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Determination of the photosynthetic oxygen release time by amperometry

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Flash-induced amperometric oxygen pulses from *Chlorella* or chloroplast samples were studied using a special electrode setup (so-called 'centrifuged electrode'). With this setup, the active particles were brought into close contact with a plane platinum cathode by (ultra)centrifugation, resulting in a fast response to any photosynthetic oxygen emission. The situation could be simply modelled and the experimental curves analyzed in terms of two independently adjustable parameters, the diffusion time and the oxygen release time. It was consistently found that the pulse kinetics is nearly diffusion-limited, and confirmed that the photosynthetic oxygen release time was in the millisecond or sub-millisecond range.

Introduction

Knowing the O_2 release time (τ_{ox}) during the functioning of the photosynthetic apparatus of higher plants has always been and is still an important piece of information, with concern to a complete description of the O_2 emitting system and, hence, its understanding. For long, the figures obtained independently through amperometric techniques by Joliot et al. [1] and Etienne [2], i.e., τ_{ox} of the order of a millisecond, were not put to discussion. Recently, however, this consensus was challenged when Plijter et al. [3] claimed that τ_{ox} was rather much longer, around 100 ms; they discussed possible reasons why much shorter values had been arrived at formerly by others and proposed that this long O_2 release time had to set a stringent limit to the turnover rate of the intact Photosystem II (and, in fact, to the whole photosynthetic electron transfer chain). Since then, a number of papers [4-6] have been published to the effect of revisiting this old problem; none of them could confirm the challengers results nor find support to their criticism of the amperometric measurements of other people.

Certainly, one of the basic problems at the heart of the controversy is a careful appraisal and correct quantitative treatment of the role of diffusion as the determining factor for the electrode kinetics in the amperometric or polarographic method. This aspect is classical among electrochemists, but less known by biologists using the O_2 electrode (a classical account of diffusion theory may be found in Ref. 7). Not less important is the role of diffusion as the universal agent of transport in the cellular environment, an aspect which is presently gathering considerable attention among biophysicists in the various fields of photosynthetic membranes functioning (see, e.g., Refs. 8 and 9).

At the time when the results of Plijter et al. [3] were published, I had been working for several years with a new configuration of O_2 electrode: the centrifuged-'well' electrode, henceforth abbreviated CWE. The starting idea is in the paradigm of 'the diffusion in a well': a molecule close to the bottom of the well, assumed to be an absorbing surface, is almost certain to get trapped at the bottom rather than to escape out of the well, provided the well is deep enough; this is readily derived from standard diffusion theory. A consequence, which I have superficially explored (unpublished data), is that, in an O_2 electrode configured as a well as above, any O_2 emission in contact with the electrode surface is quantitatively by Faraday's law - 4 electrons per O_2 molecule - expressed in the time integral of the electric current. Another, obvious consequence, which is put to use in the present work, is that the closer the O_2 source to the electrode the shorter the diffusion lag which shall deform the genuine emission kinetics. At any rate, diffusion actually sets a limit to how fast a photosynthetic O_2 emission can be measured by amperometry. Practically, in the CWE, the O_2 emitting material, originally standing as a suspension, is layered by centrifugation over the flat platinum electrode.

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Abbreviations: CWE, centrifuged (well) electrode; τ_d, diffusion time; τ_{ox}, O_2 release time.

The present report, besides presenting another result in favor of a τ_{ox} in the millisecond range, proposes a methodological and theoretical framework which, I hope, could be helpful for a more profitable use of the amperometric method in photosynthetic studies.

Theory of the amperometric oxygen pulse

A minimal model to quantify the situation of an O_2-emitting material layered onto the platinum surface in the CWE is according to the scheme of Fig. 1A. The assumptions are as follows: the active layer has a negligible thickness, is parallel to the platinum surface, at a distance X from it; X is small compared to the lateral dimension of the system and to the height of the electrolyte above the active layer. Consequently, we have to solve a diffusion problem in a one-dimensional semi-infinite medium with absorbing boundary at the origin, when at zero time one unit of O_2 per unit surface is deposited in the active layer, as a monomolecular process with rate constant k ($= 1/\tau_{ox}$), and allowed to diffuse with diffusion constant D.

