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PULSED POLAROGRAPHIC STUDY
OF THE OXYGEN-EVOLVING SYSTEM OF PHOTOSYNTHESIS IN CHLORELLA

A pulsed polarographic device was built for measuring the amount of oxygen evolved in photosynthetic systems illuminated with light flashes. The oxygen-yield sequence of Chlorella suspensions under 30 μ s flashes at a repetition rate of 2 Hz revealed that the experimental oscillatory pattern of the yield vs. number of flashes can be described theoretically with the linear four-step model (Kok's model) with the exception of the height of the yield after the first flash. The deviation of theory and experiment can be explained by the dark relaxation processes in the oxidized states (S-states) of the water-splitting enzyme, since nearly half of the reaction centers can be found in S-states having no positive accumulated charges in dark-adapted systems. Based upon the homogeneous hypothesis for the initial distribution of the transition probabilities among the S-states, the average transition probabilities are 10, 75 and 15% for the non-reactivity (misses), normal reactivity and double-reactivity (double-hits) of the reaction centers, respectively.

Introduction

Green plant photosynthesis primarily implies oxidation-reduction reactions driven by two light reactions in series, in two distinct systems, photosystem I (PS I) and photosystem II (PS II), respectively (Fig. 1). The light is absorbed by light-harvesting pigments of these systems, and the excitation energy migrates via exciton transfer to the reaction centers. The excited reaction centers (special chlorophyll-a forms) are able to donate an electron to the primary acceptor molecule (Q or X), resulting in the primary charge separation, i.e. the direct conver-

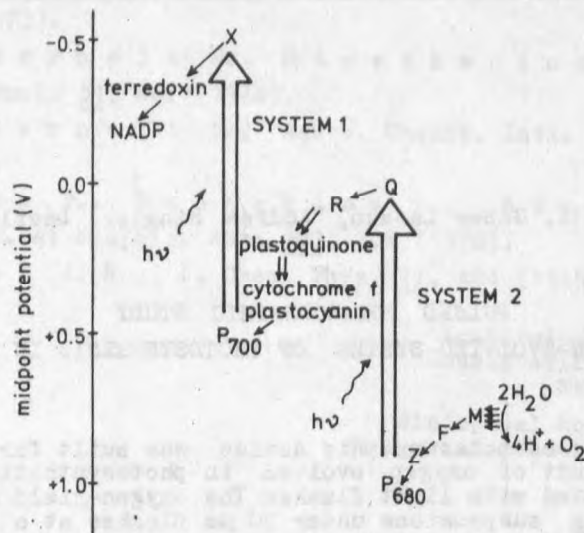


Fig. 1. Z-scheme of green plant photosynthesis, demonstrating the intermediates according to their estimated midpoint potential. The vertical arrows denote the photochemical charge separations due to the absorption of quanta $h\nu$ in the two photosystems. The further arrows show the transfer of a single electron. P_{700} and P_{680} are the primary donors, X and Q the primary acceptors of PS I and PS II respectively; M is the water splitting enzyme

Z-schemat fotosyntezy roślin zielonych. Składniki łańcucha transportu elektronów ułożone w kolejności ich potencjałów normalnych. Strzałki pionowe wskazują fotochemiczne rozdzielanie ładunku spowodowane pochłonięciem kwantu $h\nu$ w dwu fotosystemach. Następne strzałki wskazują przenoszenie pojedynczego elektronu. P_{700} i P_{680} są pierwotnymi donorami, X i Q - pierwotnymi akceptorami układów PS I i PS II; M - enzym rozszczepiający wodę

Z-схема фотосинтеза зелёных растений. Интермедиаты в последовательности их нормальных потенциалов. Вертикальные стрелки обозначают фотохимическое разделение заряда вызванного поглощением квантов $h\nu$ в двух фотосистемах. Следующие стрелки показывают перенос единого электрона. P_{700} и P_{680} являются первичными донорами, X и Q первичными акцепторами PS I и PS II; M - энзим расщепляющий воду

sion of the light energy into chemical energy; PS I produces a strong reducing agent (NADPH), performing the fixation of CO_2 into carbohydrates and oxidizes the pool of plastoquinone molecules. PS II oxidizes water, thereby evolving oxygen and reducing the plastoquinone pool.

