

On the Determination of the S-State Distribution in the Kok Model

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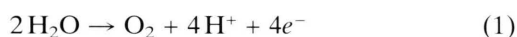
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O₂-Evolution, σ - and ζ -Analysis, Transition Probabilities, Cyanobacteria, Tobacco, *Chlorella*

We use the Markov chain theory to analyze the oscillation pattern of oxygen evolution during water oxidation in photosystem II under short saturating light flashes. We propose a method based on the standard least square deviation (test χ^2) to determine the number of S-states in the Kok model. As pointed out by Burda *et al.* (1995) this information is amongst others important for the interpretation of the role of calcium for oxygen evolution. A specific mathematical representation for a situation when the S₄ state is longer living than generally assumed is introduced which requires an explicit extension of the Kok model to five states. The higher stability is modelled by introducing additional decay channels, e.g. a nonvanishing probability for the transition of S₃ to the S₀ state and a further transition probability for the transition from S₃ to S₄. Our analysis is extended to the case of damped oscillations of oxygen evolution caused, for example, by the lack of electron acceptor or the short life time of photosystem II particles.

Introduction

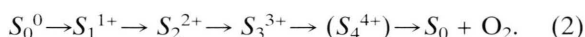
Water oxidation is a topic of great interest in photosynthesis research. The oxygen evolving complex (OEC) of photosystem II (PS II) catalyzes the light-driven reaction:



Coupling of the four-electron oxidation of water to the single electron turnover of the central reaction centre components

(Tyr Z → P680 → Pheo → Q_A) requires that the OEC is able to store four oxidizing equivalents in the Mn active site. The linear 4-step model for the water oxidizing cycle, which is now generally used, has been proposed 25 years ago by Kok *et al.* (1970). The model is based on Joliot's original observation (Joliot *et al.*, 1969) that oxygen production in dark adapted chloroplasts, measured as consequence of short light flashes oscillates with a periodicity of four and represents a damped oscillation. The transient states of the OEC (S) accumulate successively four positive charges. Each transition is driven by the absorption of a photon

by the reaction centre chlorophylls. Rapid charge separation generates a powerful oxidant (P680*) that is ultimately reduced by electrons from water:



Kok and co-workers needed an additional assumption to explain why the yield of the third flash exceeds that of the fourth flash. They concluded that S₁ is dark stable and does not revert in the usual experimental time of minutes to the ground state S₀. The S₀ state is formed exclusively by way of the reaction S₃ + hν → S₀ + O₂. Thus, a typical initial state distribution is 25% S₀ and 75% S₁ in dark adapted systems. However, Zimmerman and Rutherford (1985), Vermaas *et al.* (1984) and Dekker and Gorkom (1987) postulated that in prolonged darkness, the OEC tends to put itself in the S₁ state because of an electron carrier 'D' (tyrosine of polypeptide D₂) which oxidizes the S₀ state to S₁.

The progressive damping in the O₂ evolution sequence reflects the random redistribution of the OEC among the S states. In order to obtain satisfactory agreement between the observations and the predictions of the model, Forbush *et al.* (1971) introduced two perturbations which finally lead to the damping of the oscillation of the O₂ flash yield: (a) a failure rate α of the trapping centres in the photochemical conversion (called misses) and (b)

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double effective excitation in a fraction γ^1 of the centres which are in the S_0 and S_1 states (called double hits). The best fit with their experimental data was obtained for 10% misses and 5% double hits. Kok postulated in the original paper (Kok *et al.*, 1970) on this linear 4-step model that the miss and double hit probabilities are homogeneous (the same for all states S_i). The double hit parameter is usually attributed to a double turnover of the reaction center induced if the duration of the actinic flash is too long (Kok *et al.*, 1970). Excitation with flashes of a few microseconds duration is expected to give rise to γ values which in the first approximation are proportional to the rate constant of Q_A -reoxidation by Q_B or other exogenous electron acceptors (Messinger *et al.*, 1993). Supposing, the homogeneity of transition probabilities between S states, Jursinic (1981) postulated within this model an association of the double hit parameter γ with the miss parameter α even if the flash duration is as short as 5 ns. He explained this phenomenon as a double advancement of a state associated with one photon in the same centre which however seems to have an extremely low probability (in the same system α was significantly larger than 0). Thibault (1978) demonstrated and analyzed that a significant deviation between theoretical and experimental sequences exists, if the homogeneous Kok hypothesis model was used. The discrepancy referred to the first flash, which if omitted from the sequence eliminated all irregularities. During some time the abnormality under the first flash was interpreted to be due to an excessive amount of double hits under the first flash. Thibault (1982) demonstrated that the signal was simply due to a contribution of approximately 17% of a more reduced state S_{-1} to a regular dark adapted sequence in *Chlorella* for example.

Delrieu (1974) proposed that misses are different in different states. At any rate the progressive damping of the oscillations is mainly due to misses (Lavorel, 1978). A recurrence law assuming misses nearly exclusively on the S_2 state

(and sometimes on S_3) and without double hits seemed to give better quantitative agreement with the O_2 yield oscillations observed in *Chlorella* than for the assumption of homogeneous misses including double hits. Delrieu (1983) showed the correlation between the average α and γ factors, used for evaluating experimental data. Different experimental conditions (saturating and non-saturating flashes) gave parallel variations of the observed homogeneous miss and double hit parameters. This observation is disturbing because the opposite variation of the α and γ parameters is expected. Meunier (1993) tried to explain the interrelationship between double hits and misses, introducing an additional parameter 'the backward transitions' which seems to be of no merit. In this context backward transitions are supposed to be due either to the combination of S state deactivation and miss, or to two S state deactivations and a single turnover hit. This leads in some cases to meaningless negative deactivation probabilities.

In this paper we propose to use a standard χ^2 -test for calculating transition probabilities (σ) with the aim to determine the number of S states in the Kok model. We also introduce some modifications into the Kok model. Our model is still the linear four step model in which four quanta have to be successively absorbed leading to the storage of four positive charges. Instead of four states we work with five states. The existence of five states has been assumed already by Kok, but it is generally accepted that the fifth state S_4 is not detectable because of the very fast oxygen evolution from S_3 following the fourth flash. We pretend that the S_4 state can, under certain conditions (in modified or sometimes in natural systems like *Chlorella*) be longer living than is usually thought. In the usually studied chloroplast systems the model of four states agrees quite well with the experimental observations because the transition probability from S_3 to S_4 is very low.

