photosynthetic participation in far-red mediated high irradiance reaction was excluded as it had longer lag than the onset of enhancement in enzyme activity and its magnitude was minimal.

The role of transcription and translation in phytochorome mediated enhancement was also investigated. Far-red light had no effect on DNA level, but increased RNA level after 2 h of lag period. The inhibition of protein synthesis in cytoplasm repressed enhancement in peroxidase activity while inhibitors of RNA and organelle protein synthesis were ineffective. Cycloheximide had anomalous effect on peroxidase activity, it enhanced it in dark and far-red grown plants. It was suggested that such an effect of cycloheximide may arise due to presence of a proteinaceous inhibitor with high turnover rate and whose synthesis is more sensitive to cycloheximide than that of peroxidase. Far-red light increased total level of polyribosomes after 2 h lag period and also enhanced the <u>in vitro</u> incorporation of amino acids into protein by isolated polyribosomes, as

compared to dark grown plants. These results point that phytochrome regulation of peroxidase activity may have its locus of action at post transcriptional level.

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PHYTOCHROME REGULATION OF PEROXIDASE ACTIVITY IN MAIZE

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SUMMARY

Phytochrome (Pfr) regulates peroxidase activity in maize in a manner characteristic of photomodulation. The increase in peroxidase activity under continuous far-red (FR) light is sensitive to puromycin but not to chloramphenicol and actinomycin D. Cycloheximide (CHI) enhanced the peroxidase activity, both in dark-grown and FR-irradiated seedlings. However, the kinetics of enhancement of peroxidase activity by continuous FR light and CHI is different.

INTRODUCTION

Pfr controls activity of different enzymes in a differential manner resulting either in an increase [1] or decrease in enzyme activity [2]. The exact mechanism by which Pfr displays its action on the enzyme activity is currently under intense debate. It may control the enzyme activity either by repressing or by derepressing genes leading to de novo synthesis of enzymes [3—6], or it may activate preformed inactive enzyme molecules [7].

The kinetics of Pfr-mediated increase in enzyme activity, in the presence or after removal of Pfr from the system, has revealed the operation of two kinds of control mechanisms viz. photomodulation and photodetermination [8]. In photomodulation, the manifestation of a photoresponse intimately depends upon the continuous presence of Pfr in the system, whereas in photodetermination the response once triggered by Pfr can continue even in its absence.

In this communication we present data to show that Pfr-mediated increase in peroxidase activity in maize occurred in a manner characteristic of photomodulation and not like photodetermination as has been reported in *Sinapis alba* [9], and that this regulation can be best interpreted in terms of Pfr-mediated de novo synthesis of the enzyme.

Abbreviations: CHI, cycloheximide; d, darkness; FR, far-red; Pfr, physiologically active form of phytochrome.

MATERIAL AND METHODS

Plant material

Seeds of *Zea mays* var. Shakti, obtained from the Indian Agricultural Research Institute, New Delhi, were sown on absorbent paper moistened with distilled water in petri dishes. Seeds were germinated in the dark at 27°C ± 1°C.

Light sources and treatments

Unless otherwise indicated, light treatments were given to 6-day-old dark-grown seedlings. Red light (500 μW cm^{-2} sec^{-1}) was obtained from two 100-W lamps wrapped with a double sheet of red cellophane paper (emission maxima 650 nm) and FR light (140 μW cm^{-2} sec^{-1}) from a 300-W tungsten lamp after filtering through a CBS-750 filter (emission maxima 750 nm) covered with 2 cm of water. The temperature during irradiation was maintained at 27° ± 1°C.

Application of inhibitors

Plants were treated with actinomycin D (8 μg/ml), puromycin (100 μg/ml), CHI (5 and 20 μg/ml) and D-threo-chloramphenicol (50 and 250 μg/ml), 2 h prior to onset of continuous FR irradiation. To study the kinetics of enzyme activity in the presence of CHI, plants were transferred to petri dishes containing inhibitor solution only 5 min prior to onset of continuous FR irradiation. In all cases, control plants were supplied with distilled water only.

Extraction of peroxidase (EC 1.11.1.7)

200 mg of apical leaves were ground in 2 ml of 0.1 M phosphate buffer, pH 7.0 with 50 mg insoluble polyvinyl polypyrrolidone for 90 sec in a pre-chilled mortar and pestle at 4°C and the homogenate was centrifuged at 26 000 *g* at 2°C for 20 min. The supernatant was used for enzyme assay, gel electrophoresis and protein estimation. All extractions were done under dim green safelight.

Enzyme assay

Peroxidase activity was determined by recording a change in absorbance per 15 sec at 610 nm after adding 50 μl of supernatant to 5.0 ml of assay mixture. Assay mixture was prepared by adding 2.0 ml of benzidine solution to 100 ml of hydrogen peroxide ($3 \cdot 10^{-2}$ M). Benzidine solution was prepared according to Scandalios [10]. An arbitrary unit of peroxidase activity was chosen as change in A of 0.1 per 15 sec per mg of protein. Protein was estimated by the method of Lowry et al. [11] using bovine serum albumin as standard.