Whereas the reduction of CO_2 to carbohydrates via the Calvin cycle has been clearly elucidated, the path of oxygen has till to be explained. Although much experimental evidence has been accumulated against light-induced water decomposition (photolysis) and in favour of the charge-accumulating system on the donor side of PS II, the mechanism of O_2 evolution from H_2O is still unknown.

The present work deals with the question of the oxygen liberation, and how the technique of pulsed polarography and mathematical analysis of the transition probabilities between different oxidized states of the oxygen-evolving enzyme has helped us to get a coherent view of the processes.

Oxygen polarograph

The principle of the polarographic cell (Fig. 2) is similar to that described by J o l i o t and J o l i o t [3]. A drop of algal suspension is homogeneously spread on a platinum electrode. In order to attain a single layer of cells, the Chlorella concentration should be adjusted. In a chamber of 0.15 mm thickness with an algal concentration of $4.5 \cdot 10^8$ cell ml^{-1} , a monolayer is formed on the electrode after the cells settle to the bottom. This can be checked visually (the color of the electrode should be faintly green), electrically (the electrode response should be steady within several % after 15 min of stabilization), or microscopically. The algal suspension is covered with a cellophane membrane. Between this membrane and a plexiglass cover, the culture medium flows. The medium contains 2 M KCl to ensure sufficient electric conductance. A constant oxygen concentration in the chamber is ensured by an equilibrium between the oxygen uptake by the algae and the oxygen supply by the flowing buffer. The glass window on the cover renders possible illumination with a Xe flash tube of about 30 μs flash duration and a repetition rate of 2 Hz.

The wiring diagram of the apparatus consists of three main parts (Fig. 3). The first part ensures the suitable voltage (-0.7 V) for O_2 -deposition by polarizing the platinum electrode with respect to the Ag/AgCl electrode. This is achieved with a

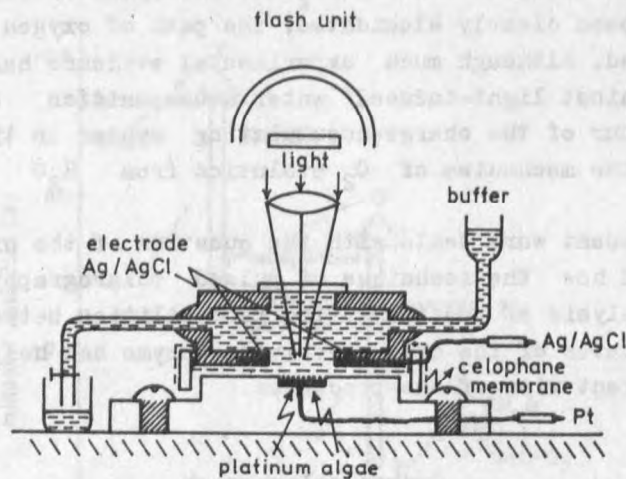


Fig. 2. Device for measuring oxygen exchange.
Explanation see in text

Urządzenie do pomiaru wymiany tlenowej. Wyjaśnienia w tekście
Устройство для измерения обмена кислорода. Объяснения в тексте

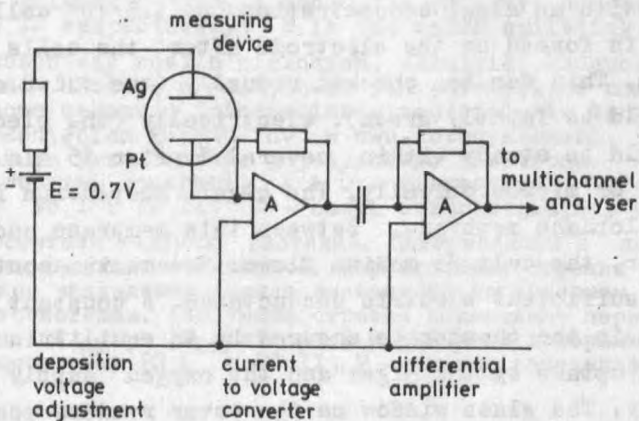


Fig. 3. Wiring diagram of the system for oxygen measurements.
Explanation see in text

