Action Spectra for \( \text{O}_2 \) Evolution by Chloroplasts with & without Added Substrate, for Regeneration of \( \text{O}_2 \) Evolving Ability by Far-red, & for \( \text{O}_2 \) Uptake \(^1\)

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Recent studies (3, 6, 10, 12) have shown that photosynthesis requires two photochemical reactions driven by different pigments. Furthermore, it has been suggested (3, 10, 12) that these two light reactions occur in series. Absorption by chlorophyll \( b \) and by chlorophyll \( a_{870} \) (so-called accessory pigments) are thought to bring about oxidation of water and evolution of \( \text{O}_2 \) with a reduction of an acceptor [\( X \), or perhaps plastoquinone (12)]. A second photochemical step, mediated by a long-wavelength form of chlorophyll \( a \), causes reduction of TP (or an artificial Hill oxidant) with the electrons coming from reduced \( X \).

If some oxidized \( X \) were available, \( \text{O}_2 \) evolution would be possible without a Hill oxidant and without the functioning of the long-wavelength chlorophyll reaction. (It may be recalled in this regard that a limited \( \text{O}_2 \) evolution upon illumination of chloroplasts suspended only in sucrose solutions has been known for over 80 years.) Such a recurring, but small, evolution of \( \text{O}_2 \) by illuminated chloroplasts was also observed in the present study. The assumption that this \( \text{O}_2 \) evolution in the absence of a Hill oxidant is a reflection of the activity of the accessory-pigment reaction can be tested by comparing the action spectra for \( \text{O}_2 \) evolution before and after the addition of a Hill oxidant. The action spectrum determined in the absence of an oxidant should have its peaks at wavelengths corresponding to the absorption of chlorophyll \( b \) and chlorophyll \( a_{870} \). In the presence of a Hill oxidant the action spectrum for \( \text{O}_2 \) evolution would be shifted to longer wavelengths and reflect the functioning of both pigment systems.

It is of interest, moreover, to investigate how chloroplasts which lack a Hill oxidant are able to continue to evolve limited amounts of \( \text{O}_2 \) over a period of several hours and to determine what factors influence this \( \text{O}_2 \) evolution.

A similar study has been made recently by de-Kouchkovsky (personal communication) who used maize chloroplasts.

Materials & Methods

Swiss chard (Beta vulgaris L. var. cicla) used in these experiments was grown in a garden at Stanford. Mature leaves were picked, rinsed with distilled water, and chilled. Preparation of chloroplasts was done in a cold room at 3 to 4° in dim green light. Leaf blades free of large midribs were ground in a solution (hereafter called grinding solution) containing 0.4 \( \text{m} \) sucrose, 0.01 \( \text{m} \) NaCl and 0.05 \( \text{m} \) \( \text{K}_2\text{HPO}_4-\text{KH}_2\text{PO}_4 \) buffer (pH 6.9). The slurry was strained through eight layers of cheese-cloth and centrifuged at 200 \( \times \) \( g \) for 2 minutes. The supernatant fluid was centrifuged at 1000 \( \times \) \( g \) for 8 minutes to sediment unwashed whole chloroplasts.

\( \text{O}_2 \) changes in chloroplast preparations were measured by a Pt electrode covered by a thin film of teflon that was permeable to \( \text{O}_2 \) but protected the electrode surface from the deleterious effect of proteins and other substances in the chloroplast preparation. Moreover, the teflon-covered Pt electrode permits the measurement of \( \text{O}_2 \) exchange in solutions such as quinone and potassium ferricyanide which would otherwise react at the bare Pt electrode.

