The Effect of Sugars on Chlorophyll Biosynthesis
in Higher Plants**†

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(Received for publication, December 31, 1959)

A study of the physical and chemical factors involved in the conversion of proplastids to mature chloroplasts in higher plants led to the investigation of biosynthesis of chlorophyll a in detached, etiolated leaves. It was found that when dark-grown leaves, which contain no chlorophyll but a low concentration of protochlorophyllide, were exposed to red or blue light, the protochlorophyllide was rapidly hydrogenated to chlorophyllide a. This step was followed by esterification with phytol in a chlorophyllase-mediated dark reaction to form chlorophyll a (1):

\[
\text{dark} \quad \xrightarrow{hv} \quad \text{protochlorophyllide} \quad \xrightarrow{+\text{phytol}} \quad \text{chlorophyllide a} \quad \xrightarrow{\text{PH}^+} \quad \text{chlorophyll a}
\]

When excised leaves were irradiated continuously with red or blue light, they accumulated chlorophyll slowly during the first hour, after which the rate of greening increased. However, after several hours of irradiation, chlorophyll formation ceased in excised leaves since the endogenous metabolites had been exhausted, and the photosynthetic apparatus was not yet functioning (2, 3).

The object of the present study was to identify the metabolites which enable immature leaves to form chlorophyll continuously when irradiated. Glucose, sucrose, and maltose were found to be equally effective in restoring to excised leaves the substrates essential for pigment synthesis which had been largely lost during a starvation pretreatment. In order to determine which sugar was most closely associated with pigment synthesis, rates of oxygen consumption were measured in (a) leaves pretreated with sugar and assayed in water, or (b) leaves pretreated with water and assayed with addition of sugars. The results indicated that during or after exposure to red light, glucose was oxidized more rapidly than sucrose. It is concluded that, while glucose is more rapidly utilized by light-exposed leaves, the rate-limiting step in chlorophyll formation lies farther along the biosynthetic path, so that the disaccharides appear to be as effective as glucose for pigment synthesis.

EXPERIMENTAL PROCEDURE

The technics used in growing seedlings of Phaseolus vulgaris, var. Great Northern, and preparing the leaves for experiment have been previously described (1). Pigment content in leaf samples was determined spectrophotometrically on the filtrate of an 80% aqueous acetone extract.

In early experiments, the rate of chlorophyll synthesis during continuous irradiation was measured. Since chlorophyllide a arises from protochlorophyllide, the latter must be continuously resynthesized during the greening process, but it cannot be measured in the presence of appreciable quantities of chlorophyll. In later experiments, therefore, it was preferable to follow directly the regeneration of protochlorophyllide in the dark after a brief exposure to red light.

For the analysis of carbohydrate content of leaves, samples were dried to constant weight at 80° in circulating air. An aliquot of finely ground leaf powder was thoroughly extracted with boiling 80% aqueous ethanol. From the residue, starch was extracted and hydrolyzed (4), then assayed by the ammonium molybdate method (5). The same assay method was used for determining reducing sugars in the ethanol extract before and after hydrolysis in 0.7 N HCl at 100° for 2.5 hours, the difference between the two values indicating sucrose content.

Manometry—For the manometric experiments, midribs were removed from treated leaves and 0.25 to 0.33 g of this material was spread out evenly over the base of a rectangular Warburg flask. The main compartment contained water, one side arm contained carbohydrate solution, and the second side arm held KOH solution and filter paper to absorb liberated CO₂. All manipulations of leaf samples were performed in a dark room over a green safelight. Manometer flasks were equilibrated at 30° for 10 minutes, then oxygen consumption was measured at 5- to 10-minute intervals for a period of 20 to 30 minutes every hour. During some manometric assays, flasks were shaken above two 15-watt red fluorescent lamps located in the water bath. The incident energy at the level of the flasks was approximately 135 microwatts cm⁻².

RESULTS

Effect of Starvation on Rate of Chlorophyll Synthesis—Excised leaves on filter paper moistened with distilled water were preincubated in dark cabinets at 25° for 18 hours. They were then irradiated with a saturating intensity of red light and the content of chlorophyll a was determined periodically. For comparison, the rate of chlorophyll synthesis in freshly excised leaves was measured. As will be seen in Fig. 1, freshly excised leaves started rapid synthesis after a short induction period. By contrast, the chlorophyll content of excised starved leaves (18 hours’ dark preincubation) even decreased slightly from the initial level for 3 hours of irradiation, probably owing to some bleaching of the...
pigment, whereas after this period, the rate of chlorophyll synthesis gradually increased.