The process of charge accumulation which leads to oxygen evolution depends on the redox conditions of the donor as well as the acceptor side of the reaction center (Shinkariyev and Wraight, 1993; Renger and Hanssum, 1988). For simplicity, in models describing this process only effective misses (and double hits) are taken into account without distinguishing the contribution of the acceptor or donor side effect to these parameters.

¹ In the original paper by Forbush *et al.* (1971) the double hit parameter was assigned as β . In the present paper we used α for the miss parameter, β for the successful single hit parameter and γ for the double hit parameter.

Material and Methods

Chloroplasts and photosystem II particles from *Nicotiana tabacum* var. John William's Broadleaf (JWB) were prepared according to the method of Berthold (Berthold and Babcock, 1981) omitting the second Tris-washing. *Chlorella kessleri* was cultured according to the conditions described by Ruppel (1952) and Kowallik (1963). The particle preparation of *Oscillatoria chalybea* was prepared as described by Bader *et al.* (1983). Chloroplast preparations of tobacco and particle preparations of *Oscillatoria* were investigated in 0.15 M Tricine buffer containing 0.3 M KCl (pH 7.5). *Chlorella* was suspended in its growth medium having a pH of about 6.3.

Amperometric measurements of O_2 -evolution have been carried out with the "Three Electrode System" described by Schmid and Thibault (1979). Saturating flashes of 8 μ s duration at half intensity were provided by the Stroboscope 1539A from General Radio (xenon flash lamp). Usually, we measured sequences of 15 flashes spaced 300 ms apart.

Mathematics of the Kok Model

The condition of the oxygen evolving complex is characterized by the distribution of its redox states S_i . The index characterizes each single electronic state of the Mn-complex and goes from 0 to n . The response of the system on a flash is modelled by transition probabilities: $p_{(i \rightarrow j)}$ between the S_i and S_j state. The probabilities $p_{(i \rightarrow j)}$ do not change within subsequent flash transitions.

Knowing the distribution of states $S_i^{(N)}$, after a certain number of flashes N , by means of the transition probabilities, we can easily find the S state occupation, $S_j^{(N+1)}$, after an additional flash:

$$S_j^{(N+1)} = \sum_{i=1}^n S_i^{(N)} p_{(i \rightarrow j)} . \quad (3)$$

It is convenient to represent the transition probabilities as elements of a certain matrix \mathbf{P} : $P_{ij=p(i \rightarrow j)}$. Then the equation (3) has the form:

$$S^{(N+1)} = S^{(N)} \mathbf{P} \quad (4)$$

where $S^{(N)}$ denotes a vector of the S state distribution with $S_i^{(N)}$ as its elements. $\mathbf{S}\mathbf{P}$ represents the

multiplication of the left matrix side by this vector². Starting from the initial state $S^{(0)}$, the states $\{S^{(0)}, S^{(1)}, S^{(2)}, \dots\}$, obtained by successive acting of \mathbf{P} , form a Markov chain. The properties of Markov chains are well known and are a direct derivative of the properties of the matrix \mathbf{P} . The fundamental feature of the matrix \mathbf{P} is that all its eigenvalues, λ 's, lie in the unit circle, i.e. $|\lambda| \leq 1$. Moreover, one of the eigenvalues is always $\lambda=1$. For practical purposes, it is sufficient to consider a subset of matrices \mathbf{P} whose eigenvalue $\lambda=1$ is not degenerated, that it has exactly one eigenvector, and all other eigenvalues lie inside a unit circle $|\lambda| < 1$. These stronger requirements ensure the existence of a unique stationary distribution: $S^* = S^* \mathbf{P}$ in which after a large number of N steps the state $S^{(N)}$ approaches S^* independently of an initial distribution $S^{(0)}$. Any initial state $S^{(0)}$ can be decomposed in terms of left (horizontal) eigenvectors, ω 's of \mathbf{P} : $S^{(0)} = S^{(*)} + \alpha_1 \lambda_1 \omega_1 + \alpha_2 \lambda_2 \omega_2 + \dots$, where an α_i is proportional to the participation of the eigenstate ω_i in the initial state $S^{(0)}$. The eigenvector to the eigenvalue $\lambda=1$ is S^* . Notice that as long as $\sum_i S_i^{(0)} \neq 0$, which is always the case in the experiments, the participation of S^* in $S^{(0)}$ is nonzero. Therefore, after N steps one gets:

$$S^{(N)} = S^{(0)} P^N = S^* + \alpha_1 \lambda_1^N \omega_1 + \alpha_2 \lambda_2^N \omega_2 + \dots \quad (5)$$

where P^N is a N -th power of the matrix \mathbf{P} . To see the main consequence of the above equation, eigenvalues are represented as $\lambda = |\lambda| \exp(i\phi)$ and τ denoted as $\tau = -(\log \lambda)^{-1}$. Rewriting yields:

$$S^{(N)} = S^* + \alpha_1 \cos(N\phi_1) \exp(-N/\tau_1) + \alpha_2 \cos(N\phi_2) \exp(-N/\tau_2) + \dots \quad (6)$$

Note that each $\tau > 0$ because $|\lambda| < 1$. The equation shows that the eigenstates disappear exponentially with the flash number N leaving only the stationary state S^* . The exponential fall-off is characterized by a kind of relaxation times τ 's. The trigonometric factors in equation (6) are absent for real eigenvalues.

The eigenvalues can be found from: $\text{Det}(\lambda - \mathbf{P}) = 0$ which is an algebraic equation of the n -th order

² The convention to have multiplication of the left matrix side comes from the equation (3) which is usually used in the theory of Markov chains. It differs, however, from that being used in the standard consideration of flash analyses (Delrieu, 1974).

with the roots λ_i :

$$0 = (\lambda - \lambda_1)(\lambda - \lambda_2) \cdots (\lambda - \lambda_n) = \lambda^n - \sigma_{n-1}\lambda^{n-1} + \sigma_{n-2}\lambda^{n-2} - \cdots + (-1)^n \sigma_0 \quad (7)$$

where $\sigma_{n-1} = \sum_j \lambda_j = \text{Tr} \mathbf{P}$, $\sigma_{n-2} = \sum_{i>j} \lambda_i \lambda_j = (1/2 \text{Tr}^2 \mathbf{P} - \text{Tr} \mathbf{P}^2)$, ..., $\sigma_0 = \lambda_1 \cdots \lambda_n = \text{Det} \mathbf{P}$. Because for any Markov transition matrix, $\lambda = 1$ is an eigenvalue, this implies that σ 's are constrained to:

$$1 - \sigma_{n-1} + \sigma_{n-2} - \cdots + (-1)^n \sigma_0 = 0 \quad (8)$$

as can be seen by inserting $\lambda = 1$ to the equation (7).

