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STUDIES ON A THERMAL REACTION ASSOCIATED WITH PHOTOSYNTHETIC OXYGEN EVOLUTION

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SUMMARY

The modulated polarographic technique of O_2 detection was applied to *Chlorella* to study the rate-limiting thermal reaction between Photosystem II and O_2 evolution. From an analysis of the operation of the polarograph at different frequencies, it was concluded that a first order thermal reaction of rate constant 305 ± 20 (S.E.) s^{-1} was consistent with the results of 22 °C. When the algae were successively studied in solutions made up with ^2H_2O and H_2O, a kinetic isotopic effect for the rate constant of 1.29 ± 0.05 (S.E.) was found. This suggests that the rate limiting step does not involve the breaking of the O–H bond in water. A temperature study of the rate constant indicated an activation energy of 5.9 ± 0.5 (S.E.) kcal $\cdot$ mole^{-1} and an entropy of activation of -25 cal $\cdot$ degree^{-1} $\cdot$ mole^{-1}. The linearity of the Arrhenius plot between 8 and 42 °C demonstrated that only one reaction was rate-limiting over this temperature range.

INTRODUCTION

The theory for the modulated O_2 electrode which was developed by Joliot et al. [1] can be used to determine the rate constant of the rate-limiting thermal reaction between the absorption of light by Photosystem II and the evolution of O_2 in algal cells (or chloroplasts). The two sets of equations which make this possible are: (a) For a thick (35 μm) layer of cells on the electrode

$$\phi = \frac{\Pi}{4} + \frac{\varepsilon\sqrt{\omega}}{\sqrt{2D}} + \tan^{-1}(\omega/k) \tag{1}$$

$$A = \frac{B\Psi(0)}{\sqrt{\omega D}} \exp\frac{-\varepsilon\sqrt{\omega}}{\sqrt{2D}} \cdot \frac{k}{\sqrt{(\omega^2+k^2)}} \tag{2}$$

(b) For a thin (6 μm) layer of cells on the electrode

$$\phi = \frac{p\sqrt{\omega}}{\sqrt{2D}} + \tan^{-1}(\omega/k) \tag{3}$$

$$A = \frac{\Psi(p)\Delta p}{D} \exp\frac{-p\sqrt{\omega}}{\sqrt{2D}} \cdot \frac{k}{\sqrt{(\omega^2+k^2)}} \tag{4}$$

where ω = angular frequency of light modulation, B = proportionality constant, ε = effective width of the layer of solution between the platinum electrode and the closest O_2 sources, D = diffusion coefficient of O_2 in buffer, $\Psi(0)$ = rate of O_2 production per unit volume amongst cells closest to the platinum electrode, $\Psi(p)\Delta p$ = rate of O_2 production in the layer of cells of thickness Δp at a distance p from the electrode, k = rate constant of the rate limiting step of O_2 evolution in photosynthesis

These equations describe the phase lag (ϕ) and the amplitude (A) of the alternating current produced in the measuring circuit of the electrode when a periodically fluctuating light of angular frequency (ω) is shone on a layer of cells lying on the platinum electrode The first two terms of Eqn 1, and the first in Eqn 3 describe, for thick and thin layers of algae respectively, the influence of diffusion on the phase lag of the signal with respect to the light The final terms in these equations describe the influence which a first order thermal reaction of rate constant k would have on the phase lag Similarly the first two factors in Eqns 2 and 4 describe the influence of diffusion on the amplitude of the signal while the final factors introduce the influence of a first order thermal reaction

By fitting Eqns 1 and 2 to their results Joliot et al [1] obtained a value of 820 s^{-1} for k with *Chlorella pyrenoidosa* Also, by performing their experiments at two different temperatures they determined a value of 7 7 kcal mole^{-1} for the activation energy

In our present study we have repeated the basic experiments performed by Joliot et al [1] to obtain a value for k We have studied the temperature dependence of k at a variety of temperatures to discover whether only one reaction is rate-limiting over the physiological temperature range Finally we measured the value of the ^2H-isotope effect with respect to the rate-limiting reaction, by immersing the algae successively in ^2H_2O and H_2O This last experiment was undertaken to test the hypothesis that the rate-limiting reaction for photosynthetic O_2 evolution involves the rupture of an O–H bond in the water molecule Failure to observe a ^2H-isotope effect of the appropriate magnitude (3–7) would be strong evidence that the above hypothesis was incorrect