Schemat elektryczny urządzenia do pomiarów tlenu.
Wyjaśnienia w tekście

Электрическая схема устройства для измерений кислорода.
Объяснения в тексте

commercial integrated operational amplifier (Fairchild, 709) with full feed-back (1 amplification). From an electrical point of view, the illuminated polarographic cell can be considered a current generator. The small current through the measuring device is transformed into electric voltage via a current-to-voltage converter, an operational amplifier with low bias current (Fairchild, 740, 2 nA), in the second part of the diagram. In order to measure only the flash response of the polarograph, i.e. to avoid the detection of the slower variations of the oxygen concentration, a differential amplifier (Fairchild, 741) is connected with the previous step. The signal is fed into a multichannel analyzer (KFKI-system, NTA 512) where it can be stored. The signal-to-noise ratio can be improved by repetition of the measurement.

The unicellular green algae (*Chlorella pyrenoidosa*, Chick-strain Emerson 3) were grown and harvested under standard conditions: 4 mW/cm² light flux, Tamiya culture medium at room temperature.

Results

Figure 4 demonstrates a typical O₂-evolution pattern (Y_n) of dark-adapted *Chlorella* cells exposed to a series of 30 μ s light flashes spaced 0.5 s apart. Several interesting observations can be made. The most striking feature is the oscillatory behavior of the O₂-yield with a periodicity of four which disappears after 3 or 4 periods due to a strong damping. Similar results were obtained first by J o l i o t et al. [4] and corroborated later by others (see e.g. [5]). After the first and the second flash practically a negligible amount of oxygen could be detected ($Y_1 \approx Y_2 \approx 0$), and the first maximum appears not after the 3rd flash but after the 4th one. The further maxima can be found after the 8th and 12th flashes, and the minima after the 6th and 10th flashes. The steady state O₂ evolution Y_{ss} is attained at the 14th or 15th flash.

In order to check whether the flash energy is high enough to saturate the oxygen evolution, the above measurements were carried out under the same experimental conditions, but with attenu-

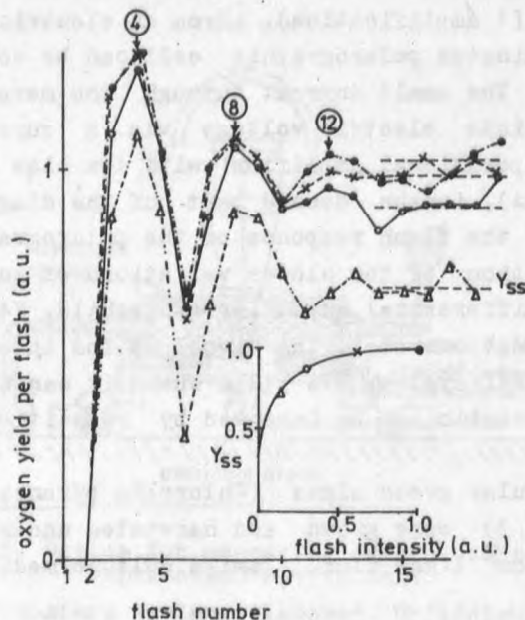


Fig. 4. Oxygen evolution in dark adapted *Chlorella* cells illuminated by a series of 30 μ s saturating flashes, as function of flash number. Y_{ss} means the steady state oxygen evolution under flashes with different intensities (in arbitrary units): \bullet - 100%, \times - 62%, \circ - 30% and Δ - 14%

Wytwarzanie tlenu przez komórki *Chlorella* adaptowane do ciemności, oświetlone serią 30 μ s sekundowych błysków nasycających w zależności od liczby błysków. Y_{ss} oznacza stacjonarny poziom wytwarzania tlenu w odpowiedzi na błyski o różnej intensywności (w jednostkach umownych): \bullet - 100%, \times - 62%, \circ - 30%, Δ - 14%

Образование кислорода в клетках *Chlorella* приспособленных к темноте, освещенных серией 30 μ s насущающих вспышек, в функции числа вспышек. Y_{ss} обозначает стационарное образование кислорода вспышками разной интенсивности произвольные единицы \bullet - 100%, \times - 62%, \circ - 30% и Δ - 14%

ated flash energy. The pattern does not change significantly: the periodicity and the locations of the maxima and minima are preserved - except for a small shift of the third maximum in the case of the smallest energy. The light-saturation curve of the steady-state oxygen evolution indicates that the highest flash intensity (100% in relative units) can be satisfactorily considered as saturating.