It is first needed to solve the problem in the case of an instantaneous O_2 emission (k infinite), which will give the reference pulse response of the CWE. The latter, to an instrumental (electrochemical) constant, is proportional to the O_2 flux per unit surface at the origin $x = 0$:

$$\Phi(t) = \left(X/2\sqrt{(\pi D t^3)}\right) \mathrm{Exp}(-X^2/4Dt) \qquad (1)$$

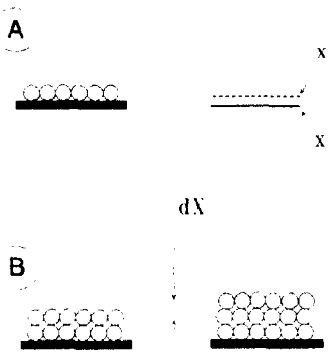

Fig. 1. Scheme of the electrode diffusion problem. (A) A layer of active cells in contact with the electrode surface (left) is modelled (right) as a source of negligible thickness (dashed line) located at a distance X from a flat perfect sink at $x = 0$ representing the electrode. (B) In the neutral layer experiment, variable amounts of inactivated cells (open circles) have been interposed between the electrode and the upper active layer (dotted symbols); the variation δX (of the distance X defined in (A)) is better known than X itself.

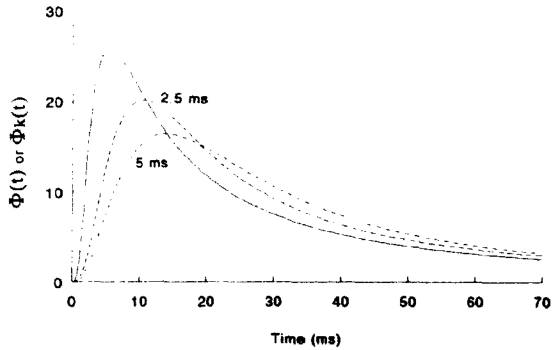

Fig. 2. Theoretical oxygen electrode pulses. In all cases, the diffusion time $\tau_d = 10$ ms. Continuous line: $\Phi(t)$ or wholly diffusion-limited O_2 pulse (O_2 release time, $\tau_{ox} = 0$); dashed lines: $\Phi_k(t)$ or O_2 pulse with significant O_2 release time lag (τ_{ox} as indicated). Notice that the $\Phi(t)$ curve crosses the $\Phi_k(t)$ curves at their maximums.

where t is time and D the O_2 diffusion constant (see Appendix I for the derivation). It will be convenient to refer to $X^2/4D$ as the diffusion time, τ_d. The time course of $\Phi(t)$ is depicted on Fig. 2; several time characteristics of the curve may be defined: t_U, the halftime of the ascending part of the curve, t_M, the time of the maximum Φ_M and t_D, the halftime of the decaying part of the curve. We note for future reference that the three above time quantities stand in a fixed mutual ratio:

$$t_U/t_M/t_D = 0.436:1:3.14 \qquad (2)$$

irrespective of the values taken by X and/or D. Other properties of the maximum of $\Phi(t)$ are noteworthy: it is found that

$$t_M = X^2/6D = 0.666\tau_d \qquad (3)$$

and that the product $t_M \Phi_M$ is invariant (see Appendix I) in other words, as expected, the O_2 pulse maximum is sharper and more rapid as the source is closer to the CWE and/or diffusion is more active. Naturally, the 'well' effect is verified, i.e., the complete time integral of $\Phi(t)$ is unity, simply meaning that all of the O_2 emitted is quantitatively absorbed (electrochemically reduced) by the platinum electrode.

Next, the O_2 flux $\Phi_k(t)$, defined for a finite rate constant k of emission at the active layer, is simply expressed as the convolution of Φ with the O_2 emission rate:

$$\Phi_k(t) = \int_0^t \Phi(t-u) k \, \mathrm{Exp}(-ku) \, du \qquad (4)$$

see Appendix II. The time course of $\Phi_k(t)$ is shown along with the corresponding $\Phi(t)$ on Fig. 2. As expected, $\Phi_k(t)$ is delayed compared to $\Phi(t)$, but its time integral is still unity; it may be proved, as seen on Fig. 2, that the maximum of $\Phi_k(t)$ lies on the falling edge of $\Phi(t)$. The time characteristics of the curve t_U, t_M and t_D are no longer in fixed ratios (cf. Eqn. 2): Fig. 3 shows their variation as a function of the dimensionless

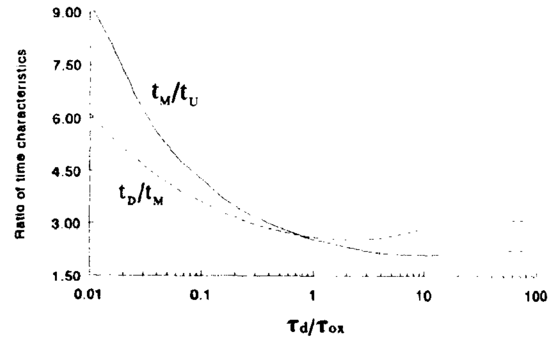

Fig. 3. Dependence of the theoretical time characteristics of the O_2 pulse upon the time parameters related to diffusion and/or O_2 release kinetics. Abscissa: ratio of diffusion time, τ_d to O_2 release time, τ_{ox}. Continuous line: ratio of pulse maximum time, t_M to pulse half-rise time, t_U; dashed line: ratio of pulse half-decay time, t_D to pulse maximum time, t_M.