Disc electrophoresis

Disc electrophoresis was carried out on 7% polyacrylamide gels at pH 8.3 using Tris—glycine buffer following the procedure of Davies [12]. The peroxidase bands were detected by incubating the gels first with $3 \cdot 10^{-2}$ M H_2O_2 for 10 min and then with benzidine for 1 min. The gels were washed with 7% acetic acid, and stored in it.

RESULTS

The established operational criterion for the involvement of phytochrome in a light mediated response requires that an induction effected by red light can be fully reversed by a subsequent irradiation with FR light [3]. The data shown in Table I fulfill this criterion for the involvement of Pfr in regulating the peroxidase activity in maize. A brief irradiation with red light of 6-day-old seedlings enhanced the peroxidase activity by 50 to 70%. This enhancement was reversed when seedlings were subsequently irradiated with FR light (Table I), while FR alone was ineffective. This response to red and FR irradiation is found to be age-dependent (Sharma, Sopory and Mukherjee, unpublished).

TABLE I

EFFECT OF BRIEF IRRADIATION OF RED AND FAR-RED LIGHT ON THE PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE SEEDLINGS

The operational criterion for the involvement of phytochrome in a photoresponse is fullfilled. Values are the mean of five separate experiments done in duplicate.

Treatments	Relative activity %
7 days d	100
6 days d + 5 min R + 24 h d	162
6 days d + 5 min FR + 24 h d	121
6 days d + 5 min R + 5 min FR + 24 h d	123
6 days d + 24 h FR	183

An alternative way to ascertain the involvement of Pfr in a photoresponse is to demonstrate the operation of high intensity reaction under continuous FR light which operates through Pfr [13,14]. On irradiating the seedlings with continuous FR light, a significant increase in peroxidase activity was noticed (Table I). The kinetics of peroxidase activity under continuous FR light (Fig. 1) showed that the enzyme activity increased steadily upto 20 h after an initial lag phase of 2 h. However, when FR light was turned off after 12 h of FR irradiation, the increased enzyme activity declines instantaneously, but was still maintained at a higher rate than the dark control. When 6.5-day-old dark-grown seedlings were transferred to FR light, peroxidase activity again increased after a lag phase of 2 h (Fig. 1). Since peroxidase occurs in plant tissues in multiple molecular forms [10,15—17], it is possible that the

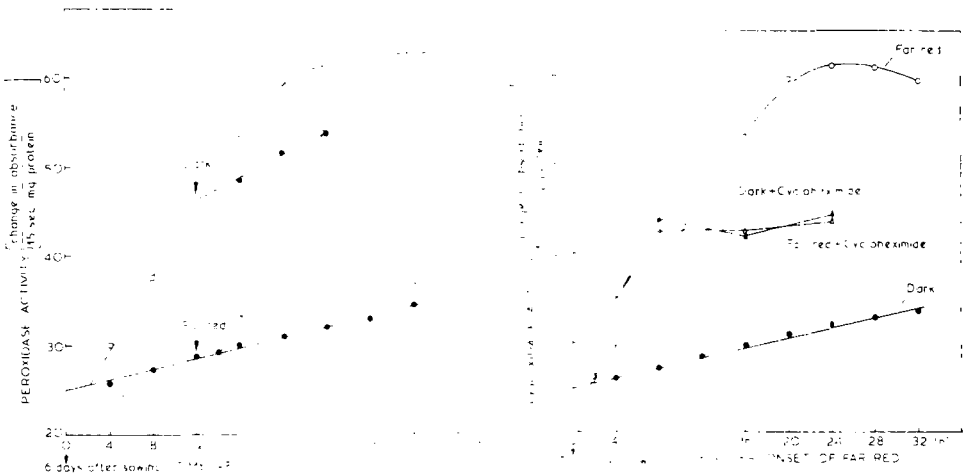

Fig. 1. Effect of continuous FR irradiation on peroxidase activity in *Zea mays*. The arrows indicate the time when the seedlings were transfered from the dark to FR (- -) and FR to darkness (• - - - •).

Fig. 2. Kinetics of peroxidase activity in the dark and in continuous FR irradiation in the presence (- -) or absence (-) of CHI 20 μg ml⁻¹ in *Zea mays*.

observed increase in peroxidase activity by FR light might be due to disappearance or appearance of certain isozymes of peroxidase. But this was not so as we have never observed any difference in isozymes in plants kept in darkness and irradiated with FR light on polyacrylamide gels.

To understand the mechanism of a Pfr-mediated increase in peroxidase activity, inhibitiors of RNA and protein synthesis were employed at different concentrations. Actinomycin D (8 μg ml) inhibited peroxidase activity by only 12% in FR-irradiated plants, whereas chloramphenicol (50 and 250 μg/ml) was totally ineffective. Puromycin (100 μg/ml) was very effective and it completely inhibited the FR-mediated increase.

CHI showed an entirely different response as compared to puromycin (Table II). At a concentration of 5 μg ml it differentially increased the peroxidase activity both in dark-grown and FR-irradiated plants, whereas at higher concentration (20 μg ml) the enhancement was less and the increase in peroxidase activity was equal in dark-grown and FR irradiated plants. If CHI (20 μg/ml) was applied to plants after 12 h of FR irradiation, at the time when plants were transferred to darkness, the increase in enzyme activity was greater (Table II).

