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Oxygen release may limit the rate of photosynthetic electron transport; the use of a weakly polarized oxygen cathode

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The kinetics of photosynthetic oxygen evolution upon flash illumination were measured electrochemically at a much less negative cathode potential than used in conventional oxygen polarography, so that the electrode current was an essentially non-disturbing probe of the oxygen concentration. This method allows a good signal-to-noise ratio at sub-millisecond time resolution with stationary suspensions of photosynthetic material and avoids a strong inhibition of photosynthesis occurring under some conditions of measurement with a bare cathode. The results contradict the generally accepted notion that oxygen release promptly follows the 1.2 ms reduction of the oxygen-evolving complex after its four-step photooxidation. A much slower process has to take place before oxygen is detected and before a next cycle of four photoreactions in Photosystem II can be completed successfully: the next three photooxidations of the complex are unaffected, but the fourth is lost for oxygen evolution. This indicates that oxygen is released only slowly from the site of water oxidation, and that water oxidation is required to stabilize the fourth charge separation. Half-times of 30–130 ms were measured for oxygen release in different batches of Photosystem II membranes, chloroplasts and algae. In some conditions oxygen release may be a significant rate-limiting step in photosynthesis.

Introduction

For time-resolved measurements of the rapid, small changes in oxygen concentration induced by flash illumination of photosynthetic material, oxygen polarography [1,2] is the only available technique. It is an electrochemical detection method: O_2 is reduced at the cathode surface by the following reactions [3]:

$$O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$$

and

$$H_2O_2 + 2e^- \rightarrow 2OH^-$$

The cathode potential is set so low that every oxygen molecule hitting the cathode is reduced. The current then depends only on the rate at which oxygen molecules arrive and should be proportional to the oxygen concentration and diffusion coefficient in the sample. The electrochemical response time is less, probably much less, than 0.5 ms [3].

Abbreviations: CCCP, carbonylcyanide 3-chlorophenylhydrazone; Chl, chlorophyll ($a + b$); Mes, 4-morpholineethanesulfonic acid; PS, Photosystem; Q_A, secondary electron acceptor in PS II.

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The method is widely used in photosynthesis research to measure the amount of oxygen evolved in response to individual light flashes, but the kinetics of the flash-induced change in electrode current have rarely been used as a source of information. Normally a large bare Pt cathode and a dense suspension or even sediment of photosynthetic material are used to obtain a reasonable current. The sample layer must be thin to allow the illumination to penetrate and, if it is not a sediment, it is usually separated from the overlying buffer solution by a filter or dialysis membrane. The geometry is further complicated by the unknown effects on photosynthetic oxygen evolution of the local conditions near the cathode surface (low potential, low O_2 concentration, H_2O_2 and OH^- production, and possibly effects of other reactions taking place at the cathode). The resulting distribution of active oxygen sources leads to complicated and partially unpredictable diffusion kinetics from which the kinetics of oxygen production are not easily extracted.

Detailed information has been obtained, however, by an indirect approach [4]. Instead of a flash, illumination was provided by weak, sinusoidally modulated light. The kinetics of the flash-induced signal discussed above will then be expressed in the modulation frequency dependence of the amplitude and phase shift of the modulated electrode current with respect to the light intensity, and can be measured with very high sensitivity. From a comprehensive quantitative analysis Joliot et al. [4] concluded that the half-time of oxygen production in Chlorella was 0.8 ms. A distance between the cathode and the nearest active oxygen sources of about 2 μm had to be assumed. By the same technique, Sinclair and Arnason [5,6] later found a half-time of 2 ms in Chlorella and 3 ms in spinach chloroplasts.

Similar conclusions were obtained more directly by Etienne [7], using a fast turbulent flow of a Chlorella suspension through a capillary tube and measuring the oxygen concentration at a variable distance downstream of a small, brightly illuminated spot. This elegant method avoids diffusion gradients in the medium and exposure of the cells to possibly inhibitory effects at the cathode surface. As a result the kinetics of oxygen release from the cells are immediately obvious from the data. Half-times of 1.5 and 2.2 ms at 26°C were obtained with Chlorella cells of less than 3.5 and of an average 5 μm diameter, respectively. On the basis of these data, measured activation energies, and a simple model calculation, it was concluded that these half-times were close to the intrinsic half-time of oxygen evolution, but somewhat increased by the time required for diffusion of oxygen out of the cells.

A half-time of less than 1.5 ms suggests that oxygen is promptly released upon reduction of the water-oxidizing complex in Photosystem II (PS II). This reduction takes place after every four successive photoreactions of PS II, which increase the oxidation state of the complex by four equivalents [8]:

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} (S_4) \longrightarrow S_0$$
$$2H_2O \quad O_2 + 4H^+$$

The higher S-states are reduced to S_1 in minutes and subsequent illumination by a series of short, saturating flashes results in a characteristic oscillation of the oxygen yield, with maxima on flash numbers 3, 7, 11, etc. For each transition Bouges-Bocquet [9] has determined the dark time required for the next flash to be successful. The slowest step was the $S_3 \rightarrow S_0$ transition, which had a half-time of 1.2 ms. Direct measurements on the oxidized species involved later confirmed that this half-time reflects the reduction of the oxygen-evolving complex [10–13].

In order to verify the supposed kinetic correlation between oxygen release and the $S_3 \rightarrow S_0$ transition, we designed an electrochemical cell which allows oxygen measurements simultaneously with the ultraviolet absorbance measurements described in Ref. 12. To minimize differences between conditions in the bulk suspension and in the immediate vicinity of the cathode surface, we used very weak polarization, so that a negligible fraction of the oxygen molecules hitting the cathode was actually reduced. Upon flash illumination the current then simply increased by an amount proportional to the oxygen produced and, because inactivation of photosynthetic material near the cathode surface was prevented, the current increase was much larger than might have been anticipated on the basis of measurements at the

strong polarization traditionally used. At a chlorophyll concentration of about 1 mM a good signal-to-noise ratio was easily obtained in the ms time range. The first and unexpected conclusion is that oxygen evolution is much slower than the reduction of the oxygen-evolving complex and must in some conditions be a significant rate-limiting process in photosynthesis.

