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# Rapid Respiratory Changes Due to Red Light or Acetylcholine during the Early Events of Phytochrome-mediated Photomorphogenesis[1]

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## ABSTRACT

**Two millimeter long secondary root tips of etiolated mung bean (*Phaseolus aureus*) plants were given 4 minute consecutive treatments of darkness, red light, far red light, and acetylcholine during darkness. We studied the effects of these treatments on exogenous ($H^+$) changes, ATP utilization, $O_2$ uptake, $P_i$ levels, and ATPase activity. Red light and acetylcholine increased the level of $P_i$, $O_2$ uptake, and exogenous $H^+$, but decreased ATP concentrations. Darkness and far red light caused the amount of ATP to increase and decreased the $O_2$ uptake and $P_i$ level. $O_2$ uptake of both excised root tips and isolated mitochondria was promoted by acetylcholine levels of the same order of magnitude that promoted the other photomimetic phenomena. ADP-O ratios indicated that acetylcholine did not cause an appreciable decrease in ATP synthesis. The total ATPase activity remained constant throughout all treatments. Ouabain caused no adhesion to negatively charged glass in the dark, while the inhibitors valinomycin, atractyloside, digitoxin, gramicidin, and oligomycin caused immediate adhesion. All of the inhibitors prevented release from the glass. In red light ouabain increased adhesion, whereas the other inhibitors caused caused immediate and complete adhesion.**

**These data seem to imply that one of the functions of the phytochrome-mediated response to red light in roots, regulated by acetylcholine, is to cause the rapid utilization of ATP pools; far red light appears to inhibit this utilization.**

Phytochrome-mediated morphogenic responses have been extensively reviewed (19, 21), but much less is known about the reactions at the cellular and biochemical level. There are two different theories as to the mode of action of phytochrome in the cell. The first, based both on inhibitor studies and long term measurements of RNA synthesis, suggests that phytochrome acts through a gene induction mechanism (39). The other theory supports the view that phytochrome action seems to control permeability and other membrane bound events (20).

Support for the theory that phytochrome acts directly on the regulation of membrane permeability has been reported in a number of papers in which very rapid physiological, biochemical, or biophysical responses were linked to membrane bound mechanisms (1, 11, 17, 22, 28, 29, 31, 32, 52). Lateral root initiation has been shown to be inhibited in both peas and mung beans by red light (12, 28), and this inhibition can also be induced by ACh[2] in the latter system (28). If ACh regulates phytochrome responses in mung bean roots, phytochrome mediated by ACh may cause changes in membrane permeability similar to those found in the excitable membranes of animal cells. There, ACh acting on its receptor may induce conformational changes leading to alterations in membrane permeability (40), possibly allowing either ions or hormones to enter and exit the cell.

This laboratory has presented evidence that ACh mediates a variety of phytochrome responses in bean roots that seem to involve the cell membranes (28, 29). Since ACh acts on membranes, and because exogenous ATP is required for far red-induced release from a negatively charged glass surface, it seemed appropriate to look for phytochrome-related ATP metabolism. The results of that study are presented here.

## MATERIALS AND METHODS

**Analytical Assays.** All the analytical assays were done with 6-day-old etiolated or 11-day-old light-grown mung bean (*Phaseolus aureus*) secondary root tips that were given consecutive treatments of 4 min dark; 4 min dark + 4 min red; 4 min dark + 4 min red + 4 min far-red; or 100 $\mu$M ACh during 4 min of darkness. The irradiation was performed in chambers that have been previously described (55) at 50 $\mu w/cm^2 \cdot sec$ at 660 nm and 150 $\mu w/cm^2 \cdot sec$ at 730 nm.

Twenty 2 mm long tips of secondary roots were harvested from 5-day-old etiolated mung bean plants for measurement of ATPase activity. They were frozen and ground in a cold mortar with 5.0 ml of 20 mM tris-maleate buffer at pH 7.4. The homogenate was divided into 2 aliquots of 2.5 ml each and incubated with 1 ml of 1.0 mM ATP. The ATPase activity in one aliquot was stopped at zero time by adding 1 ml of 1 N trichloroacetic acid. The rate of ATPase activity was linear for the first 30 min, therefore, each aliquot was incubated at 27 C for 30 min. The homogenate containing 1 ml of 1 N trichloroacetic acid was then neutralized with 1 N NaOH and centrifuged for 5 min at 2700*g*. Three milliliters of the supernatant fluid were added to 2 ml of chromogenic reagent (30) and allowed to develop for 15 min at 27 C. The resulting blue color was measured at 650 nm on a Coleman-Hitachi double beam spectrophotometer. A reagent blank was prepared by adding 3 ml of a solution containing 1 ml of 1 mM ATP and 3.5 ml of ion-free water to 2 ml of chromogenic reagent.

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[2] Abbreviation: ACh: acetylcholine.