The enhancement in peroxidase activity by CHI followed a different kinetics than that obtained with FR light (Fig. 2). The increase in peroxidase activity in CHI was apparent after a lag phase of 2 h and activity reached a maximum in 8 h. In continuous FR the lag phase was also 2 h but the maximum activity was attained in 20 h and the increase in activity was greater than in CHI.

TABLE II

EFFECT OF CYCLOHEXIMIDE ON PHYTOCHROME-MEDIATED INCREASE IN PEROXIDASE ACTIVITY IN APICAL LEAVES OF MAIZE

Values are the mean of three separate experiments done in duplicate.

Treatment		Peroxidase activity units/mg protein	Relative activity %
(1)	7 days d (H_2O)	33.0	100
(2)	6 days d (H_2O) + 24 h d (CHI 5 μg/ml)	56.2	170
(3)	6 days d (H_2O) + 24 h d (CHI 20 μg/ml)	44.6	135
(4)	6 days d (H_2O) + 24 h FR (H_2O)	61.0	183
(5)	6 days d (H_2O) + 24 h FR (CHI 5 μg/ml)	67.8	205
(6)	6 days d (H_2O) + 24 h FR (CHI 20 μg/ml)	44.0	133
(7)	6 days d (H_2O) + 12 h FR (H_2O) + 12 h d (H_2O)	53.0	161
(8)	6 days d (H_2O) + 12 h FR (H_2O) + 12 h d (CHI 20 μg/ml)	65.0	197

DISCUSSION

Although some literature exists on the control of peroxidase activity in plants by hormonal and environmental factors [15—17], very meagre information is available on the effects of light on peroxidase activity. Graham et al. [18] observed an enhancement in peroxidase activity on transferring detached leaves of maize to light, but they did not study the possible involvement of Pfr in this response. Our experiments on the reversibility of red light-mediated enhancement of peroxidase activity by FR light, demonstrate the involvement of Pfr in mediating the peroxidase activity. This is further supported by the fact that the enzyme activity also increased in the presence of continuous FR light.

The study of kinetics of peroxidase activity in the presence of continuous FR light brought forth another interesting fact.

In a similar study done earlier on *Sinapis alba*, it was shown that the Pfr control of peroxidase activity is a case of photodetermination [9], but our results suggest that, in maize, it is a case of photomodulation. This is clear from Fig. 1 where transferring of FR-irradiated seedlings to darkness (which invariably leads to decline in Pfr level) resulted in an immediate decrease in the rate of peroxidase activity. Thus, in two different systems, the mode of regulation of peroxidase by Pfr is different. The present work also rules out the possibility of enhancement of enzyme activity due to changes in isozymes.

The insensitivity of Pfr-mediated increase to actinomycin D and chloramphenicol excludes the possibility of increase in mRNA or translation of mRNA on 70S ribosomes of organelles. On the other hand, the effect of puromycin in nullifying the Pfr-enhanced peroxidase activity supports the contention, that the

increase may be due to fresh synthesis of enzyme molecules on the cytoplasmic 80S ribosomes. It has been reported that Pfr leads to activation of 80S ribosomes in maize [19]. Therefore, it is possible that the enhanced activity of peroxidase could be due to activation of ribosomes in the presence of Pfr.

The enhancement in peroxidase activity in the presence of CHI suggest the existence of an inhibitor of peroxidase with a rapid turnover rate. Similar suggestions have been made earlier for phenylalanine ammonia lyase [20–22] and invertase [23]. CHI may inhibit the synthesis of this inhibitor protein thus resulting in an enhancement of peroxidase activity due to reduction in its rate of inactivation. The differential response noted by different concentrations of cycloheximide (Table II) is rather difficult to explain. It is possible, however, that at low concentration (5 μg ml) CHI may stop inactivation but may not hinder the synthesis of peroxidase thereby resulting in enhanced activity. This may be the reason for the differential increase observed in peroxidase activity in plants kept in darkness and those treated with FR light (Table II). At a higher concentration (20 μg/ml) of CHI, enzyme synthesis may also be inhibited thereby leading to an overall reduction in magnitude of enhancement. An almost identical response was noted in sunflower leaf discs by Creasy et al. [24], where on comparing CHI-mediated inhibition at different concentration they showed that inactivation was much more sensitive to inhibitor than phenylalanine ammonia lyase formation. From kinetics of enzyme activity in the presence of CHI and under continuous FR it seems that they do not have a common mode of operation. CHI probably increased peroxidase activity by inhibiting enzyme inactivation, while in FR light fresh enzyme synthesis takes place.