Materials and Methods

Chloroplasts were isolated from spinach leaves, freshly picked or obtained from local shops, by grinding, filtration and centrifugation. The isolation buffer contained 20 mM Mes (pH 6.0), 15 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose. PS II membranes were prepared from these chloroplasts according to the method of Berthold et al. [14], except that the second Triton X-100 incubation was omitted. The membranes were suspended at about 3 mM Chl in the abovementioned Mes buffer for storage in liquid nitrogen. Before measurement they were diluted to 1 mM Chl in Mes buffer without sucrose and 100 mM NaCl was added for conductivity.

Oxygen measurements were carried out in a special cuvette built for simultaneous oxygen and absorbance measurements. It consists of a black plastic front plate with a 10×14 mm quartz window transmitting the flash and the measuring beam. The cathode is a 45% transmittance gold minigrid (500 wires per inch, Buck Bee Mears, St. Paul, MN) covering the window, and the anode consists of two rectangular Ag spades mounted on either side of the gold mesh, next to the window, in the front plate. The Au surface in the illuminated part of the cuvette amounts to nearly 3 cm^2, which ensures large flash-induced signals, and the Ag|Au surface ratio is 0.3. The back wall of the cuvette consists of a larger quartz window, clamped on an 0.4 mm spacer surrounding the ensemble of electrodes. This quartz plate must be removed for sample replacement. Although this is an inconvenient procedure and introduces scatter between data obtained with different samples, this arrangement was chosen because it allowed the short optical path length, optimal for absorbance measurements at the high sample concentration optimal for oxygen measurements. The electrodes are connected to the polarizing circuit by a triaxial cable, the outer mantle of which is connected to the metal cuvette compartment of the spectrophotometer for electromagnetic shielding. The measurements shown in the figures were done with this cuvette.

For some measurements a different electrochemical cell was used. This was the bottom part of a Joliot electrode [4] described in detail by Den Haan [15]. It consists essentially of a shallow plexiglass beaker (depth adjustable to zero) with a circular platinum cathode, surrounded by a broad ring-shaped Ag|AgCl anode, both mounted flush with the bottom surface. The Ag|Pt surface ratio is 11. The set-up is mounted horizontally; the sample covers both electrodes as a thin layer and is exposed to air over the whole surface. A diaphragm prevents direct illumination of the anode. This cell allows sedimentation of the sample on the electrodes before measurement and is suitable for measurements at strong polarization. It was also used for routine checks at weak polarization: by placing a sheet of tissue paper over the electrodes and pipetting just enough sample on that to soak the whole surface, a sufficiently stable dark current was obtained in seconds. The signal-to-noise ratio was lower, but sample replacement much easier than with the gold mesh electrode. The kinetics of flash-induced oxygen evolution in a homogeneous suspension measured at weak polarization were found to be identical with both electrochemical cells.

The electrochemical cells were polarized by a floating voltage difference between anode and cathode, without reference potential. The cathode and anode potentials were measured as a function of the applied polarization voltage in separate measurements with an additional Pt probe and a standard Ag|AgCl reference microelectrode. All potentials indicated in the text are given relative to the standard hydrogen electrode. The measured anode potential was 110 mV (at 125 mM Cl^-) and changed very little in the range of polarization voltages used, except when potentials of the gold mesh cathode of less than -500 mV were required. The conductivity of the sample became limiting at ion concentrations below 100 meq/l, decreasing the sensitivity of measurement. For this reason 100 mM NaCl was normally added. Unless

otherwise indicated, the sample temperature was about 5 °C.

Changes of the electrode current were recorded via current-to-voltage conversion, d.c. offset, amplification, and analog-to-digital (a/d) conversion, using the same apparatus as in Ref. 12. Averaging and base-line correction were done in an LSI 11 computer. The base-line correction was performed by linear extrapolation of the signal before illumination; measurements were carried out long enough after the onset of the polarization voltage to ensure an essentially linear decrease of the dark current in the time range of the measurement. In practice, the delay needed for this reason between the onset of the polarization voltage and the start of the measurement ranged from 20 s at weak polarization to 5 min at strong polarization. Typically, the total decrease of the dark current during the sweep-time was of the same order of magnitude as the average current increase per flash. Saturating red or blue xenon flashes of 10 μs half-width were filtered by Schott RG665 + Calflex C or Schott GG455 + Corning CS4-96 + Calflex C, respectively. Flashes of 532 nm and 15 ns half-width at a repetition rate of 50 Hz were obtained from a frequency-doubled Nd-YAG laser (JK Lasers) and were about 90% saturating.

Results

The electrode current change upon flash illumination of a suspension of PS II membranes, measured at different cathode potentials, is shown in Fig. 1A. The decrease of the dark current during the time of measurement has been subtracted as described in the Materials and Methods section. Fig. 1B is a semilogarithmic plot of the peak amplitudes (triangles) and the amplitudes at 10 ms after the flash (circles, normalized to the peak amplitude at −300 mV). A new sample was used for each measurement.

At strong polarization, as the voltage reaches the value where every oxygen molecule hitting the cathode is reduced, both the signal decay rate and the peak amplitude should become independent of the voltage. Instead, the amplitude becomes very small. This phenomenon effectively prevented measurements at strong polarization of flash-induced oxygen evolution in the relatively dilute (1 mM Chl) suspensions used. It was unrelated to the fact that the anode potential increased substantially at these cathode potentials, because the same effect was observed with the Joliot electrode (see Materials and Methods). With that electrode a reasonable signal amplitude at strong polarization could be measured only after sedimentation of the sample, whereas at weak polarization sedimentation was not required. After a measurement at strong polarization a less negative cathode potential only led to an even smaller signal. The amplitude was restored only after stirring of the sample or, partially, after a long wait. These observations indicate that oxygen evolution near the cathode is inhibited by conditions created at strong polarization. The cause of the inhibition is not yet clear; anaerobic conditions did not seem to inhibit oxygen evolution at weak polarization. The inhibition is not limited to a few micrometer near the cathode surface, since this would cause a delay of the signal rather than an attenuation. Typically, in a first measurement on a sample sedimented on the Joliot electrode the peak amplitude at −700 mV was several times lower than at −100 mV, whereas the Tafel coefficient (see below) would predict a more than 10-times higher signal amplitude.