quantity τ_d/τ_{ox} ($= kX^2/4D$). From the above, it is rather obvious that, if the process is only nearly diffusion-limited (the diffusion time τ_d being commensurate to the O_2 release time, τ_{ox}), the analysis of the O_2 pulse cannot determine τ_{ox} properly, but can only impose an upper limit for it.

Materials and Methods

Chlorella pyrenoidosa Emerson was grown on Bristol medium [10] at 20°C, 1% CO_2 and medium intensity white fluorescent light; the culture was harvested twice weekly. The above growth conditions resulted in small cells of rather uniform size. *Chlorella sorokiniana*, a strain of naturally small cell size, was grown as in Ref. 11. Pea was grown in artificial light for 2 or 3 weeks; chloroplasts were isolated from the leaves as in Ref. 12. For the polarographic experiments, cells are suspended in their growth medium and chloroplasts in buffer C of Ref. 12; the medium or buffer is supplemented with KCl (0.2 M final).

For a control of the material deposited over the platinum electrode, the algal cell volume was determined by centrifugation of an aliquot in a hematocrit tube and the average cell size was determined by inspection at the microscope. In the case of chloroplasts, the amount deposited was chosen by reference to the algal material on a chlorophyll basis. The precision of the hematocrit method was poor (approx. 10%); as an indirect check of the amount deposited to build a single cell layer, the following procedure was used. A cell suspension in the electrode bucket of concentration c_1 with a height of suspension h_1 is compared with another suspension of concentration c_2 examined under the microscope after settling in a microchamber of known thickness h_2 (appropriate spacer between slide and cover slip); the cell densities of the deposited layers in the two situations are indentical if c_1 is chosen equal to c_2h_2/h_1. By trial and error, it is easy to determine c_1 corresponding to a single cell layer.

The CWE has been introduced and used previously [13,14], and described in detail in Ref. 14. It is recalled that by centrifugation of the CWE with a swinging bucket type rotor the biological particles are smoothly layered over the platinum surface. In the so-called neutral layer experiments, whose precise motivation will be explained later, the active layer A is separated from the electrode with a given amount B of 'neutral' material – thermodenatured sample: 50° C for 10 min (cf. Fig. 1B). To achieve this configuration, the particles are deposited in two steps: first the B layer, then the appropriate amount of A is very gently pipetted on top of the previous supernatant before another centrifugation.

Because the layered material is topped by 1-2 cm of air-saturated electrolyte, the amperometric d.c. signal drifts strongly after switching the electric polarization on, due to the formation of an extensive O_2 gradient, thus practically forbidding any measurement before 3-5 min have elapsed. The measurement protocol was usually as follows (although with variants): light pulses were given at a low repetition rate (e.g., a 1 s period) and a sequence of, e.g., 10 pulses were averaged and recorded as in Ref. 14; the low repetition rate, although permitting some deactivation between pulses, was necessary for ensuring that the shape of each O_2 pulse was negligibly affected by the previous ones. Other operating details are given in Results.

The O_2 pulse has been analyzed by optimally fitting the synthetic function $\Phi_k(t)$ to the data using a Simplex algorithm [15] with two adjustable parameters, the diffusion time τ_d and the O_2 release time τ_{ox} (see Theory and Appendix IV). The Simplex analysis program was run on each experimental curve several times with random choice of the unknown parameters as initial seeds. In general, a majority of runs on a given data set gave solutions closely clustered (see standard deviation between runs for τ_d and τ_{ox} on Table I); however, a minority of the runs resulted in different solutions with higher τ_{ox}, which were simply discarded because they were a minority and they yielded a poorer fit. When this happened, the behavior was a kind of bistability, in that the results of analysis were clustered into two distinct, narrow groups, but not broadly dispersed. Overall, less than 6% of runs were thus discarded (analysis of 31 curves, with 10 runs per curve).

The theory of the amperometric O_2 pulse and the Simplex analysis have been controlled as follows: the diffusion-limited flow of O_2 molecules towards the platinum electrode has been simulated as a random walk by the Monte Carlo method (see Appendix III) and then analyzed with the Simplex program. The agreement (Fig. 4) is satisfactory.