The inorganic phosphate concentration of 40 2 mm secondary root tips was measured after irradiation by grinding them in a cold mortar with 2.5 ml of tris-maleate buffer, pH 7.4, containing 1 ml of 1 N trichloroacetic acid to stop ATPase activity. The solutions were neutralized, centrifuged, and color development was measured at 750 nm using the chromogenic reagent of Chen *et al.* (7). The development of the color was complete after 2 hr, and a standard phosphate curve was prepared by using $Na_3PO_4 \cdot 12\ H_2O$ (30).

To measure oxygen uptake, two methods were used. In the first, 100 1.0 cm etiolated secondary root tips (about 100 mg) were excised under a safelight and placed in 5 ml of 20 mM tris-maleate buffer at pH 7.4. They were then transferred under the safelight to a Warburg flask fitted with a single side arm and containing 0.3 ml of tris-maleate buffer which kept the root tips moist. A piece of filter paper moistened with 0.20 ml of 20% KOH was placed in the center well and 0.25 ml of 0.1 mM ACh was placed in the side arm. Three prepared flasks were fastened to a Gilson respirometer. Two separate flasks containing 0.5 ml of buffer were used as a thermal barometer and an equilibrium reference, and the water bath was set for 27 C. Following 15-min equilibration, the manometer valves were closed, and readings were taken every minute for 15 min. At the end of the 15-min dark exposure, the flasks were removed from the constant temperature bath under a green safelight, and the aluminum foil was taken off. The flasks were quickly rewrapped with two layers of red cellophane for red light exposure and then returned to the constant temperature bath. For far red exposure, blue cellophane was wrapped over the red. Irradiation was provided by two 20 w rheostatically controlled incandescent lamps placed 27 cm above the water bath with a mirror placed in the bottom of the bath to reflect light toward the flasks. A sheet of aluminum foil, placed so that it would not stop light from reaching the flasks, reflected excess heat given off by the lights.

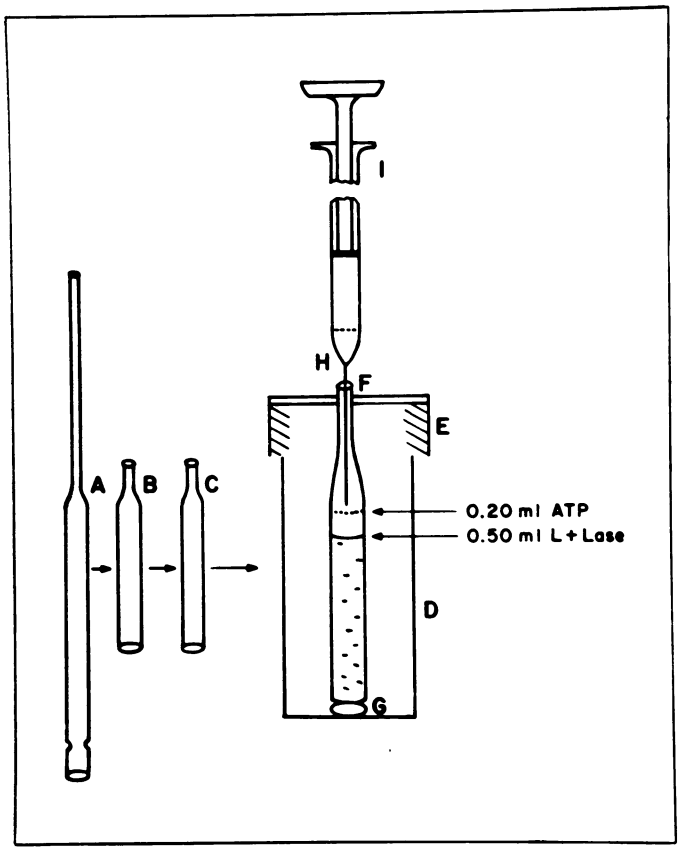


FIG. 1. Diagram of luciferin-luciferase ATP assay system performed with a Packard Tri-Carb scintillation counter. A: Disposable pipette; B: pipette cut to desired length; C: pipette with sealed end; D: standard scintillation vial; E: vial cap with hole drilled in it; F: end of vial protruding from cap; G: vial held in place with clay; H: syringe placed in secondary vial neck; I: hypodermic syringe.

Exposures to dark, red, and far red light were repeated several times, allowing the flasks to equilibrate for 5 min before each light exposure. Following the light exposures, the flasks were removed from the bath, tipped to mix the ACh with the excised root tips, and allowed to equilibrate for 5 min.

