Oxygen Concentration in Isolated Chloroplasts during Photosynthesis

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ABSTRACT

The O₂ concentration in intact and osmotically disrupted isolated spinach (Spinacia oleracea, L.) chloroplasts during photosynthesis was estimated. The chloroplasts were allowed to reduce 3-phosphoglycerate, CO₂, or ferricyanide in light until the rate of O₂ production was linear. When the light was turned off O₂ evolution from the chloroplasts continued for a few seconds. This prolonged O₂ evolution is due to an O₂ surplus inside the chloroplasts which equilibrates with that in the medium. From this surplus the O₂ concentration inside the chloroplasts at the moment when the light had been switched off was calculated. In all experiments the O₂ concentration inside the photosynthesizing chloroplasts was higher than that outside, but was dependent upon the O₂ concentration of the chloroplast medium. At low external O₂ concentration (30 μM) the ratio of the internal to the external O₂ concentration was about 5, whereas at concentrations corresponding to those in air-saturated water this ratio was close to 1. With osmotically broken chloroplasts this ratio was 1.2 at 30 μM O₂ and almost 1 from 150 μM onward. When the O₂ surplus found in broken chloroplasts during photosynthesis was related to the volume of the thylakoids, a ratio of about 2.3 was observed.

At least three reaction complexes of chloroplasts are thought to depend on the internal O₂ concentration in these organelles. First, the Warburg effect, an inhibition of photosynthesis by higher oxygen tensions, has given rise to a series of physiological studies that were dedicated to an understanding of the responsible biochemical mechanism (5, 6, 21, 22).

The second reaction complex depends on the photosynthetic reduction of O₂ to H₂O₂, well known as the Mehler reaction (7, 8, 16–18, 20), which has now been shown to proceed via the superoxide radical anion and might be at least partly mediated by an O₂-reducing factor (7, 8).

Third, O₂ has been shown to react in the ribulose-1,5-diphosphate oxygenase reaction (1, 2). Although the internal O₂ concentration of chloroplasts, which might depend on the photosynthetic O₂ production as well as on the O₂ tension outside the chloroplasts, is important for the metabolism of the cell, no reports are to be found in the literature on its magnitude.

Here, we attempted to estimate the internal O₂ concentration of photosynthesizing isolated chloroplasts and to determine how much this O₂ content can be influenced by the O₂ tension of the suspension medium of the chloroplasts.

MATERIALS AND METHODS

Preparation of Chloroplasts and Measurement of Oxygen Concentration in Chloroplast Suspension during Hill Reaction.

Spinach leaves (Spinacia oleracea L., vital R) were homogenized with an Ultraturrax for 5 sec in medium containing 0.33 mM sorbitol, 5 mM MgCl₂, 10 mM Na₃P₂O₇, 4 mM ascorbate adjusted at pH = 6.6 with HCl (4). After filtration through muslin the suspension was centrifuged for 1 min at 2,000g. The pellet was suspended in the buffer B described by Jensen and Baslam (14). Centrifugation at 30g (1 min) removed whole cells and greater particles. From the supernatant the chloroplasts were spun down at 500g (1 min) and resuspended in the same buffer (3). All steps were performed at 0°C. The percentage of intact chloroplasts, as measured by the ferricyanide method (10), was usually greater than 90.

For photosynthetic CO₂ or PGA reduction, chloroplasts equivalent to 90 to 225 μg of Chl were added to 1.5 ml of O₂-free buffer C (14) containing either 7.9 mM nonradioactive NaHCO₃ or 1 mM PGA. The temperature of the suspension was maintained at 20°C, the O₂ concentration of the suspension was measured with a Clark electrode and was adjusted to desired level by flushing with N₂ or O₂. The suspension was illuminated with white light at an intensity of 8,000 to 80,000 lux. After the experiment had been terminated the volume of the chloroplasts was determined as packed chloroplasts by centrifugation of an aliquot of the suspension in hemocytometer tubes. The volume of the plastid space in the pellet was determined as sorbitol-permeable space (5%) with sorbitol dehydrogenase (Boehringer, Mannheim) and subtracted from the volume of the pellet. The volume determined was of the same order of magnitude as would have been calculated by the method of Heber and Kirk (9) from the Chl content. Osmotically broken chloroplasts were prepared by the addition of chloroplasts equivalent to 90 to 225 μg of Chl to 1.5 ml of 15-fold diluted buffer C (14). The electron acceptor in the Hill reaction with broken chloroplasts was 3 mM K-ferricyanide.