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Phytochrome Regulation of Peroxidase Activity in Maize
II. Interaction with Hormones, Acetylcholine and cAMP[1])

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With 4 figures

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Summary

Peroxidase activity in leaves of etiolated maize seedlings is controlled by phytochrome. When supplied exogenously, kinetin, gibberellin and acetylcholine enhanced peroxidase activity in dark but cAMP and IAA were ineffective. With red light (5 minutes) irradiation, the enzyme activity slightly enhanced in presence of hormones and acetylcholine but not with cAMP. Under continuous far-red light (24 hours), in presence of hormones, acetylcholine and cAMP enhancement was less in magnitude than control. The application of ABA, CCC and inhibitors of cAMP and acetylcholine metabolism did not significantly affect the enzyme activity in dark or in red light (5 minutes) irradiated seedlings. Under continuous far-red light, these compounds reduced the magnitude of enhancement by only 20–30 %. The results suggest that phytochrome regulation of peroxidase activity in maize is not regulated via hormones, acetylcholine or cAMP.

Key words: Phytochrome, peroxidase, maize, hormones, cAMP, acetylcholine.

Introduction

The mechanism of action of phytochrome has been under intense debate since last decade (MOHR, 1972; SMITH, 1975). The association of phytochrome with membranes (MARMÉ et al., 1974) and its regulation of hormonal level *in vivo* (BLACK and VLITOS, 1972; KANDELER, 1974) and *in vitro* (COOKE et al., 1975; EVANS and SMITH, 1976) have promoted the view that phytochrome action may be mediated through hormones, where phytochrome may exert control as a permease associated with membranes (SMITH, 1970, 1975; EVANS and SMITH, 1976). The involvement of hormones in phytochrome mediated responses has been shown in certain cases e. g. via ethylene production in hook opening (KANG and RAY, 1969) and carotenoid synthesis (KANG and BURG, 1972) and via gibberellin production in leaf unrolling of cereals

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(Lovtys and Wareing, 1971; Cooke and Saunders, 1975). Other workers have not found any involvement of gibberellin in phytochrome responses (Drumm et al., 1971) and it has been suggested by others too that phytochrome and hormones act as two independent factors (Mohr, 1972; Bajracharya et al., 1975).

Besides hormones, in other systems, acetylcholine (Jaffe, 1970) and cAMP (Rasmussen et al., 1973; Weintraub and Lawson, 1972) have been observed as photomimicking phytochrome mediated response and their endogenous level is also shown to be under phytochrome control (Jaffe, 1970; Janystin and Drumm, 1975). However, many other workers did not find any such involvement of either acetylcholine (Saunders and McClure, 1973; Satter et al., 1972; Kasemir and Mohr, 1972) or cAMP (Kirshner et al., 1975).

The activity of peroxidase in plants is regulated by an interplay of hormonal, environmental and genetic factors (Galston and Davies, 1969). Nearly all hormones have been shown to affect peroxidase activity (Hagen, 1963; Lavee and Galston, 1968; Galston and Davies, 1969; Gaspar et al., 1973). In an earlier investigation, we have demonstrated that in apical leaves of maize phytochrome controls the activity of peroxidase. In this study we have tried to find out the possible involvement of hormone(s), acetylcholine or cAMP in phytochrome mediated increase in peroxidase activity.

Material and Methods

Seeds of *Zea mays* var. Shakti were germinated in petri dishes on absorbent paper moistened with distilled water upto six days at 27 ± 1 °C in complete darkness. The treatments of various chemicals was given by transferring the plants (six day old) to different petri dishes supplied with respective solutions 30 minutes prior to onset of red or far-red irradiation. In all cases the control seedlings were supplied with distilled water only.

The light source for obtaining red and far-red light was a 500 watt tungsten reflector lamp (Westinghouse, U.S.A.). Red light (500 $\mu W\ cm^{-2} Sec^{-1}$) was obtained by filtering through a CBS-650 filter (emission maxima, 650 nm), and far-red light (140 $\mu W\ cm^{-2} Sec^{-1}$) after filtering through 8 cm of constantly flowing tap water and CBS-750 filter (emission maxima, 750 nm). Handling of plant material and subsequent manipulations were done under dim green safe light.

For enzyme extraction, apical leaves were homogenized in 0.1 M phosphate buffer, pH 7.0 (10 ml buffer per gm fresh weight) with insoluble polyvinylpolypyrrolidone (2.5% w/v) in mortar and pestle at 4 °C in cold room. The homogenate was centrifuged at 26 000 × g for 30 minutes at 2 °C. The supernatant was used for enzyme assay, protein estimation and gel electrophoresis.

Peroxidase activity (E.C. 1.11.1.7.) in supernatant was assayed by recording a change in absorbance at 610 nm at 15 seconds interval after adding 50 µl of supernatant to 3 ml of assay mixture. The assay mixture contained, benzidine 2.4×10^{-3} M, hydrogen peroxide 3×10^{-2} M, acetic acid 7×10^{-2} M, pH 3.8. Benzidine solution was prepared according to Scandalios (1964). One unit of enzyme activity was defined as change in absorbance of 0.1 at 610 nm per 15 seconds (1 unit = 0.1 A 610/15 seconds). Protein was estimated after dissolving the trichloroacetic acid (5% final conc.) precipitate in 1 N NaOH by the procedure of Lowry et al. (1951) using bovine serum albumin as standard.