The electrode assembly used in these studies was adapted from a bare Pt electrode described by French et al. (7). The use of Ag-Ag\(_2\)O as a reference electrode in 0.5 \( \text{m} \) KOH under a plastic film was suggested by Clark et al. (2) and by Carritt and Kanwisher (1). The electrode shown in figure 1 combines features of the Clark (or Kanwisher-Carritt) membrane-covered electrode with the Blinks-Haxo electrode principle (8). The base was clear lucite and was designed so it could be positioned reproducibly under the beam from a monochromator. The Pt electrode, 1 \( \times \) 15.3 mm, was set flush into the lucite. A rectangular Ag-Ag\(_2\)O reference electrode, 22.6 \( \times \) 28.2 mm, with the center cut out was made from pure silver 0.8 mm thick, and was mounted so that it was also flush with the Pt electrode. The lucite was grooved underneath the Ag-Ag\(_2\)O electrode to make a pool for the KOH. The area of the Ag-Ag\(_2\)O electrode was about 75 times that of the Pt electrode. Both electrodes were covered with a 6.4 \( \mu \) thick film of teflon held down by a rubber ring. A thin film of KOH over the surface of the electrodes was trapped under the teflon. Connection between the KOH pool and the KOH under the teflon film was achieved through channels cut at intervals in the space between the electrodes as shown in the insert of figure 1. Leads soldered to the undersides of the electrodes were brought out under the rubber ring, sealed with beeswax, and attached to posts on the base.

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Chloroplasts were spread in a thin layer on the teflon directly above the Pt electrode and covered with a piece of moistened dialysis membrane held in place by a rubber gasket and cover (fig 1). Fluid circulated through the cover and over the membrane as shown in figure 2. A centrifugal pump with a hold-up volume of about 3 ml driven by a synchronous motor provided a flow rate of 410 ml/min. Gas concentration was adjusted and held constant by a gas exchanger made of 4 cm glass tubing partly filled with sections of small tubing. The idea of using a flowing system came originally from Professor Jack Myers (7). This system provided a constant flow of fluid of constant O₂ tension over the chloroplasts, and thus changes of O₂ tension at the electrode may be regarded as resulting from chloroplast activity. About 50 ml of fluid was required to fill this circulating system.

The Ag₂O coating was formed originally by polarizing the Pt electrode at —0.8 v with reference to the Ag electrode and leaving it in the air for a day. The resulting Ag-Ag₂O reference electrode was then ready for use. Since the teflon membrane is permeable to CO₂, the electrode assembly, when not in use, was kept in a desiccator over NaOH pellets to prevent CO₂ from neutralizing the KOH in the electrode.

When in use, the Pt electrode was polarized at —0.8 v by a circuit with 1.35 v Mallory (RM-42RT) mercury batteries which also powered a balancing circuit. Voltage across a 100-ohm resistor in series with the electrode was measured as described previously (7) by a Beckman model 14 chopper amplifier.

The electrode without the cover showed a light response and caused the recorder pen to go in the same direction as that caused by O₂ production. This signal apparently resulted from a light reaction at the Ag-Ag₂O electrode. When the cover was in place the Ag-Ag₂O electrode was shaded and there was no longer a response to light with the highest intensities used in these experiments.

The experiments were conducted at room temperature (19-21°) using unwashed whole chloroplasts suspended in grinding solution.

Results

The Time Course of O₂ Evolution at 650 μ. Figure 3 shows curves of O₂ evolution by chloroplasts exposed to the same intensity of 650 μ light under anaerobic and aerobic conditions. The ana-
Fig. 3. O₂ exchange with illuminated chloroplasts under anaerobic (A) and aerobic (B) conditions. The solution used in the circulating system was 0.4 m sucrose, 0.01 M NaCl, and 0.05 M K₂HPO₄·KH₂PO₄ buffer (pH 6.9). A dark interval of 17 minutes separated the last exposure of part A from the first exposure in part B. During this time air, instead of purified nitrogen (99.999% N₂), was bubbled in the gas exchanger. The dashed line traces the course of the dark base line which drifts upward in B since a complete equilibrium has not yet been obtained in the fluid bubbled with air. Deflections above the base line correspond to net O₂ evolution and deflections below to net O₂ uptake. Intensity of the 650 μm beam = 803 ergs cm⁻²sec⁻¹.

erobic curve (part A, fig 3) shows a high initial rate (spike) followed by a rapid decrease to a steady-state net O₂ evolution after 3 minutes. The subsequent spike after 3 minutes of darkness was smaller than the initial one and smaller also than a later one following 10 minutes of darkness.

Fig. 4. O₂ production spikes from brief exposures to 650 μm given at dark intervals after a previous 4-minute exposure to this 650 μm beam. The light exposures were on only long enough to permit the spike to reach its highest value. The height of the O₂ production spike produced by the initial 4-minute exposure was 62 units. The intensity of the 650 μm was 803 ergs cm⁻²sec⁻¹. Gas phase, N₂.

