Rapid Phytochrome-mediated Changes in the Uptake by Bean Roots of Sodium Acetate [1-14C] and Their Modification by Cholinergic Drugs*

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Summary. 4 min of red light increases the uptake of sodium acetate[1-14C] by excised, etiolated secondary roots of Phaseolus aureus Roxb. 4 min of far-red light reverses this effect. AMO-1618, which inhibits acetylcholinesterase activity, enhances the red-light effect, while d-tubocurarine, which blocks the animal acetylcholine receptor, inhibits it. Red light also increases basipetal translocation of the label. When the metabolic fate of the label was determined in dark-held roots, 36% of the label remained as acetate, 48% evolved as [14C]CO2, 3% partitioned with acetylcholine, and 3% effluxed from the roots. The rest of the label was associated with the coarse residue left after extraction. The major effect of red light was to increase the uptake of the label in the acetate fraction.

We interpret these observations to mean that the phytochrome mechanism immediately causes an increase in uptake of the label during brief irradiation with red light. Because of our previous demonstration that both red light and acetylcholine increase respiration, it is probable that the increased absorption of the label is a process requiring respiratory energy. These data support the concept of phytochrome as a membrane-bound functional system that in bean roots is mediated by the acetylcholine mechanism.

Introduction

The demonstration, in several laboratories, that phytochrome is able to mediate a number of very rapid responses (Fondeville et al., 1967; Forward, 1970; Yunghans and Jaffe, 1970) has generated the hypothesis that the primary function of this pigment is to change the selective permeability of cell membranes (Hendricks and Borthwick, 1970). This idea has been supported by the demonstration of phytochrome-mediated changes in salt flux (Jaffe and Galston, 1967), and phytochrome-regulated

* Abbreviations: Acetylcholine = ACh; acetylcholinesterase = AChE; adenosine triphosphate = ATP; 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methyl chloride = AMO-1618; tetraphenyl boron = TPB; D = darkness; FR = far-red; R = red.

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proton efflux (Jaffe, 1970; Yunghans and Jaffe, 1972) and depolarizing potentials in bean roots (Jaffe, 1968; Newman and Briggs, 1971; Racu- sen et al., 1971). It seemed probable that if phytochrome mediated changes in the ability of cell membranes to pass solutes, then roots which are organs specifically adapted for absorption and release should display uptake characteristics that could be altered by red and far red light. Because the bean root system seems particular well suited to such a study (Yunghans and Jaffe, 1970), we have designed experiments to measure the uptake of sodium acetate\[1-^{14}C\] by these organs during brief irradiation with red and far red light.

**Materials and Methods**

The secondary roots of 7-day-old, dark grown seedlings of *Phaseolus aureus* Roxb. were used as previously described (Jaffe, 1968; Yunghans and Jaffe, 1970). For each determination, 20 roots were lined up with their tips at the end of a glass microscope slide. Another slide was fastened over these, exposing the apical 1 cm. These root tip "sandwiches" were the experimental preparations and were used in a dark room maintained at 23°C. For each determination, one or more sandwiches was placed in 12 ml of 2 μCi sodium acetate\[1-^{14}C\] (4.3 × 10⁶ dpm) solution in water or 10 m M phosphate buffer, pH 5.5 (both of which gave similar results) in a 50-ml beaker. The irradiation regimes were: 4 min D, 4 min R light, 8 min D, 8 min R, or 4 min R followed by 4 min FR light. The roots on the slide were removed after intervals of 4 or 8 min, blotted dry with a piece of filter paper, rinsed with 50 ml distilled water, and again dried. The sandwiches were then placed in light-tight boxes and frozen for 1 h before homogenization.

Radioactive acetate and ACh were recovered and counted according to the method of Fonnum (1969). The exposed 10 mm of roots were cut from the slide with a sharp scalpel and ground in 1 ml water with a hand homogenizer. The solution was centrifuged 10 min at 2000 × g, the supernatant was removed and saved, and the pellet resuspended in 1 ml and again centrifuged. All separation steps were done in the cold to minimize metabolism of the label.

