Comparable preparations of isolated nuclei have since been obtained from viable embryos of oats, barley and rye by means of the procedure described here.

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INDUCTIVE CONTROL OF INDOLEACETIC ACID OXIDASE ACTIVITY BY RED AND NEAR INFRARED LIGHT [1,2]

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Many plant processes, including seed germination, photoperiodic flower induction, and the development of stems and leaves, can be affected by brief exposures to low energies of light. Action spectra for such light effects resemble each other closely, with red light (ca 660 mμ) showing maximum effectiveness. In addition, the effects of red light can usually be prevented by exposure to near infrared[3] radiation (ca 730 mμ) sufficiently soon after red light treatment. These characteristics have led to the belief that one photoreaction and pigment system may control widely diverse processes (2, 3, 9, 19). Although the relation between this photoreaction and a biochemical system which might control plant growth is unknown, there is considerable evidence that auxin metabolism may be involved (5, 7, 11, 12, 13). Since the indoleacetic acid (IAA) oxidase system may be a controlling factor in auxin metabolism (6), its relation to red light action has been investigated in the present work. The results indicate that the IAA oxidase activity of certain tissues is controlled by a low energy photoreaction apparently typical of those controlling photoperiodism and the other processes mentioned.

[3] Wassink and Stolwijk (19) have pointed out why "infrared" is preferable to the term "far-red" to designate this region of the spectrum.

GENERAL PROCEDURES

Most of the experiments were conducted with peas (*Pisum sativum* L. var. Alaska) obtained from Associated Seed Growers, Inc., New Haven, Connecticut. The seedlings were grown in vermiculite for 7 days at 26° C in darkness, with occasional dim green light during handling. Some were also exposed to 2 to 2500 kiloergs/cm² of red light during a brief period 5 minutes to 40 hours before harvest. Red light was supplied by Sylvania red fluorescent tubes with maximum emission at 640 mμ and none below 580 mμ. Energy measurements were made with a phototube light meter whose construction and calibration is described elsewhere (5).

Tissues harvested for IAA oxidase assays were weighed, placed immediately at – 16° C, and stored at that temperature until use (1 to 10 days) with little or no resultant loss in activity. For assay, the frozen tissue was rapidly reduced to a powder with a chilled mortar and pestle and ground further with 0.02 M phosphate buffer, pH 6.1. The preparation was centrifuged in the cold to remove cellular debris and the clear supernatant extract made to volume and assayed. Usually 600 mg fresh weight of tissue were made to a final volume of 25 ml.