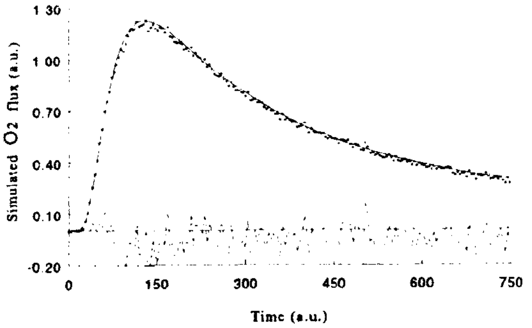

Fig. 4. Monte Carlo simulation of the O_2 pulse (see text and Appendix). *Dots*: simulation; continuous line: $\Phi_k(t)$ fitted to Monte Carlo points by the Simplex program; dashed line: fitting error (enlarged 5 times).

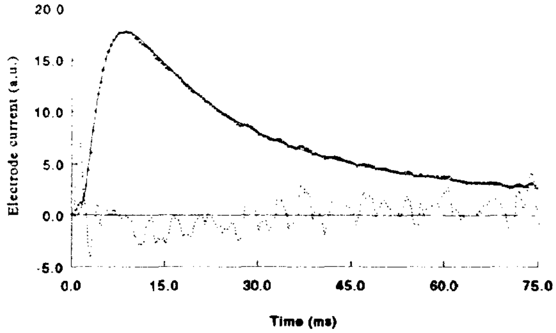

Fig. 5. Flash-induced O_2 pulse from *Pea* chloroplasts and analysis in terms of $\Phi_k(t)$. Chlorophyll equivalent layer density: 8.1 $\mu g\ cm^{-2}$; electrode polarization: −0.9 Volt; temperature 25°C. Dots: average of 5 O_2 pulses at third flash of sequences separated by 1 s. Continuous line: $\Phi_k(t)$ fitted with the Simplex program (see also Table I, Chloroplasts, #2). Dotted line: fitting error (enlarged 10 times).

Results

Representative instances of experimental curves compared with the Simplex analysis are shown on Fig. 5 for *Pea* chloroplasts and Fig. 6 for *Chlorella pyrenoidosa*. The analysis in the case of chloroplasts (Fig. 5) shows that τ_{ox} is much shorter than τ_d resulting in the 2 functions $\Phi(t)$ and $\Phi_k(t)$ being nearly identical (see Theory). It may be noted that the fit is far from optimal in the case of *Chlorella* cells (Fig. 6A). This misfit has been observed more or less regularly with cell samples. That it has probably a biological origin is suggested by comparing with Fig. 6B and C, showing neutral layer experiments (with an identical layer of