The supernatant from the second centrifugation was added to that of the first and 1 ml of the combined supernatant (containing labeled authentic ACh and sodium acetate) was then added to 5 ml of sodium tetraphenyl borate in benzyl alcohol (5 mg TPB:1 ml benzyl alcohol) in a test tube. The test tubes were stoppered, shaken vigorously for 20 s and then centrifuged at 2000 × g for 10 min. Two distinct layers resulted; the water-soluble acetate in the upper layer and lipid-soluble cations, such as ACh, in the alcohol fraction (Fig. 1). 0.5 ml of the water fraction or 1 ml of the alcohol fraction were added to 10 ml Aquasol (New England Nuclear Co. Boston, Mass., USA) for counting in a Packard Tricarb liquid scintillation counter. Corrections for 100% efficiency and volume losses were made in the final calculations.

In order to test the effect of cholinergic drugs on the phytochrome mediated uptake of labeled sodium acetate, the root sandwiches were placed in solutions of 10⁻³ or 10⁻⁵ M AMO-1618, or d-tubocurarine for 30 min prior to the 4-min uptake experiment in red light.

The efflux into the bathing solution, of previously absorbed sodium acetate \[1-^{14}C\] was determined, in order to determine if efflux had any effect on irradiation
Extract 20 roots in 1 ml water
[centrifuge at 2000xg] for 10 min

Resuspend and wash pellet

Supernatent fluid

pellet

2 ml

1 ml combined supernatent fluid + 5 ml of TPB in benzyl alcohol
(5 mg/ml), and shake
[centrifuge at 2000xg] for 10 min

Alcohol
95.2% Ac
1.5% ACh

Water
4.8% ACh
98.5% Ac

Fig. 1. Flow sheet for the partitioning of sodium acetate and acetylcholine. After homogenizing and washing with distilled water, the supernatant obtained by the final centrifugation of the homogenate was vigorously shaken in the biphasic system of water:TPB in benzyl alcohol. When the two phases were separated by centrifugation, most of the acetate was in the upper water phase and most of the ACh was in the lower alcohol phase.

induced changes in uptake. 20 roots on a slide were allowed to take up the label for 8 min. The roots were then blotted dry, rinsed, blotted dry once more, and the preparation placed in 12 ml unlabeled acetate (6 µg/12 ml). This concentration was similar to that of the label within the roots. The preparation was gently stirred by a magnetic bar, and 0.2 ml aliquots of the bathing solution were taken during intervals of dark, red and far-red light treatments. The amounts removed were added to 10 ml Aquasol and counted.

In order to detect the amount of sodium acetate[1-14C] that was metabolized through respiration to [14CO2], one root-tip sandwich was placed in a beaker of labeled sodium acetate. This was placed in a cubic, closed plastic container measuring 100 mm per side. 3 ml of 10% hyamine hydroxide in methanol were included in a dish and magnetically stirred. 4-min treatments of darkness or of red light were given, and at the end of these the preparation was pulled from the labeled solution by a thread from the outside of the chamber. At this time 0.1 ml of concentrated H2SO4 was added to the bathing solution to drive off any dissolved CO2. After 4 more minutes, the mixture of hyamine hydroxide and hyamine carbonate was removed from the chamber, and 1 ml aliquots were added to 10 ml of a toluene-
based scintillation fluid. The effect of quenching by the hyamine was eliminated by using an internal standard during counting.

The basipetal translocation of the labeled acetate was followed by allowing the roots to take up the label as usual in either 4 min of darkness or red light. After rinsing and drying, the roots were divided into 4 equal, linear quarters. Each sample was dissolved for 48 h in protosol (New England Nuclear Co.). The radioactivity of the dissolved samples was then measured in the liquid scintillation counter.

**Results**

The excised secondary roots easily took up the labeled acetate (Table 1). Table 1 shows the ultimate fate of the [1-14C] label when roots were extracted after 4 min D, or 4 min of R irradiation. The initial pellet

<table>
<thead>
<tr>
<th>Fraction</th>
<th>dpm/20 roots</th>
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<tbody>
<tr>
<td></td>
<td>4 min darkness</td>
</tr>
<tr>
<td>Pellet</td>
<td>434 ± 78a</td>
</tr>
<tr>
<td>Water soluble</td>
<td>1360 ± 107a</td>
</tr>
<tr>
<td>Soluble in alcohol and TPB</td>
<td>100 ± 13a</td>
</tr>
<tr>
<td>Effluxed into cold bathing solution</td>
<td>103 ± 48a</td>
</tr>
<tr>
<td>Evolved as [14C]-labeled CO₂</td>
<td>1831 ± 213a</td>
</tr>
</tbody>
</table>