The assay used, described in more detail elsewhere (10), consists of colorimetric determinations of residual IAA in reaction mixtures incubated at 26° C. Each 10-ml reaction mixture contained 0.5 to 5 ml extract, 2 ml 0.01 M phosphate buffer, pH 6.1, and a
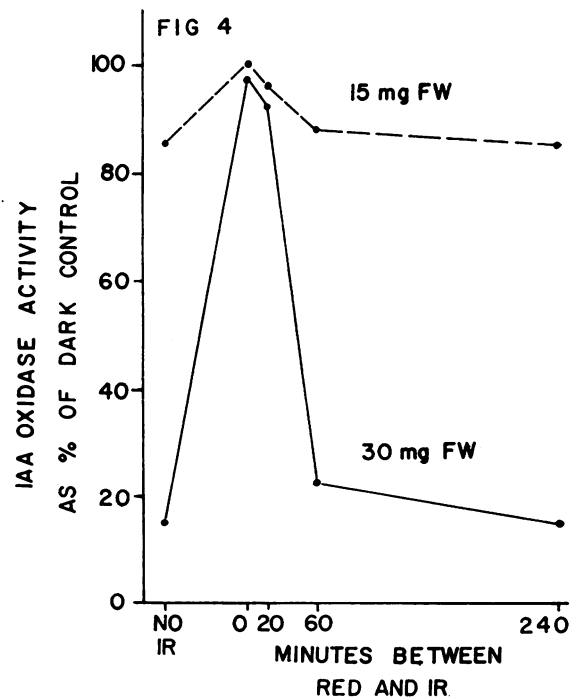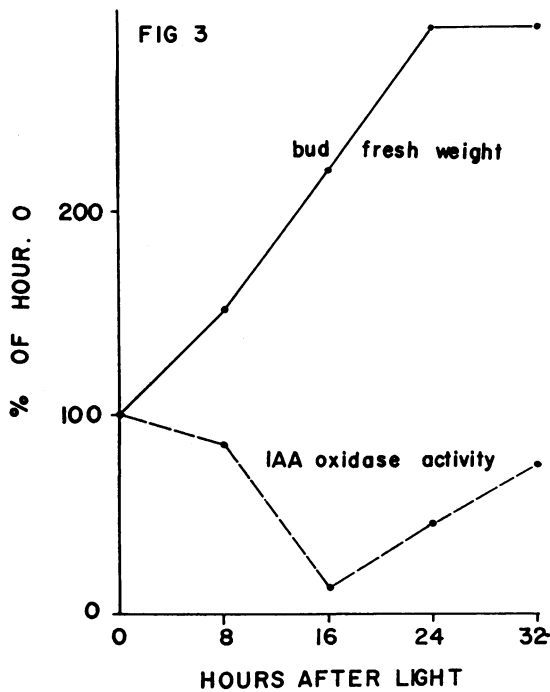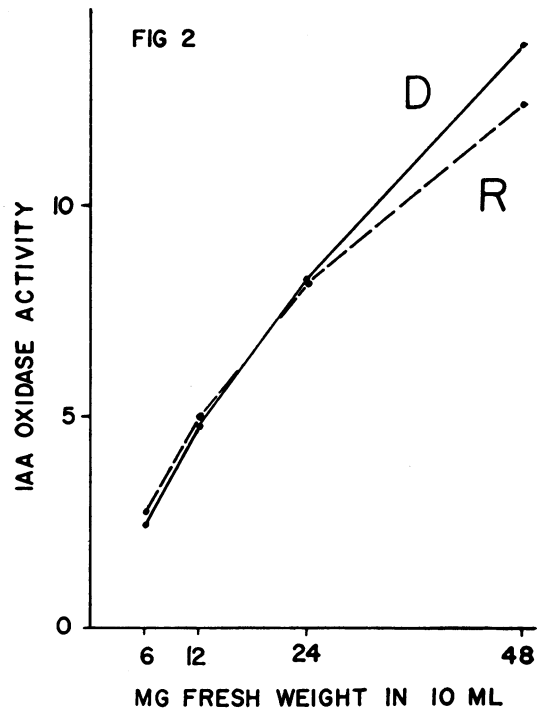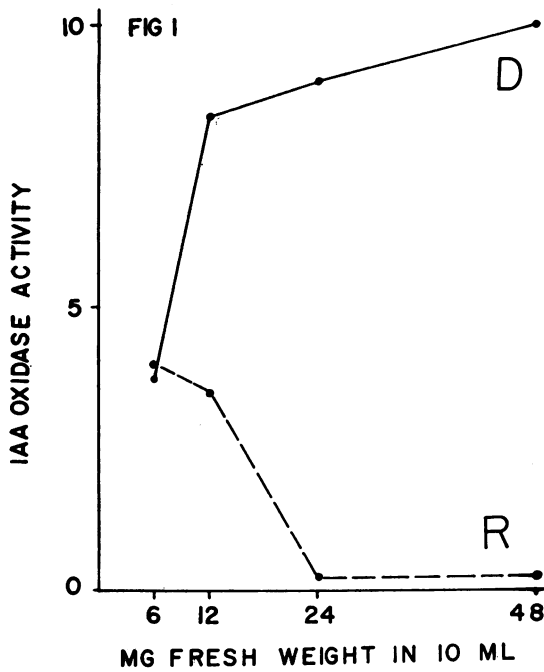
FIG. 1 (*upper, left*). IAA oxidase activity (μgm/ml IAA destroyed in 60 min in a 10-ml reaction mixture started at 35 μgm/ml) as a function of crude extract concentration representing the indicated tissue fresh wts. D, extract from buds of dark-grown plants. R, extract from buds of plants exposed to 750 kiloergs/cm² red light 16 hrs before harvest.

starting IAA concentration of 35 μgm/ml (2×10^{-4} M). Either 10^{-4} M 2,4-dichlorophenol (DCP) or 10^{-4} M $MnCl_2$ was usually present as well, as cofactor for the enzyme system (7, 10). Activity without either Mn or DCP was too low for reliably significant readings, and was rarely tested after the preliminary experiments. Activity was expressed as μgm/ml IAA destroyed in 20 or 60 minutes, either on a fresh weight basis or as specific activity (6) on a protein nitrogen basis.