Figure 3B compares the effect of exposing chloroplasts to the same intensity of 650 μm light under aerobic conditions. Upon illumination, the O₂ production spike is seen again. However, the rate of O₂ production declines in the light reaching a steady-state net O₂ uptake after about three minutes. The chloroplasts show an O₂ gulp when the light is turned off. The dark base line, which slowly drifts upwards, is attained again after about three minutes.

Chloroplasts retained their ability to evolve O₂.

Fig. 5 (upper). Action spectrum for the production of the O₂ spike by Swiss chard chloroplasts. The circulating solution used is described in the legend of figure 3. The action spectrum was determined by using equal incident quanta (477 ergs cm⁻²sec⁻¹ at 650 μm). At points below 580 μm the light intensities were limited by the output from the monochromator. These points were adjusted upwards appropriately to correspond with other points in the action spectrum. Temp = 19.5°, gas phase, N₂.

Fig. 6 (lower). Relative height of the O₂ production spike as a function of 650 μm intensity for chloroplasts used for the action spectrum given in figure 5.
for many hours even in some cases after being left on the electrode overnight in the dark.

The Recovery of the O₂-evolving Capacity in the Dark. Chloroplasts were exposed to 650 μm light for 4 minutes. In the following dark period short exposures to 650 μm were given just long enough (about five sec) to allow the spike of O₂ production to reach its highest point. This short exposure sampled the recovery without retarding it appreciably. Figure 4 shows that the O₂ production spike has recovered half of its original height after about 1.5 minutes in the dark under anaerobic conditions. It can be seen that the build-up continues for a considerable time in the dark, and that a maximum was not attained after 16 minutes.

The Action Spectrum for O₂ Evolution. The action spectrum for the production of the O₂ spike was measured anaerobically for chloroplasts suspended in grinding solution which does not contain a Hill oxidant. For this purpose a 650 μm-reference beam was turned on, as described above, just long enough to allow the maximum rise in the O₂ spike, about five to ten seconds. This was repeated after 1 minute dark intervals until a constant response was attained. A similar five to ten second exposure to the monochromator beam was then given, again just long enough for the peak rate to be reached. After a 1 minute dark interval short exposures to the 650 μm-reference beam were given as before, and were followed by an exposure to the monochromator beam set at a different wavelength. The ratio: O₂ spike produced by exposure to the monochromator beam to the O₂ spike from preceding 650 μm-reference beam was used to plot the action spectrum shown in figure 5. Peaks occur around 650 and 480 μm which correspond to regions of maximum absorption by chlorophyll b. A shoulder can be seen at about 680 μm which would correspond to chlorophyll a absorption. A check of the O₂ production spike vs. 650 μm intensity showed that, at 650 μm and at the intensity used for the action spectrum measurements, the response fell on the linear region of the curve (fig 6).

Effect of Light on the Recovery of O₂-evolving Capacity. The O₂ production brought about by the 650 μm beam was influenced by a previous light exposure. To study this effect, short exposures to 650 μm light were given an anaerobic preparation at 1-minute intervals until a constant response was attained (fig 7 upper left). A 2-minute exposure to the 650 μm beam was then given. This exposure was followed by a 1-minute dark interval whereupon a far-red light (730 μm) was turned on for 3 minutes. Near the end of the far-red interval a 650 μm exposure was briefly superimposed on the far-red. The effectiveness of 730 μm in increasing the 650 μm O₂-spike may be expressed as the ratio of the height of the 650 μm O₂-spike during an exposure to 730 μm.

![Diagram](http://www.plantphysiol.org)
Fig. 8. Time courses of \( \text{O}_2 \) production in 480 \( \mu \text{m} \) light (883 ergs cm\(^{-2}\)sec\(^{-1}\)) for Swiss chard chloroplasts suspended in solution given in figure 3. 730 \( \mu \text{m} \) background light (459 ergs cm\(^{-2}\)sec\(^{-1}\)) also increases the \( \text{O}_2 \) production spike from 480 \( \mu \text{m} \) light. Gas phase, \( \text{N}_2 \). See text for details.