Isozymes of peroxidase were separated by polyacrylamide gel electrophoresis at 4 °C. Samples (100 μl), which were prepared by mixing supernatant and glycerol (2 : 1 ratio), were layered on the top of the gel. The cationic and anionic isozymes were resolved by the methods of REISFIELD et al. (1962) and DAVIES (1964) respectively. Gels were stained by first immersing them in benzidine-acetic acid mixture (2.4×10^{-4} M and 7×10^{-3} M respectively) for 10 minutes and then with hydrogen peroxide (3×10^{-2} M). Intense blue bands which later turned brown were obtained. Immediately gels were washed and stored in 7 % acetic acid.

Abbreviations

GA, gibberellic acid; Na_2cAMP, adenosine 3'5' cyclic monophosphoric acid sodium salt; DB_2cAMP, N^6O^2-dibutryl adenosine 3'5' cyclic monophosphoric acid; IAA, indole-3-acetic acid; CCC, 2-chloroethyl trimethylammonium chloride; ABA, abscisic acid; PRB-8, α-chloro-B-(3 chloro-o-tolyl) propoinitrile; AMO-1618, 2 isopropyl-4-dimethyl amino-5-methylphenyl-piperidine carboxylate methylchloride; ACh, acetylcholine chloride; CCh, choline chloride; Kn, kinetin; ES, eserine sulphate; AT, atropine; TH, theophylline.

Results

The experiments were done under three different conditions. Firstly, the effects of hormones, acetylcholine and cAMP were seen in dark grown seedlings, secondly, in

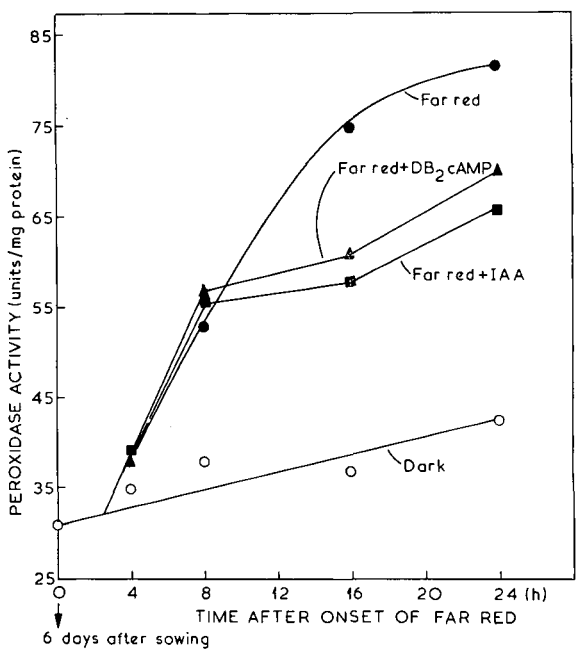

Fig. 1: The kinetics of peroxidase activity in apical leaves of maize under continuous far-red light (●——●) and in darkness (○——○) and also in presence of DB_2cAMP 10^{-5} M (▲——▲) and IAA 10^{-4} M (■——■) under continuous far-red light.

seedlings exposed to red light for 5 minutes and thirdly, under continuous far-red irradiation for 24 hours. Under the same three conditions the effects of inhibitors of hormones and acetylcholine and cAMP metabolism were seen on peroxidase activity.

Effects of different hormones on peroxidase activity in dark

As is seen in Table 1, in presence of GA, Kn and ACh the peroxidase activity increased after 24 hours, the optimum concentration being 10^{-6} M, 10^{-5} M and 10^{-5} M respectively. This effect of ACh was specific as there was no effect of choline chloride at the same concentration. There was no effect of IAA, at 10^{-7} M to 10^{-4} M, and cAMP, at 10^{-8} M to 10^{-5} M. Since it has been reported that the uptake of Na_2cAMP is not efficient (ROBINSON et al., 1971), we also tested DB_2cAMP in the same concentration range and found similar results thereby ruling out the possibility of hindered uptake in our system. In further experiments only optimal concentration of effective hormones and ACh were employed while IAA and DB_2cAMP were used at 10^{-4} M and 10^{-5} M respectively.

Interaction of hormones, ACh and cAMP with red and continuous far-red irradiation in controlling peroxidase activity

The exposure of seedlings to red light for 5 minutes resulted in enhanced peroxidase activity. With hormones during the red light treatment, the peroxidase

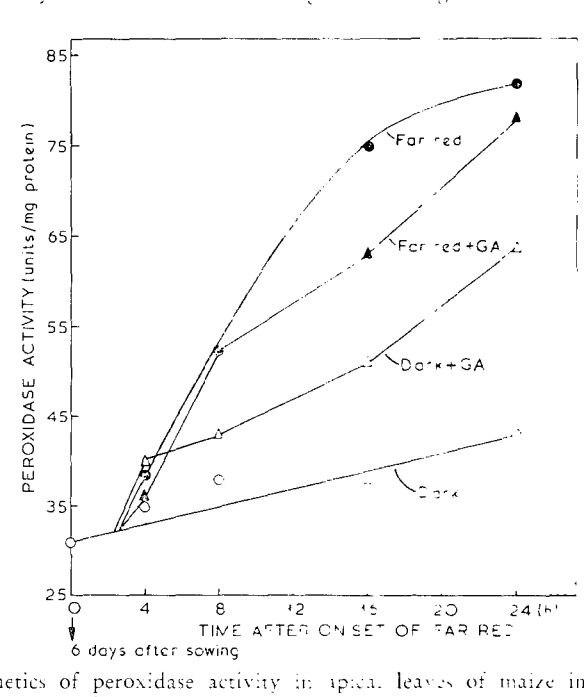

Fig. 2: The kinetics of peroxidase activity in apical leaves of maize in presence of GA 10^{-6} M under continuous far-red-light (▲– —▲ and darkness (○– —○).