At the most negative cathode potential shown, the kinetics of the flash-induced current transient are 'normal', taking the thickness of the sample layer into account. The decay of the signal becomes very slow when the cathode potential becomes much less negative than required for efficient oxygen reduction. In the range of −50 to −475 mV the signals were superimposable by assuming a Tafel coefficient of 0.16, somewhat lower than reported in Ref. 3. (The Tafel coefficient is the slope of the straight line in Fig. 1B multiplied by RT/F.) Since the cuvette used is closed, the sample ultimately becomes anaerobic even at weak polarization. To minimize this effect, a cathode potential of −100 mV was used in all measurements shown below. At this potential the estimated probability of reducing an oxygen molecule hitting the cathode was about 1% (at 5 °C and non-limiting sample conductivity) and the oxygen concentration at the cathode surface was depressed by approx. 10% at the time of measurement. The latter value was estimated by numerical

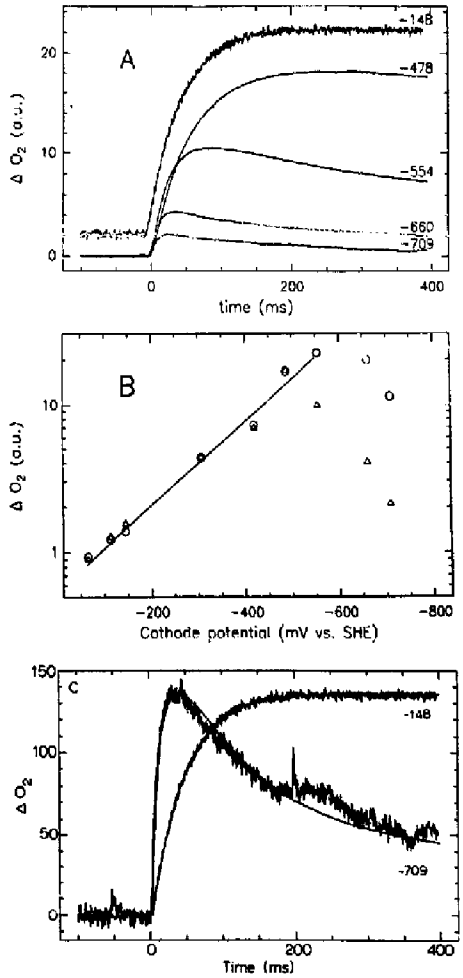

Fig. 1. (A) Cathode-potential dependence of the flash-induced oxygen signal. A suspension of dark-adapted PS II membranes was illuminated by three saturating flashes at 1 s intervals and the electrode current change caused by the third flash was measured and corrected for a decrease of the dark current as described in Materials and Methods. At cathode potentials higher than −500 mV only the amplitude was potential dependent. The trace measured at −148 mV was multiplied by 13.4 and offset for clarity. (B) The peak amplitudes (triangles) and the amplitudes at 10 ms after the flash (circles, normalized to the peak amplitude at −300 mV) are shown on a logarithmic scale; the slope of the line corresponds to a Tafel coefficient of 0.16. Average of 3–5 traces. (C) The traces at −148 and −709 mV, normalized to the same amplitude, are shown together with curves calculated (see Appendix) for the weak and strong polarization limits, assuming first-order oxygen evolution kinetics with the same time constant in both cases.

simulation of the oxygen diffusion perpendicular to the cathode; it could not be derived from the dark current, because the polarogram of the dark current (not shown) indicated that at −100 mV it was largely due to other reactions than oxygen reduction, especially at short times after the onset of the polarization voltage.

The 30 ms half-rise time of all signals in Fig. 1 except those showing a significant decay suggests that the apparently shorter rise time of the latter is due only to convolution with the decay kinetics. Fig. 1C shows that this is indeed the case. Taking the rise time of the signal at −148 mV as the intrinsic time constant of oxygen evolution, the kinetics expected at strong polarization were calculated as described in the Appendix. The result is seen to explain the risetime of the trace measured at −709 mV quite well. The same pronounced influence of the decay kinetics on the apparent rise time is seen in the data in Ref. 16 (there erroneously attributed to a capacitance on the cathode surface; at a polarization weak enough to avoid a significant decay of the signal we found no influence on the kinetics by a limiting conductivity). We conclude that the kinetics of oxygen production are – expectedly – independent of the polarization voltage. In this batch of PS II membranes detectable oxygen seemed to appear in 30 ms after a flash; in other batches rise-times of up to 130 ms have been found. A rise half-time near 1.3 ms would be expected if oxygen production were rate-limited by the reduction of the oxygen evolving complex in this preparation [12], which consists of membrane sheets with the PS II donor side freely exposed to the medium [17]. The observed rise is much slower and was therefore studied in detail.

Fig. 2A shows that the flash-induced current increase is really due to photosynthetic oxygen. Dark-adapted PS II membranes were illuminated by a series of 15 blue saturating flashes. The first flash induced only a short-lived negative transient, which will be discussed below. Subsequent flashes caused the same transient to a progressively smaller extent, and in addition a stepwise increase of the current with a half-time of 0.1 s on all steps. The amplitudes of the successive steps (Fig. 2B) exhibit a damped oscillation with maxima on flash numbers 3, 7, and 11, characteristic of the four-step

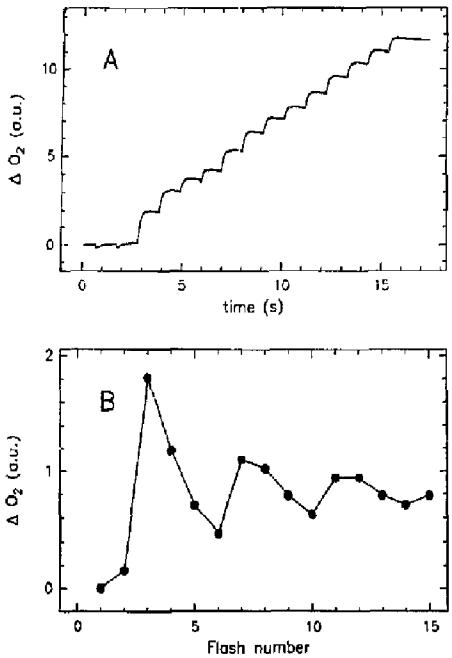

Fig. 2. (A) oxygen evolution upon illumination of dark-adapted PS II membranes by a series of saturating flashes, measured at a cathode potential of −100 mV. (B) The current increase caused by each flash is plotted. Average of four traces.