Fig. 9. The effect of 730 \( \mu \text{m} \) exposure time on the \( \text{O}_2 \) production spike brought about by a 650 \( \mu \text{m} \) light superimposed on 730 \( \mu \text{m} \). After 730 \( \mu \text{m} \) light had been on for the time indicated, a 650 \( \mu \text{m} \) beam was turned on long enough for the spike to reach its highest value. Both beams were then turned off. The ordinate is \( \mu \text{m} \) divided by the height of the 650 \( \mu \text{m} \) \( \text{O}_2 \)-spike before an exposure to 730 \( \mu \text{m} \) (in this case 1.37). A control, with an equivalent dark period placed between the 650 \( \mu \text{m} \) exposures, (lower part of fig 7) gives a ratio of 0.77.

A similar time-course curve for \( \text{O}_2 \) production and enhancement of the height of the spike is also observed when a 480 \( \mu \text{m} \) beam is substituted for 650 \( \mu \text{m} \) (fig 8, compare with fig 7).

Figure 10 shows the increase in the 650 \( \mu \text{m} \) \( \text{O}_2 \)-evolution spike brought about by a previous exposure to 730 \( \mu \text{m} \) for varying times. Since the build-up by 730 \( \mu \text{m} \) light is relatively slow, a simultaneous exposure to 730 \( \mu \text{m} \) and to 650 \( \mu \text{m} \) light does not give noticeable enhancement. The effect of the intensity of 730 \( \mu \text{m} \) light on the increase of the 650 \( \mu \text{m} \) \( \text{O}_2 \)-spike is shown in figure 10. With the 1-minute exposure used, the effect was half saturated at about 240 ergs cm\(^{-2}\)sec\(^{-1}\). At higher intensities there is an appreciable amount of \( \text{O}_2 \) evolution sustained in 730 \( \mu \text{m} \) light and a corresponding drop in its effectiveness in causing increased \( \text{O}_2 \) production from 650 \( \mu \text{m} \).

The Action Spectrum for the Light-induced Recovery. The action spectrum for the effectiveness expressed as the ratio: height of the 650 \( \mu \text{m} \) \( \text{O}_2 \) spike after a 730 \( \mu \text{m} \) exposure (or dark period for dark control) /height of the 650 \( \mu \text{m} \) \( \text{O}_2 \) spike before a 730 \( \mu \text{m} \) exposure. The intensity of the 730 \( \mu \text{m} \) beam was 570 ergs cm\(^{-2}\)sec\(^{-1}\) while the 650 \( \mu \text{m} \) beam was 803 ergs cm\(^{-2}\)sec\(^{-1}\). Temperature 20.1°. Gas phase, \( \text{N}_2 \).
of light in increasing $O_2$ production from a 650 nm beam was plotted from the same set of measurements that was used for figure 5. A point on the ordinate of figure 11 corresponds to the ratio: height of the $O_2$ production spike from the 650 nm-reference beam (given 1 min after a previous exposure to monochromatic light) divided by the height of the $O_2$ production spike from the 650 nm-reference beam. The red peak in this action spectrum occurs around 730 nm. The far-red peak positions which were found in other measurements of this action spectrum occurred at 713, 720, 725, and 729 nm. The position of the blue peak is not certain, but it is clear that blue-green light can also bring about a response similar to that of far-red light. A dark control for figure 11, with a dark period substituted for a mono-

![Image of action spectrum graph](image)

**Fig. 11 (upper).** The action spectrum for the ability of a previous light exposure to increase the $O_2$-production spike upon exposure to 650 nm light. The data for this figure and for figure 5 were obtained from the same experiment. The values for the ordinate were determined as described in the text.

**Fig. 12 (lower).** $O_2$-evolution spikes obtained from a 650 nm beam given at varying dark intervals after a 1-minute 730 nm exposure (or dark period for dark control). Intensity of the 730 nm beam = 459 ergs cm$^{-2}$sec$^{-1}$, 650 nm = 803 ergs cm$^{-2}$sec$^{-1}$.

chromatic exposure, gave a ratio of increased 650 nm $O_2$ production equal to 1.03.