EXPERIMENTAL

PRELIMINARY—IAA OXIDASE INHIBITION IN BUDS: Experiments were conducted to see what tissues, if any, would exhibit changed IAA oxidase activity after exposure to red light. The test plants were exposed to a total energy of 750 kiloergs/cm² during one hour and returned to darkness. Terminal buds and apical 1-cm sections of the terminal internode were harvested 16 to 18 hours after the light treatment, and their activities compared with that of totally dark-grown controls harvested at the same time. The 16 to 18 hour interval between illumination and harvest was chosen since it is the optimal time for expression of the changed auxin sensitivity in the internode section test (5). When assays were conducted at extract levels representing 50 mg fresh weight in each 10-ml reaction mixture, in the presence of 10^{-4} M Mn or DCP, the IAA oxidase activity of buds of red-illuminated plants was depressed to 5 to 15 % of that of the dark control buds on a fresh weight basis. Specific activities maintained the same relationships, since the ratio of protein nitrogen to fresh weight did not change significantly following illumination. An 85 to 95 % inhibition of activity in the buds could thus be attributed to the red light treatment. This inhibition was observed in assays either with DCP or Mn alone, or with both present in various proportions. Since corresponding experiments with the internode sections failed to show differences of any magnitude or consistency between dark-grown and red-illuminated tissues, bud tissues only were used in most of the subsequent experiments.

EXTRACT DILUTION—EVIDENCE OF AN INHIBITOR: For a more detailed comparison of the activities of extracts from red-illuminated and dark-grown buds, the effect of extract concentration in the reaction mixture was studied. The results of a typical experiment are shown in figure 1. Amounts of extract representing 6, 12, 24 or 48 mg fresh weight were used in standard 10-ml reaction mixtures, all with 10^{-4} M DCP. At the higher extract levels, the inhibitory effect of prior red light treatment was very evident.

At lower levels, however, both activities approached the same value. The fact that the activity of extract from red-illuminated buds was so markedly increased by dilution suggested the presence of an inhibitor. To test this possibility, attempts were made to increase the activity of the extract by dialysis.

Centrifuged extracts of both red-illuminated and dark-grown buds were prepared, 600 mg fresh weight being made up to 25 ml of extract. One 10-ml portion of each was dialyzed in a cellophane membrane against 6 l of 0.02 M phosphate buffer, pH 6.1, at ~ 2° C. Another 10-ml portion of each was stored in a cellophane membrane at the same temperature as a control. After 24 hours, the extracts were assayed at various concentrations in the presence of 10^{-4} M DCP. The activities of the dialyzed extracts are shown in figure 2. The undialyzed controls supplied the data used in figure 1. Two effects of dialysis are evident from a comparison of the figures. The activities of both types of extract were greatly increased, and the difference between them was almost entirely removed. Apparently both extracts contained a dialyzable inhibitor, or inhibitors, but with a higher level present in that from the red-illuminated buds.

In order to test this hypothesis further, dialysates from both types of extract were assayed for inhibitory effects. Extracts of red-illuminated and dark-grown buds were prepared as usual. Ten ml of each was placed in a cellophane dialysis membrane and dialyzed against 10 ml of buffer in a large test tube at ~ 2° C for 24 hours with vigorous shaking. The extracts were discarded, and various amounts of the buffered dialysates added to standard reaction mixtures containing 10^{-4} M DCP and a highly active enzyme preparation from internode tissue. The dialysates from extracts of red-illuminated buds were at least 4 times as inhibitory as those from dark controls, on a fresh weight basis. Similar results were obtained by adding boiled extracts instead of dialysates as sources of inhibitor.

The simplest conclusion is that the inhibition of activity induced by red light treatment is due entirely to increased levels of a dialyzable, heat-stable inhibitor (or inhibitors). In the absence of any direct test for the inhibitor, however, further experiments were conducted by comparing bud extracts from variously-treated plants at fresh weight levels of 24 or 30 mg per 10-ml reaction mixture, and always in the presence of 10^{-4} M DCP.