In the second method for measuring oxygen uptake, 20 to 40 1 cm long root tips were excised and placed in 2.5 ml of phosphate buffer, pH 7.0, in a reaction vial over a magnetic spin bar. A Clark polarographic electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) was used for the measurement of dissolved oxygen. The electrode was in circuit with an amplifier and a variable gain chart recorder. To test the effect of ACh on oxygen uptake, 0.2 ml of ACh in distilled water was added to the reaction mixture containing the root tips. The resulting rate of oxygen uptake was compared to the rate before adding the ACh, and the datum expressed as percentage change.

Mitochondria were isolated from light-grown secondary bean roots as follows. One gram of root tips was used for each preparation. All steps of the extraction procedure were performed at about 4 C, and all solutions and glassware were prechilled to that temperature. The chilled root tips were homogenized for 45 sec in a mortar with 10 ml of grinding buffer per gram of tissue. The grinding buffer consisted of 0.5 M sucrose, 1 mM EDTA, 67 mM potassium phosphate buffer at pH 7.2, plus 0.75 mg of bovine serum albumin per ml. The homogenate was strained through a double layer of fine mesh nylon fabric. The filtrate was centrifuged for 15 min at 3000 rpm using a Sorvall SS-34 rotor. The pellet was removed, and the supernatant fluid was removed by aspiration, and fresh grinding buffer was added; the suspension was centrifuged for 2 min at 20,000*g*. The mitochondrial pellet was then resuspended for use in 0.5 ml of the basal reaction medium. This consisted of 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate buffer, and 10 mM tris-HCl buffer at pH 7.2, plus 0.75 mg of bovine serum albumin per ml. Tightly coupled mitochondria were obtained by the method of Ikuma (24), and ADP-O ratios were obtained by the method of Estabrook (9).

Oxygen uptake by excised root tips was measured in the dark and in the light, and extracted mitochondria in the light. For the experiments in the light, the reaction was carried out in a growth chamber (Sherer-Gilette Co., Marshall, Mich.) lighted by fluorescent lamps which were reflected via a mirror to the reaction chamber. The reaction vessel was enclosed in a glass-walled circulating water bath which was maintained at 27 C.

ATP was measured by the luciferin-luciferase reaction (54). Thirty 2-mm secondary root tips were placed in 2 ml of ion-free water for irradiation. At the end of each exposure, 1 ml of 1 N trichloroacetic acid was added, and the tips were ground in a cold mortar. The homogenates were neutralized as before and centrifuged for 5 min at 2500*g*. The supernatant fluids were pipetted into clean test tubes and kept in an ice bath for ATP analysis. ATP concentration was measured by first adding 0.5 ml of refrigerated luciferin-luciferase into a specially prepared cuvette positioned in a scintillation vial (Fig. 1). Extract (0.2 ml) was injected into the cuvette at the same time that the elevator

to the counting chamber was lowered. Luminescence decayed very rapidly (Fig. 2); hence, measurements were integrated over the first 6 sec by the instrument. Counting was done for 6 sec on a Packard Tri-Carb Model 2002 liquid scintillation counter, set for a window of A25, B10, and a gain of 6.6, at manual operation. The instrument was specially modified by the company to eliminate the lag preceding the start of counting. A standard curve was made for each experiment using freshly prepared ATP solutions (Fig. 3). Measurements of exogenous $H^+$ changes were done by the method of Jaffe (28).

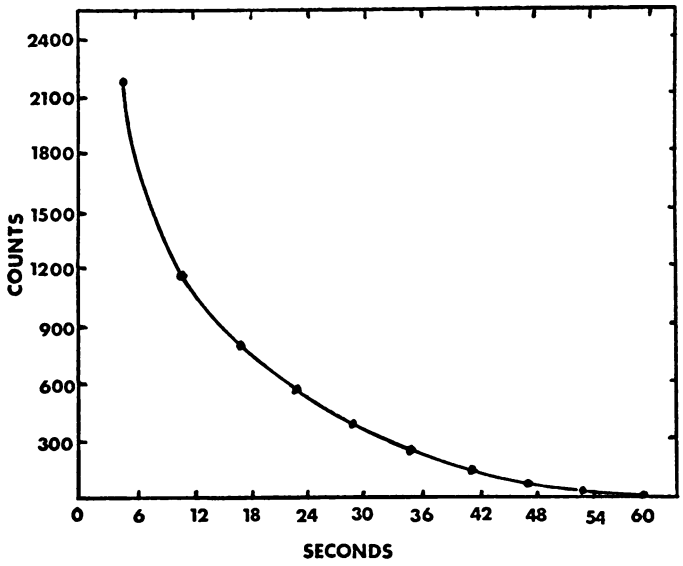


FIG. 2. Kinetics of luminescence decay in the luciferin-luciferase (L-Lase), ATP reaction. The reaction cuvette contained 0.5 ml of freshly prepared L-Lase and 0.2 ml freshly prepared 10 nM ATP.