Determination of Internal Oxygen Concentration of Photosynthesizing Chloroplasts. Intact chloroplasts which photoreduce CO₂ or PGA at a constant rate produced a small increment in the O₂ concentration of the suspension after the light had been turned off (Fig. 1, curve a). This increment must be caused by the equilibration of a higher internal O₂ concentration within the photosynthesizing chloroplasts with that of the external medium.

When the light was switched off at time T (Fig. 1), the internal O₂ concentration of the chloroplasts (Cₕ) can be calculated from equation 1:

\[ Cₕ = (Cₖ - C₉) \times \frac{\text{volume of the suspension}}{\text{volume of the chloroplasts}} \]

where C₉ equals the external O₂ concentration (of the suspen-
The measured values of electrode inertia were obtained subsequent to switching off the light. Curve a represents the trace of the recorder; curve b shows the calculated curve of the postillumination increase of the response which is due to the inertia of the electrode alone, i.e., would have been recorded if no internal O₂ surplus had existed.

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Time course of O₂ evolution (R) in a single experiment as measured with a Clark electrode. Intact chloroplasts were allowed to reduce PGA in the light and the O₂ increment (Rₕ - Rₖ) in the suspension was determined after switching off the light. Curve b represents the trace of the recorder; curve a shows the calculated curve of the postillumination increase of the response which is due to the inertia of the electrode alone, i.e., would have been recorded if no internal O₂ surplus had existed.

The increment in the O₂ concentration (C₄ₑₓₙ - C₄ₑₓₙ) cannot be measured directly, however, since the Clark electrode reacts with some delay and requires about 5 sec for a response of more than 90%. Therefore, the increment in the O₂ concentration which was recorded after the light had been turned off was composed of a real increment and of an apparent one due to the inertia of the electrode (Fig. 1, curve b). The latter can be calculated exactly if the response of the electrode to a brief O₂ pulse is approximated by a simple mathematical expression. Figure 2 shows such a response which was produced by injection of 50 μl of air-saturated water into a 20 mm solution of pyrogallol (pH 9), which is known to reduce O₂ immediately. The time course of this response (D) can be described by equation 2:

\[
D_{\text{exn}} = \frac{A \left[ \alpha (1 - e^{-t'}) + \beta (1 - e^{-t}) \right] e^{-\alpha t}}{1 + e^{-\alpha t}} \text{ for } 0 \leq t \leq T
\]

where A, α, and β are electrode parameters which must be obtained by fitting equation 2 to the actual response. T is the lag period of the response and t is the actual time which had elapsed since the onset of the pulse. Parameters of the response were determined after each experiment. From Figure 2 the conformity of the record of the impulse with the time course calculated with equation 2 can be seen.

The response of the electrode (Rₖ) to the O₂ production of the chloroplasts may be considered as composed of a series of subsequent single pulses and therefore can be described by equation 3:

\[
R_{\text{exn}} = \int_{t} dt' \cdot C_{\text{exn}}(t') \cdot D(t - t')
\]

where the integration variable t means an intermediate time, and Rₖ is measured in μm.

In performing the integration one has to keep in mind that the term for C₄ₑₓₙ is for t ≤ T and t ≥ T, respectively. Provided that an exponential transition between C₄ₑₓₙ and C₄ₑₓₙ (Fig. 1) takes place, the O₂ concentration at time t [C₄ₑₓₙ] can be obtained from equation 4:

\[
C_{\text{exn}} = \frac{C_{\text{exn}}(t)}{1 + e^{-\alpha T}} \text{ for } T \leq t \leq T
\]

where T comprises the lag phase of the photosynthetic O₂ production as well as the lag period of the electrode response.

is a phenomenological velocity constant for the diffusion of O₂ from the chloroplast. Inserting C₄ₑₓₙ from equation 4 into equation 3 one obtains equation 5:

\[
R_{\text{exn}} = \frac{A \cdot C_{\text{exn}}}{1 + e^{-\alpha T}} \left[ \frac{\alpha (1 - e^{-\alpha t})}{\alpha (1 - e^{-\alpha T})} \right] \text{ for } 0 \leq t \leq T
\]