Knowing the transition matrix \mathbf{P} and the initial S state distribution $S^{(0)}$ one can reproduce the whole chain of states. In practice we face the problem differently. Usually one wants to learn as much as possible about the matrix \mathbf{P} from the experimentally obtained sequence of amplitudes, corresponding to the linear combination of the state population: $Y^{(N)} = \sum_i S_i^{(N-1)} p_{i \rightarrow 0}$ which is proportional to the number of O_2 molecules, evolved during water oxidation under the N -th flash.

One of the direct consequences of the equation (7) is that $n+1$ sequential states of any quantity, being a linear combination of eigenstates ω 's, like for example Y 's, are related by:

$$Y^{(N+n)} - \sigma_{n-1} Y^{(N+n-1)} + \sigma_{n-2} Y^{(N+n-2)} - \cdots + (-1)^n \sigma_0 Y^{(N+0)} = 0 \quad (9)$$

Because Y 's are obtainable in direct measurements the last equation can be used to reduce the number of unknown transition probabilities σ 's. Thus, by collection of experimental data for n equations one can determine all sigmas $\sigma_0, \dots, \sigma_{n-1}$. This type of analysis is commonly referred to as 'sigma analysis' (Thibault, 1978), (Lavorel, 1976). The simplest way of obtaining an input for n linearly independent equations is to extract experimentally a sequence of $2n$ amplitudes. They give the equations:

$$\sum_{j=0}^{n-1} (-1)^{(n-j)} Y^{(i+j)} \sigma_j + Y^{(i+n)} = 0, \text{ for } i=1, \dots, n \quad (10)$$

The equation for the vector σ is linear: $\mathcal{Y}\sigma + Y = 0$, where the elements of the matrix \mathcal{Y} are $\mathcal{Y}^{ij} = Y^{(i+j)}$. Its solution is $\sigma = -\mathcal{Y}^{-1}Y$. This procedure can lead numerically to instabilities of the solution. Firstly, because the elements of the matrix \mathcal{Y} are highly correlated by the fact that they represent the same experimental values. A small fluctuation $\delta Y^{(i+j)}$ of

the value of one amplitude changes several elements of the matrix \mathcal{Y} . This can introduce a large error in the inverse matrix \mathcal{Y} and thereby in σ 's. Secondly, as can be seen from equation (6), in a long sequence, the amplitudes Y 's approach exponentially a stationary value Y^* . More precisely, the participation of eigenstates to different eigenvalues λ disappears in any state of the system exponentially with the typical relaxation time $\tau = -1/\ln|\lambda|$. When $|\lambda|$ is relatively close to zero, one has no chance to detect this eigenstate already after a few steps, n , because in this case n becomes very quickly much larger than τ . At the stage when the difference between two amplitudes in the sequence is of the order of the experimental resolution or the typical scale of statistical fluctuations, a further use of experimental values of Y 's in the equation (10) introduces a huge uncontrollable error to the sigma values.

The set of N equations (10) forms a minimal set needed to estimate all n unknown values of σ 's. A more stable way of determining σ 's would require to enlarge the number of equations, thus, finding σ -values which minimize the effect of the statistical fluctuation. Such a strategy is based on the standard concept of the χ^2 -test and has already been used in the flash analyses described by Thibault (1978) and Delrieu (1983). We here propose a slightly modified version of this analysis. Consider the following function

$$\Psi_0 = \sum_{i=1}^N \left(\sum_{j=0}^{n-1} (-1)^{(n-j)} \mathcal{Y}^{ij} \sigma_j + Y^{(i+n)} \right)^2 \quad (11)$$

where $Y^{(i)}$ are values of amplitudes, and as before $\mathcal{Y}^{ij} = Y^{(i+j)}$ are n amplitudes with $j = 0, \dots, n-1$. The optimal choice of σ 's minimizing the fluctuations corresponds to the saddle point of the function Ψ_0 , that is, the best values of σ 's are obtained by minimizing Ψ_0 with respect to σ 's.

The main difference between this approach and that from the equation (10) is that \mathcal{Y}^{ij} is not any more a square matrix, but a matrix of the size $n \times N$ where N can in general be larger than n . In particular, for $n=N$ the minimum of Ψ_0 is realized by the same σ 's which solve (10).

In experiments one should take N as large as possible since then one can much easier distinguish between the fit quality and statistical fluctuations. As mentioned before, we cannot take too many amplitudes in a sequence because the differences between

amplitudes disappear within the sequence. Therefore, in order to gather as many experimentally independent entries as possible for the expression on the right hand side of (11) it is better to take the data from many sequences. To avoid a linear dependence of the data in (11) one should utilise the fact that σ 's do not change if one changes the initial state, and that one can produce independent sequences by different numbers of preflashes.

In the usual χ^2 -test the size of the squares under the sum in the ψ definition with the uncertainty of their estimation is normalized to the number of freedom degrees (13). This gives the quantitative information on how good a proposed fit is. We do this by comparing each term $\sum_j (-1)^{(n-j)} Y^{ij} \sigma_j + Y^{(i+n)}$ in the sum (11) with the committed error by determining its value. The error comes from both $Y^{(i)}$ and Y^{ij} . In this respect the situation differs from that usually encountered in χ^2 -tests, where one compares the difference between a theoretical value of the fitted function and an experimental value with the error which comes only from the experimental value. The theoretically fitted part would introduce no errors. Here we have no clear distinction between experimental and theoretical values because they are mixed in squares. In other words, the errors are also contained in $Y^{ij} \sigma_j$. Let us assume that the error or uncertainty of the value of each amplitude is equal to the resolution of the system, which we denote by Y and which is independent of the value of ΔY . Therefore, the maximal error is:

$\Delta_{\text{MAX}} \{ \sum_j (-1)^{(n-j)} Y^{ij} \sigma_j + Y^{(i+n)} \} = |\Delta Y| \{ 1 + \sum_{j=0}^{n-1} |\sigma_j| \}$
In the real error estimation a statistical nature out of the error should be taken into account assuming that some errors are mutually cancelled out. Then we obtain the following expression for the total error:

$$\Delta \{ \sum_j (-1)^{(n-j)} Y^{ij} \sigma_j + Y^{(i+n)} \} = \Delta Y \sqrt{1 + \sum_{j=0}^{n-1} \sigma_j^2} \quad (12)$$

which can be used to redefine the test function Ψ_0 (11) to:

$$\begin{aligned} \Psi &= \frac{1}{(N-n)} \frac{1}{(\Delta Y)^2 \{ 1 + \sum_{j=0}^{n-1} \sigma_j^2 \}} \Psi_0 = \\ &= \frac{1}{(N-n)} \frac{1}{(\Delta Y)^2 \{ 1 + \sum_{j=0}^{n-1} \sigma_j^2 \}} \sum_{i=1}^N \left(\sum_{j=0}^{n-1} (-1)^{(n-j)} Y^{ij} \sigma_j + Y^{(i+n)} \right)^2. \quad (13) \end{aligned}$$

The meaning of the expression in front of Ψ_0 is twofold. It shifts a solution of the saddle point

equations from that for Ψ_0 . By comparing the square sizes with the error sizes, it gives an estimation of the quality of the fit for σ 's and of statistical fluctuation.