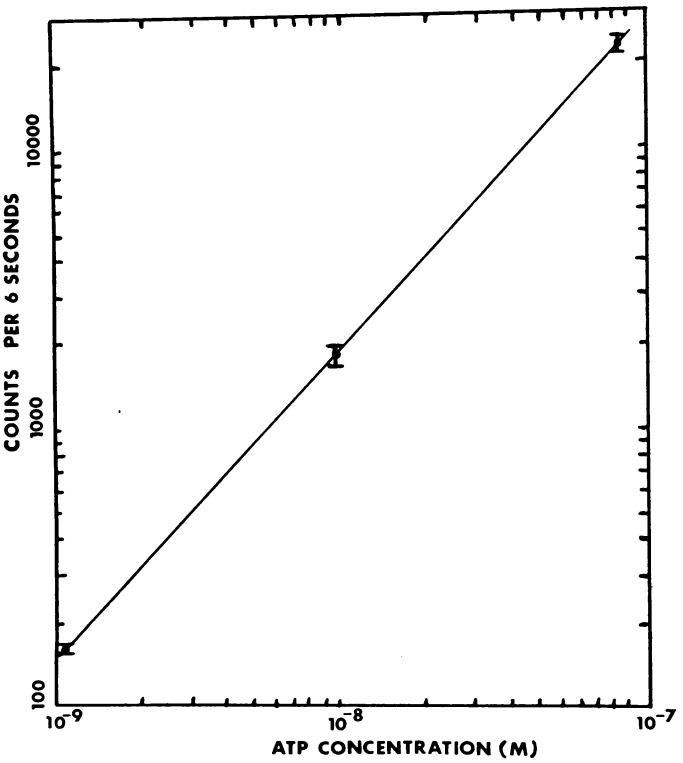


FIG. 3. ATP standard curve using the luciferin-luciferase assay performed on a Packard Tri-Carb scintillation counter. The reaction cuvette contained 0.5 ml of freshly prepared L-Lase and 0.2 ml of freshly prepared ATP. The vertical lines indicate the standard errors.

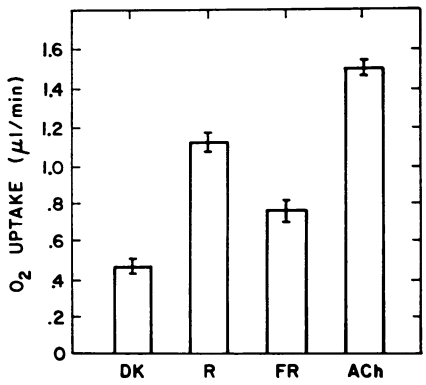


FIG. 4. $O_2$ uptake at 27 C in 100 1 cm long mung bean secondary roots, treated with either 15 min dark (DK), 15 min DK + 15 min red (R), 15 min DK + 15 min R + 15 min far red (FR), or 15 min of 0.1 mM ACh in the dark. The data represents the results of five experiments. The vertical bars indicate standard errors.

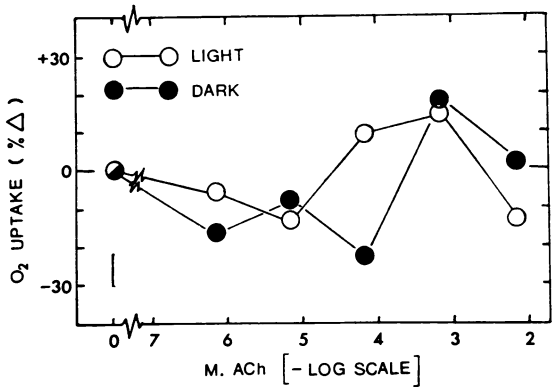


FIG. 5. Oxygen uptake by excised roots of light-grown plants respiring either in the light or in the dark. $O_2$ uptake was measured for 3 min, ACh was added with a syringe in 0.2 ml of distilled water, then $O_2$ uptake was measured for 3 more min. The difference in $O_2$ consumption was obtained from these data and plotted as the percentage change of the first 4-min measuring interval.

**Adhesion and Release.** The effects of 5 µM solutions of ADP, AMP, or cyclic AMP as substitute for ATP were tested on the photoreversible adhesion and release of root tips to a negatively charged glass surface (55). Inhibitors that affect respiration and ion flux were added in the dark or immediately before red and far red irradiation. Those used were valinomycin, 10 µM; gramicidin, 0.10 µM; oligomycin, 0.25 µM; atractyloside, 10 µM; digitoxin, 10 µM; ouabain, 25 µM.

## RESULTS

Measured manometrically, oxygen uptake increased in red light and ACh but decreased in far red light to a level comparable to that found in darkness, a reversal of the red light and ACh response (Fig. 4).