Under the condition \( T \leq t \leq T + \gamma \), \( e^{-\alpha(t-T)} \) (see equation 2) is negligible compared to 1 if \( T = T \) is chosen large enough, i.e., at least 15 sec. In this case the response at the moment T is given by equation 6:

\[
R_{\text{exn}} = \frac{A \cdot C_{\text{exn}}}{1 + e^{-\alpha T}} \left[ \frac{\alpha (1 - e^{-\alpha t})}{\alpha (1 - e^{-\alpha T})} \right] \text{ for } T \leq t \leq T + \gamma
\]

From equation 6 C₄ₑₓₙ can be calculated because all other terms can be determined. However, the exact determination of A, which characterizes the amplitude of the single impulse, is rather difficult. Therefore, it is desirable to eliminate this term from the equation. For this purpose a second relation between Rₖ and C₄ₑₓₙ must be established. For \( t \geq T + \gamma \) the first integral of equation 5 is negligible for the same reason as mentioned above if \( t \) is chosen large enough. This is possible since the chloroplasts did not consume measurable amounts of O₂ in the dark. The second integral of equation 5 gives, under this condition, equation 7:

\[
R_{\text{exn}} = \frac{C_{\text{exn}}}{1 + e^{-\alpha T}} \left[ \frac{\alpha (1 - e^{-\alpha t})}{\alpha (1 - e^{-\alpha T})} \right] \text{ for } T \leq t \leq T + \gamma
\]

By division of \( R_{\text{x}} / R_{\text{F}} \), A is eliminated and \( C_{\text{exn}} \) can be calculated from the resulting equation 8:

\[
C_{\text{exn}} = \frac{R_{\text{exn}}}{R_{\text{F}} \cdot \frac{1}{1 - e^{-\alpha T}} \left[ \frac{\alpha (1 - e^{-\alpha t})}{\alpha (1 - e^{-\alpha T})} \right]} \text{ for } T \leq t \leq T + \gamma
\]

Under the conditions employed in this work the single terms of equation 8 were in the following order of magnitude: \( T \approx 30 \text{ sec}, \gamma = 1 \text{ sec}, \gamma = 5 \text{ sec}, \alpha = 0.8, \text{ and } \beta = 1; C_{\text{exn}} \) varied according to the O₂ concentration at the beginning of the experiment between 30 and 300 μM. Therefore, \( C_{\text{exn}} \) depended mainly on the magnitude of \( C_{\text{exn}} \), \( R_{\text{F}} \), and \( R_{\text{exn}} \). Because in most cases the difference between \( R_{\text{F}} \) and \( R_{\text{exn}} \) amounted only to several mm, the values of \( C_{\text{exn}} \) showed considerable scattering. Therefore, all types of experiments were repeated at least five times, each as a series of about 30 to 40 single measurements.

**Determination of PGA Concentration inside Chloroplasts.** To prove whether the effect of O₂ on its permeation through

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Response of the Clark electrode to a single short O₂ impulse produced by the injection of 50 μl of air-saturated water (mark: H₂O) into an O₂-free 0.02 M solution of pyrogallol (pH 9). The electrode was calibrated immediately before the experiments with O₂-free water and air-saturated water (0.28 μM O₂/ml) at 20 C. Responses to a given amount of O₂ were completely reproducible. Dotted line gives actual response; asterisks mark time course calculated with equation 2.
the chloroplast membranes was artificial and due to an effect on
the permeation velocity of the Hill acceptor, the PGA concen-
brations inside the chloroplasts were determined at a constant
PGA concentration of the medium but with different external
O₂ concentrations. For this purpose chloroplasts were incubated
as described above in PGA containing buffer C (14) and
removed after 1 min from the medium by centrifugation through
a layer of silicone oil (15) into 5% perchloric acid. After
neutralization of the lower phase with KOH and removal of the
insoluble material by centrifugation, the amount of PGA in the
chloroplast extract was determined. The PGA amount associ-
ated with the chloroplasts, as measured after centrifugation
through silicone oil, was about 200 nmol/mg of Chl, whether it
was determined prior or subsequent to chloroplast illumination,
and was not influenced by the external O₂ concentration. When
the same determination was carried out for control reasons with
osmotically broken chloroplasts, only 15% of the amount of
PGA found with intact chloroplasts was observed with the
plastids. There the predominant portion of PGA must be inside
these organelles and a limitation of the O₂ evolution rates
carried away from the PGA deficiency inside the intact plastids can be ruled out.