Comparison of the rate of greening of freshly excised leaves with that of leaves remaining attached to the seedling is provided in Fig. 2. The results indicate a gradual loss of ability to form chlorophyll in the excised leaf compared with a leaf attached to the whole seedling. Further tests showed the difference between the two conditions to be largely owing to the presence or absence of cotyledons, since in leaves to which only one of the two cotyledons remained attached, greening occurred more rapidly than in leaves on whole seedlings. This stimulatory effect of the cotyledon reached a maximum at about 4 hours of dark preincubation, when the ability to form pigment in 1 hour's assay irradiation was 17% higher than at the beginning of the preincubation (Fig. 3). At this time the chlorophyll-forming capacity of excised leaves similarly preincubated in the dark had dropped 24% below their initial capacity. After the leaf-cotyledon preparation had reached its peak of pigment-forming ability, the latter gradually diminished parallel to the continuous decline seen in excised leaves. The stimulatory effect due to the presence of a cotyledon was abolished by incubation in nitrogen instead of air, or by lowering the temperature of preincubation to 15°; the optimal temperature was about 30°.

Nature of the Cotyledonary Factor—Progressive loss of pigment-forming ability of excised leaves during preincubation in the dark is illustrated in Fig. 4. The chlorophyll-synthesizing ability of the leaves decreased rapidly in the dark for 7 hours, then leveled off at 50% of the initial value. Many compounds were tested for the ability to preserve the pigment-forming system during the dark starvation period at the level of freshly excised leaves. Growth regulators (kinetin $10^{-4}$, $10^{-6}$ M; indoleacetic acid, $10^{-8}$ to $10^{-4}$ M; 2,3,5-triiodobenzoic acid, $10^{-4}$, $10^{-8}$ M), heavy metals (Co$^{2+}$, Mn$^{2+}$, $10^{-3}$ M), KNO$_3$ ($10^{-2}$ M), amino acids (arginine, $10^{-2}$ M; glycine or tryptophan, $10^{-4}$ to $2.5 \times 10^{-4}$ M), or carboxylic acids (acetate, pyruvate, citrate, succinate, $2.5 \times 10^{-4}$ M) had no effect. Glycine and succinate have been demonstrated to give rise to porphyrins in diverse tissues (6, 15). Only carbohydrates were active, and of those, sucrose was slightly superior to glucose. Although fructose also gave some stimulation, an equimolar mixture of glucose and fructose was no better than glucose alone. Inorganic orthophosphate ($10^{-1}$ M, pH 6.7) was slightly inhibitory by itself, and reduced the stimulation due to 0.2 M sucrose by over 90%. The optimal sucrose concentration was found to be 0.20 to 0.25 M. As seen in Fig. 4, sucrose at this concentration maintained the pigment-forming system of excised leaves at its initial level throughout the 24-hour test period. However, sucrose had no stimulatory effect when the cotyledon was left attached to the leaf, indicating that the endogenous carbohydrate level was saturating.

The results of an examination of the carbohydrate levels in the leaves after various pretreatments are shown in Table 1. Leaves starved by incubation on water lost one-third of their reducing sugar. An attached cotyledon caused the concentrations of sucrose and starch to increase in the leaf, while the presence of 0.2 M sucrose raised only the starch level of excised leaves but not the internal sucrose concentration.

Specificity of Sugars—Many sugars were tested for their ability to stimulate protochlorophyllide synthesis in starved excised leaves during a 3-hour dark assay. Control and test samples were irradiated with red light (615 to 700 nm) from a bank of red fluorescent lamps for 4 minutes (incident energy = 10 milli-
joules cm$^{-2}$) to give maximal conversion of protochlorophyllide to chlorophyllide $a$ (1). Residual protochlorophyllide was then extracted from the control samples, while test samples of leaves were kept in the dark for 3 hours on filter paper wetted with distilled water or test solution. At the end of this dark incubation period, the pigments were extracted. Newly synthesized protochlorophyllide was calculated as the pigment present after dark incubation minus that found in the controls extracted before dark incubation. Although the pigment synthesis in controls kept on water during the assay was small and therefore subject to large variation (0.80 ± 0.40 μg per g fresh weight), the 3-hour period resulted in the greatest percentage increase with active compounds. The results, for three concentrations (Table II), are shown as percentage increase (or decrease) over the control. Glucose was found to be as effective as sucrose, and maltose was equally effective at 0.2 M, suggesting that the disaccharides were probably giving rise to glucose as the immediate active compound. Cellobiose was much less active, although it differs from sucrose only in having a β-linkage replacing the α-linkage.