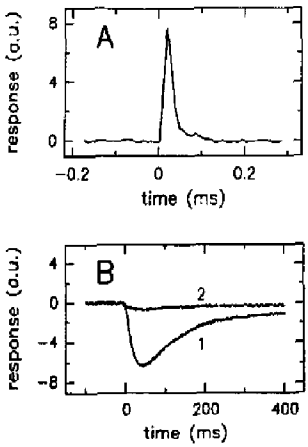

Fig. 3. Flash-induced electrode current transients, measured without photosynthetic material in the buffer solution. (A) Flash illumination of the Ag|AgCl anode, with a flash of 10 μs half-width. (B) Normal optical configuration, flash illumination of the Au cathode with a blue flash (curve 1) and a red flash (curve 2) of similar intensity.

redox cycle of the water oxidizing complex [18,19].

The electronic response time was most easily checked by flash illumination of the Ag|AgCl anode. The 20 μs transient then observed (Fig. 3A) shows that the electronic bandwidth was at least 50 kHz (in slower measurements the bandwidth was adjusted to the sample frequency of the a/d converter by a low pass filter). In the normal optical configuration no trace of this signal was detected, showing that scattered light reaching the anode was negligible. Instead, illumination of the cathode in the usual Cl^--containing media caused a negative transient signal (Fig. 3B). Such transients have been reported earlier [20,21]. This signal was dependent on the Cl^- concentration and was much larger after blue (trace 1) than after red flashes (trace 2). It may be caused by a transient oxygen consumption involved in a metal-catalysed reaction chain initiated by photodissociation of Cl_2 [22]. The Cl^--dependent 'artifact' is seen to be

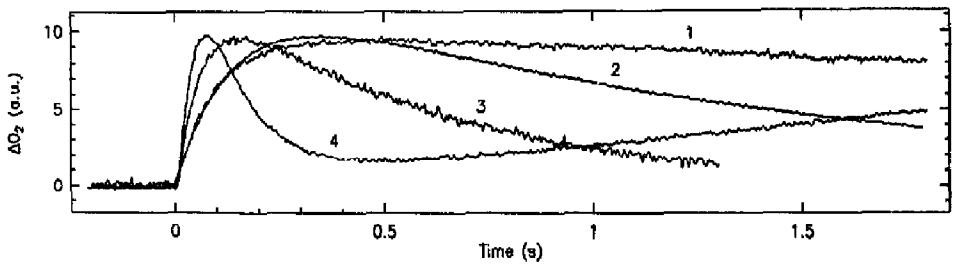

Fig. 4. Flash-induced oxygen concentration changes at 19°C in suspensions of (1) PS II membranes; (2) chloroplasts; (3) *Chlorella vulgaris* on the last of 50 saturating flashes fired at 2 Hz, the contribution by preceding flashes, obtained by extrapolation of the kinetics after the 49th flash according to the measured kinetics from 500 ms after the 50th flash, has been subtracted; (4) the same algae on the third flash after dark-adaptation. The traces have been normalized to the same amplitude.

comparatively small in Fig. 2A, where blue flashes were used. In principle it can be removed by subtraction of the signal observed without oxygen-evolving particles in the cuvette, if otherwise identical conditions can be created at the cathode surface. In practice this method was not very reliable. This signal is also much faster than the appearance of photosynthetic oxygen, confirming that the time resolution of measurement, for reactions at the surface of the electrodes, was more than adequate.

The 0.1 s rise time of photosynthetic oxygen could not be explained by assuming a minimum distance between the cathode and the nearest active oxygen sources because an unlikely large distance would have to be assumed and because the rise time was independent of the viscosity (0–400 mM sucrose), sample concentration (5-fold change), and cathode potential (−50 to 475 mV, Fig. 1).

The slow rise is also not due to some peculiarity of the PS II membranes. Fig. 4, measured at 19°C, suggests that a faster rise in other material may be due to convolution with a flash-induced oxygen consumption. As shown in trace 1, PS II membranes yield a similar slow rise at this temperature. Trace 2 shows that spinach chloroplasts exhibit similar kinetics, except for a pronounced decrease at the end of the trace which is probably due to oxygen reduction by PS I [23,24]. Since these chloroplasts probably did not retain ferredoxin, oxygen presumably reacted with reduced electron acceptors in PS I directly. The rise time in PS II membranes was slightly shorter than in the chloroplasts from which they were prepared, but the difference was small compared to the differences between batches of the same material: a wide range of half-rise times has also been observed in chloroplasts. Trace 3 shows the kinetics in *Chlorella vulgaris* under steady-state illumination by saturating flashes fired at 2 Hz, revealing a faster rise but also a more rapid oxygen consumption. The algae, which were grown in batch cultures, also showed a wide range of rise times of the oxygen signal. Induction phenomena complicate the kinetics in algae after dark adaptation: trace 4 shows the kinetics after the third flash fired on dark-adapted *Chlorella vulgaris*. The kinetics of oxygen evolution may still be as slow, but the signal appears to be more rapid due to convolution with a fast oxygen consumption [25]. In addition there is a flash-induced change in the slope of the baseline (a baseline correction was always applied, see Materials and Methods), which may be due to a decrease of the 'chlororespiration' rate [26]. The use of a weakly polarized cathode will greatly simplify the study of such phenomena. The observed rise time in trace 4 still exceeds that expected [4–7] by at least an order of magnitude.

Even more convincing is the observation that PS II cannot make oxygen again before the slow process has occurred. In the experiments of Fig. 5A dark-adapted PS II membranes were first illuminated by three xenon flashes at 1 s intervals, to generate the S_0 state and oxidize water. After a variable dark time Δt a series of six laser flashes, spaced at 20 ms, was fired to produce a second cycle of the S-states (allowing for misses and the incomplete saturation by the laser flashes). The total amount of oxygen produced was significantly depressed at Δt values less than a few hundred ms. No Δt dependence was observed when two instead of three xenon flashes or two instead of six laser flashes were used (not shown). The Δt dependence must be due to the oxygen yield of the last few flashes and be attributed to PS II centers which were in the S_0 state during Δt. Apparently the ability of these centers to produce oxygen a second time during the flash series was impaired if Δt was short. The inhibition was relieved with a half-time corresponding to that of oxygen release in the same preparation (C; only the slope matters; the amplitude was arbitrarily normalized). It follows that the measured oxygen release kinetics reflect a process at the site of water oxidation itself.