RESULTS

Determination of Oxygen Surplus inside Photosynthesizing
Chloroplasts. Isolated intact chloroplasts were allowed to reduce
exogenous PGA or CO₂ photosynthetically at various O₂ con-
centrations of the suspension medium. The reaction was fol-
lowed by potentiometric measurement of the O₂ production
and reached linearity after a few sec (Fig. 1). This indicated
that an equilibrium between O₂ production and O₂ efflux from
the chloroplasts had been established. After about 30 sec (T)
the light was turned off and Cᵢ,₁ and Cᵢ,₂ were determined
according to equations 8 and 1.

In virtually all experiments with intact chloroplasts a surplus of
O₂ inside the chloroplasts was observed as compared with the
O₂ concentration of the suspension medium. This surplus is
plotted against the external O₂ concentration (Fig. 3, curve A
and x). The maximal internal O₂ surplus was observed at the
lowest O₂ concentrations employed, where the rates of O₂
evolution by the chloroplasts were found to be relatively low
(Fig. 4).

An increase in the external O₂ concentration resulted in a
stimulation of the rates of O₂ production, but inversely the
internal O₂ surplus, which was more or less constant up to 100
μM O₂, decreased drastically. Although at O₂ concentrations in
the suspension higher than 320 μM the precision of the measure-
ment of R₈ and R₆ was insufficient to yield useful data.
extrapolation of the curve suggests that at 380 to 400 μM O₂
practically no O₂ surplus inside the chloroplasts would have been
found.

At a given external O₂ concentration the magnitude of the
internal O₂ surplus was practically independent from the rate of
photosynthetic O₂ production. This is shown in Figure 3 where
curve A gives the results from a chloroplast preparation yielding
a maximal rate of 20 μM/mg of Chl-hr (Fig. 4, curve A).25
whereas curve B was obtained with a considerably more active
preparation which produced O₂ at a corresponding rate of 90
(Fig. 4, curve B). Furthermore, Figures 3 and 4 show that the
O₂ surplus inside the plastids as well as the rates of O₂ evolution
were similar, whether PGA or CO₂ (both curves C) was admin-
istered as the Hill oxidant.

When the experiment was performed with osmotically dis-
rupted chloroplasts which produced O₂ at the same rates as
whole chloroplasts (Fig. 4, curve D) only a very small O₂ surplus
was detectable at low external O₂ concentrations (Fig. 3,
dotted line). At higher concentrations, practically no incre-
ment in the O₂ concentration could be found after the light had
been switched off. Since these broken chloroplasts had not been
freed from their stroma by centrifugation but obviously altered
only with respect to the state of their membranes, this
strongly reduced internal O₂ surplus indicates that not the
density of the chloroplast stroma but that of the membranes is
the main permeation barrier for the diffusion of O₂.

DISCUSSION

For practical purposes it is useful to calculate the ratio of
the internal O₂ concentration of the photosynthesizing chloroplasts
to the external concentration and to plot this ratio against the
latter concentration. The results given in Figure 3 are therefore
shown as the ratio Cᵢ,₁/ᵢ,₂ in Figure 5.

For intact chloroplasts (—) this ratio was always found to
be as large as 5 at the lowest external O₂ concentrations

![Fig. 3. Internal O₂ surplus at time T of intact (-- — —) and osmotically disrupted (---) photosynthesizing chloroplasts as influenced by the O₂ concentration of the medium. Line A was obtained as a regression curve of the asterisks shown upon PGA reduction at low light intensity. In line B the corresponding regression curve from a similar experiment with high light intensity is shown. Line C is the regression curve from an experiment with CO₂ as the Hill acceptor, and Line D shows the O₂ surplus in osmotically broken chloroplasts upon ferricyanide reduction (regression curve from the points shown). Curves correspond to those shown in Figure 4.](www.plantphysiol.org)

![Fig. 4. Rates of photosynthetic O₂ production by intact (curves A [x], B, and C) and osmotically disrupted chloroplasts (curve D) upon reduction of PGA, CO₂, and ferricyanide, respectively, as influenced by the O₂ concentration of the medium. Lines shown have been calculated as a regression curve. Curve A (B): O₂ evolution by intact chloroplasts upon PGA reduction at 8,000 (80,000) lux; curve C: O₂ evolution by intact chloroplasts upon CO₂ reduction at 80,000 lux; curve D: O₂ production by osmotically broken chloroplasts upon ferricyanide reduction at 80,000 lux. In B, C, and D only regression curves were shown which refer to the ordinate scale given on the right side. A similar inhibition of photosynthetic O₂ evolution at low external O₂ concentrations was observed by Miyachi and Okabe (19).](www.plantphysiol.org)
employed (30 μM). No difference could be observed when PGA was replaced by CO₂ as the Hill oxidant.