Secondary root tips from light-grown plants give the same phytochrome response as do those from dark-grown plants (55). When ACh was added to the reaction mixture of such light-grown root tips, the rate of oxygen uptake, as measured with the oxygen electrode, was affected both in the light and in the dark (Fig. 5). Lower concentrations of ACh either

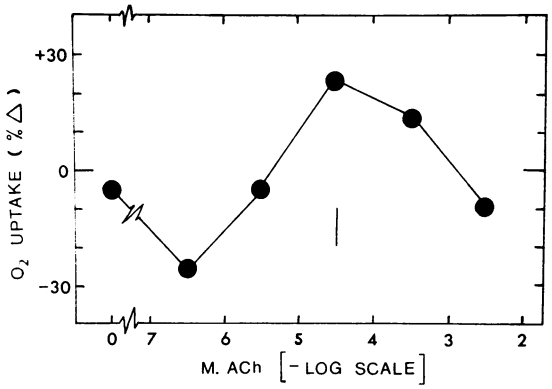


FIG. 6. Oxygen uptake by suspensions of the mitochondrial fraction of bean root tissue. The data was obtained and computed as in Figure 5. The vertical bar indicates the average standard error.

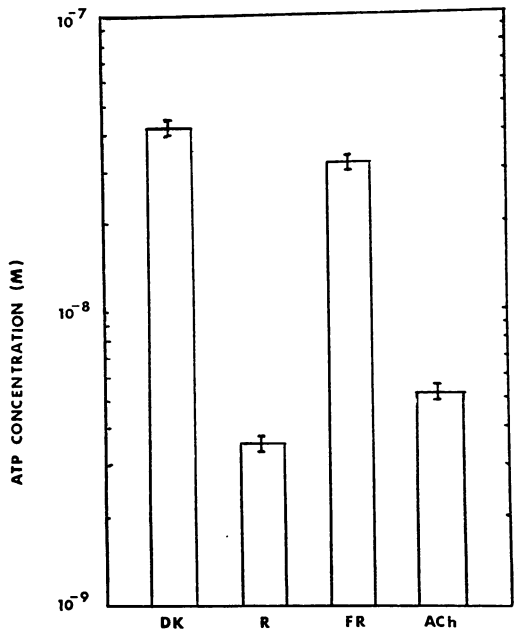


Fig. 7. ATP concentration in 30 2 mm long mung bean secondary root tips treated with either 4 min dark (DK), 4 min dark and 4 min red light (R), 4 min red light and 4 min far red light (FR), and bathed 4 min in 0.1 mM ACh. The vertical bars indicate the standard errors.

retarded or had little effect on respiration and will not be discussed here, as the concentrations were not consistent with those used in other experiments. Eighty micromolar to 80 mM ACh increased $O_2$ uptake. This is consistent with the increase of oxygen uptake caused by 100 mM ACh, measured by Warburg manometry (Fig. 4). Although the roots exposed to both light and darkness responded to ACh, those in the light were enhanced by lower concentrations. This might be because of differences in ACh uptake due to light-activated changes in membrane permeability, or to changes in the respiratory mechanism. The respiratory activity of the mitochondrial fraction of bean root tissue was similarly affected by ACh (Fig. 6). ACh (330 mM) retarded and 33 mM ACh optimally increased oxygen uptake in isolated mitochondria. About 25 times more ACh was needed in the reaction medium to optimally increase respiration in the excised roots than in the mitochondria. This was probably due to the difficulty of getting the ACh into the roots.

The dosage response of bean root mitochondria to ACh is very similar to that of rat brain mitochondria (53, 54). Thus, it is possible that the well known role of ACh in regulating nerve membrane permeability to ions functions via the mitochondria in the nerve cells.

ATPase activity did not vary in any of the treatments. The absorbance of the $P_i$-chromogenic complex after 30 min incubation was: dark, 0.035; red light, 0.035; far red light, 0.035; and ACh, 0.037. All ATPase is therefore apparently not directly affected by red and far red light or ACh. ATP concentration was found to decrease 12-fold in red light and in ACh, and returned to slightly less than the dark level in far red light (Fig. 7). The substitution of various nucleotide phosphates, ADP, AMP, and cyclic AMP for ATP during the adhesion and release of root tips confirms the need for ATP in release under far red light. Partial adhesion and release was observed in red light with cyclic AMP and AMP and adhesion, but no release was observed with ADP (Fig. 8). $P_i$ concentration increased in red light and ACh but decreased in far red light (Table I). However, the concentration of $P_i$ was much higher than that released by ATP hydrolysis, indicating a possible involvement of other $P_i$ releasing systems triggered by red light or ACh.

When ADP-O ratios (9) were computed by examining state III and IV respiration of coupled mitochondria treated during steady state II respiration with buffer or 10 μM ACh, no changes in ATP synthesis due to ACh were observed (Table II).