Before considering a specific example we discuss how a damped oscillation of a flash pattern of oxygen evolution, obtained by short flashes can be analyzed. Insufficient amount of acceptor in the investigated system usually causes a decrease of amplitudes and quick damping. As a first approximation the basic equation (3), describing the electron transfer between the states, can stay the same. The only difference relies on the fact that the number of electrons, which can be further transferred, decreases with the flash number and therefore the amount of the oxygen yield decreases as well. It seems very plausible to assume that the amplitudes fall exponentially $\exp(-n/T) = x^n$, where n is the flash number, T is a certain characteristic constant, and $x = \exp(-1/T) \leq 1$. Therefore, the only modification of the model is to re-scale the amount of the created oxygen by the factor x^n : $Y^{(n)} \rightarrow x^n Y^{(n)}$ which is equivalent to the statement that only a portion x^n of all oxygen evolving centres are able to yield O_2 . This means that the equation (9) has the form:

$$Y^{(N+n)} - x^{n-1} \sigma_{n-1} Y^{(N+n-1)} + x^{n-2} \sigma_{n-2} Y^{(N+n-2)} - \dots + (-1)^n x^0 \sigma_0 Y^{(N+0)} = 0. \quad (14)$$

Equation (14) is equivalent to Eqn. (9) but in the places of σ 's stand effective variables $\zeta_{n-i} = x^{n-i} \sigma_{n-i}$. It means that exactly the same amplitude analysis for the new variables ζ 's as before for σ 's can be carried out, but $\zeta_i \leq 1$.

Data analysis

In this section we show the transition probabilities ζ_i calculated by minimizing the function defined in Eqn.(13). We consider 15 amplitudes of three different patterns: *Nicotiana tabacum* var John William's Broadleaf, *Chlorella kessleri* and *Oscillatoria chalybea*. The collected values of ζ_i obtained using 4- and 5- ζ (σ) analyses³ and the values of σ_i calculated from two different models

³ Instead of the name σ -analysis we will use ζ -analysis to emphasize that the sum of the S states does not need to be normalized to unity (it decreases in time of the measurement), see previous section.

for the transitions between the S states are shown in Tables I, II and III. In the program calculating ζ_i probabilities we impose constraints, which keep the parameters in the range: $0 \leq |\zeta_i| \leq 4$. The $\Delta\zeta_i$ errors are estimated by assuming that the uncertainty in the measurements of oxygen yield was 1%.

The sum of the calculated ζ_i for tobacco chloroplasts, *Chlorella* and *Oscillatoria* is equal 1 within the errors and therefore the obtained values of ζ_i directly correspond to σ_i .

The σ_i probabilities calculated for *Chlorella kessleri* and *Nicotiana tabacum* chloroplasts in the 4- ζ analysis (Tables I and II) are in a good agreement with those reported by Lavorel (1976) and Thibault (1978) for *Chlorella* and chloroplasts.⁴ It can be seen that $|\sigma_3|$ for *Chlorella* is larger than $|\sigma_3|$ for tobacco and $|\sigma_0|$ is lower for

Table I. 4 - σ and 5 - σ analysis for chloroplasts of *Nicotiana tabacum* var. John William's Broadleaf. σ_i values are derived from Eqn. (14), minimizing the function (13) and σ_i is calculated on the basis of the homogeneous (Eqns (17) and (18)) and heterogeneous (Eqns (19) and (20)) model.

<i>Nicotiana tabacum</i> var. John William's Broadleaf (dark adaptation time - 15 min., buffer - pH 7.5)			
4 - σ analysis	Calculated from α, β, γ	Calculated from α_i , where $i=0, \dots, 3$	
σ_0	-0.5284 ± 0.0558	-0.4669	-0.5568
σ_1	0.0000 ± 0.0024	0.0700	0.0000
σ_2	0.0129 ± 0.0735	0.1196	0.0146
σ_3	0.4408 ± 0.1115	0.5680	0.4578
χ^2	1.52	-	-
<i>Nicotiana tabacum</i> var. John William's Broadleaf (dark adaptation time - 15 min., buffer - pH 7.5)			
5 - σ analysis	Calculated from α, β, γ, c	Calculated from α_i , where $i=0, \dots, 4$ and d	
σ_0	0.0000 ± 0.0132	-0.0227	-0.0006
σ_1	-0.5206 ± 0.0486	-0.5016	-0.5685
σ_2	0.0000 ± 0.0006	0.0494	0.0000
σ_3	0.0000 ± 0.0367	0.1186	0.0043
σ_4	0.4421 ± 0.0572	0.5626	0.4349
χ^2	1.52	-	-

⁴ We use the reverse notation of σ : $\sigma_{n-i} \Leftrightarrow \sigma_i$ in comparison to the work of other authors (Lavorel (1976), Thibault (1978) and Delrieu (1982)).

Table II. 4 - σ and 5 - σ analysis for cells of *Chlorella kessleri*. Values of σ_i are derived from Eqn. (14), minimizing the function (13) and σ_i is calculated on the basis of the homogeneous (Eqns (17) and (18)) and heterogeneous (Eqns (19) and (20)) model.