activity increased over the control except with DB_2cAMP which repressed the enzyme activity. The magnitude of enhancement, however, was marginal over red light irradiated water control and in none of these cases it was additive. In presence of continuous far-red irradiation (24 hours), the hormone, ACh and DB_2cAMP treated seedlings showed lesser enhancement than the control. This repression was more with IAA and ACh and least with GA (Table 2).

Kinetics of peroxidase activity in presence of hormones, ACh, cAMP in dark and under continuous far-red light

Seedlings irradiated with continuous far-red light showed an enhanced activity of peroxidase after a lag phase of 2 hours and reaching a maximum between 20 to 24 hours (Fig. 1) (see also SHARMA et al., 1976). The presence of IAA and DB_2cAMP had no effect on the kinetics of peroxidase activity in dark but under continuous far-red light repressed the enhancement after following a similar kinetics upto 8 hours. Gibberellic acid and kinetin also reduced the magnitude of enhancement in presence of continuous far-red light after 8 hours but at 24 hours the repression was marginal. Under dark, however, they followed a different kinetics (Fig. 2 and 3). With GA,

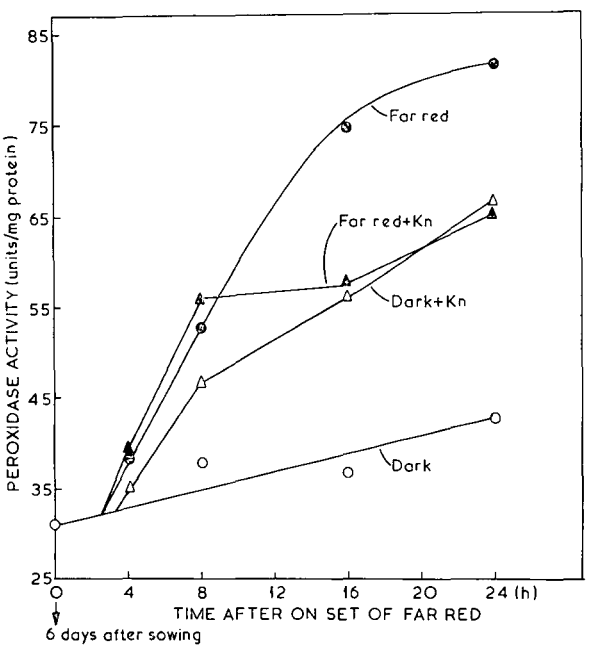

Fig. 3: The kinetics of peroxidase activity in apical leaves of maize in presence of kinetin 10^{-5} M under continuous far-red-light (▲——▲ and darkness (△——△).

peroxidase activity increased after a lag of 2 hours and the rate of enhancement was slower than with far-red alone. Kinetin enhanced the peroxidase activity steadily after a lag phase of 4 hours. Acetylcholine also enhanced the peroxidase activity in dark after 4 hours but under far-red light it repressed the enhancement after 4 hours (Fig. 4).

Interaction of hormone antagonists and inhibitors of cAMP and ACh metabolism with 5 minutes red light and continuous far-red light in controlling peroxidase activity

The data presented in Table 3 shows that most of the antagonists did not exert any significant effect on peroxidase activity in dark. The irradiation with 5 minutes of red light, which resulted in enhanced peroxidase activity was, however, inhibited by 20 % by CCC. Under this condition, ABA, atropine and eserine sulphate had no effect whereas theophylline potentiated the enhancement. Under continuous far-red light (24 hours) all these compounds inhibited the peroxidase activity by 20–35 %, atropine being the most effective. However, none of them could repress the enzyme activity totally.

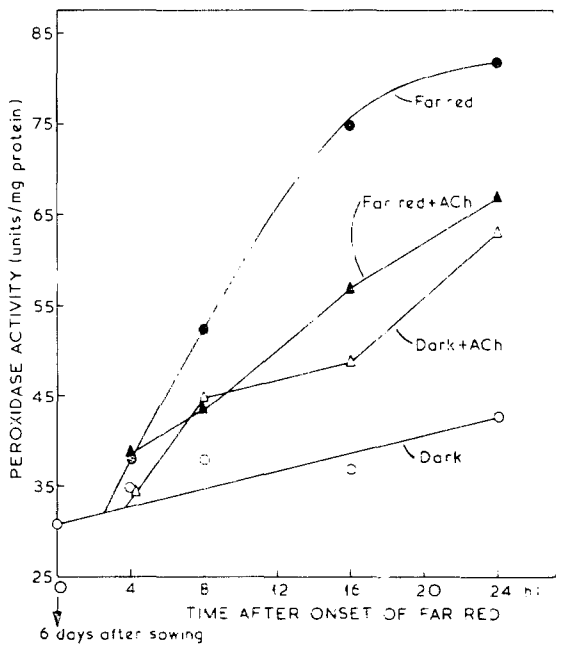

Fig. 4: The kinetics of peroxidase activity in apical leaves of maize in presence of ACh 10^{-5} M under continuous far-red-light (▲ —▲) and darkness (○ — ○)

Effect on isozymes of peroxidase

There was no qualitative difference observed in isoperoxidase pattern in plants supplied with hormones, cAMP, ACh and their inhibitors in dark or treated with 5 minutes of red light or under continuous far red light. There were 3 bands of cationic isozymes and 8 anionic isozymes. There was no change in number of bands and their Rf values.