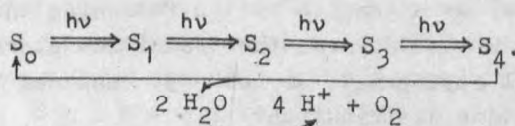
The O_2 -yield sequence of *Chlorella* grown under synchronized (16 h light and 8 h dark) and asynchronized conditions was practically unchanged.

It was found that the ratio Y_4/Y_3 was very sensitive to the dark-adaptation of the algae. When the sample was kept in darkness for 30 s after steady flashing, Y_3 became equal to, or even greater than Y_4 if the time for dark-adaptation was less than 30 s.

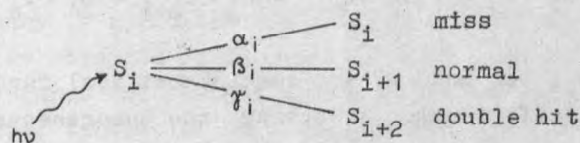
The O_2 -response did not follow the exciting flash promptly, but had a delay of about 15 ms, in good agreement with the finding of Zankel [10] based on delayed fluorescence measurements.

Discussion

The kinetic property of the damped oscillation can be successfully explained by a linear four-step model, first introduced by Kok et al. [6]. According to this model, each PS II centre occupies cyclically five states, S_0-S_4 , associated with the accumulation of four positive charges; the subscripts refer to the number of positive oxidizing equivalents stored on the "S"-complex. The transition from one state to the next requires the absorption of one photon:



Since the O_2 -response follows 15 ms after the flash, one can argue that the transition from one state to an other occurs via a metastable state with a rate constant $k \approx (15 \text{ ms})^{-1}$. To explain the damping in the oscillatory pattern, Kok assumed that even with saturating flashes, a center has a small probability of not reacting ("misses"). Depending on the flash duration, there is a given probability for a center to react twice ("double hits"):



α_i , β_i and γ_i denote the probabilities of misses, normal reactions and double hits, respectively. The quantitative analysis of Kok's model was first elaborated by Delrieu [1, 2], and later developed by Lavorel [7], Lavorel and Lemasson [8] and Thibault [9]. In the quantitative treatment, Kok's model is considered a Markoff-process. The effect of a flash may be described as the operation of a matrix \tilde{K} on a state vector $S^{(n)}$, resulting in a new state vector $S^{(n+1)}$:

$$S^{(n+1)} = \tilde{K}S^{(n)}. \quad (1)$$

The components of $S^{(n)}$ are defined as the relative concentrations of centers in states S_0 , S_1 , S_2 and S_3 after n flashes, and the elements of \tilde{K} are the transition probabilities between different states:

$$\tilde{K} = \begin{bmatrix} \alpha_0 & 0 & \gamma_2 & \beta_3 \\ \beta_0 & \alpha_1 & 0 & \gamma_3 \\ \gamma_0 & \beta_1 & \alpha_2 & 0 \\ 0 & \gamma_1 & \beta_2 & \alpha_3 \end{bmatrix} \quad (2)$$

Let us focus our attention to the following three important questions. 1. How to determine the transition elements? 2. Is the matrix-model appropriate or not? 3. What can be said about the initial S-state distribution?

Ad 1 and 2. Unfortunately, the direct determination of the matrix elements of \tilde{K} in Eq. (1) is not possible. However, four quantities, with limited content of information, are readily derived from the experimental data (Y_n values). The general recurrence law deduced from the eigenvalue problem of the matrix \tilde{K} ($\tilde{K}x = \lambda x$) is

$$Y_{n+4} - \sigma_1 \cdot Y_{n+3} + \sigma_2 \cdot Y_{n+2} - \sigma_3 \cdot Y_{n+1} + \sigma_4 \cdot Y_n = 0, \quad (3)$$