**Effect of Sugars on Oxygen Consumption of Leaves**—To gain further insight into the role of sugars in stimulating pigment synthesis in excised leaves, the rate of oxygen consumption after various pretreatments was measured under several conditions. In the first of these experiments, excised leaves in the presence of test sugars were preincubated in the dark for 18 hours, washed in distilled water, blotted, weighed, and finally placed in manometer vessels in the dark. The rate of oxygen consumption in the dark in the absence of added substrate was determined at hourly intervals; the results are shown in Fig. 5. Starved leaves (water) maintained a fairly constant rate which declined slowly after 3 hours. Addition of 0.25 M glycerol during pretreatment stimulated oxidation by about 15%. After preincubation on 0.25 M sucrose or glucose, the initial rate of oxygen consumption was 39% above the starved control, the rate then declined and at the fifth hour it was only 15 to 20% above the control. Evidently, endogenous reserve materials formed during pretreatment on glucose or sucrose are quite rapidly depleted during incubation in the absence of external oxidizable substrate.

The effect of red light was tested because of its demonstrated ability to induce developmental changes in tissues of higher plants (16–19). By comparing the position of each curve of Fig. 6 with the corresponding one of Fig. 5, one can see the effect of red light during the manometric assay without added substrate on leaves pretreated in the dark with various carbohydrates. Red light increased the initial rate of oxygen consumption of starved leaves about 15% above that of starved leaves assayed in the dark, but the stimulation due to red light gradually diminished until the fifth hour, when the rate was the same as that in

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**Fig. 4.** Effect of sucrose during dark pretreatment of excised bean leaves on subsequent ability to form chlorophyll.

**Table I**

<table>
<thead>
<tr>
<th>Leaf preparation</th>
<th>Incubation medium</th>
<th>Reducing sugar mg glucose/g dry wt.</th>
<th>Additional reducing sugar mg/g dry wt. after hydrolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly excised</td>
<td>0 hours</td>
<td>13.1 ± 0.9</td>
<td>13.5 ± 0.95</td>
</tr>
<tr>
<td>Cotyledon attached</td>
<td>16 hours</td>
<td>9.1</td>
<td>31.3</td>
</tr>
<tr>
<td>Excised</td>
<td>16 hours</td>
<td>8.65 ± 0.81</td>
<td>14.6 ± 1.85</td>
</tr>
<tr>
<td>Excised</td>
<td>16 hours 0.2 M sucrose</td>
<td>31.8 ± 2.4</td>
<td>34.1 ± 3.5</td>
</tr>
</tbody>
</table>

* Hydrolysis in 0.7 N HCl for 2.5 hours at 100°, indicating sucrose content.
† Standard deviation.
‡ Not determined.
TABLE II
Comparison of carbohydrates in stimulation of protochlorophyllide synthesis in starved excised leaves
Numbers shown in table are means of replicate experiments. Negative numbers signify inhibition.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Increase in protochlorophyllide formed in 3 hours over water control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 10 )</td>
</tr>
<tr>
<td>Glycerol</td>
<td>%</td>
</tr>
<tr>
<td>Glucose</td>
<td>245</td>
</tr>
<tr>
<td>Fructose</td>
<td>82</td>
</tr>
<tr>
<td>Galactose</td>
<td>71</td>
</tr>
<tr>
<td>Mannose</td>
<td>63</td>
</tr>
<tr>
<td>Sucrose</td>
<td>270</td>
</tr>
<tr>
<td>Maltose</td>
<td>240</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>95</td>
</tr>
<tr>
<td>Melibiose</td>
<td>40</td>
</tr>
<tr>
<td>Lactose</td>
<td>22</td>
</tr>
<tr>
<td>Melezitose</td>
<td>35</td>
</tr>
<tr>
<td>Raffinose</td>
<td>32</td>
</tr>
</tbody>
</table>

Fig. 5. Endogenous rates of oxygen consumption in the dark of excised leaves pretreated 18 hours in the dark on substrates indicated (concentration, 0.25 M). Temperature, 30\(^\circ\); gas phase, air.

Leaves tested in the dark. Glycerol-pretreated leaves were stimulated by red light to about the same extent as starved leaves. Leaves preincubated on glucose showed an even greater stimulation (25% initially) by the red light. However, it is most significant that leaves preincubated on sucrose were completely unaffected by the red light during the manometric assay. Further experiments were conducted to determine the cause of this observed difference in susceptibility to red light stimulation by leaves preincubated on glucose from those which had been kept on sucrose.

Leaves were starved on water, then tested for oxidation of added glucose or sucrose (final concentration, 0.25 M). From the observed rates, the rate of oxygen consumption by a control without added carbohydrate was subtracted, and the increments over the control are presented in Fig. 7. Leaves kept in the dark during both pretreatment and manometric assay showed little difference between the rate of oxidation of glucose or of sucrose (Curves 1 and 2). Leaves that had been exposed to low irradiance of red energy (0.4 microwatts cm\(^{-2}\), 620 to 700 mp) during the 18-hour pretreatment, then assayed in the dark, showed a marked difference in their ability to oxidize glucose or sucrose. During the first hour of contact with glucose, oxidation proceeded at an increasing rate (Curve 4), and thereafter the rate remained constant through the assay, at a level 15% above the corresponding
dark-pretreated sample. The oxidation of sucrose (Curve 3) was initially very slow, even below the dark-pretreated sample, but after the third hour, the rate began to increase until after 5 hours it nearly equaled the rate of glucose oxidation. When red light and carbohydrate were presented simultaneously to leaves starved in the dark, glucose was oxidized rapidly and at a linearly increasing rate throughout the assay period (Curve 6), whereas sucrose oxidation (Curve 5) was slower during the first 2 hours but then increased parallel to the rate for glucose.