TIMING OF RED LIGHT TREATMENT: In the preceding experiments, 16 to 18 hours was interposed between red light exposure and harvest, on the assumption that a maximum effect would result. The

FIG. 2 (upper, right). Like figure 1, except with dialyzed extracts, and activity measured over 20 min.

FIG. 3 (lower, left). IAA oxidase activity and bud fresh weight at various times after red light treatment (225 kiloergs/cm²). Activity measured as in figure 1, with 12 mg FW per reaction mixture; value for hour 0, 13.4. Average bud fresh weight, hour 0, 6.1 mg.

FIG. 4 (lower, right). The effect of time elapsed (in darkness) between red and near infrared (IR) treatment on the subsequent IAA oxidase activity. Red light 75 kiloergs/cm² given 16 hrs before harvest. IR, 4 min. Dark control activity, (as in fig 1) for 30 and 15 mg fresh weight levels, 17.0 and 16.3, respectively.

effects of red light timing were next studied directly by harvesting tissues immediately, or 4, 8, 16, 24, 32 or 40 hours after 225 kiloerg/cm² light treatment (15 min). Such experiments can be performed in two ways: All plants can be illuminated at the same time, with harvests at various times afterwards; this has the advantage of exposing all plants at the same developmental stage, but the disadvantage of harvesting plants of dissimilar ages. Conversely, groups of plants can be illuminated at different times and all harvested at the same time, with the opposite considerations. Fortunately, both types of experiments agreed. Results of an experiment of the first kind described are shown in figure 3. IAA oxidase activity and average bud fresh weight are presented for comparison as percentages of the zero time value, i.e., immediate harvest. Data for the dark controls remained substantially constant during the experimental period, and activity at zero time did not differ from the dark controls. Of the times chosen, illumination 16 hours before harvest produced the maximum inhibition of IAA oxidase in the buds. Tissues harvested as long as 32 hours after light (40 in other experiments) still showed lower activity than the dark controls.

The bud weight data in figure 3 are of particular interest. While the minimum IAA oxidase activity was always obtained about 16 hours after illumination in such experiments, bud size continued to increase for at least 24 hours, and in some experiments 32 or 40 hours, after illumination. In more general terms, the maximum effect of light was expressed sooner in the inhibitor level than in bud weight, suggesting that the process controlling inhibitor level may be more closely linked to the photoreaction. While this conclusion may not be justified, figure 3 at least indicates clearly that the results reported here are not merely consequences of assaying morphologically different tissues, as they might be if a simple inverse relation existed between bud size and IAA oxidase activity. In further experiments, inhibition has

TABLE I

IAA OXIDASE ACTIVITY OF PEA BUDS 16 HOURS AFTER EXPOSURE OF INTACT PLANTS TO RED AND NEAR INFRARED (IR) RADIATION IN SEQUENCE*

TREATMENT	IAA OXIDASE ACTIVITY**	INHIBITION, %
Dark controls	8.3	(0)
IR only	8.0	4
Red only	0.7	92
Red-IR	7.4	11
Red-IR-Red	1.0	88
Red-IR-Red-IR	7.6	8

* Red light exposure 2 min, total energy 30 kiloergs/cm². IR exposure 2 minutes (high intensity—see text). Less than 1 min elapsed between successive exposures.
** Activity measured as μgm/ml IAA destroyed in 20 minutes. Each 10-ml reaction mixture contained 35 μgm/ml IAA at the start, 10⁻⁴ M DCP, and bud extract equivalent to 24 mg fresh wt.

been obtained as little as three hours after illumination, before any weight increase or gross morphological change could be detected.

RED LIGHT DOSAGE: In order to determine the energy required to produce a measurable inhibition, groups of plants were exposed to 2, 20 or 200 kiloergs /cm² within a 15-minute period 16 hours before harvest. The activities of bud extracts were then compared with that of the dark controls at fresh weight levels of 24 and 12 mg per reaction mixture. Measured at the 24-mg level, all dosages produced about 90 % inhibition on both fresh weight and specific activity bases. Measured at the 12-mg level, the corresponding inhibitions were 2, 11 and 48 %. While the effects of dilution indicate the impossibility of obtaining quantitative data without a direct assay for the inhibitor in question, it is still evident that the effects of as little as 2 kiloergs/cm² of red light are easily detectable under the proper conditions.