where σ_1 , σ_2 , σ_3 and σ_4 are quasisymmetrical functions of the transition coefficients. Accepting the homogeneous hypothesis ($\alpha_i = \bar{\alpha}$, $\beta_i = \bar{\beta}$ and $\gamma_i = \bar{\gamma}$), they can be expressed as

$$\begin{aligned}
\sigma_1 &= 4\bar{\alpha}, \\
\sigma_2 &= 6\bar{\alpha}^2 - 2\bar{\gamma}^2, \\
\sigma_3 &= 4\bar{\alpha}^3 - 4\bar{\alpha}\bar{\gamma}^2 + 4\bar{\beta}^2\bar{\gamma}, \\
\sigma_4 &= \bar{\alpha}^4 - \bar{\beta}^4 + \bar{\gamma}^4 - 2\bar{\alpha}^2\bar{\gamma}^2 + 4\bar{\alpha}\bar{\beta}^2\bar{\gamma}.
\end{aligned} \tag{4}$$

The σ_i ($i = 1, 2, 3, 4$) values can be directly calculated from the experimental sequence Y_n : they are the solutions of a system of four linear equations, where five consecutive Y_n values enter each equation as coefficients. The calculations can be conveniently performed on a programmable pocket calculator (Texas Instruments, SR 52) and the results are given in Table 1. The numbers (n) in the first column denote the starting values of the trains of flashes applied for calculation of σ_i in Eq. (3). The last column contains the $\sigma_1 - \sigma_2 - \sigma_3 - \sigma_4$ values calculated from different trains of flashes. This expression serves as a relatively simple method for checking the validity of the model: it should always be equal to 1, as $\lambda = 1$ is an eigenvalue of the matrix \tilde{K} . A very surprising observation is that the first flash acts in a specific way not consistent with the model. The effects of the following flashes (up to the 8th), however, are in reasonably good agreement with the model. If $n \geq 8$, we again get deviation from the predictions of Kok's model; this discrepancy, however, can be attributed to the inaccuracy of the measurement in this domain, mainly due to the strong damping. The reason for the deviation of the first flash behavior is unknown. Similar conclusions can be drawn from the analyses of Lavorel [7] and Thibault [9].

The problem of calculation of the transition probabilities from the σ_i values (Eq. (4)) arises from the non-linear and overdetermined properties of the equations (even a normalization relation, $\bar{\alpha} + \bar{\beta} + \bar{\gamma} = 1$, should be connected with the set of equations under (Eq. (4))). A graphical optimum calculation was carried out to obtain the average transition probabilities: $\bar{\alpha} = 0.10$, $\bar{\beta} = 0.75$ and $\bar{\gamma} = 0.15$. Not all the quanta of light absorbed by the system advance the S-states forward. There is an inefficiency of approximately 10%, which correspond fairly well to the value obtained by others [7] with simulation methods.

Ad 3. As regards the S-state distribution of the dark-adapted cells we refer to Fig. 4: no O_2 -responses could be detected

Table 1

Values of $\sigma_i S$ derived from Eq. (3) using trains of flashes starting with the n-th flash
 Wartości $\sigma_i S$ otrzymane z równania (3) dla serii błysków poczynając od n-tego błysku
 Величины $\sigma_i S$ полученные с уравнения (3) для ряда вспышек начинающегося с n-той вспышки

n	σ_1	σ_2	σ_3	σ_4	$\sigma_1 - \sigma_2 + \sigma_3 - \sigma_4$
2, 3, 4, 5,	0.466	-0.047	0.128	-0.347	0.988
3, 4, 5, 6,	0.422	-0.083	0.097	-0.390	0.993
4, 5, 6, 7,	0.376	-0.117	0.094	-0.410	0.998
2, 4, 6, 7,	0.393	-0.024	0.228	-0.343	0.988
①, 2, 3, 4,	0.442	-0.368	0.185	-0.443	1.437
①, 3, 5, 7,	0.088	-0.749	0.113	-0.248	1.198
①, 4, 5, 6,	0.127	-0.522	0.150	-0.041	0.839
5, 6, 7, ⑧,	0.547	-0.115	0.159	-0.092	0.893
6, 7, ⑧, ⑨,	0.561	-0.159	0.246	-0.240	1.205
7, ⑧, ⑨, ⑩,	0.444	-0.068	0.150	-0.457	1.117
2, 5, 7, ⑧,	0.319	-0.174	0.266	-0.118	0.876