<i>Chlorella kessleri</i> (dark adaptation time - 15 min, pH 6.3)			
4 - σ analysis	Calculated from α, β, γ	Calculated from α_i , where $i=0, \dots, 3$	
σ_0	-0.3995 ± 0.0459	-0.3440	-0.4042
σ_1	0.0000 ± 0.0018	0.0960	0.0010
σ_2	0.0847 ± 0.0995	0.2363	0.04712
σ_3	0.6912 ± 0.1155	0.7962	0.6419
χ^2	1.32	-	-
<i>Chlorella kessleri</i> (dark adaptation time - 15 min, pH 6.3)			
5 - σ analysis	Calculated from α, β, γ, c	Calculated from α_i , where $i=0, \dots, 4$ and d	
σ_0	0.0000 ± 0.0129	-0.0973	-0.0521
σ_1	-0.4187 ± 0.0265	-0.3958	-0.4316
σ_2	0.0000 ± 0.0018	0.0869	0.0076
σ_3	0.0781 ± 0.0700	0.3454	0.1280
σ_4	0.6608 ± 0.0875	0.9600	0.7409
χ^2	1.09	-	-

Chlorella than for tobacco. For *Oscillatoria chalybea* the transition probabilities $|\sigma_i|$ are very similar to those obtained for *Chlorella* (Tables II and III). The 5- ζ analysis carried out for all these patterns shows that $|\sigma_4|$ for tobacco is lower and $|\sigma_1|$ is larger than for *Chlorella* and *Oscillatoria*. Disregarding the later introduced model of the oscillation patterns σ_3 and σ_4 (in the 4- ζ analysis and 5- ζ analysis) correspond to the efficiency of the sequential transitions between S states (chloroplasts have the highest efficiency).

Introduction of a S_4 state with definite life time in *Chlorella* significantly improves the fit quality but gives no change in χ^2 for tobacco and *Oscillatoria*. Using this ζ -analysis one cannot yet come to the conclusion that state S_4 should be introduced into the model in the case of tobacco chloroplasts and the cyanobacterium.

The assumption of a very fast transition of $S_3 \rightarrow S_0$ via the S_4 state gives good numerical results compared to the experimental data. We have also tried 6- ζ analyses for the same data but this leads to bad standard least square deviations in comparison to the situation where 5- S states are taken

Table III. 4 – σ and 5 – σ analysis for thylakoid preparations of *Oscillatoria chalybea*. Values of σ_i are derived from Eqn. (14), minimizing the function (13) and σ_i is calculated on the basis of the homogeneous (Eqns (17) and (18)) and heterogeneous (Eqns (19) and (20)) model.

<i>Oscillatoria chalybea</i> (dark adaptation time – 15 min, buffer – pH 7.5)			
4 – σ analysis	Calculated from α, β, γ	Calculated from α_i , where $i=0, \dots, 3$	
σ_0	-0.3912 ± 0.0515	-0.2513	-0.3805
σ_1	0.0000 ± 0.0117	0.1256	0.0071
σ_2	0.0990 ± 0.1224	0.3611	0.1351
σ_3	0.7038 ± 0.1410	0.9841	0.7475
χ^2	1.29	–	–

<i>Oscillatoria chalybea</i> (dark adaptation time – 15 min, buffer – pH 7.5)			
5 – σ analysis	Calculated from α, β, γ, c	Calculated from α_i , where $i=0, \dots, 4$ and d	
σ_0	-0.0006 ± 0.0079	-0.0506	-0.0027
σ_1	-0.3815 ± 0.0594	-0.3165	-0.3849
σ_2	0.0117 ± 0.0226	0.1114	0.0073
σ_3	0.0816 ± 0.1308	0.3664	0.1323
σ_4	0.7111 ± 0.1494	0.9892	0.7428
χ^2	1.29	–	–

into account: 2.31 for tobacco, 1.18 *Chlorella* and 1.35 *Oscillatoria*.

The σ -analysis (and here analogously ζ -analysis) leaves the indetermination in assigning the transition probabilities $p_{(i \rightarrow j)}$, as long as no additional constraints are imposed (Lavorel, 1976). There are many models which can lead to the same values for σ_i and in the frame of a certain model many sets of transition parameters fulfil the same equations for σ_i . Therefore, we have carried out the numerical analysis of the oxygen yield Y_n in flash sequences by using two completely different models which are widely used: (i) the model with equal misses and non-vanishing equal double hits (Forbush *et al.*, 1971), and (ii) the model with unequal misses without double hits (Delrieu, 1974), and have then calculated σ_i . Miss and double hit factors as well as the initial distribution of the S_i states were free parameters in the fits. We imposed extra constraints upon parameters to keep them in the physical range, to avoid for example negative probabilities or state populations.

The elements of the matrix \mathbf{A} given by Eqn. (15) represent the transition probabilities (β) between

states assuming equal misses (α) and double hits (γ) for each state, with $\alpha + \beta + \gamma = 1$. There are five states $S_0 \dots S_4$ and therefore the matrix \mathbf{A} must be 5×5 . We introduced the parameter c which controls the splitting between the fast transition from $S_3 \rightarrow S_0$, via S_4 and the parameter $(1-c)$ which controls the slow transition with a longer living state S_4 . It has been postulated by Forbush *et al.* (1971) that the ground state S_0 cannot be formed by deactivation in the dark but is only formed during illumination. The decay of the fifth state takes place without light absorption (in contrast to the transition $S_3 + h\nu \rightarrow S_0$) and therefore we assume that S_4 decays to S_i which is the most stable state in darkness (Velthuys and Visser, 1975). If $c = 1$ and $\alpha(1,3) \leftrightarrow \alpha(5,3)$, the matrix corresponds exactly to the model proposed by Forbush *et al.* (1971).

$$\mathbf{A} = \begin{pmatrix} \alpha & 0 & 0 & \beta c & 0 \\ \beta & \alpha & 0 & \gamma & 1 \\ \gamma & \beta & \alpha & 0 & 0 \\ 0 & \gamma & \beta & \alpha & 0 \\ 0 & 0 & \gamma & \beta(1-c) & 0 \end{pmatrix} \quad (15)$$

Another possibility was proposed by Delrieu (1974). She proposes for the S -state transitions unequal misses α_i , assuming that double hits were nonexistent ($\gamma = 0$), because she was able to show that in experiments carried out with short 3–10 μ s flashes double hits became neglectable (Delrieu, 1974 and 1983). In this case the \mathbf{B} matrix (16) has five dimensions and its elements are misses ($\alpha_{i=0, \dots, 3}$) and the transition probabilities leading to the higher states ($\beta_{i=0, \dots, 3}$). The sum of elements in each column is equal to 1. The meaning of the d parameter is exactly the same as c in the previously described model.

$$\mathbf{B} = \begin{pmatrix} \alpha_0 & 0 & 0 & \beta_3 d & 0 \\ \beta_0 & \alpha_1 & 0 & 0 & 1 \\ 0 & \beta_1 & \alpha_2 & 0 & 0 \\ 0 & 0 & \beta_2 & \alpha_3 & 0 \\ 0 & 0 & 0 & \beta_3(1-d) & 0 \end{pmatrix} \quad (16)$$

The $\beta_{(3 \rightarrow 4)} = \beta_3(1-d)$ for $d=1$ is vanishing, if the state S_4 immediately decays. Then \mathbf{B} is reduced to the obtained transition matrix 4×4 proposed by Delrieu (1974).