METHODS

Experimental material

Chlorella vulgaris and *Chlorella pyrenoidosa* were obtained from the Carolina Biological Supply Co and grown in the laboratory under constant aeration in a Warburg–Buck medium Thick and thin layers of cells were deposited on the platinum electrode as described by Sinclair [2] A medium which contained 0 1 M KCl, and 0 05 M potassium phosphate buffer, pH 6 4, was perfused through the O_2 electrode at between 5 and 10 ml/min

The modulated electrode

The electrode used was described by Joliot and Joliot [4] and the methodology was similar to that of Sinclair [2] except that a PAR lock-in amplifier, model 129, with low-noise transformer, model 190, was used to measure the signal and a model 222 chopper system was used to modulate the light We found that the model 129 gave significantly better accuracy than the HR-8 used by Sinclair. We estimated that a phase measurement could be repeated with a precision of ± 1° However, the measurement of the phase of the light at the electrode introduced a constant error which in any given experiment could have been as large as ± 5° Allowance was made for this by having a floating intercept at zero frequency in the computer fit of the phase results

Estimate of rate constant by computer

The model based on O_2 diffusion and a thermal reaction was fitted to the phase results by a least-squares method The computer used estimates of the rate constant, k, the expression $\varepsilon/\sqrt{2D}$ or $p/\sqrt{2D}$ and the phase at zero frequency, ϕ_0, to calculate phase values according to Eqns 1 or 3 and then calculated the sum of squares of the deviations between the theoretical and experimental values This sum was minimized by an iterative procedure in which k, $\varepsilon/2D$ and ϕ_0 were allowed to vary by small increments The values of the three parameters which yielded the minimum were taken as the best fit values This procedure was sometimes repeated with alternative starting values for the three parameters to verify the results The programme also provided the standard deviation of the phase results from the best fit values

Temperature experiments

Most early experiments were performed at room temperature (22 °C) but in the study of temperature on the rate constant, special modifications were made which allowed close control and observation of the temperature Water from a Lauda constant-temperature bath (Type K2RD) was passed through a glass coil immersed in the solution reservoir and through a hollow metal plate on which the polarograph was mounted The temperature of the *Chlorella* was monitored with a small thermistor positioned just below the platinum electrode It was found that this arrangement routinely kept the temperature constant to ±0 2 °C Once the temperature had stabilized a normal survey of the effect of modulation frequency on current emplitude and phase was performed Such experiments were carried out at various temperatures between 8 and 42 °C

Kinetic isotope experiments

In the kinetic isotope experiments, the effect of using ^2H_2O instead of H_2O in the medium was investigated The ^2H_2O was obtained from I C N in a 99 8 % pure form to which was added precisely the same salts as for the water based medium At the start of an experiment all traces of water were carefully removed from the apparatus and then the *Chlorella* cells, previously spun down and resuspended in ^2H_2O-based medium, were injected into the bottom compartment When the ^2H_2O medium was allowed to flow through the apparatus it rapidly exchanged with any water in the lower compartment, after which a scan of the influence of modulation frequency on the current was made Later the ^2H_2O buffer was replaced with the corresponding H_2O solution and the experiment repeated The data were analysed with the computer programme as described above

RESULTS

Experiments at 22 °C with Chlorella

The mean value of k obtained from the phase results was 305±20 (S E) s^{-1}

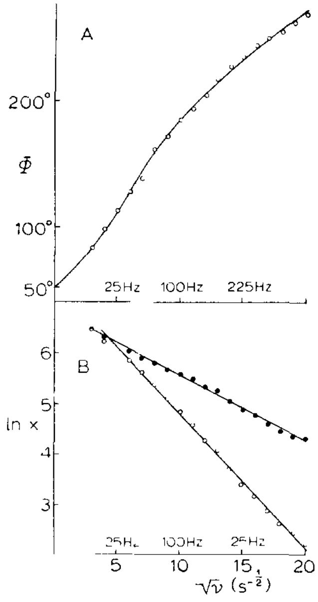


Fig 1 A The phase lag between the current and the light modulation plotted against the square root of the modulation frequency (open circles), obtained with a 35-μm layer of *Chlorella* The solid line represents the curve generated by the equation

$$\phi = \phi_0 + \frac{\varepsilon}{\sqrt{2D}}\sqrt{\omega} + \arctan\left(\frac{\omega}{\kappa}\right), \text{ for } k = 330\ \mathrm{s}^{-1},$$