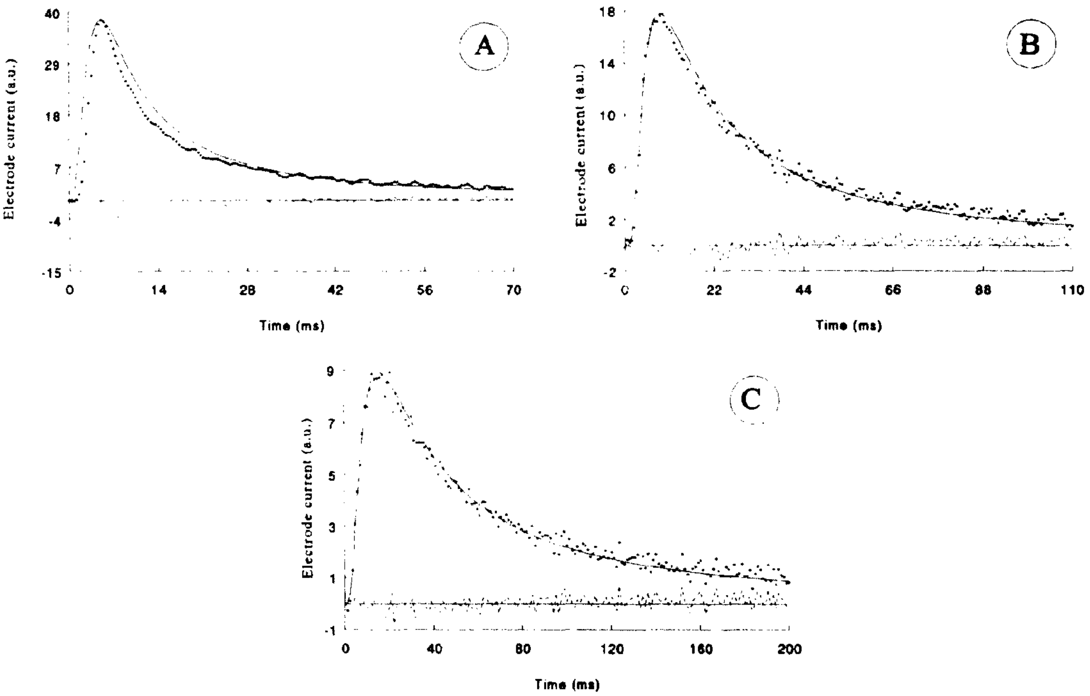

Fig. 6. Flash-induced O_2 pulse from *Chlorella pyrenoidosa* cells alone (A) or with interposition of neutral layers (B and C), analysis in terms of $\Phi_k(t)$. Chlorophyll equivalent layer density: 1.6 $\mu g\ cm^{-2}$ for the active top layer, 4.5 and 9.0 $\mu g\ cm^{-2}$ neutral layer for B and C respectively; electrode polarization: −0.9 V; temperature 20°C. Other experimental conditions as in Fig. 5. See also Table I, *Chlorella*, Nos. 1–3.

TABLE I

Analysis of O_2 pulse in chloroplasts and whole cells

Material	No. [a]	τ_d (ms) [b] (S.D) [c]	τ_{ox} (ms) [b] (S.D.) [c]	Fitting error [d]	Condition
Chloroplasts	1	10.80 (0.02)	0.5 (0.047)	0.17	Chl 4.05 μg cm^{-2} 5*X* [e]
		9.50 (0.03)	0.88 (0.02)	0.13	10*X*
	2	13.00 (0.04)	0.35 (0.005)	0.15	Chl 8.1 μg cm^{-2}, 5*X*, t = 5 min
		13.00 (0.08)	0.27 (0.06)	0.15	t = 10 min
		12.50 (0.014)	0.50 (0.06)	0.15	t = 35 min
Chlorella	1	5.37 (0.03)	1.37 (0.01)	2.2	70 μl N [f]
	2	12.95 (0.03)	0.67 (0.01)	0.2	70 μl N + 200 μl TD [f]
	3	25.8 (0.1)	0.18 (0.06)	0.22	70 μ N + 400 μl TD

[a] Numbers refer to different samples.

[b] τ_d = diffusion time; $\tau_{ox} = O_2$ release time.

[c] Standard deviation for 10 runs of the analysis simplex program.

[d] If Y_i is a data point, y_i the corresponding item in model and N the total of data point, the fitting error is defined as: $\sqrt{\{(Y_i - y_i)^2/N\}}$.

[e] Chlorophyll equivalent of the material deposited on the electrode; nX = record is sum of n pulses.

[f] N: normal; TD: thermodenatured; 100 μl is equivalent to 2.26 μg cm^{-2} chlorophyll.

active material). This type of experiment amounts to increasing the source to sink distance, therefore the O_2 pulse kinetics becomes diffusion-limited and unaffected by the O_2 emission kinetics details properly. In these cases, the fit (with function $\Phi(t)$) is almost perfect.

Table I displays in detail a selection of the analyzed data for the purpose of illustrating several effects. In the first place, it is to be noted that the precision (standard deviation among the runs in a single analysis) is much better for τ_d (< 1%) than for τ_{ox} (several %); as discussed in Theory, this simply reflects the fact that τ_{ox} is inherently poorly determined when it is appreciably shorter than τ_d. The first group of data in Table I is concerning two samples of the same chloroplast preparation. The amount of material in sample No. 2 is twice that in sample No. 1; comparison of τ_d values and pulse amplitudes suggests that the lower dose is already close to the minimal layer saturation. With sample No.2, the effect of the time elapsed while standing on the platinum electrode (with the electric polarization switched on) is shown: although the O_2 emitting activity had dropped by 27% after 35 min compared to after 5 min, the time characteristics of the O_2 pulse (τ_d and τ_{ox}) are essentially unchanged; this indicates that, whatever the cause of the activity loss, it occurs as a all or none process.

The next group of data in Table I is relative to the same experiment as Fig. 6A,B,C above; as the thickness of the neutral layer increases (absent in sample No. 1, one unit, two units neutral layer in sample Nos. 2 and 3, respectively), τ_d significantly increases due to a longer diffusion path from source to sink (see Fig. 1B), while τ_{ox} remains insignificant.

Comparing the two groups of results derived from different plant materials reveals no significant difference for τ_{ox}, whereas τ_d appears to be somewhat shorter for cells as compared to chloroplasts, due perhaps in part to the high viscosity of the buffer (0.4 M sucrose) in the case of chloroplasts.