NEAR INFRARED REVERSIBILITY: A 1000-watt incandescent light, separated from the plants by 10 cm of water and 4 thicknesses each of dark red and dark blue cellophane, was used as the source of infrared radiation (2, 3). The distance from the filament to the plant tops was approximately 58 cm. Preliminary experiments showed that 30 seconds of such irradiation was sufficient to cause over 90 % reversal of the effects of 30 to 75 kiloergs/cm² red light, if given immediately after the red light treatment. When several rapid alternations of red and infrared (IR) were carried out, the final effect depended upon the last radiation given. In the data presented in table I, the activities of buds from plants receiving IR alone, or red followed by IR, or red-IR-red-IR, were essentially like the dark controls, while the treatment red-IR-red caused the same inhibition as red alone.

Since 30 seconds of IR caused essentially complete reversal, the intensity of the source was reduced to less than 1/3 with a variable-voltage transformer in order to obtain dose-response data. Such data, however, like those with red light, are rendered difficult to interpret by the effects of enzyme dilution in the assay. In a typical experiment, all radiations were given about 16 hours before harvest, and the buds were assayed at 30 and 15 mg FW per 10-ml reaction mixture (with 10⁻⁴ M DCP). Seventy-five kiloergs/cm² red light (5 minutes) inhibited activity 96 % as assayed at the higher fresh weight level, and 40 % at the lower. Ninety seconds of (reduced intensity) IR given immediately after the red was sufficient to remove inhibition as assayed at the lower level, while 4 to 5 minutes was required at the higher.

In spite of such complications it was possible to obtain fairly precise data on the length of time during which the red light effect remains reversible by IR. For the experiment summarized in figure 4, all plants (except the dark controls) were first exposed to 75 kiloergs/cm² of red light (5 min). Some were exposed to 4 min of IR immediately afterwards, while others received IR 20 minutes, one hour, or four hours after the end of the red light. Bud tissues were har-

vested 16 hours after the initial (red) radiation and assayed as usual at 30 and 15 mg fresh weight levels. It is evident from figure 4 that, at 26° C, the red light effect is only very slightly reversible by IR given more than 1 hour afterwards. This is true even according to the assay at the lower fresh weight level, in which the apparent inhibition due to red light was small.

PEA INTERNODES AND EXCISED BUDS: Although no effect of red light on the IAA oxidase activity of internode tissue was observed in the preliminary experiments, the results with buds suggested that a wide range of extract concentrations should be tried before reaching this conclusion. Accordingly, the effect of 750 kiloergs/cm² given 16 hours before harvest was re-examined. Apical 1-cm portions of the 3rd (terminal) internodes from illuminated and control plants were compared at fresh weight levels from 12.5 to 200 mg per reaction mixture, with 10^{-4} M DCP. The initial observation was confirmed.

The effect of red light on the IAA oxidase activity of excised buds was also investigated. Six hundred-mg lots of buds were excised from 6-day-old dark-grown peas and placed in beakers with 0.02 M phosphate buffer, pH 6.1. Some were then exposed to 750 kiloergs/cm² red light, the others retained in darkness. The tissues were recovered after 16 hours, and later assayed as usual. While activity declined slightly as a result of excision, no effect of the red light could be detected. Neither the inclusion in the medium of 2 % sucrose plus 0.05 M KNO₃, nor of cotyledons from germinating peas, would confer any light-sensitivity in subsequent experiments.

BEAN AND LUPINE BUDS: Other plant materials were cursorily investigated for two reasons. It seemed desirable to see whether the results obtained with peas might be generally applicable to other plants. It would also be extremely useful for further experiments to obtain the response in excised tissues which might then be exposed to various substances and rapid changes in temperature.

The entire hypocotyl was removed from 2-day-old Black Valentine Bean (*Phaseolus vulgaris* L.) seedlings grown in darkness at 26° C, leaving only the two cotyledons and the enclosed plumule. One cotyledon was also removed, and the remaining tissue placed, plumule upwards, in a Petri dish with buffer. Twenty-five such plumules with cotyledons were used for each experimental treatment with radiation or darkness. Eighteen hours after radiation, the plumules (mainly young leaf tissue) were harvested for the usual assays.