The elements of matrices **A** and **B** as well as the initial distribution of the S_i states fitted to the experimental data for 4- and 5-S states are given in Tables IV and V.

Figures 1, 2 and 3 show the oxygen flash yield for tobacco, *Chlorella* and *Oscillatoria*, respectively. Theoretical data are marked by open squares. For *Nicotiana tabacum* chloroplasts and *Oscillatoria chalybea* the quality of the fit is comparable in the 4- and 5-S state system, whereas the best fit for *Chlorella kessleri* is obtained in a 5-S state system.

The σ_i probabilities for each of these models can be easily calculated. In the homogeneous hypotheses we obtain the following expressions for σ_i values:

a) for the four dimensional matrix **A** ($S_{i=0,\dots,3}$)

$$\begin{aligned}\sigma_3 &= 4\alpha \\ \sigma_2 &= 6\alpha^2 - 2\gamma^2 \\ \sigma_1 &= 4\alpha^3 + 4\beta^2 - 4\alpha\gamma^2 \\ \sigma_0 &= \alpha^4 - \beta^4 + \gamma^4 + 4\alpha\beta^2\gamma - 2\alpha^2\gamma^2\end{aligned}\quad (17)$$

b) for the five dimensional matrix **A** ($S_{i=0,\dots,4}$)

$$\begin{aligned}\sigma_4 &= 4\alpha \\ \sigma_3 &= 6\alpha^2 - \gamma^2 \\ \sigma_2 &= 4\alpha^3 + \beta^2\gamma + 2c\beta^2\gamma + (2-c)\beta\gamma - 2\alpha\gamma^2 \\ \sigma_1 &= \alpha^4 - (1-c)\beta^3 - c\beta^4 + \gamma^4 + 2(2-c)\alpha\beta\gamma \\ &\quad + (1+2c)\alpha\beta^2\gamma - \alpha^2\gamma^2 \\ \sigma_0 &= (2-c)\alpha^2\beta\gamma + c\beta\gamma^3 - (1-c)\alpha\beta^3.\end{aligned}\quad (18)$$

Assuming unequal misses, the σ_i are given by these equations:

a) for the four dimensional matrix **B** ($S_{i=0,\dots,3}$)

$$\begin{aligned}\sigma_3 &= \alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 \\ \sigma_2 &= \alpha_0\alpha_1 + \alpha_0\alpha_2 + \alpha_0\alpha_3 + \alpha_1\alpha_2 + \alpha_1\alpha_3 + \alpha_2\alpha_3 \\ \sigma_1 &= \alpha_0\alpha_1\alpha_2 + \alpha_0\alpha_1\alpha_3 + \alpha_0\alpha_2\alpha_3 + \alpha_1\alpha_2\alpha_3 \\ \sigma_0 &= \alpha_0\alpha_1\alpha_2\alpha_3 - (1-\alpha_0)(1-\alpha_1)(1-\alpha_2)(1-\alpha_3)\end{aligned}\quad (19)$$

b) for the five dimensional matrix **B** ($S_{i=0,\dots,4}$)

$$\begin{aligned}\sigma_4 &= \alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 \\ \sigma_3 &= \alpha_0\alpha_1 + \alpha_0\alpha_2 + \alpha_0\alpha_3 + \alpha_1\alpha_2 + \alpha_1\alpha_3 + \alpha_2\alpha_3 \\ \sigma_2 &= \alpha_0\alpha_1\alpha_2 + \alpha_0\alpha_1\alpha_3 + \alpha_0\alpha_2\alpha_3 + \alpha_1\alpha_2\alpha_3 \\ \sigma_1 &= \alpha_0\alpha_1\alpha_2\alpha_3 - (1-\alpha_0)(1-\alpha_1)(1-\alpha_2)\beta_3d \\ &\quad - (1-\alpha_1)(1-\alpha_2)\beta_3(1-d) \\ \sigma_0 &= -\alpha_0(1-\alpha_1)(1-\alpha_2)\beta_3(1-d).\end{aligned}\quad (20)$$

All the calculated σ_i for tobacco, *Chlorella* and *Oscillatoria* are collected in Tables I, II and III, respectively.

In order to demonstrate how to obtain σ_i values using the ζ -analysis, we carried out measurements on isolated PSII particles from *Nicotiana tabacum* var. John William's Broadleaf. The oscillation pattern is damped (see Fig. 4) because of lack of acceptor. In this case $\sum_i (-1)^n \zeta_i + 1 = \eta \approx 0$. To estimate the real transition probabilities we have to normalize ζ_i by multiplying them by powers of x : $\sigma_{n-i} = \zeta_{n-i} x^{-(n-i)}$, where x is a solution of the Eqn. (8). As η is close to zero, we can simplify the problem of solving this order equation by introducing ε . One can estimate $x = 1 - \varepsilon$ (where ε is very close to 0) and neglect the higher powers of ε . One gets:

1) for 4 S states

$$\varepsilon = \frac{\eta}{\zeta_1 - 2\zeta_2 + 3\zeta_3} \quad (21)$$

2) or for 5 S states

$$\varepsilon = \frac{\eta}{-\zeta_1 + 2\zeta_2 - 3\zeta_3 + 4\zeta_4}. \quad (22)$$

Knowing ε , it is easy to calculate the parameter T which characterizes the time of the inactivation of PS II particles:

$$T = -\frac{1}{\ln(1-\varepsilon)}. \quad (23)$$

We have obtained $\varepsilon = 0.12 \pm 0.08$ and $T = 8 \pm 5$ in the case of 4-S states, and $\varepsilon = 0.09 \pm 0.05$ and $T = 11 \pm 9$ in the case of 5-S states. It should be noted that T is expressed in units which represent the length of the time interval between flashes (in our experiments 300 ms). We have also made an attempt to fit experimental data by using the heterogeneous model with and without the S_4 state (Table VII). Based on the obtained parameters we calculated the σ_i values (Table VI). At first glance one could worry that the value for the T constant differs too much from the one obtained by the ζ -analysis, but has to recall that it appears under a logarithm and therefore has a large error.