One might think of using the diffusion time, τ_d, of the above data to estimate the diffusion coefficient, D, of the composite medium (membrane, stroma, enveloppe, etc.) responsible for the delay in the O_2 response (see Theory). Unfortunately, there is too much uncertainty in our knowledge of X for this approach to be practicable. The difficulty has been partly circumvented in performing experiments of the neutral layer type already referred to above. The thermal denaturation used in preparing the neutral material is deemed not to modify appreciably D. As already pointed out, by interposing layers of neutral material between the O_2 source and the platinum electrode the process becomes diffusion-limited, hence described by the simple function $\Phi(t)$, as a consequence, $\sqrt{t_M}$ must be a linear function of X, now including the neutral layer(s) (see Eqn. 3), and Fig. 7 shows that this is indeed verified, except towards the origin where a positive deviation reveals the influence of τ_{ox}. The slope of the linear section of the curve allows for an estimation of D, not dependent on any assumption on the structural geometry of the O_2 emitting system. The estimated diffusion coefficient is appreciably lower (about $2 \cdot 10^{-6}$ cm^2 s^{-1}) than the corresponding figure for O_2 in water (about 10^{-5} cm^2 s^{-1}). Another independent test of the

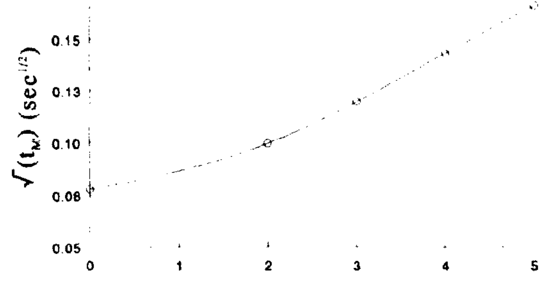

Fig. 7. Square root of t_M, the time of maximum of the O_2 pulse in a neutral layer experiment with *Chlorella sorokiniana* as a function of the number of active layers (see also Table II). Chlorophyll equivalent layer density: 1.2 μg cm^{-2} for the active top layer, 1.7 μg cm^{-2} per added neutral layer. Other experimental conditions as in Fig. 5.

TABLE II

O_2 pulses with interposition of neutral layers (cf. Fig. 7)

	t_U/t_M [a]	t_D/t_M [a]
No. of neutral layers [b]:		
2	0.425	3.40
3	0.413	3.24
4	0.450	2.99
5	0.452	2.90
Average	0.435	3.13
Theory [c]	0.437	3.136

[a] t_U, t_M and t_D: time abscissae for half-rise, maximum and half-decay of O_2 pulse respectively.

[b] A layer is 1.73 μg cm^{-2} chlorophyll equivalent.

[c] cf. Eqn. 2.

theory is given by the ratios t_U/t_M and t_D/t_M of points in the linear section of Fig. 7 (Table II), which average value is in effect very close to that predicted by theory.

The above data indicate that the O_2 release time is of the order of milliseconds or less, in confirmation of most earlier estimations.

Discussion and Conclusion

This work has been motivated by the desire of constructing the simplest experimental setup for studying with the amperometric technique the various aspects of light induced O_2 emission or absorption in photosynthetic systems.

From the experimental point of view, the O_2 pulse data that are reported here are similar in shape and time characteristics to that which was obtained by most other groups (Refs. 3-5 and 16) although the geometry and electrochemical conditions of the electrode used were different. Thus, one may be confident that the results are not much deformed due to unexpected experimental side-effects. The CWE here used has distinctive features offering advantages not found in other setups: first, the amount of biological material under study can in principle be controlled and precisely reproduced from run to run; second, using a minimal layer of active material deposited on the electrode by centrifugation provides the best sensitivity and time resolution yet warranting a homogeneous illumination. The CWE has a decisive advantage when one is working with small particles (chloroplast sub-particles [13], bacterial cells [14]), that would barely sediment in a reasonable time over the electrode surface in the classical setup. A serious drawback however in this system is the risk of anaerobiosis to which the biological material is submitted due to the depth of the CWE bucket, the O_2 consumption by the electrode under polarization and eventualy by any biological effect (e.g., respiration). The problem has been discussed in Ref. 16. We had found this problem of concern in a former study of the light-induced modification of respiration in photosynthetic bacteria [14]; however, it is shown here (see Table I) that the kinetic factors underlying the O_2 pulse are not significantly affected by letting the cells or organites stand in layered configuration for a long period.