In these experiments, 75 kiloergs/cm² of red light (the lowest energy tried) affected IAA oxidase activity precisely as in the intact pea buds. Attempts at infrared reversal, however, were completely unsuccessful over a wide dosage range. IR given alone acted in the same way as red light—it induced a great decrease in IAA oxidase activity. When a different IR filter system was used, consisting of a Corning HR Red-Purple-Ultra filter (15) and two thicknesses of red cellophane, the same results were still obtained.

An experiment was performed with buds of 5-day-old seedlings of *Lupinus alba* handled in the same way as the beans. Activity of the extract was extremely high compared with peas or beans, even in the absence of DCP or added manganese, and no evidence of an inhibitor could be detected in dark-grown tissue. Exposure to 750 kiloergs/cm² red light had no effect on activity.

DISCUSSION

The major experimental findings can be briefly summarized. The IAA oxidase activity of intact pea buds, as assayed in extracts, was inductively inhibited by total energies of red light as low as 2 kiloergs/cm² given to the intact plants at some time before harvest. The inhibition was reversible by near infrared radiation given immediately after the red light. The inhibition appears to be due entirely to increased levels of a thermostable, dialyzable inhibitor (or inhibitors) originally present in dark-grown plants as well.

Tang and Bonner (18) were the first to report that light exposure reduced the IAA oxidase activity of etiolated peas by causing an increase in the level of an inhibitor. In their experiments, inhibitor concentration increased proportionally to the number of hours of diffuse daylight given. The present results differ chiefly in showing that IAA oxidase inhibition can be controlled by low energies of photoperiodically active radiations.

A number of reports have appeared in which the auxin metabolism of etiolated or semi-etiolated material was modified by red light and near infrared treatment. Kent and Gortner (11) found that the auxin sensitivity of the split pea epicotyl test was greatly increased by exposure of the plants to red light 32 hours before use. Galston and Baker (5) showed that the auxin sensitivity of pea internode sections was reduced by exposure to about 2000 kiloergs/cm² red light 16 to 18 hours before the test. Goldacre et al (7) found the IAA oxidase activity of extracts of entire pea epicotyls, assayed in the absence of added DCP or manganese, to be much higher when plants were grown under continuous red light rather than in darkness. Liverman and Bonner (13) were able to show that the response of Avena coleoptile sections to auxin was promoted by red light; this effect was reversible by infrared, but only when the IR was given in the presence of auxin. Most recently, Klein et al (12) have used red light to decrease the sensitivity of excised hypocotyl hooks of etiolated bean seedlings to IAA.

The use of different materials treated in different ways, and the absence of any experimental attempts to relate one investigation to another, leaves the relationship of all these results completely obscure. This applies with equal force to the present work, and it is possible only to point out certain major differences between it and the reports with which it might be compared. The total light energies needed here were lower by a factor of 1000 than those employed in (5) and (7). The materials tested, the phenomena exam-

ined, or both, were different from those in any of the preceding investigations. No IR reversals were attempted in (5) (7) or (11). In view of this situation, any attempt to discuss the present work in terms of that cited would be sheer speculation. It will be more fruitful to consider the possible physiological significance of the results presented, and also whether they afford any further insight into the red-infrared response.

Several points suggest that the red light induced increase in inhibitor may be of physiological significance. In itself, the inductive nature of the phenomenon is worth noting together with the fact that the maximum inhibition precedes maximum bud weight attained. The extreme sensitivity of bud (leaf) tissue as compared with the internode is of interest since Parker et al (14) reported that the internode response of etiolated peas was relatively slight, and irregular, compared to that of the leaves. The low light intensities needed also suggest a close relationship to de-etiolation (2, 14). No attempt was made to determine the most effective region in the red, and the fluorescent source emitted all wavelengths between 580 and about 710 mμ, so it is probable that considerably less than 2 kiloergs/cm^2 would be effective at the most active wavelength. Finally, the rapid " escape from photochemical control," after which the effect of red light is no longer reversible by infrared, is similar to that obtained by Downs (3) in the photoperiodic induction of Xanthium and Biloxi soybean.