$\varepsilon = 3\ 0\ \ 10^{-4}$ cm and $\phi_0 = 50°$ The standard deviation between the experimental and calculated points was 2 3° B The amplitude results of the same experiment as in A For the open circles $x = A\sqrt{\nu}$ and for the closed symbols $x = (A\sqrt{\nu}\ \ k/\sqrt{(k^2+\omega^2)})$ with $k = 330\ \mathrm{s}^{-1}$, both are plotted against $\sqrt{\nu}$ According to the theory described in the text, the upper line should only reflect the influence of oxygen diffusion while the lower line should also reflect the influence of a thermal rate-limiting reaction From the slope of the upper line a value of 3 2 10^{-4} cm was derived for ε

for 21 experiments at 22 °C with extreme values of 160 s^{-1} and 510 s^{-1} A typical best fit value for ϕ_0 was 45 ° with a 35-μm layer of *Chlorella* and for ε a typical value of 3 μm was obtained with $D = 2\ 0\ \ 10^{-5}\ \mathrm{cm}^2\ \ \mathrm{s}^{-1}$ As illustrated in Fig 1a the theoretical curve gave a very good description of the experimental results at all frequencies with a standard deviation (S D.) between the theoretical and experimental values of 2 3 ° The values of k and ε from the phase results gave a good description of the amplitude results as shown in Fig 1b Similar results were obtained from the two species of algae Work with 6-μm layers of *Chlorella* yielded results which were well described by Eqns 3 and 4 which gave values of k similar to those found with thick layers

Temperature experiments

The temperature dependence of k computed from the phase results of a thick layer of *Chlorella* is shown in the Arrhenius plot in Fig 2a A least squares line is drawn through the points, from which an activation energy, E_a, of 5 2 kcal mole^{-1} was calculated for this experiment The mean of the activation energies for 14 experi-

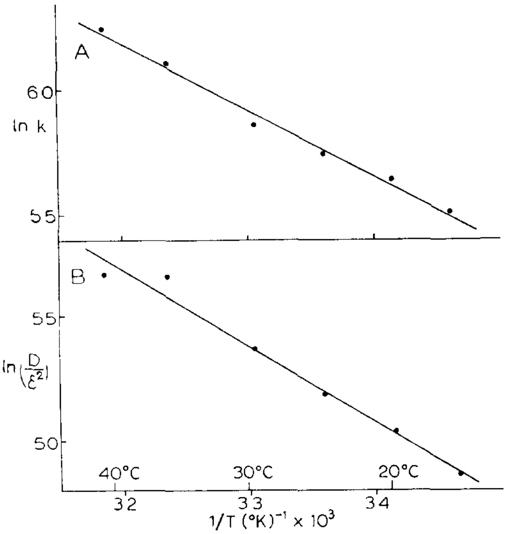


Fig 2 A The natural logarithm of the rate constant k, derived from the phase data versus $1/T$, obtained for a 35-μm layer of *Chlorella* A least squares line is drawn through the points from which an activation energy of 5 2 kcal mole^{-1} was calculated B The natural logarithm of D/ε^2 from the same phase data, also plotted against $1/T$ The least squares line through these results corresponds to an activation energy of 6 0 kcal mole^{-1}

ments was 5 9±0 5 (S E) kcal mole^{-1} In the temperature range studied (8–42 °C), there did not appear to be any significant curvature in the Arrhenius plots, which indicated that only one rate process was limiting