Discussion

Hormones and phytochrome influence a variety of similar developmental processes in plants, still the participation of hormones in phytochrome mediated responses is controversial (MOHR, 1972; SMITH, 1975). Hormonal regulation of enzyme activity has been studied in detail, for example, in case of GA induced amylase formation in barley aleurone layers (MANN, 1975). The involvement of hormones in phytochrome regulated enzyme activity, however, has not been investigated in detail. In the present study we have made an attempt to study the role of hormones, ACh and cAMP in phytochrome mediated regulation of peroxidase activity in maize. Any particular hormone, in order to qualify as a mediator in phytochrome action, should satisfy the following criteria: 1. The *in vivo* titer of hormone should be either controlled by phytochrome and/or phytochrome should modify its distribution, transport or change the affinity of target of action. 2. Exogenously supplied hormone should mimick the action of phytochrome and kinetics of response induced by exogenous hormone should be similar to the kinetics of photoresponse. 3. In case phytochrome does not alter *in vivo* titer of hormone but change the affinity of target of action than in presence of exogenous hormone phytochrome action should either be enhanced or repressed. 4. The inhibitors which are known to affect the hormonal level or counter their action should nullify the phytochrome response.

In the present investigation we have not studied the first criterion. Phytochrome controls of hormonal level, transport and distribution are, however, well known (BLACK and VLITOS, 1972; KANDELER, 1974). In cereals, phytochrome leads to an increase in extractable gibberellin activity (LOVEYS and WAREING, 1971) and its transport out of etioplasts (EVANS and SMITH, 1976; COOKE et al., 1975). The control of level of other hormones has not been investigated in cereals. Regarding phytochrome mediated change in the affinity of target of action, it can be speculated in the basis of its association to particulate fraction in maize after red light irradiation (QUAIL et al., 1973).

When the other three criterion were studied, it was found that none of the hormones tested could absolutely be considered as a mediator in phytochrome controlled peroxidase activity in apical leaves of maize. The exogenous supply of GA, Kn and ACh enhanced the peroxidase activity in dark whereas cAMP and IAA were ineffective (Table 1). The magnitude of enhancement was, however, always less than that obtained by 5 minutes of red light or continuous far-red light alone (Table

Table 1: Effect of different hormones, ACh and cAMP on peroxidase activity in apical leaves of maize in dark. Six days old etiolated seedlings were supplied with hormone solutions and the enzyme activity was measured after 24 hours. The results are mean of 4 experiments done in duplicate. Values are expressed as percentage of water control which is taken as 100.

Concentration	Treatments						
	GA	IAA	Kn	ACh	CCh	Na_2 cAMP	DB_2 cAMP
10^{-8} M						107	109
10^{-7} M	125	107	131			114	109
10^{-6} M	147	122	137			109	116
10^{-5} M	134	122	146	146	122	118	96
10^{-4} M	134	122	142	128	125		

Table 2: Interaction of hormones, ACh and cAMP with 5 minutes of red light and continuous far-red light (24 hours) in regulating peroxidase activity in maize. Treatments were given to six days old etiolated seedlings and enzyme activity was measured after 24 hours. Results expressed are mean of four experiments done in duplicate.

Treatments	Dark		Red light (5 minutes)		Far red light (24 hours)	
	Units[1])	RA[2])	Units	RA	Units	RA
Water control	43	100	73	170	82	190
DB_2cAMP 10^{-5} M	43	100	65	151	72	162
GA 10^{-6} M	64	148	78	181	78	181
IAA 10^{-4} M	43	100	76	176	66	153
Kn 10^{-5} M	67	155	79	183	75	174
ACh 10^{-5} M	64	148	77	179	67	155

[1]) Units per mg protein.
[2]) Relative activity.

2). Moreover, the kinetics of enhancement obtained by these hormones was different and usually had a longer lag phase of 4 hours, except GA, where it was 2 hours, and the rate of enhancement was much slower than continuous far-red light, which also had a shorter lag time of 2 hours (SHARMA et al., 1976). The results with ACh are different than those reported in spinach, where the enhancement in peroxidase activity after ACh, red and far-red light treatment was apparent after only a few minutes (PENEL and GREPPIN, 1973).

In presence of 5 minutes of red light and continuous far-red light the effect of hormones, ACh and cAMP was different than in dark (Table 2; Figs 1-4). Except DB_2cAMP, in 5 minutes red light, all others had a slight enhancing effect on peroxidase activity. Under continuous far-red irradiation, the enhancement was inhibited by all these regulators except with GA, which had only a marginal effect.