On the assumption that the results of in vitro IAA oxidase assays are representative of in vivo activities, and on the further assumption that bud growth in the dark is limited by endogenous auxin level, the data presented might be used to explain the increased bud growth induced by red light. The first assumption, however, is debatable. Although there is a considerable body of literature on natural IAA destruction, only two reports have been primarily concerned with its physiological significance. While Galston and Dalberg (6) obtained evidence which related increased IAA oxidase to tissue ageing and cessation of growth in peas, the results of Briggs et al (1) call into question the entire concept of using in vitro extract assays as estimates of in vivo activity. In the fern *Osmunda cinnamomea* L, tissues which would transport auxin without destroying it would destroy it rapidly when converted into extracts. In addition, tissues were also found in which IAA would disappear rapidly, but which would not inactivate it as extracts. Some support for the in vivo significance of IAA oxidase has been derived from the observations of Goldacre et al (7) that 2,4-dichlorophenol (DCP), which enhances the IAA oxidase activity of extracts, also enhances the disappearance of IAA from solutions incubated with pea stem sections. This observation was re-examined and confirmed in the course of the present work, but preliminary results also showed that IAA destruction continues at a high rate for some time after removal of the tissues, suggesting that much if not all of the IAA destruction in such a system is due to leakage of

enzymatic material from the short (5-mm) sections used. In view of such findings, any direct physiological interpretation of the present results would be indefensible at this time.

The few experiments with bean and lupine tissues are also relevant to considerations of physiological significance. The ease with which a red light effect similar to that on peas could be demonstrated in bean buds contrasts with the complete inability of near infrared to reverse the action of red. This is the more remarkable since the action of red light on hypocotyl hooks of the same bean variety is clearly infrared-reversible (12); in the present experiments, however, IR acted in the same way as red itself. While no satisfactory explanation for this result is at hand, it may be related to the occasional reports (3, 19) that in cases where brief exposures to IR cause reversal of a red light effect, longer exposures may fail to do so. Whatever the mechanism, this apparent reinforcement of red light action by IR appears to be completely dominant in the bean system studied. Since no evidence of inhibitor was found in the etiolated Lupinus buds, the absence of a red light effect on their IAA oxidase activity is not surprising; it is possible that more thorough investigation would modify these results. In summary, it is evident that the results obtained with peas are probably not directly applicable to other plants, even within the same family.

While the simplest explanation for the present results appears to be an increased inhibitor level induced by red light, as stated earlier, it is not necessarily the correct one. Both naturally occurring inhibitors (4, 8) and activators or cofactors (16, 20) for IAA oxidase have been reported, but not identified. In view of the complexity of the IAA oxidase system, and of the uncertainty as to the precise nature and role of its components, alternate explanations of the increased inhibition caused by dialysates from illuminated plants might be proposed. Even assuming the inhibition to be due to increased inhibitor, the mechanism of inhibitor action is not known. Kinetic observations indicate that it acts by introducing an extended lag period. Thus the inhibition values recorded are only approximations of inhibitor level, since it proved impractical to evaluate the length of the lag period for each extract tested.

Whatever the mechanism of the inhibition, it is clear that the effect of the red light on IAA oxidase activity has been on the dialyzable, heat-stable components rather than on the protein fraction. The results represent a clear-cut biochemical change controlled by photoperiodically active radiation. The syntheses of a tomato cuticle pigment (15) and of certain anthocyanins (17) are known to be controlled by red and infrared light. To this group can now be added the unknown " inhibitor." The control of inhibitor level, and of the other compounds mentioned, suggests an approach to the identification of the light-sensitive system. Retracing their origin to the particular stage at which the light-reaction is the controlling factor would represent a real advance in light

physiology. As an initial step, attempts to identify the chemical basis of the inhibition are now in progress.

SUMMARY

The IAA oxidase activity of buds of etiolated peas, as assayed in extracts with the addition of 2,4-dichlorophenol or manganous ion, was greatly inhibited by 2 to 750 kiloergs/cm^2 red light given to the intact plants during a brief period at some time before harvest. Maximum inhibition with a given energy was obtained by illuminating about 16 hours before harvest. The inhibition was reversible by near infrared radiation given immediately after the red light, but not more than one hour afterwards. The activities of extracts from dark-grown and red-illuminated buds could be made the same by dilution, or by dialysis, suggesting that the inhibition may be due entirely to increased levels of a dialyzable inhibitor. The IAA oxidase activity of young internode tissue was insensitive to the low energies used. The results may be of significance in the physiology of de-etiolation and of other processes controlled by red and near infrared radiations.

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