The ratio of $C_i^{\text{in}}/C_e^{\text{ex}}$ depended only on the external O₂ concentration and on the intactness of the plastids. An influence of the rate of photosynthetic O₂ evolution on this ratio could never be observed.

The maintenance of strongly unbalanced O₂ concentrations on both sides of the chloroplast envelope was not to be expected, since the nonpolar O₂ molecule should not be retained by biomembranes. But the chloroplast envelope obviously represents a permeation barrier for O₂. However, in order to explain the data shown in Figures 3 and 4, it must be assumed that O₂ itself is able to improve the permeability of the membrane, since at higher external O₂ concentrations the internal O₂ surplus decreased strongly. Furthermore, it is necessary to assume a sidedness of the membranes with respect to this O₂ effect. At an external O₂ concentration of 100 μM the internal concentration is 220, according to a $C_i^{\text{in}}/C_e^{\text{ex}}$ ratio of 2.2; however, at an external O₂ concentration of 220 μM the internal concentration is only 275 (instead of $220 \times 2.2 = 484$) and the ratio is 1.2. This suggests that the effect of the external O₂ concentration on the permeability of the membranes is much more pronounced than that of the internal concentration. The results shown do not permit conclusions to be drawn with respect to the biochemical background of this effect. However, the effect could be reversed by lowering the external O₂ concentration, provided that the chloroplasts were still intact.

The possibility that the described O₂ effects are artificial caused by the use of an artificial chloroplast medium cannot be ruled out. We can, however, exclude the idea that they are due to an O₂ effect on the permeation of the Hill acceptor because: (a) the O₂ surplus was virtually unchanged whether PGA or CO₂ was supplied to the chloroplasts; and (b) a significant O₂ effect on the concentration of PGA inside the chloroplast could not be detected.

At an external O₂ concentration corresponding to that of air-saturated water chloroplasts are highly permeable to O₂. This indicates that in vivo the O₂ concentrations inside the photosynthesizing chloroplast and the cytoplasm may be more or less equal. Therefore, the velocity of the O₂ exchange between the cell and its surroundings should determine the internal O₂ concentration of the chloroplasts.

The experiment with osmotically broken chloroplasts showed a much lower resistance of the chloroplast envelope to permeation by O₂, yielding a ratio $C_i^{\text{in}}/C_e^{\text{ex}}$ near unity (Fig. 5, dotted line). This finding supports the results obtained with intact chloroplasts, because it must also be expected from theoretical considerations. If the osmotic shock results in a good permeability of the chloroplast membrane for otherwise nonpermeable substances, such as ferricyanide or even dextran (11), the permeation barrier for dissolved gases should be decreased even more. It might be argued that the calculated internal surplus from inside to outside the chloroplasts does not actually exist, and that the O₂ liberated upon darkening of the suspension resulted from a decomposition of photosynthetically produced H₂O₂. In this case, the so-called surplus must increase with the external O₂ concentration because the H₂O₂-producing Mehler reaction is known to depend directly on the O₂ concentration of the medium (5). On the contrary, the surplus was found to decrease with higher O₂ concentrations. A very active chloroplastic ascorbate peroxidase has been detected (manuscript in preparation) which obviously prevents accumulation of H₂O₂ in the plastids. Whether the remaining relatively low permeation resistance ($C_i^{\text{in}}/C_e^{\text{ex}} = 1/11$ at about 100 μM O₂) represented the diffusion barrier of the thylakoid membranes can only be decided if a permeation resistance of the osmotically disrupted chloroplast envelope can be ruled out. In this case, the “inner space” of the chloroplasts would be the volume of the thylakoids, *i.e.* roughly 10% of the total volume of the chloroplasts (12, 13). If this is so, a ratio of the external to the internal O₂ concentrations of about 2.3 would have resulted at the lowest O₂ concentration tested, thus being roughly 50% of the ratio found with intact chloroplasts. This would signify that the thylakoids as well as the chloroplast envelope contribute to the permeation barrier found in intact chloroplasts.

**LITERATURE CITED**