Among the groups which have studied the amperometric O_2 pulse, only Plijter et al. [3] have offered a theoretical quantitative treatment of its kinetics embodying all its relevant factors, in similarity to what is proposed here. However, there is little point in comparing both treatments, as they refer to quite different geometries. The analysis given in [4] is only empirical (linear combination of two exponentials) and therefore carries little interpretive value. The considerations in Ref. 5 are correct in my opinion, but remain purely qualitative. The theoretical model proposed in the present work and detailed in the Appendix seems to explain quite correctly the experimental data obtained with the CWE (Figs. 5 and 6). It is also gratifying to find that independent controls provided by the model, such as the ratios t_U/t_M and t_D/t_M (Table II) and the relation: $\sqrt{t_M}$ proportional to X for large X's (Fig. 7) in the neutral layer experiment, also confirm the model.

It could have been argued a priori (see Ref. 5) that the biological structure is too complicated and poorly known to allow one to attempt such a theory. In fact, the present results show that the simple and naive model of an active layer of negligible thickness at a distance X of a sink, in a medium of diffusion coefficient D is a reasonable first approximation.

A by-product of the present approach is an estimation of the average diffusion coefficient of O_2 for in vivo plant systems (see discussion of Fig. 7). No doubt knowing this more precisely should be useful, but would require further work, in particular for a better determination of the specific density of the biological material.

In conclusion, the results presented here add to the weight of other previously published evidences in favor of a photosynthetic O_2 release time of the order of a millisecond, but possibly less. It is as yet unclear for what reasons Plijter et al. [3] got a much longer range of values. I may tentatively offer the following explanation: firstly, in spite of the layer immediately in contact with the platinum surface prevailing for inducing the electrode response, still the fact that the active material was distributed over a sizeable thickness in their setup contributes to a lengthening of the O_2 pulse; secondly, the very low polarization used by the above authors has probably the same effect as an increase of X, the minimal distance from the source to the electrode (see Appendix I). The experimental and theoretical approach which I have used, as needed for experimenting with the CWE, in my opinion indicates that the process as observed in the usual conditions (chloroplast fragments, whole microalgal cells, etc.) is probably diffusion-limited, which means that a precise value of the O_2 release time shall not be found using the amperometric method, unless one is using much smaller objects, for instance O_2 emitting sub-particles as in Ref. 13.

Acknowledgement

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Appendix

I. The one-dimensional semi-infinite system sufficient to describe the 'well (centrifuged)' electrode in its generality is expressed by the following set of equations:

$$\frac{\delta c(x,t)}{\delta t} = D\frac{\delta^2 c(x,t)}{\delta x^2} \qquad \text{(A-1a)}$$

$$D\left.\frac{\delta c(x,t)}{\delta x}\right|_{x=0} = \alpha c(0,t) \qquad \text{(A-1b)}$$

$$c(x,0) = \text{Dirac}(x - X) \qquad \text{(A-1c)}$$

$$c(L, t > 0) = 0 \qquad \text{(A-1d)}$$

where $c(x,t)$ is the O_2 concentration at distance x from the electrode and time t and D is the O_2 diffusion coefficient in the given (biological) medium. Eqn. A-1a is Fick's second law. Eqn. A-1b expresses the electrochemical condition at the electrode: the O_2 flux, $\Phi(t)$, (first member which is Fick's first law) is equal to the O_2 reduction rate at the electrode (second member: first order reaction); at the polarographic plateau, α is infinite, $c(0,t) = 0$, but the O_2 flux remains finite. In Eqn. A-1c, 'Dirac' is the Dirac function; the equation means that at $t = 0$ there is unit density of O_2 in the source plane, at distance $x = X$ from the electrode and zero concentration elsewhere. Eqn. A-1d sets the condition at the top of the well, a distance L from the electrode. Note that nothing is lost in generality by assuming a zero O_2 concentration everywhere (except at $x = X$) instead of a more realistic initial distribution.

The above partial differential system is solved with the help of the Laplace transform along the time axis, whereby $\Phi(t)$ is transformed into $\pounds\Phi(p)$, with p being a dummy variable. Several simplifying assumptions are made. Firstly, as L is very much larger than X, one may put $L = \infty$, then:

$$\pounds\Phi(p) = \frac{\alpha\,\text{Exp}(-rX)}{Dr + \alpha} \qquad \text{(A-2a)}$$

where

$$r = \sqrt{(p/D)} \qquad \text{(A-2b)}$$

Secondly, if D/α is small, but not zero (a condition of electrode polarization slightly below the polarographic plateau), the Laplace transform Eqn. A-2a is approximated by:

$$\pounds\Phi(p) = \text{Exp}(-r(X + D/\alpha)) \qquad \text{(A-3)}$$

in other words, it is as if X was replaced by $X + D/\alpha$, i.e., as if the real electrode was replaced by a virtual one at $x = -D/\alpha$. We shall see that increasing the source to electrode distance slows down the O_2 signal. Then, if Eqn. A-3 can be extrapolated to low electrode polarization as used in Ref. 3, the result could be giving the appearance of a long O_2 release time. At the polarographic plateau, $D/\alpha = 0$ and Eqn. A-3 is reduced to:

$$\pounds\Phi(p) = \text{Exp}(-rX) \qquad \text{(A-4)}$$

Accordingly, with the two above assumptions, the inverse Laplace transform of Eqn. A-4 is:

$$\Phi(t) = \left(X/2\sqrt{(\pi D t^3)}\right)\text{Exp}(-X^2/4Dt) \qquad \text{(A-5)}$$