**A Comparison of Dark and of Light-induced Recovery.** An obvious question is whether the slow dark recovery of $O_2$-evolving capacity and the more rapid light-induced recovery are similar or different processes. Do they both eventually give rise to the same level or are they additive? These questions were investigated by observing the response to short exposures (5-10 sec) of a 650 nm-reference beam given at varying dark intervals after a 730 nm exposure (fig 12). 650 nm exposures were given at 0, 5, 20, 40, and 80 seconds after a previous 1 minute exposure to 730 nm. A one minute dark period was substituted for the 730 nm light in the dark control. The recovery brought about by 730 nm remains after the light is turned off and is added to that brought about by a dark period. This suggests that a period of darkness or an exposure to 730 nm light produces the same effect.

**A Low Intensity Photooxidative Process and its Action Spectrum.** $O_2$ production by these chloroplasts was inhibited by DCMU$^2$. Figure 13 shows the effect of adding this herbicide to an aerobic circulating solution. The 650 nm light was again turned on at 36 seconds after adding the DCMU. The $O_2$ spike is smaller than before and a rapid drop in $O_2$ evolution is seen during the 650 nm exposure. A smaller $O_2$ gulp is also seen when the light is turned off. Subsequent exposures to 650 nm light result in a net uptake of $O_2$. The light-dependent $O_2$ uptake becomes reproducible after five minutes after poisoning and the $O_2$ gulp disappears.

These effects are interpreted as follows: while the light is on $O_2$ is produced from a substrate that is rapidly used up. At the same time a respiratory stimulation is induced by the formation of a product of the light reaction. When the light is turned off the respiratory stimulation persists until this product is used up, thus giving the $O_2$ gulp. DCMU poisons the $O_2$ evolution but not the photostimulated respiration which can now be measured without interference by $O_2$ evolution.

The action spectrum for $O_2$ uptake was determined for this chloroplast suspension by the procedure for the automatic recording of action spectra using equal numbers of incident quanta (7). Figure 14 shows that the peak in the red region for this effect occurs at 690 nm. $O_2$ uptake as a function of intensity of 690 nm is given in figure 15. Since the curve is half saturated at about 250 ergs cm$^{-2}$ sec$^{-1}$ the action spectrum was determined by using a quantum flux equivalent to that shown by the position of the arrow (67.2 ergs cm$^{-2}$ sec$^{-1}$) on the saturation curve. Upon addition of trichloroacetic acid (TCA) to the circulating solution to a final concentration of 1.2 $\%$, the chloroplasts turned olive brown and $O_2$ uptake was abolished at the light intensity used for

\[ \text{DCMU, } 2\text{-(3,4-dichlorophenyl)-1,1-dimethylurea} \]

was kindly supplied by Dr. H. J. Thorne, E. I. Du Pont & Co., Wilmington.
Fig. 13. Swiss chard chloroplasts exposed to 650 mμ light before and after the addition of DCMU. The final concentration of the DCMU (added dissolved in 0.2 ml 95% ethanol) was \( 2.7 \times 10^{-5} \text{ M} \). The same intensity 650 mμ beam (803 ergs cm\(^{-2}\)sec\(^{-1}\)) was used for all exposures. Gas phase, air.

Fig. 14. The action spectrum of \( \text{O}_2 \) uptake for the aged (24 hr) chloroplasts which had been treated with DCMU as described in figure 13. See text for details.

Fig. 15. \( \text{O}_2 \) uptake as a function of 690 mμ light intensity for the chloroplast suspension treated with DCMU as described in figure 13.

The effect of potassium ferricyanide on \( \text{O}_2 \) production. A comparison was made between the \( \text{O}_2 \) production of chloroplasts to which no Hill oxidant had been supplied and the \( \text{O}_2 \) production after the addition of DCMU. There remained, however, a large \( \text{O}_2 \) uptake when the TCA-treated chloroplasts were exposed to bright white light. This \( \text{O}_2 \) uptake may be a result of photooxidation reactions similar to those described by Franck and French (5) whereas the present photooxidation process is measurable at low intensity (i.e. with a quantum yield roughly comparable to that of photosynthesis). This process is believed to be that described as respiratory stimulation in Porphyridium by French and Fork (6).
The effect of potassium ferricyanide on the shape of the time course curves of $O_2$ evolution and on the relative rate of $O_2$ evolution in the steady state. Potassium ferricyanide (final concentration of $4.3 \times 10^{-3}$ M) was added to the circulating solution during the 17-minute dark interval. Gas phase, $N_2$. The intensity of the 650 $\mu$m beam used was $= 803$ ergs cm$^{-2}$sec$^{-1}$; the 730 $\mu$m beam $= 459$ ergs cm$^{-2}$sec$^{-1}$.