 contained 11% and 10% of the label, respectively. This pellet probably contains large membrane fragments and some large organelles, as well as the cell-wall material. Because of the unknown nature of these constituents, the nature of the radioactivity bound in this fraction cannot be rigorously interpreted at this time. 36% of the label was recovered in the water fraction from the D homogenate, and 40% from the R-irradiated material. However, the absolute amount of label retained in the water fraction of R-irradiated roots was significantly greater than that from the D treatment. This increase represents a true enhancement of uptake by R light rather than a decrease in efflux of the label since the amount of label effluxed in the same length of time was negligible during both
Table 2. The effect of red and far red light on the uptake of sodium acetate[1-14C] by excised bean roots

Each datum indicates the amount of label in the appropriate fraction, and the method of partitioning the label is as shown in figure one. Each datum is followed by its standard error and the 95% confidence limit was determined with a "t" test; in each column, data followed by different letters are significantly different from one another at the 5% level.

<table>
<thead>
<tr>
<th>Irradiation regime</th>
<th>dpm per 20 roots</th>
<th>H₂O-soluble fraction</th>
<th>TPB-benzyl alcohol fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 min dark</td>
<td>1360±107 a</td>
<td>100±13 a</td>
<td></td>
</tr>
<tr>
<td>4 min red</td>
<td>1892±161 b</td>
<td>133±16 b</td>
<td></td>
</tr>
<tr>
<td>8 min dark</td>
<td>3578±470 c</td>
<td>378±44 c,d</td>
<td></td>
</tr>
<tr>
<td>8 min red</td>
<td>4771±249 d</td>
<td>434±51 d</td>
<td></td>
</tr>
<tr>
<td>4 min red + 4 min far red</td>
<td>3039±264 c</td>
<td>341±40 c</td>
<td></td>
</tr>
</tbody>
</table>

treatments (Table 1). The sums of the water fractions plus the effluxed label are 1463±155 dpm for 20 dark-held roots, and 1948±163 for R-irradiated roots, and are significantly different. A very large percentage of the absorbed label was respired and released as [14C]CO₂: 47% in the D and 45% by the R-irradiated roots. There was no significant increase in the absolute production of labeled CO₂ during R irradiation over what was produced in the D. The alcohol fraction, which presumably contains strongly cationic lipoidal substances such as ACh, contained significantly more label in the R-irradiated samples than in those obtained from D-held roots. Calculating the label in just the alcohol and water layers, there was 6.9% in the D and 6.6% in R-irradiated samples, in comparison with 1.5% using authentic compounds (Table 2). However, we do not feel that the small amount of label partitioned into the alcohol fraction can be meaningfully interpreted at this time.

Because of the uncertainty of the composition of the pellet fraction, we omitted it from the data when calculating the uptake, leaving the alcohol and water layers. Table 2 shows that the roots took up sodium acetate[1-14C] faster in R light than in D, and that the effect of R light could be reversed by FR light. Thus, during the second 4 min of R irradiation, the roots took up 2878±160 dpm of labeled sodium acetate, but when 4 min of R light was followed by 4 min of FR light, the value was 1147±130 dpm.

When the D-held roots were divided into four equal linear fractions, there was 3.1 times as much label in the apical portion as in the basal quarter (Fig. 2). In those roots that had been irradiated with R light, however, there was only 1.4 times as much label in the apical as in the
Fig. 2. Translocation of the label in excised secondary roots during 4 min of darkness or red light. 20-mm-long roots were excised, placed in a glass sandwich and the tips submerged in 12 ml of bathing solution containing 2 μCi of sodium acetate[1-14C]. After 4 min the roots were rinsed, divided into four equal, linear quarters and each quarter sample was placed in 1 ml of Protosolv for 48 h. 1-ml aliquots of this were added to 10 ml Aquasolv scintillation fluid, and counted. A secondary root is sketched below the abscissa to indicate the regions of the root that were used. In the experiment illustrated by the figure, the total dpm from roots in the dark were 1659, and from those in the red, 2044. In this experiment, the extracts were not fractionated, and total radioactivity in the crude extract in each sample is shown in the figure.