**DISCUSSION**

The results of the present study indicate that certain carbohydrates, principally glucose and sucrose, can stimulate the biosynthesis of chlorophyll in excised, starved leaves exposed to red light. The experiments on oxygen consumption of leaves pretreated with red light and assayed in darkness, or on leaves kept in the dark and then assayed in red light, suggest that the red light enhances the oxidation of glucose or a closely related metabolite in the leaf, but that utilization of sucrose involves a lag phase during which a readily oxidizable metabolite is presumably formed.

An effect of sugars in stimulating chlorophyll formation was reported by Palladin (20). He found that some etiolated leaves, such as those of wheat, contain adequate stores of carbohydrate for greening, whereas others, such as those of bean and lupine, require exogenous sugar. Gautheret (21) noted a stimulation by glucose or fructose of greening in excised barley roots, and assumed that the accumulated sugars were essential for the formation of the chlorophyll molecule. A stimulation by light of sugar consumption in disks of green, detached cotton plant leaves was observed by Phillips and Mason (22). When these disks were incubated on a medium low in sucrose (0.1%), diffuse daylight stimulated the uptake of sugar over that in the dark during a 12-hour period. There was a marked increase in sucrose content of the disks but only a slight increment in reducing sugars. The presence of air during the light exposure favored the formation of reducing sugars without affecting the sucrose level. Our etiolated leaf preparations, when incubated aerobically in the dark on a higher sucrose concentration, showed a large increase in reducing sugar content.

A linear rate of sugar uptake for over 24 hours by mature tobacco leaf disks incubated on C14-labeled sucrose or invert sugar was reported by Porter and May (23). Sucrose enters the cell intact, since little label appeared in the moiety of sucrose not originally radioactive in the sucrose fed. Glucose disappeared from the medium 66% faster than fructose. In most of our experimental conditions, glucose was respired more rapidly than sucrose, although the effectiveness for pigment formation was the same for the two sugars. Presumably the rate-limiting step in pigment synthesis is located at a metabolic stage much later than the initial uptake and conversion to respiratory substrate. Porter and May also found that the respiration rate on sucrose showed an initial lag, then increased continuously for 6 hours, as our experiments with a higher sugar concentration confirmed. Putman and Hassid (24) noted that green leaves in the dark rapidly transferred radioactive label from fed sugars to other compounds, especially amino and hydroxy acids, before equilibration with the large nonradioactive sugar pool in the cell.

Pennell and Weatherley (25) reported that, in leaf disks of *Atropa belladonna*, the maximal sucrose uptake was observed at 15% (0.44 M), a concentration double that found optimal in our work on pigment synthesis. Fructose, glucose, and galactose were taken up at comparable rates, but utilization of sucrose was slower, and of maltose still less, whereas glycerol consumption was similar to that of sucrose. For pigment synthesis, however, we noted that glucose, sucrose, and maltose were equally effective at 0.20 M, whereas fructose gave only 40% of maximal stimulation, and glycerol only 14%.

Whether metabolic products of the sugars were incorporated directly into the pigment molecules, or served as energy sources for the synthesis could not be decided. The porphyrin precursors, glycine, acetate or succinate (6-15), tested singly or in combination, had no effect on excised leaf preparations.

**SUMMARY**

When excised, etiolated bean leaves are starved by preincubation on water in the dark, they exhibit a lag in chlorophyll synthesis on exposure to red or blue light. This lag can be overcome by leaving a cotyledon attached to the leaves, or by adding glucose or sucrose during the pretreatment. The presence of sugar during the preincubation increases the subsequent endogenous respiration in the dark. Exposure of sugar-pretreated leaves to red light during manometry further stimulates oxidation of endogenous, accumulated substrates in glucose-treated leaves, but not in those incubated with sucrose. Starved leaves irradiated during the assay oxidized added glucose more rapidly than sucrose.

It is concluded that, although metabolites of glucose may be oxidized more rapidly than those of sucrose, both sugars are equally effective in supporting chlorophyll synthesis, since the rate-limiting step in pigment formation lies farther along the biosynthetic pathway.

**REFERENCES**


The Effect of Sugars on Chlorophyll Biosynthesis in Higher Plants
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