The relative rate of $O_2$ evolution and on the relative rate of $O_2$ evolution in the steady state. Ferricyanide concentration was the same as that used in figure 16. At 675 $\mu$m the quantum flux was 365 ergs cm$^{-2}$sec$^{-1}$. Gas phase, $N_2$. 

**FIG. 16.** The effect of potassium ferricyanide on the shape of the time course curves of $O_2$ evolution and on the relative rate of $O_2$ evolution in the steady state. Potassium ferricyanide (final concentration of $4.3 \times 10^{-3}$ M) was added to the circulating solution during the 17-minute dark interval. Gas phase, $N_2$. The intensity of the 650 $\mu$m beam used was $= 803$ ergs cm$^{-2}$sec$^{-1}$; the 730 $\mu$m beam $= 459$ ergs cm$^{-2}$sec$^{-1}$.

The ability of a previous 730 $\mu$m exposure to increase the 650 $\mu$m $O_2$ spike is also seen. During a dark interval of 17 minutes potassium ferricyanide was added to the circulating solution. A subsequent exposure to the same 650 $\mu$m beam produced a steady-state rate of $O_2$ evolution which was about 25 times higher than that obtained previously. Moreover, the time course of $O_2$ evolution at 650 $\mu$m in the presence of ferricyanide lacks the $O_2$-production spike. A previous exposure to 730 $\mu$m no longer stimulates $O_2$ production in the 650 $\mu$m beam. In some experiments with ferricyanide the time course for $O_2$ production exhibited a long-term induction effect with a protracted $O_2$ spike. In these cases, however, steady-state $O_2$ production was attained after about three minutes in the light.

**FIG. 17.** The action spectrum for $O_2$ evolution in Swiss chard chloroplasts to which potassium ferricyanide had been added. The ferricyanide concentration was the same as that used in figure 16. At 675 $\mu$m the quantum flux was 365 ergs cm$^{-2}$sec$^{-1}$. Gas phase, $N_2$. 

The action spectrum for $O_2$ evolution with Potassium Ferricyanide. The action spectrum for $O_2$ evolution with ferricyanide was obtained by using automatic procedures mentioned above and is shown in figure 17. It has a main red peak at 678 $\mu$m and a broad shoulder in the 640 to 650 region. A check of $O_2$ production as a function of intensity of 675 $\mu$m light showed that the action spectrum was determined well within the linear region of the saturation curve.
fects described in figure 3 for whole chloroplasts were observed with fragments which also retained an ability to evolve \( \text{O}_2 \) for many hours after isolation.

**Discussion**

It is clear that illuminated chloroplasts are able to evolve \( \text{O}_2 \) even though no Hill oxidants are provided. The components responsible for this \( \text{O}_2 \) evolution must be bound to the chloroplasts since a continual dialysis of water-soluble substances takes place into the circulating medium which passes over the chloroplasts on the electrode.

This endogenous evolution of \( \text{O}_2 \) apparently results largely from the functioning of chlorophyll b since peaks at 650 and 480 \( \text{m} \mu \) in the action spectrum for the \( \text{O}_2 \)-production spike were measured using chloroplasts without an added Hill-oxidant. The shoulder in this action spectrum at 680 \( \text{m} \mu \) indicates that at least one form of chlorophyll a may be active. The proximity of the \( \text{O}_2 \)-evolving step in photosynthesis to the accessory pigment system has been postulated recently by Witt and co-workers (12), by Losada et al. (10) and by Duyens’ group (3). Witt’s scheme, derived from studies on absorption changes, suggests that electrons from water reduce an unknown substance, \( \text{X} \), (plastoquinone) and that the accessory pigment system is closely connected to this reduction. The oxidation of this reduced compound (\( \text{X}^- \)), in turn, ultimately depends upon the activation of chlorophyll a whereby electrons, removed from \( \text{X}^- \), ultimately reduce TPN (or an artificial electron acceptor such as ferricyanide).