Table 3. The effect of AMO-1618 and d-tubocurarine on sodium-acetate[1-14C] uptake by bean roots

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uptake in the combined water and alcohol fractions (dpm/20 roots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 min darkness, no addendum</td>
<td>1460 ± 227a</td>
</tr>
<tr>
<td>4 min red, no addendum</td>
<td>2025 ± 177b</td>
</tr>
<tr>
<td>4 min red, 10 μM AMO-1618</td>
<td>3198 ± 325c</td>
</tr>
<tr>
<td>4 min red, 1 mM d-tubocurarine</td>
<td>1395 ± 165a</td>
</tr>
</tbody>
</table>

basal quarter. Thus the sodium acetate[1-14C] was not only taken up at a greater rate in R light than in D, but was also basipetally translocated more rapidly.
The effects of the cholinergic drugs on uptake of the label is shown in Table 3. AMO-1618, which is capable of inhibiting both animal (Newhall, 1969) and plant cholinesterases (Riov and Jaffe, in press), caused an enhancement of the red light effect. If endogenous ACh mediates the R-light effect (Jaffe, 1970), inhibiting the AChE present in the roots might enhance the effect by inhibiting the endogenous acetylcholinesterase (Riov and Jaffe, in press) and allowing the ACh to persist and continue to act. The ACh animal receptor antagonist, d-tubocurarine, inhibited the R-light enhanced uptake, presumably by occupying the ACh recognition site on the receptors at the expense of the ACh in the root cells.

Discussion

Jackson et al. (1970) have shown that the acetate ion, rather than the salt, can be absorbed by roots. However, from our data it is not apparent whether the label is absorbed as the salt or as the organic anion. Our study demonstrates that the absorption and translocation of labeled sodium acetate by bean roots is under phytochrome control, and that this control can be reversed or enhanced by the appropriate cholinergic drugs. The increased uptake during brief R irradiation is probably not due to an increased acetate gradient caused by the size of the intracellular acetate pool, since there is but a relatively small increase in respiration of the label. This apparent, but not significant R-light-induced increase in $[^{14}\text{C}]\text{CO}_2$ production is undoubtedly related to the parallel increase in oxygen consumption which we have previously demonstrated to occur during treatment with ACh or R light (Jaffe, 1972; Yunghans and Jaffe, 1972). Since ACh has a similar effect on cell-free preparations of mitochondria (Yunghans and Jaffe, 1972), the increase in uptake of the label can be said to occur together with an increase in respiration. We have shown that part of this increase in respiratory activity is accompanied by a sudden, rapid decrease in the ATP content of the roots (Yunghans and Jaffe, 1972). Thus, it seems that the R light induced increase in uptake of sodium acetate$[^{1-14}\text{C}]$ is an energy-requiring process and that the source of the energy is probably ATP synthesized during respiration.

It is further possible that the energy used in this phytochrome-mediated process is responsible for the increased translocation of the label during R irradiation. Since such translocation probably occurs as a result of dynamic action in the phloem cells, ACh regulated activity of mitochondria is probably involved there also.

Although phytochrome-mediated absorption and translocation of solutes have been reported by other workers (Goren and Galston, 1966; Köhler et al., 1968), those changes occurred hours after irradiation. Further, whereas our previous reports involved phytochrome-mediated
fluxes of inorganic ions (Jaffe and Galston, 1967; Jaffe, 1970, 1972), the present study is the first demonstration of rapid phytochrome-mediated enhancement of the absorption and translocation of an organic salt during irradiation with R light. The rapidity of this effect, and of its reversal by FR light, indicate how closely it must be related to those primary events immediately following phototransformation of the phytochrome holochrome.

Since the most rapidly measured of these events is the development of a phytochrome-mediated bioelectric potential (Jaffe, 1968; Newman and Briggs, 1971; Racusen et al., 1971), and because there is evidence that ACh mediates the phytochrome mechanism in mung-bean roots (Jaffe, 1970), it is not surprising that d-tubocurarine inhibits and AMO-1618 enhances the R-light effect. In fact, it seems probable that ACh regulates the R-light-induced increase in uptake of sodium acetate [1-14C], just as it does the phytochrome-mediated efflux of H+ ions from bean roots (Yunghans and Jaffe, 1972). A similar relationship has recently been reported by Evans (1972); in this case ACh stimulates the growth of Avena coleoptiles and atropine inhibits the stimulation.

Thus we conclude that by regulating such processes as absorption across the cell membrane and translocation up the root, phytochrome controls developmental and growth changes in the roots of the mung-bean plant.

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References


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