Table IV. Transition parameters and the initial S_i state distribution estimated according to Eqn. (15) for chloroplasts of *Nicotiana tabacum* var. John William's Broadleaf, cells of *Chlorella kessleri* and thylakoid preparations of *Oscillatoria chalybea*.

	<i>Nicotiana tabacum</i> (JWB)	<i>Chlorella kessleri</i>	<i>Oscillatoria chalybea</i>
α	0.1420	0.1991	0.2460
β	0.8314	0.7738	0.7217
γ	0.0266	0.0272	0.0322
S_0	0.0255	0.3682	0.2478
S_1	0.9277	0.6285	0.6731
S_2	0.0309	0.0000	0.0043
S_3	0.0158	0.0033	0.0745

	<i>Nicotiana tabacum</i> (JWB)	<i>Chlorella kessleri</i>	<i>Oscillatoria chalybea</i>
α	0.1407	0.2400	0.2473
β	0.8333	0.7445	0.7288
γ	0.0139	0.0155	0.0239
c	0.7165	0.0038	0.4537
S_0	0.0669	0.2638	0.2361
S_1	0.8349	0.7301	0.6769
S_2	0.0782	0.0039	0.0154
S_3	0.0000	0.0000	0.0717
S_4	0.0000	0.0000	0.0000

Table V. Transition parameters and the initial S_i state distribution estimated according to Eqn. (16) for chloroplasts of *Nicotiana tabacum* var. John William's Broadleaf, cells of *Chlorella kessleri* and thylakoid preparations of *Oscillatoria chalybea*.

	<i>Nicotiana tabacum</i> (JWB)	<i>Chlorella kessleri</i>	<i>Oscillatoria chalybea</i>
α_0	0.0281	0.0064	0.0020
α_1	0.0032	0.0275	0.1402
α_2	0.4238	0.5611	0.5098
α_3	0.0028	0.0469	0.0956
S_0	0.0250	0.2586	0.1641
S_1	0.8418	0.7200	0.6798
S_2	0.1231	0.0255	0.0802
S_3	0.0000	0.0000	0.0760

	<i>Nicotiana tabacum</i> (JWB)	<i>Chlorella kessleri</i>	<i>Oscillatoria chalybea</i>
α_0	0.0041	0.1217	0.0111
α_1	0.0048	0.0483	0.1330
α_2	0.4249	0.5229	0.5116
α_3	0.0011	0.0480	0.0871
β_3	0.9963	0.9490	0.9069
d	0.7295	0.0090	0.3605
S_0	0.0350	0.2832	0.1654
S_1	0.8266	0.6735	0.6691
S_2	0.1271	0.0231	0.0898
S_3	0.0108	0.0002	0.0696
S_4	0.0000	0.0000	0.0000

Nicotiana tabacum var. John William's Broadleaf, PSII particles
(dark adaptation time – 15 min., buffer – pH 7.5)

4 – ζ analysis	σ_i Calculated from ζ_i	Calculated from α_i , where $i=0, \dots, 3$
ζ_0 –0.2685 \pm 0.0582	σ_0 –0.2685 \pm 0.0582	–0.3944
ζ_1 0.0000 \pm 0.0098	σ_1 0.0000 \pm 0.0098	0.0003
ζ_2 0.0129 \pm 0.0092	σ_2 0.0000 \pm 0.0092	0.0264
ζ_3 0.5377 \pm 0.0781	σ_3 0.7373 \pm 0.0540	0.6316
χ^2 1.93	–	–

Nicotiana tabacum var. John William's Broadleaf, PSII particles
(dark adaptation time – 15 min., buffer – pH 7.5)

5 – ζ analysis	σ_i Calculated from ζ_i	Calculated from α_i , where $i=0, \dots, 4$ and d
ζ_0 0.0000 \pm 0.0127	σ_0 0.0000 \pm 0.0127	–0.0010
ζ_1 –0.2813 \pm 0.0618	σ_1 –0.3063 \pm 0.0210	–0.3818
ζ_2 0.0000 \pm 0.0015	σ_2 0.0000 \pm 0.0015	0.0010
ζ_3 0.0000 \pm 0.0374	σ_3 0.0000 \pm 0.0374	0.0491
ζ_4 0.5119 \pm 0.1014	σ_4 0.6936 \pm 0.0356	0.6549
χ^2 1.52	–	–

Table VI. Values of ζ_i and σ_i derived from Eqn. (14), minimizing the function (13). σ_i is calculated on the basis of the heterogeneous model (Eqns (19) and (20)) for photosystem II particles of *Nicotiana tabacum* var. John William's Broadleaf.

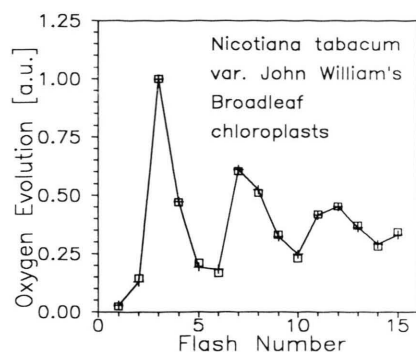


Fig. 1. Flash induced oxygen yield pattern in chloroplasts of *Nicotiana tabacum* var. John William's Broadleaf suspended in Tricine HCl buffer, pH 7.5, observed after 15 minutes of sedimentation and dark adaptation. Squares are the theoretical values evaluated on the transition matrix **B** (Eqn. (16)) assuming 4-S states (Table V).

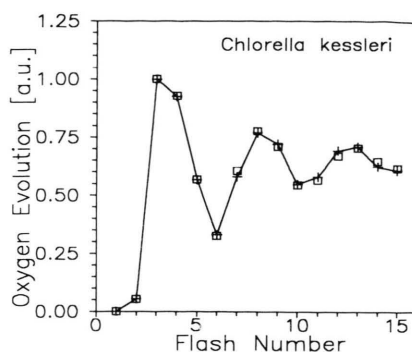


Fig. 2. Flash induced oxygen yield pattern in *Chlorella kessleri* suspended in growth medium, pH 6.3, observed after 15 minutes of sedimentation and dark adaptation. Squares are the theoretical values evaluated on the transition matrix **B** (Eqn. (16)) assuming 5-S states (Table V).