Hill (9) discovered that substantial quantities of \( \text{O}_2 \) could be produced from chloroplasts supplied with an appropriate hydrogen acceptor. In the present experiments, likewise, the addition of ferri-cyanide to the circulating solution allowed steady-state \( \text{O}_2 \) production to proceed at a rate which was about 25 times faster than the low endogenous \( \text{O}_2 \) production observed under anaerobic conditions. Not only was the rate of \( \text{O}_2 \) production higher with ferri-cyanide but also the action spectrum for \( \text{O}_2 \) production had a major peak at 678 \( \text{m} \mu \) in a region of absorption by chlorophyll a and a shoulder around 640 to 650 \( \text{m} \mu \) corresponding to chlorophyll b absorption. This action spectrum may be compared with a normal action spectrum for \( \text{O}_2 \) evolution by green plants (cf *Ulva lactuca* of Haxo & Blinks, 8).

The \( \text{O}_2 \)-production spike seen initially upon illumination would depend upon the amount of a natural electron acceptor which is present in an oxidized form. If no endogenous (or artificial) Hill oxidant is present, the unknown component would accumulate in the reduced state in the light and the rate of \( \text{O}_2 \) production would decrease. The time course of \( \text{O}_2 \) evolution in chloroplasts without added ferri-cyanide has, initially, a burst of \( \text{O}_2 \) production which drops rapidly in the light to a low steady-state \( \text{O}_2 \) evolution (anaerobic chloroplasts). This small net \( \text{O}_2 \) evolution could be supported by a slow endogenous re-oxidation of the unknown reduced compound.

A re-oxidation of the unknown reduced compound apparently continues in the dark since a sufficiently long dark period replenishes the capacity of chloroplasts to produce the \( \text{O}_2 \) spike again. Repeated brief exposures to 650 \( \text{m} \mu \) (shown in the upper, right hand part of fig 7) show this progressive build-up of the height of the \( \text{O}_2 \)-production spikes after a previous exposure to light.

A dark period, as well as an exposure to certain wavelengths of light, can bring about an increased \( \text{O}_2 \)-production spike. The action spectrum for the increase of a 650 \( \text{m} \mu \) \( \text{O}_2 \)-production spike shows that far-red light (730 \( \text{m} \mu \)) and blue-green light (ca. 500 \( \text{m} \mu \)) are both effective in this regard. As was seen in figure 12, an exposure to 730 \( \text{m} \mu \) light and a dark period are apparently additive in their effect on the 650 \( \text{m} \mu \) \( \text{O}_2 \) spike.

The far-red peak around 730 \( \text{m} \mu \) in the action spectrum suggests that \( \text{O}_2 \) production might be influenced by a phytochrome system. If exposure to 730 \( \text{m} \mu \) or a dark period brings the chloroplasts into a state favorable for \( \text{O}_2 \) evolution, an exposure to 650 \( \text{m} \mu \), absorbed strongly by the 600 \( \text{m} \mu \)-phytochrome, would convert the chloroplasts back again to a condition where \( \text{O}_2 \) evolution is retarded. The sharp drop in the rate of \( \text{O}_2 \) evolution during a 650 \( \text{m} \mu \) exposure would be understandable if this mechanism were operating. However, the phytochrome system seems not to be involved since the time course curve of \( \text{O}_2 \) evolution at 480 \( \text{m} \mu \) (not absorbed by the reversible phytochrome system) also shows a decline in the rate of \( \text{O}_2 \) evolution during the light period (fig 8). The stimulatory effect of pretreatment with 730 \( \text{m} \mu \) light was nevertheless seen with subsequent 480 \( \text{m} \mu \) exposures.

At present, it would seem reasonable to attribute the stimulation of \( \text{O}_2 \) production by far-red to a light reaction which in some way influences the re-oxidation of the unknown reduced component discussed previously.