Exogenous hydrogen ion concentration increased in root tips exposed to red light and ACh and decreased in dark and

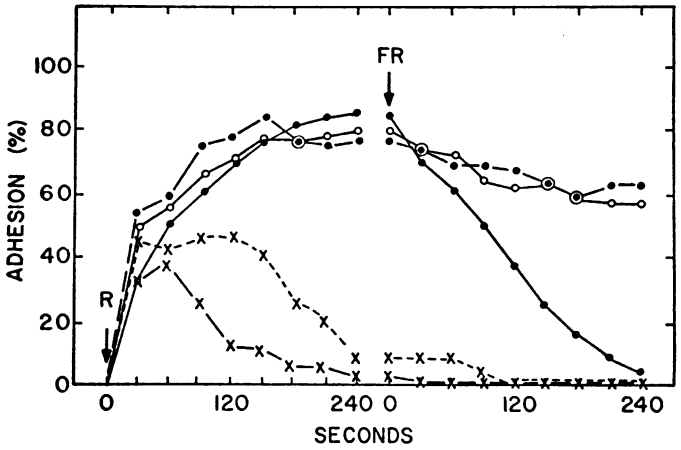


FIG. 8. The effects of substituting 5 μM either ADP (●——●); AMP (×---×); cyclic AMP (×——×), or no addendum (○——○), for ATP (●——●), on the adhesion and release of mung bean secondary root tips to glass. Average standard error, ±1.7.

Table I. *Inorganic Phosphate in Mung Bean Secondary Root Tips*

Root tips were treated with either 4 min dark, 4 min dark and 4 min red light, 4 min red light and 4 min far red light, or bathed 4 min in 0.1 mM ACh. Each datum is followed by the standard error.

| Inorganic Phosphate in Roots | |
|---|---|
| Exposure | $P_i$/4 min |
| *μmoles/100 2 mm root tips* | |
| Dark | 0.55 ± 0.01 |
| Red light | 7.95 ± 0.01 |
| Far red light | 0.50 ± 0.02 |
| ACh | 7.50 ± 0.03 |

far red light (Fig. 9). There was, however, no change in the ambient hydrogen ion concentration when the experiment was done with boiled roots, a piece of cotton thread, or nothing between the electrodes. The addition of ACh itself caused a change in pH, and the data in the figure are corrected for this. Inhibitors were used to further investigate a possible relationship of ATP metabolism and ion transport with adhesion and release. Each inhibitor, gramicidin, oligomycin, atractyloside, digitoxin, valinomycin and ouabain prevented root tip release in far-red light (Fig. 10, A–F). Gramicidin, oligomycin, atractyloside, digitoxin, and valinomycin increased the rate of root tip adhesion in red light and dark. Ouabain did not cause adhesion in the dark (Fig. 10F), but increased it slightly in red light.

## DISCUSSION

ATP concentration, exogenous $H^+$ changes, and $O_2$ uptake are regulated by the photoreversible conversion of phytochrome, and ACh is able to mimic red light in these responses. We propose that the hormone ACh couples the phytochrome to these energetic processes and that acetylcholine is directly involved in the early events following the photoconversion of phytochrome (28, 29). Many long and short term phytochrome-controlled responses, such as changes in RNA, ion transport, synthesis of Calvin cycle enzymes, histogenesis in pea tissue, changes in bioelectric potential, $K^+$ uptake, auxin-induced lateral root initiation, chlorophyll synthesis, and electrolyte efflux from *Albizzia pinna* presumably involve utilization of energy from ATP (10–13, 23, 26, 27, 31, 32, 44, 51). ATP is required for root tip release (49, 55) in far red light, as shown by the unsuccessful substitution of ADP for ATP, although this requirement can be partially satisfied by cytidine triphosphate (55). Only partial adhesion and release occurred in red light when either form of AMP was substituted for ATP. This may be caused by AMP acting as an inhibitor and occupying active sites on ATPase, thus preventing its proper functioning.

The data obtained from the addition of inhibitors during the red, far red photoreversible adhesion and release of root tips suggest that the uptake of cations into the cell are involved (34). Atractyloside, gramicidin, valinomycin, and digitoxin increase $K^+$ uptake and membrane permeability to $K^+$ (2, 5, 16, 43). Ouabain, an ATPase inhibitor (6), did not stimulate root tip adhesion in the dark, suggesting that ATP is only needed for ion transport and root tip release. Oligomycin (25) accelerated adhesion in the dark, indicating that ion uptake or release may be taking place. In older parts of the root oligomycin inhibits $K^+$ influx (90%), but in the terminal 1 cm portion of the root, $K^+$ influx is stimulated about 200% (personal communication, Dr. T. K. Hodges).