In most cases, the repression in rate of enhancement became apparent only in later phase of far-red irradiation. In DB_2cAMP, IAA and Kn it was apparent after 8 hours whereas with ACh it started after 4 hours.

Hormone mediated repression of photoresponses has been reported earlier also in a few cases e. g. in pea, where both auxins and GA negated phytochrome mediated responses (RUSSEL and GALSTON, 1968; PURVES and HILLMAN, 1958). Similarly phytochrome mediated enhancement of phenylalanine ammonia lyase, anthocyanin and carotenoid synthesis was shown to be blocked by exogenous supply of PRB-8, a synthetic growth regulator (DIERICKX and VENDRING, 1973). In mustard seedlings, GA repressed, when supplied exogenously, the far-red controlled amylase activity (DRUMM et al., 1971). With ACh too, a complex interaction with light treatments has been reported in moss callus (HARTMANN, 1974). In the present studies also, since hormones, irrespective of their stimulatory effect on peroxidase activity in darkness, are repressing the enzyme activity under continuous far-red light, it seems that in latter case some secondary processes are triggered by hormone application which compete with far-red induced photoresponse, thereby limiting the rate of enhancement.

The application of chemicals, which oppose or interact with the action of hormones also did not give any results in favour of participation of ACh, cAMP or hormones in phytochrome control of peroxidase activity. Eserine, an inhibitor of ACh esterase (FLUCK and JAFFE, 1975) did not affect red light mediated enhancement (Table 3). We have not tested the effect of AMO-1618, another inhibitor of ACh esterase, which was shown to stimulate the red light mediated uptake of ^{14}C-acetate in mung bean root tip (JAFFE and THOMA, 1973). However, another inhibitor, atropine, which blocks ACh receptors (GOODMAN and GILMAN, 1965), also failed to affect the red light mediated enhancement of peroxidase activity, though in *Avena* coleoptiles it negated the action of ACh (EVANS, 1972). Under continuous far-red light, both eserine and atropine repressed the enhancement in enzyme activity.

Table 3: Interaction of antagonists of hormones and inhibitors of ACh and cAMP metabolism with 5 minutes of red light and continuous far-red light (24 hours) in regulating peroxidase activity in apical leaves of maize. Treatments were given to six days old seedlings and enzyme activity was measured after 24 hours. Results are mean of four experiments done in duplicate.

Treatments	Dark		Red light (5 minutes)		Far-red light (24 hours)	
	Units[1])	RA[2])	Units	RA	Units	RA
Water control	43.0	100	71.0	166	82.0	190
ABA 10^{-4} M	43.0	100	71.0	166	69.0	160
CCC 10^{-4} M	43.0	100	63.0	147	72.0	167
Es 10^{-3} M	49.5	115	69.0	160	73.0	170
AT 10^{-4} M	46.0	107	73.0	170	66.5	155
TH 10^{-3} M	40.0	93	76.5	178	72.0	167

Theophylline, an inhibitor of phosphodiesterase (ROBINSON et al., 1971), was ineffective in dark but inhibited far-red mediated enhancement.

Similarly, the inhibitors of hormones also had no definite effect. Red light enhancement of peroxidase activity was not found to be sensitive to ABA, at a concentration where it blocks the action of most hormones (MILBORROW, 1974). CCC, an antagonist of GA biosynthesis (DENIS et al., 1965) had a little effect under red light which may be due to its inhibitory action on protein synthesis (KINSMAN et al., 1975). Under continuous far-red light also the repression by ABA and CCC was never more than 20 to 30%. In mustard seedlings also, BAJRACHARYA et al. (1975) could not find any specific role of ABA in phytochrome mediated photomorphogenesis.

Light has been shown to promote the formation of catalase isozymes in *Sinapis* (DRUMM and SCHOPFER, 1974) and hormones can alter the activity of peroxidases by a qualitative and quantitative change in isozyme pattern (GASPAR et al., 1973). In our experiments, no qualitative difference was discernible with respect to different treatments in anionic or cationic isozymes of peroxidase on polyacrylamide gels.

The involvement of hormones in phytochrome mediated photoresponse can be unequivocally considered only when a particular hormone satisfies all the critria mentioned before. In the present investigations, although some of the compounds enhanced the peroxidase activity in dark, they cannot be considered as a mediator in phytochrome response because, 1. the enhancement in dark was always less than that obtained by 5 minutes of red light or under continuous far-red light alone; 2. the kinetics of enhancement was different with longer lag phase; 3. the inhibitors tested could not bring down the far-red mediated enhancement more than 20–30%. It is very likely, therefore, that the enhancement induced by hormones and their interactions with phytochrome mediated photoresponse results from their independent action of these factors on a similar process. Any generalization on participation of hormones, ACh or cAMP in phytochrome mediated photoresponse should be, therefore, carefully interpreted and based on rigrous analysis and not merely by showing the mimicking effect e.g. as has been done in case of cAMP (RAST et al., 1973; WEINTRAUB and LAWSON, 1972) and ACh (PENEL and GREPPIN, 1973).

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