Fig. 5B shows experiments on chloroplasts preilluminated to randomize the S-state distribution. After the preillumination, a series of seven laser flashes spaced at 20 ms was fired, in order to obtain a situation where all centers that were in the S_3 state after the flash series had performed an $S_3 \rightarrow S_0$ transition (and oxidized water) during the flash series. After a variable dark time Δt a single xenon flash was fired. In this case the Δt dependence of the final oxygen yield must be due to the oxygen yield of the last flash, attributed to centers that were in S_3 during Δt. The outcome is the

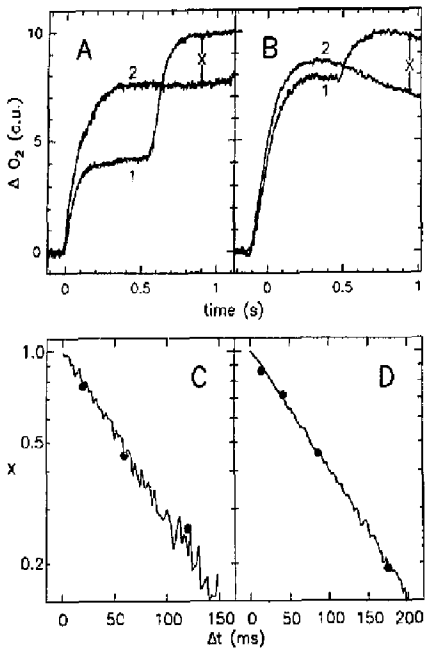

Fig. 5. Inhibition of oxygen evolution by oxygen not yet released. (A) Dark-adapted PS II membranes; illumination sequence: three xenon flashes spaced at 1 s, dark-time Δt, six laser flashes spaced at 20 ms. (B) Preilluminated chloroplasts; illumination sequence: seven laser flashes spaced at 20 ms, darktime Δt, one xenon flash. In both A and B time zero marks the start of Δt and curves 1 and 2 are examples of measured traces for $\Delta t \approx 500$ ms and $\Delta t = 20$ ms, respectively. (C) and (D) Normalized logarithmic plots of the difference (×) between the final oxygen yield obtained with $\Delta t = 500$ ms and that obtained with the indicated Δt values (circles); the lines show the kinetics of the flash-induced oxygen signal $(1 - \Delta O_2/\Delta O_{2\,max})$ in the same preparation.

same as in the previous experiments: at short Δt a significant depression of the final oxygen yield is obtained (B), and the effect disappears at increasing Δt with the half time of oxygen release in the same sample (D). This result confirms that of Fig. 5A and C and shows in addition that the S_3 state may be reached before oxygen release, and that oxygen release in S_3 has the same rate constant as that in S_0. In separate fluorescence measurements (not shown), using a gated photomultiplier and 100 kHz modulated measuring light as in [27], it was checked that the inhibition was not associated with an accumulation of Q_A^-.

The extent of the inhibition before oxygen release may be estimated by plausible simulations of the S-state turnover by the laser flashes and taking into account the same exponential oxygen release kinetics during both Δt and the laser flash series. The observed effect is larger than simulated even for a 100% inhibition. Also the small difference in Fig. 5B between the final oxygen yield with $\Delta t = 20$ ms (trace 2) and that before the last xenon flash in trace 1, relative to the oxygen yield of that flash, indicates a remarkably large inhibition at short Δt. We do not wish to draw quantitative conclusions on this basis, but it seems clear that, before oxygen release has taken place, the chances of making oxygen by the next S-state cycle must be small. In the experiment of Fig. 5B trace 1, the oxygen yield of the xenon flash (less than 1 O_2 per S_3 due to misses) relative to that of the series of seven laser flashes (more than 1 O_2 per center due to release between flashes) indicates that nearly half of the centers were in S_3 when the xenon flash was fired. This suggests, qualitatively, that the inhibition of oxygen production was accompanied by an accumulation of the S_3 state.

Three conclusions follow from these experiments: (1) the advance of the S-states up to S_3 does not require oxygen release; (2) the oxygen release time is about the same in S_3 as in S_0; (3) a flash-induced charge separation in the state S_3 before oxygen release has taken place is lost for S-state turnover and oxygen production. The third conclusion implies that the measured oxygen release time is due to a process in PS II and predicts that this process limits the rate at which PS II can deliver electrons to the plastoquinone pool or to artificial electron acceptors added.

Fig. 6 shows Arrhenius plots of the temperature dependence of oxygen release in PS II membranes (1) and in chloroplasts (2), revealing activation energies near 0.25 eV (24.3 kJ/mol). The flash-induced oxygen reduction in chloroplasts (3) had an activation energy of 0.71 eV. It must be mentioned that the signal amplitudes in PS II membranes increased about twofold per 10 degrees, presumably due to increased diffusion and the activation energy of oxygen reduction at the cathode surface. Preferably such changes of the sensitivity should be compensated by adjustment of the polarization voltage, but at the voltage used here a more than

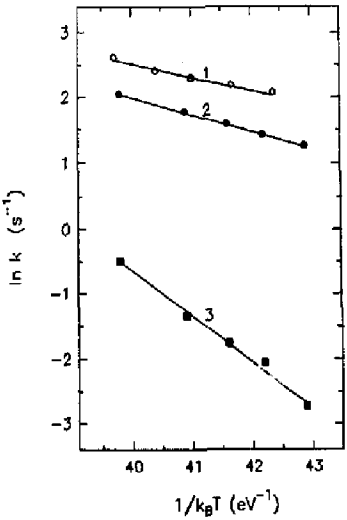

Fig. 6. Temperature dependence of oxygen release in PS II membranes (1) and chloroplasts (2) and of oxygen reduction in chloroplasts (3). The measured traces were fitted by the difference of two exponentials. The Arrhenius plots of the rate constants thus obtained show activation energies near 0.25 eV for oxygen release and 0.71 eV for oxygen reduction.

tenfold increase of the rate of oxygen reduction by the cathode would be needed to produce a significant effect on the measured kinetics (Fig. 1B).