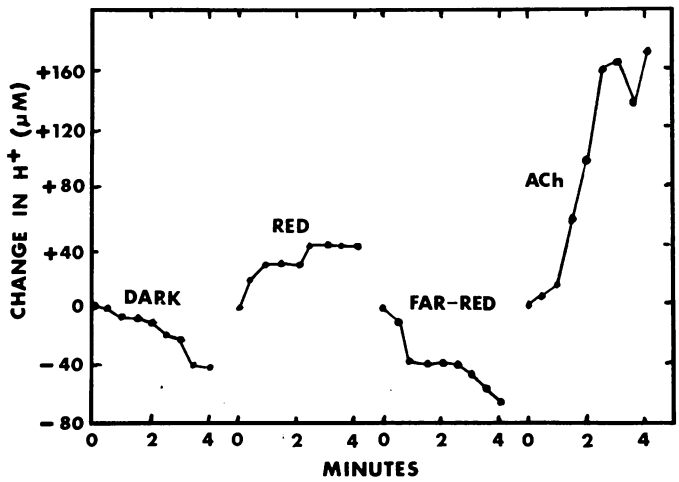


FIG. 9. Kinetics of exogenous $H^+$ concentration in the solution bathing the mung bean root tips treated with 4 min dark (DK) followed by 4 min red light (R), 4 min far-red light (FR), and 4 min 0.1 mM ACh.

$Ca^{2+}$ seems to be involved in these red and far red responses (29, 55). Exogenous $H^+$ changes, possibly due to $H^+$ efflux during treatment with red light and ACh, may be due to an accumulation of $Ca^{2+}$ in the cell. Millard *et al.* (38) have shown an equivalent release of $H^+$ for each $K^+$ ion transported into the cell, and $Ca^{2+}$ binding and uptake has been associated with a large $H^+$ release in liver mitochondria (45, 46). Hanson and Miller (15) have suggested that phosphate bond energy is expended in cation transport, and this also seems to be true for animal mitochondria (35). An analogous response has been proposed for brown fat metabolism in hibernating and young rodents. As a result of the action of the neurohumor epinephrine, energy is released and is used for fat hydrolysis (36, 42, 48).

In studying the effects of ACh on rat and rabbit brain and rabbit liver mitochondria, Vdovichenko found a 20% increase in $O_2$ consumption and a decrease in P/O ratio at ACh concentrations of 10 μM to 100 μM (53). Demin (8) found concentrations of acetylcholine between 0.68 μM to 6.8 mM stimulated $O_2$ uptake in guinea pig cerebral cortex tissue, and it was later observed that ACh concentrations of 68.4 μM to 0.68 mM stimulated $O_2$ uptake in guinea pig brain (54). We have found a large, similar increase in $O_2$ uptake in plant roots and mitochondria, but no change in ATP synthesis, indicating a possible increase in the utilization of ATP. The fact that ACh does not cause a change in the ADP-O ratio indicates that there is probably very little, if any, uncoupling of oxidative phosphorylation, a possibility that was suggested in a previous report (29).

Synthetic analogues of ACh have been studied by other workers and have been shown to produce effects similar to those that we found with ACh. For example, the chlorine substituted choline derivative, chlorocholine chloride, retards the growth of roots in peas and rice (4, 41). Heatherbell *et al.* (18) found that oxygen uptake was stimulated in mitochondria of etiolated pea roots by chlorocholine chloride at

Table II. *Estimation of ATP Synthesis*

Tightly coupled mitochondria were prepared using 10 mM succinate as substrate. After 3 min of state II respiration, either buffer or ACh (final concentration of 10 μM) was added to the reaction mixture. After 3 more min, ADP (final concentration of 0.1 mM) was added, initiating state III respiration. The rates of state III and state IV respiration and the fraction III/IV were computed for both the control and ACh-treated mitochondria.

| Experiment | State III $O_2$ Uptake/State IV $O_2$ Uptake | |
|---|---|---|
| | Buffer control | 10 μM ACh |
| 1 | 1.76 | 1.82 |
| 2 | 1.60 | 1.73 |
| 3 | 1.58 | 1.45 |
| 4 | 1.38 | 1.50 |
| 5 | 1.56 | 1.31 |
| 6 | 1.73 | 2.14 |
| 7 | 2.18 | 1.70 |
| 8 | 1.70 | 2.14 |
| Average | 1.69 | 1.72 |