An \( \text{O}_2 \)-production spike was also seen upon initial illumination of a chloroplast suspension under aerobic conditions (fig 3B). Here, however, a small net \( \text{O}_2 \) uptake was seen after steady state conditions had been attained. In some experiments no net \( \text{O}_2 \) exchange was observed. Mehler (11) has shown that \( \text{O}_2 \) can act as a Hill oxidant. In this case \( \text{O}_2 \) is both produced and consumed and no gas exchange is apparent. In the present experiments darkening of chloroplasts under aerobic conditions resulted in a gulp of \( \text{O}_2 \). This could result if an \( \text{O}_2 \) production and an \( \text{O}_2 \)-uptake reaction were both occurring in the light. If \( \text{O}_2 \) production were to stop more quickly upon darkening than an \( \text{O}_2 \)-uptake reaction, a gulp of \( \text{O}_2 \) would be observed. It seems probable that an \( \text{O}_2 \)-consuming reaction takes place in the light concurrently with \( \text{O}_2 \) production. The steady-state level of \( \text{O}_2 \) exchange would depend upon which reaction predominates. Under anaerobic conditions a net \( \text{O}_2 \) production can be sustained, and no \( \text{O}_2 \) gulp occurs upon darkening. Under aerobic conditions, however, \( \text{O}_2 \) exchange varies from posi-
tive to negative depending upon which reaction is greater.

A light-dependent O₂ uptake is still seen after O₂ evolution has been poisoned by DCMU (Fig 13). This uptake of O₂, in contrast to the uptake observed by Mehler after ethanol-catalase had been added as a trap for the H₂O₂, did not depend on the functioning of the O₂-evolving system. Green fragments of red algal chloroplasts which no longer evolved O₂ after phycoerythobilin pigments had been leached out likewise showed a light-dependent uptake of O₂ (4).

O₂ uptake as a function of 690 μm light intensity in chloroplasts poisoned with DCMU was linear only at very low light intensities. This O₂ uptake at low light intensities was abolished after chloroplasts were treated with trichloroacetic acid, in contrast to the O₂ uptake seen in denatured chloroplasts exposed to bright white light. The former process may depend upon enzymic reactions, the latter on physical, photooxidative processes (5).

The action spectrum for the low light intensity O₂-uptake reaction was complex. It had, however, a main peak at 690 μm corresponding to the absorption of a long-wavelength form of chlorophyll a.

The functioning of the low intensity O₂ uptake reaction may depend upon a reduction of O₂ mediated largely by chlorophyll a. This reaction would require an endogenous supply of electrons from a reduced compound other than that supplied by the accessory pigment reaction [X⁻ in Witt's scheme (12)].

Summary

Swiss chard (Beta vulgaris L. var. cichar) chloroplasts (& chloroplast fragments) suspended in a sucrose, NaCl solution in phosphate buffer with no added Hill oxidant retained, for many hours, a limited capacity to evolve O₂ upon illumination. After about three minutes in light a steady rate of O₂ production was sustained under anaerobic conditions which was much lower than the height of an initial O₂ burst. Under aerobic conditions the O₂ burst was followed by a net O₂ uptake in the light under steady state conditions, and an O₂ gulp was observed upon darkening.

Addition of potassium ferricyanide to the chloroplasts allowed O₂ evolution to proceed at a rate about 25 times higher in the steady state than the endogenous rate.

A comparison was made between the action spectra for O₂ evolution from chloroplasts with and without an added Hill oxidant (ferricyanide). The action spectrum for the evolution of O₂ by chloroplasts without ferricyanide followed the absorption of light by chlorophyll b and had peaks at 650 and 480 μm while the action spectrum for O₂ production in the presence of ferricyanide had a peak at 678 μm and a shoulder around 650 μm. These results are interpreted as indicating a close connection between O₂ evolution and the accessory pigment system.

The production of O₂ from chloroplasts at 650 μm light with and without ferricyanide could be increased by a previous exposure to certain wave-lengths of light. The action spectrum for the effectiveness of light to increase the O₂ production upon excitation of chlorophyll b had a maximum in the red at 730 μm. Moreover, a dark period after a previous exposure to 650 μm light also resulted in a higher O₂ production spike from a subsequent 650 μm exposure.

The stimulating effect of a dark period (& perhaps far-red light) on the O₂ production spike is interpreted as being a result of a slow endogenous re-oxidation of a substance reduced upon oxidation of water. This effect of a dark period and of 730 μm light on the 650 μm O₂-production spike was not seen when ferricyanide acted as an electron acceptor.

Chloroplasts treated with 3-(3,4-dichlorophenyl)-1,1-diethylurea, which suppresses O₂ evolution, exhibited a light-dependent uptake of O₂. The action spectrum for this O₂ uptake had a main peak in the red region at 690 μm.

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