As mentioned earlier, a rather wide range of oxygen release times has been observed in different batches of chloroplasts and the PS II membranes isolated from them. However, apart from temperature and pH-values higher than 7.5 (which caused a slower release, up to twofold at pH 9) no conditions were found which influenced the release time in one batch. Among the additions tested were 1 mM NH_4Cl, 10 μM CCCP and 5 mM $NaHCO_3$. Treatments used to remove the extrinsic polypeptides of PS II led to a more rapid oxygen release (to be published).

Finally, it may be noted that in all measurements the exponential rise of the oxygen signal was preceded by a significant lag, usually in the range of 2–10 ms. This lag is not only due to superposition of the Cl^--dependent artifact of Fig. 3B. Subtraction of the signal measured on the first flash after dark adaptation (cf. Fig. 2A) or without oxygen-evolving material did not yield reproducible results but always left a small lag phase. Extrapolation of the later exponential kinetics on a semilogarithmic plot was unreliable, too, because the recovery from the artifact often took place in the same time range as the release of photosynthetic oxygen (Fig. 3B), but also this method always left a small lag phase unexplained. Most likely this is due to the $S_3 \rightarrow S_0$ transition, because one has to assume that oxygen release cannot begin before water is oxidized.

We conclude that, upon flash illumination of PS II, polarographically detectable oxygen appears as a result of two sequential processes. The first process is water oxidation concomitant with the reduction of the manganese complex to S_0 and may be expressed in the lag phase of the signal. The subsequent release of a free dioxygen molecule is a hitherto not recognized and remarkably slow process, which is not required for the continued turnover of the S-states up to S_3, but has to take place before the next $S_3 \rightarrow S_0$ transition can occur.

Discussion

The results show that oxygen release by PS II takes much longer than previously assumed and has to take place before the next $S_3 \rightarrow S_0$ transition of the oxygen evolving complex can occur. The latter observation rules out an artifactual origin of the measured oxygen release kinetics and is most easily explained by assuming: (1) that oxygen release is required before new water molecules become available for oxidation by the complex; and (2) that water oxidation is required for the stabilization of a charge separation in the S_3 state. If such a charge separation is lost by reversed or cyclic electron flow it also cannot contribute to the reduction of plastoquinone or added electron acceptors. This may explain why maximum oxygen evolution rates in isolated PS II particles fail to live up to the expectations based on a presumed 1.2 ms turnover halftime (a sample containing 60 Chl/PS II should then yield 10 mmol O_2 per mg Chl per h), in spite of considerable efforts to optimize these rates.

The oxygen release time did not appear in Bouges-Bocquet's classical study on the S-state turnover times [9] because the time available for a full cycle of the S-states was always long enough in those experiments. The transport of 6–8 electrons upon illumination at −30 °C, in a frozen medium, also suggests that the S-state transitions (at least) up to S_3 do not require the presence of water at its oxidation site [28]. Ultraviolet absorbance changes suggested that the S-state transitions up to S_3 involve the oxidation of manganese and therefore not of water [29,30] (although for the $S_0 \rightarrow S_1$ transition this conclusion is still under debate [31]). Isotope exchange experiments have shown that the oxygen produced after a flash is derived from water which is still exchangeable in the S_3 state present before that flash [32,33].

Our findings should be reflected as well in the kinetics of electron transport at the onset of saturating continuous illumination. Starting mainly from the S_1 state after dark adaptation, the first six photoreactions of PS II should occur with the turnover times determined by Bouges-Bocquet [9], and the seventh turnover should fail until the oxygen made on the third turnover is released. This induction phenomenon was studied quantitatively two decades ago by several authors. An initial burst of PS II activity ('oxygen gush') takes place [34] until electron transfer from the plastoquinone pool to PS I, slowed down by the acidification of the thylakoid lumen [35], becomes rate-limiting [36]. The oxygen gush is strongly biphasic: the fast phase, indicating a PS II turnover time of less than 2 ms, indeed amounts to one oxygen molecule [34] and six electrons arriving in the plastoquinone pool (which can accommodate 14) [37] and hence at P-700 [36], or at an artificial electron acceptor added [38]. After this, the PS II turnover rate drops by an order of magnitude [34,38]. Stiehl and Witt [37] could fit the sudden decrease of the plastoquinone reduction rate after three of the seven molecules were reduced by assuming that this rate was proportional to the square of the oxidized fraction of the pool. Their explanation of this dependence, by a dismutation of two semiquinones produced in parallel, was ruled out by the discovery of the two-electron gate mechanism by Bouges-Bocquet [39] and Velthuys and Amesz [40]. To our knowledge, no other explanation for the end of the oxygen gush fast phase has yet been offered. Our conclusions provide this explanation.

The earlier evidence for a fast oxygen release [4,7] was described already in the Introduction. We observed a rather wide range of rate constants of oxygen release and cannot exclude the possibility that oxygen release sometimes does take only a few milliseconds. However, as we have not yet seen release times of less than a few tens of milliseconds, we doubt the validity of the earlier, less direct evidence for a much faster release. Convolution with light-induced oxygen reduction may have played a dominant role. In the modulated oxygen measurements [4] a phase shift and attenuation by the time required for the $S_3 \rightarrow S_0$ transition is expected and may erroneously have been attributed to oxygen release, but it is not obvious how a slower oxygen release could have been missed altogether. The analysis required the ad hoc assumption of a minimum distance of 2 μm between the cathode and the nearest oxygen sources, for which we found no evidence yet. We do find rather short apparent rise times after dark adaptation (Fig. 4, trace 4) and light-induced oxygen uptake was not considered in Ref. 4. The light intensity used was low enough to expect a nearly dark-adapted state.

The seemingly clear-cut evidence for a fast oxygen release obtained by Etienne [7], described in the introduction, leaves little room for doubt. The algae were presumably light-adapted. If our conclusions are correct, the flow dynamics at the end of the capillary, near the cathode surface, must have caused a surprisingly large underestimation of the time between illumination and measurement. The original data have been searched in vain for possible indications to that effect. On the other hand, the data do not include an independent experimental confirmation of the calculated delay between illumination and polarographic detection.