after the first and the second flashes:

$$Y_1 = Y_2 = 0, \quad (5)$$

additionally

$$Y_3 = (1 - \bar{\alpha})^2 (1 - S_0^{(0)}) \quad (6)$$

and

$$Y_4 = (1 - \bar{\alpha})^3 S_0^{(0)} + 3\bar{\alpha}(1 - \bar{\alpha})^2 (1 - S_0^{(0)}) \quad (7)$$

may be derived from Eq. (1) using the homogeneous hypothesis. From the last two equations the following expression is deduced:

$$S_0^{(0)} = \frac{1}{1 + \frac{(1 - \bar{\alpha}) \cdot \frac{Y_3}{Y_4}}{1 - 3\bar{\alpha} \cdot \frac{Y_3}{Y_4}}}. \quad (8)$$

Substituting the experimental data ($Y_3/Y_4 = 0.9$), we obtain 0.47 and 0.53 for $S_0^{(o)}$ and for $S_1^{(o)}$, respectively ($S_2^{(o)}$ and $S_3^{(o)}$ are equal to 0 according to Eq. (5)). This would mean that practically half of the centers are in state S_0 and half in S_1 in a dark-adapted sample. The relatively high ratio S_0/S_1 we obtained can be attributed either to a thorough dark-adaptation, or to some speciality of the oxygen-evolving system of our *Chlorella* strain, or more specifically to the rapid relaxation processes of the higher S states.

Acknowledgements

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BADANIA UKŁADU
FOTOSYNTETYCZNEJ GENERACJI TLENU METODĄ POLAROGRAFII IMPULSOWEJ

Skonstruowano urządzenie do badania metodą polarografii impulsowej ilości tlenu wytwarzanej w układach fotosyntetycznych oświetlanych błyskami światła. Określono sekwencję wydajności tlenowej zawiesin *Chlorella* oświetlanych błyskami 30-μsekundowymi powtarzanymi z częstością 2 Hz i stwierdzono, że sekwencja taka może być opisana teoretycznie za pomocą liniowego modelu czterostopniowego (model Koka), który jednak nie daje właściwych przewidywań wydajności tlenu po pierwszym błysku. Odchylenie teorii od eksperymentu społecznego może być wyjaśnione procesami relaksacji ciemnej w stanach utlenionych (stanach S) enzymu rozszczepiającego wodę, gdyż stwierdzono, że prawie połowa centrów reakcji o stanach S_0 w układach adaptowanych do ciemności nie posiada nagromadzonych ładunków dodatnich. W oparciu o hipotezę homogennego rozkładu początkowego prawdopodobieństw przejść pomiędzy stanami S otrzymano następujące przeciętne prawdopodobieństwa przejść pomiędzy centrami reakcji: 10% dla niereaktywności (chybienia), 75% dla normalnej reaktywności i 15% dla podwójnej reaktywności (podwójne trafienia).

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ПУЛЬС-ПОЛЯРОГРАФИЧЕСКИЕ ИССЛЕДОВАНИЯ
ФОТОСИНТЕТИЧЕСКОЙ СИСТЕМЫ ОБРАЗОВАНИЯ КИСЛОРОДА У *CHLORELLA*

Построили пульс - полярографическое устройство для измерения количества кислорода образованного в фотосинтетической системе иллиминированной вспышками света. Последовательность выхода кислорода сиспензии *Chlorella* при 30 ммс вспышках, повторяемых с частотой 2 Гз можно описать теоретически при помощи линейной четырехступенчатной модели (модель Кока) которая, однако, не охватывает величины выхода после первой вспышки. Отклонение теории от эксперимента можно объяснить процессами темновой релаксации в окисленных состояниях (S-состояния) энзима расщепляющего воду, так как, почти половину центров реакции можно найти в S-состояниях в системах адаптированных в темноте без накопленных положительных зарядов. Полагая гомогенное начальное распределение вероятностей перехода по S-состояниям, нагнали среднее вероятности перехода: 10% для нормальной реактивности и 15% для двойной реактивности двойные удары центров реакции.