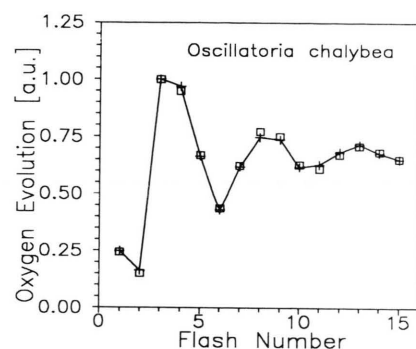


Fig. 3. Flash induced oxygen yield pattern in thylakoid preparations of *Oscillatoria chalybea* suspended in Tricine HCl buffer, pH 7.5, observed after 15 minutes of sedimentation and dark adaptation. Squares are the theoretical values evaluated on the transition matrix **B** (Eqn. (16)) assuming 4-S states (Table V).

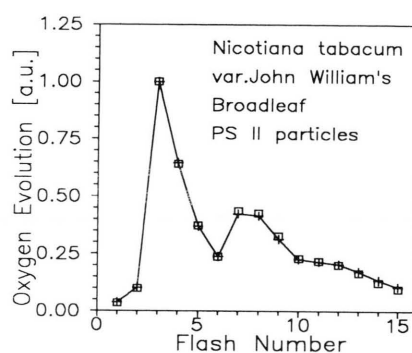


Fig. 4. Flash induced oxygen yield pattern in photosystem II particles of *Nicotiana tabacum* var. John William's Broadleaf suspended in Tricine HCl buffer, pH 7.5, observed after 15 minutes of sedimentation and dark adaptation. Squares are the theoretical values evaluated on the transition matrix **B** (Eqn. (16)) assuming 4-S states and taking into account the decrease of the number of active centres during the measurement (Table VII).

<i>Nicotiana tabacum</i> (JWB) PS II particles	
α_0	0.0030
α_1	0.0167
α_2	0.5877
α_3	0.0243
S_0	0.0361
S_1	0.8877
S_2	0.0794
S_3	0.0000
T	50

<i>Nicotiana tabacum</i> (JWB) PS II particles	
α_0	0.0032
α_1	0.0434
α_2	0.5724
α_3	0.0361
β_3	0.9644
d	0.1338
S_0	0.0131
S_1	0.8917
S_2	0.0821
S_3	0.0124
S_4	0.0000
T	70

Table VII. Transition parameters and the initial S -state distribution estimated according to Eqn. (16) for photosystem II particles of *Nicotiana tabacum* var. John William's Broadleaf.

Conclusions

The method which allows to determine the number of S states in the Kok model is based on the determination of the standard least square deviation (test χ^2). The ζ -analysis carried out for *Chlorella kessleri* shows that the description of the system is much better, if the existence of five states is assumed. In the case of chloroplasts from *Nicotiana tabacum* and thylakoid preparations from *Oscillatoria chalybea* the 4- S state model is as good as the 5- S state one (our results and Bader *et al.*, 1983). A mathematical analysis of the oscillation pattern of oxygen evolution taking into account 5- S states has already been carried out for the case of *Chlorella* (Thibault, 1982) and cyanobacteria (Bader *et al.*, 1983; Schmid *et al.*, 1994). This has been done, however, by use of a different transition matrix. It is quite possible that the introduction of another description of the transitions will allow to omit the 5th state. In the present paper we show, independently on any model, that the 5- ζ analysis describes much better the experimental data obtained for *Chlorella*, clearly showing that an additional state should be taken into account. We associate the 5th S state with the S_4 state. According to the literature, the S_4 state is not detectable because of its very short life time in comparison to other S_i states. Speculation on the life time of S_4 is a problem. Strzałka *et al.* (1990) and Schulder *et al.* (1992) have recently determined the release time for oxygen i.e. the time for the $S_3 \rightarrow S_0$ transition to be less than 0.5 msec which leaves over only a very short life time for S_4 . In reality, there is not much time left at all for the existence of this state. However, it looks as if a fraction of the S_4 state which would be more stable or longer living (within the time frame exposed) than usually assumed, influences the pattern of oxygen evolution.

In this paper we propose the combination and or extension of two models widely used in the literature (the one with equal misses and double hits for all transitions and the one with non equal misses, without double hits). An extra parameter c has been introduced into the homogenous model and d into the heterogeneous model. Both factors represent the probability for the fast transition from $S_3 \rightarrow S_0$. $(1-c)$ in the homogenous and $(1-d)$ in the heterogeneous model are the fractions of the

decay of S_3 via the longer living S_4 state. S_4 decays to S_1 as the final state, as this transition takes place in darkness. The light driven reaction of oxygen evolution in the case of *Chlorella* would almost exclusively go via the longer living S_4 state, whereas in *Oscillatoria* ~60% of the reaction and in tobacco only ~30% of the reaction go via this S_4 state (Tables IV and V). It is generally valid that the increase of misses is associated with less efficient forward transitions of the S_i states and it seems natural that the probability for the appearance of a longer living S_4 state increases as well. The two modes of S_3 transition (proposed by us) under short saturating flashes yielding O_2 -evolution might be associated with two different S_3 conformational states as has been postulated by Renger and Hanssum (1992) for OEC's in PSII membrane fragments from spinach and by Koike *et al.* (1987) in the thermophilic cyanobacterium *Synechococcus vulcanus*. Our model might be consistent with the suggestions of two types of electron transfer through photosystem II. The heterogeneous character of the S_3 transition may result not only from two different conformational states of S_3 but also from the heterogeneity of the secondary donor of PSII as well as from the not homogenous acceptor side of PSII (Drechsler and Neumann, 1989; Joliot and Joliot, 1979; Hodges and Barber, 1986; Shinkariev and Wraight, 1993). Our model resembles that of Thibault in the sense that it has also 5- S states. However, the role of the additional 5th state is completely different. In the Thibault model the additional state corresponded to a reduced state S_{-1} created by dark deactivation of S_2 .

The σ_i values calculated on the basis of the two different models might be an indication that the heterogeneous model describes oxygen evolution better than the homogenous one. Other measurements confirm that the transitions between S states show different kinetics (for a review see Debus, 1992). Moreover, the model with equal misses and finite double hits shows parallel variations of the miss and double hit factors (Delrieu, 1983) (see also our results).

In this paper we extend the σ -analysis to the more general ζ -analysis which is also valid in the case of damped oscillations of O_2 yield due to inactivation of the oxygen evolving centres during the measurements (decrease of electron acceptor or death of the investigated system). With the ex-

ample of PS II particles from tobacco we demonstrate how one can obtain realistic σ_i values. The attempt, how to take the decrease of the apparent number of active centres at each flash of a series into account, has been proposed by Delrieu and Rosengard (1987). Our approach would be more general, because the ζ -analysis of the experimental data secures the Markovian character of the

oxygen evolution process, is independent on any model of oxygen evolution and provides a parameter characterizing even the inactivation time of PSII particles.

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