Fitting the flash-induced kinetics on the assumption of a fast oxygen release has not led to reasonable results. Maróti et al. [22] postulated a high diffusion barrier in Chlorella. Meunier and Popovic [16] postulated a large electrical capacitance on the cathode surface. An interesting case is the seaweed Ulva, in which nearly all chloro-

phyll is confined to a layer from about 10–20 μm below the surface on either side of the symmetrical thallus [41]. Fitting the flash-induced kinetics on the assumption of a fast oxygen release in this case produced a clearly unrealistic calculated distribution of PS II [42]. The shape of the calculated distribution would perhaps well be explained by a slow oxygen release. In conditions approaching the situation that all oxygen sources are in contact with a strongly polarized cathode, the electrode current should become equal to the rate of oxygen evolution and hence to the first derivative of the oxygen concentration changes measured with a weakly polarized cathode in a homogeneous suspension (see Appendix). Oxygen measurements on a thin layer of PS II membranes centrifuged onto a strongly polarized cathode [43] tend to confirm this prediction.

Our data indicate that oxygen evolution may be severely inhibited under the normal conditions of measurement with a bare oxygen cathode. However, Joliot (personal communication) does not observe that, the only obvious difference being that in his electrochemical cell the sample is well protected from chemical influences of the anode. This point requires further investigation. The consequences of the inhibition for the interpretation of earlier data are probably limited, because the inhibition does not obviously modify the period-4 oscillation in the oxygen yield induced by a series of flashes. In the sometimes very detailed interpretations of such oscillations, however, artifacts caused by this inhibition cannot be excluded. Oxygen measurements on homogeneous suspensions with a weakly polarized cathode are better defined, allow kinetic deconvolution of oxygen production and consumption processes, and provide access to a new and probably important parameter in photosynthesis, the oxygen release time.

Our conclusions suggest that the rate of photosynthetic electron transport may not always be limited by the reoxidation of the plastoquinone pool. The overall turnover time found in intact systems, first studied by Emerson and Arnold in 1932 [44], is usually in the same range as the 30–130 ms, 4 electron turnover allowed by oxygen release. If oxygen release is indeed a physiologically relevant rate-limiting step in photosynthesis, the wide range of its rate constant in our measurements may reflect meaningful variations under physiological control, and warrants a more systematic search for the origin of these variations.

Appendix

The measuring system used may be described by a one-dimensional semi-infinite sample solution at $x > 0$ with the electrode at $x = 0$. If oxygen is released instantaneously at $t = 0$ the diffusion kinetics are described by the following set of equations:

$$\frac{\partial}{\partial t}C(x,t) = D\frac{\partial^2}{\partial x^2}C(x,t) \tag{A-1a}$$

$$D\frac{\partial}{\partial x}C(x,t)\Big|_{x=0} = \alpha C(0,t) \tag{A-1b}$$

$$C(x>0,0) = C_0 \tag{A-1c}$$

in which C is the oxygen concentration, D the diffusion constant and α a proportionality constant for the electrode. α approaches 0 for weak polarization and infinity for high polarization. The solution of Eqn. A-1 is [45]:

$$C(x,t) = C_0\{\mathrm{erf}(x/2\sqrt{Dt}) + \exp(\alpha x/D + \beta) \times(1-\mathrm{erf}(x/2\sqrt{Dt} + \sqrt{\beta}))\} \tag{A-2}$$

in which $\beta = \alpha^2 t/D$ and $\mathrm{erf}(x) = 2/\sqrt{\pi}\int_0^x \exp(-y^2)\,\mathrm{d}y$. This yields for the cathode current I:

$$I(t) = \frac{\partial}{\partial x}C(x,t)\Big|_{x=0} = (\alpha/D)C_0\exp(\beta)(1-\mathrm{erf}(\sqrt{\beta})) \tag{A-3}$$

For the limiting case $\beta \downarrow 0$ (i.e. weak polarization and not extremely long t) Eqn. A-3 reduces to

$$I_{\mathrm{weak}}(t) \approx \alpha C_0/D \tag{A-4}$$

On the other hand, using the asymptotic expansion

$$\sqrt{\pi}z\exp(z^2)(1-\mathrm{erf}(z)) = 1 + \sum_{n=1}^{\infty}(-1)^n(1\cdot 3\cdot\ldots\cdot(2n-1))(2z^2)^{-n} \tag{A-5}$$

the limiting case $\beta \to \infty$ (i.e. strong polarization and not extremely short t) Eqn. A-3 reduces to

$$C_{\text{strong}}(t) \approx C_0/\sqrt{\pi D t} \tag{A-6}$$

Note that in both cases the shape of the function is independent of D.

When oxygen release after a flash is a first-order process with time constant τ, the observed signal is described by the convolution of the oxygen release and the electrode response $\int_0^t \exp(-s/\tau) I(t-s)\,\mathrm{d}s$, so

$$I_{\text{weak}}(t) \approx 1 - \exp(-t/\tau) \tag{A-7a}$$

$$I_{\text{strong}}(t) \approx \exp(-t/\tau) \int_0^{\sqrt{t/\tau}} \exp(y^2)\,\mathrm{d}y \tag{A-7b}$$

Numerical calculation shows that the latter function has a peak at $t = 0.85\tau$ and reaches $(1 - 1/\mathrm{e})$ of its peak level at $t = 0.14\tau$. Thus the rise time of oxygen evolution is about 7 times the rise-time of the signal in this case. At longer times it decays with $1/\sqrt{t}$.

If the sample is a layer of finite thickness on a strongly polarized cathode, both the rise and decay of the signal become even more rapid. In the limiting case that all oxygen sources are at $x = 0$ all oxygen released is immediately consumed by the electrode, i.e. the electrode response function is

$$I_{\text{strong}}(t) \approx \delta(t) \tag{A-8}$$

yielding for the convolution with oxygen evolution:

$$I_{\text{strong}}(t) \approx \exp(-t/\tau) \tag{A-9}$$

So in this case the oxygen release time equals the signal decay time. Any observed rise-time cannot originate from oxygen release and must be ascribed to a lag phase in oxygen release.