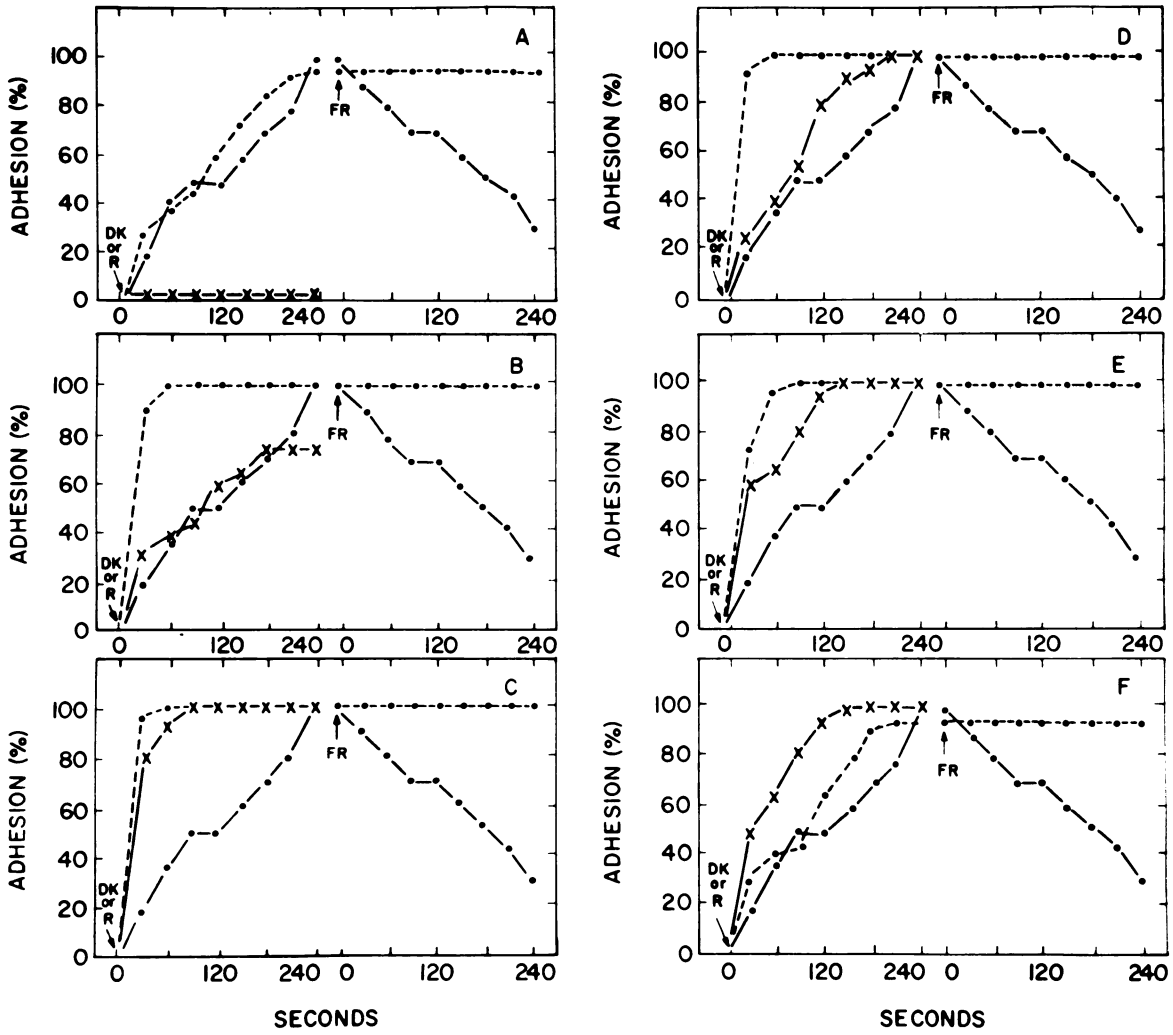


FIG. 10. The effects of various inhibitors on the adhesion and release of excised root tips from a negatively charged glass surface. Inhibitor in the dark (×——×); red and far red light minus inhibitor (●——●); red and far red light plus inhibitor (●- - -●). A: 10 μM gramicidin, SE = ±1.2; B: 2.5 μM oligomycin, SE = ±0.4; C: 1 mM atractyloside, SE = ±1.4; D: 1 mM digitoxin, SE = ±1.7; E: 10 μM valinomycin, SE = ±1.1; F: 2.5 mM ouabain, SE = ±1.2.

a concentration of 8.3 mM to 1.6 mM. The mode of action of such plant growth retardants remains unknown (50), but it has been proposed that growth retardants are antagonistic to gibberellins and auxins (14, 33, 37), although this interpretation has been criticized (3). Heatherbell *et al.* (18) suggest that ATP, necessary for cell division, elongation, and auxin synthesis is decreased in response to the growth retardants.

Therefore, we propose the following scheme which seems to be supported by our data. ACh is synthesized and released in the root cells as a result of irradiation with red light (28) and moves at random to many target sites. One of the latter is the cell membrane where ACh facilitates ion transport. Another target site is the mitochondrial membrane, where ACh may change membrane configurations (29) and where oxygen consumption is increased and the rate of ATP utilization is apparently increased. This may play a role in the long and short term morphological effects observed in plants exposed to red light or ACh. In any event, the bond energy of ATP may then in part be used for the active transport of monovalent cations (35), and perhaps in part to other energy requiring biochemical mechanism, such as a synthetic process. Far red light induces the destruction or prevents further release of ACh (28), thus reversing the effects of red light on respiration.

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