The maximum of this function is found at $t = t_M$ such that:

$$\Phi(t_M) = 6^{3/2} D\,\text{Exp}(-3/2)/\left(2\sqrt{\pi}X^2\right) \qquad \text{(A-6a)}$$

$$t_M = X^2/(6D) \qquad \text{(A-6b)}$$

(in other words, the larger the source to electrode distance, the slower the pulse reponse) and the product $\Phi(t_M)t_M$ is invariant, independent of X and D:

$$\Phi(t_M)t_M = \sqrt{(3/(2\pi))}\ \text{Exp}(-3/2) \qquad \text{(A-6c)}$$

this constant is approx. 0.1542 molecule of O_2. This refers obviously to the situation when initially 1 molecule of O_2 per unit surface was deposited in the source plane X and when, consequently, the total time integral of $\Phi(t)$ would yield also 1 molecule of O_2 per unit surface. Thus, as the recording of the above time integral is difficult practically, Eqn. A-6c affords a convenient estimation of this quantity (provided the O_2 emission is diffusion-limited):

$$O_2\ \text{flash yield} = \Phi(t_M)t_M/0.1542 \qquad \text{(A-7)}$$

II. In case of a non-instantaneous O_2 emission with rate constant k, since the delay in arrival of an O_2 molecule to the electrode is the sum of a reaction time at the source and a diffusion time, the resulting function $\Phi_k(t)$ now is the convolution of $\Phi(t)$ with the rate of O_2 emission $k\,\text{Exp}(-kt)$:

$$\Phi_k(t) = \int_0^t \Phi(t-u)k\ \text{Exp}(-ku)\,\mathrm{d}u$$

$$= \int_0^t \Phi(u)k\ \text{Exp}(-k(t-u))\,\mathrm{d}u \qquad \text{(A-8)}$$

By differentiation of Eqn. A-8 with respect to t, one gets the useful relation:

$$\frac{\mathrm{d}\Phi_k(t)}{\mathrm{d}t} = k(\Phi(t) - \Phi_k(t)) \tag{A-9}$$

hence, at the maximum of $\Phi_k(t)$ (left member = 0), $\Phi_k(t) = \Phi(t)$, i.e., the top of the $\Phi_k(t)$ curve lies on the falling edge of the $\Phi(t)$ curve, as illustrated in Fig. 2. Eqn. A-9 has also been used for the numerical calculation of $\Phi_k(t)$.

III. The diffusion-limited O_2 pulse (calculated above as $\Phi(t)$) has been simulated by Monte Carlo (e.g., Ref. 17) with the following scheme. An ensemble of 10^6 'O_2 particles' (implemented by running the Monte Carlo experiment 200 times with an array of 5000 particles) is initially located at distance $x = X$ from the 'electrode'. This distance is a dimensionless quantity (0–255 interval) stored in a memory location representing a particle. For every time unit (dimensionless), each particle in turn is allowed to change position by one unit towards or away from the electrode with equal probabilities. Whenever a particle reaches the electrode ($x = 0$), it is 'absorbed', i.e., withdrawn from the ensemble; similarly if the particle escapes the system ($x = 255$). For a total of 255 time units, the number of particles absorbed by the electrode per unit time is recorded as a function of time, in analogy to $\Phi(t)$ (cf. Fig. 4).

IV. The pulse analysis uses the elementary Simplex algorithm [15] to fit the experimental data to $\Phi_k(t)$ with the two adjustable parameters τ_d and τ_{ox}, the agreement being appreciated by least-squares. The $\Phi_k(t)$ function is calculated with the help of Eqns. A-5 and A-9. Because of the large amount of numerical calculation involved, the following optimization has been found helpful. The differential Eqn. A-9 integrated with the Euler method, initially with an integrating step equal to the input data time step, say dt. Simplex is allowed to run iteratively until a minimal steady-state of the fitting function is reached. Then dt is divided by 2 and the process resumed It finally comes to a stop either when no improvement in the quality of fit is observed after changing dt or when the initial dt has been divided by 8.

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