In the remaining limiting case of all oxygen sources at $x = 0$ on a weakly polarized electrode the electrode response function is determined by diffusion away from the electrode, thus goes with $1/\sqrt{t}$, similar to the case of the semi-infinite solution at strong polarization (Eqn. A-5). Thus the observed signal $I_{\text{weak}}(t)$ is then described analogously to Eqn. A-7b:

$$I_{\text{weak}}(t) \approx \exp(-t/\tau) \int_0^{\sqrt{t/\tau}} \exp(y^2)\,\mathrm{d}y \tag{A-10}$$

Of these four cases those described by Eqns. A-7a and A-9 yield the most straightforward results. The latter case, a thin layer with a strongly polarized cathode, however, suffers from the condition that the measurements must be performed anaerobically, as the cathode oxygen concentration is kept 0 ($\alpha = \infty$ in Eqn. A-1b). Moreover, the condition that all oxygen sources are at $x = 0$ cannot be approached if these sources have appreciable dimensions (e.g., cells). In the other case, a semi-infinite suspension with a weakly polarized cathode, this complication will introduce a lag phase which, if much shorter than τ, is more easily corrected for. We conclude that a system consisting of a suspension in combination with a weakly polarized cathode is to be preferred on theoretical grounds, in addition to its practical advantages.

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References

1 Kolthoff, I.M. and Lingane, J.J. (1952) Polarography, 2nd Edn., Interscience Publishers, New York.
2 Fatt, I. (1976) Polarographic Oxygen Sensors, CRC Press, Cleveland, OH.

3 Davies, P.W. (1962) in Physical Techniques in Biological Research, Vol. IV (Nastuk, W.L., ed.), pp. 137–179, Academic Press, New York.
4 Joliot, P., Hofnung, M. and Chabaud, R. (1966) J. Chim. Phys. 63, 1423–1441.
5 Sinclair, J. and Arnason, T. (1974) Biochim. Biophys. Acta 368, 393–400.
6 Arnason, T. and Sinclair, J. (1976) Biochim. Biophys. Acta 430, 517–523.
7 Etienne, A.-L. (1968) Biochim. Biophys. Acta 153, 895–897.
8 Joliot, P. and Kok, B. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 387–412, Academic Press, New York.
9 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785.
10 Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) FEBS Lett. 61, 286–289.
11 Velthuys, B.R. (1981) in Proceedings of the 5th International Photosynthesis Congress (Akoyunoglou, G., ed.), Vol. 2, pp. 75–85, Balaban International Science Services, Philadelphia, PA.
12 Dekker, J.P., Plijter, J.J., Ouwehand, L. and Van Gorkom, H.J. (1984) Biochim. Biophys. Acta 767, 176–179.
13 Cole, J. and Sauer, K. (1987) Biochim. Biophys. Acta 891, 40–48.
14 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
15 Den Haan, G.A. (1976) Thesis, University of Leiden.
16 Meunier, P.C. and Popovic, R. (1988) Photosynth. Res. 15, 271–279.
17 Dunahay, T.G., Staehelin, L.A., Seibert, M., Ogilvie, P.D. and Berg, S.P. (1984) Biochim. Biophys. Acta 764, 179–193.
18 Joliot, P., Barbieri, G. and Chabaud, R. (1969) Photochem. Photobiol. 10, 309–329.
19 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
20 Joliot, P. and Joliot, A. (1981) Biochim. Biophys. Acta 638, 132–140.
21 Marôti, P., Laczkó, G. and Szalay, L. (1984) Acta Physica Hungarica 55, 175–184.
22 Gmelins Handbuch der anorganischen Chemie, 8th Edn. (1958), Vol. O 3, p. 649, Verlag Chemie GmbH, Weinheim/Bergstraße.
23 Schmid, G.H. and Thibault, P. (1979) Z. Naturforsch. 34c, 414–418.
24 Swenson, S.I., Colbow, K. and Vidaver, W.E. (1986) Plant Physiol. 80, 346–349.
25 Radmer, R. and Ollinger, O. (1980) Plant Physiol. 6, 723–729.
26 Peltier, G., Ravenel, J. and Verméglio, A. (1987) Biochim. Biophys. Acta 893, 83–90.
27 Meiburg, R.F., Van Gorkom, H.J. and Van Dorssen, R.J. (1983) Biochim. Biophys. acta 724, 352–358.
28 Joliot, A. (1974) Biochim. Biophys. Acta 357, 439–448.
29 Dekker, J.P., Van Gorkom, H.J., Wensink, J. and Ouwehand, L. (1984) Biochim. Biophys. Acta 767, 1–9.
30 Dekker, J.P. and Van Gorkom, H.J. (1987) J. Bioenerg. Biomembr. 19, 125–142.
31 Lavergne, J. (1987) Biochim. Biophys. Acta 894, 91–107.
32 Radmer, R. and Ollinger, O. (1986) FEBS Lett. 195, 285–289.
33 Bader, K.P., Thibault, P. and Schmid, G.H. (1987) Biochim. Biophys. Acta 893, 564–571.
34 Joliot, P. (1965) Biochim. Biophys. Acta 102, 116–134.
35 Rumberg, B. and Siggel, U. (1969) Naturwissenschaften 56, 130–132.
36 Kok, B., Joliot, P. and McGloin, M. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), Vol. II, pp. 1042–1056, H. Laupp, Jr., Tübingen.
37 Stiehl, H.H. and Witt, H.T. (1969) Z. Naturforsch. 24 b, 1588–1598.
38 Forbush, B. and Kok, B. (1968) Biochim. Biophys. Acta 162, 243–253.
39 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256.
40 Velthuys, B.R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94.
41 Koeman, R.P.T. and Van den Hoek, C. (1981) Brit. Phycol. J. 16, 9–53.
42 Meunier, C.P., Swenson, S.I. and Colbow, K. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 737–740, Martinus Nijhoff, Dordrecht.
43 Miyao, M., Murata, M., Lavorel, J., Maison-Peteri, B., Boussac, A. and Etienne, A.-L. (1987) Biochim. Biophys. Acta 890, 151–159.
44 Emerson, R. and Arnold, W. (1932) J. Gen. Physiol. 15, 391–420.
45 Crank, J. (1956) The Mathematics of Diffusion, Oxford University Press, London.