In Fig 2b a plot of ln D/ε^2 vs $1/T$ is shown, which was computed from the phase results of the experiment described in Fig 2a The results are again fairly linear Following the theory of Eyring and Olander [4] we have expressed the temperature dependence of D/ε^2 as an activation energy for diffusion (E_D) whose mean was 5 7±0 03 (S E) kcal mole^{-1} for 14 experiments Investigation of the cell size at 20 °C and 40 °C did not reveal any size alteration significant to our results The value of E_D can be compared with one computed from values quoted in Himmelblau's review [4] which was 6 0 kcal mole^{-1} Thus the temperature dependence of D obtained from the computer fit is consistent with known diffusion data

Experiments in ^2H_2O

In Fig 3, the dependence of ϕ on $\sqrt{\nu}$ for a 6-μm layer of *Chorella* at 22 °C is shown for ^2H_2O- and water-based media The rate constant in ^2H_2O was calculated initially from the results in Fig 2 as 232 s^{-1} with an S D between the experimental and theoretical values of 1 3 °C After 1 5 h in ^2H_2O another experiment was undertaken with nearly identical results This time k was 230 s^{-1} and the S D was 1 2° After perfusion with H_2O buffer for 1 h the rate constant calculated from the results in Fig 2 was 289 s^{-1} with an S D of 0 8° The results with four different samples of algae gave $k_{H_2O}/k_{^2H_2O}$ = 1 29±0 05 (S E)

Because ^2H_2O is slightly more viscous than H_2O, diffusion is slower and the phase of the O_2 signal for a particular frequency has a higher value A calculation

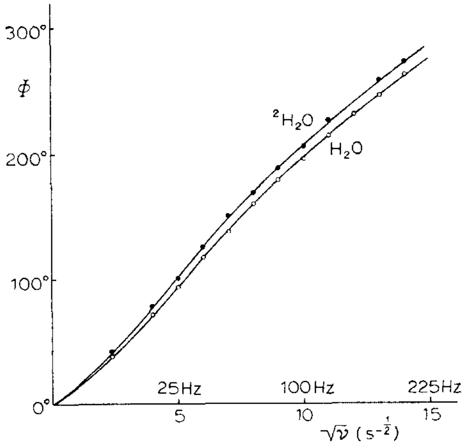


Fig 3 The effect of substituting ^2H_2O for water in the experimental medium, for a 6-μm layer of *Chlorella vulgaris* at 22 °C The closed circles are the phase values obtained shortly after suspension of the algae in ^2H_2O-based buffer Virtually the same results were observed 1 5 h later After 1 h in H_2O-based solution, the phase values represented by the open circles were obtained The upper line is the best fit curve for ^2H_2O, generated for $k = 232\ s^{-1}$, $p/\sqrt{D} = 0\ 136\ s^{-\frac{1}{2}}$, and $\phi_0 = 0°$ The standard deviation between the experimental and theoretical points was 1 3° The lower line is the best fit curve for the experiment in water for which $k = 289\ s^{-1}$, $p/\sqrt{D} = 0\ 129\ s^{-1}$, $\phi_0 = 0°$ and the S D = 0 8°

based on the assumption that ε has the same value in H_2O and ^2H_2O gave 1 3±0 1 (S E) for the ratio of the diffusion coefficients in H_2O and ^2H_2O based buffers This figure can be compared with that obtained by independent physical measurements on water and ^2H_2O (There is probably little difference in the ratio $D_{H_2O}/D_{^2H_2O}$ for buffer or pure solvent) Taking values of the viscosity of water and ^2H_2O from Katz and Crespi [5], and assuming that the ratio of the diffusion coefficients of oxygen in the two liquids is inversely proportional to the ratio of the viscosities, we obtain a value of 1 24 for $D_{H_2O}/D_{^2H_2O}$

DISCUSSION

The theory of the operation of the modulated O_2 polarograph which was developed by Joliot et al [1] has been supported by an important experimental study by these authors which yielded considerable evidence in favor of their theory. In a later study Sinclair [2] disputed their value of k and proposed a higher value. The present work confirms the finding of Joliot et al and revealed that if Sinclair had examined higher frequencies he would have found the non-linearities which are strong evidence of a lower rate constant

We have found two new pieces of evidence which support Joliot's theory of the modulated electrode As mentioned in the results section the derived values for the activation energy of O_2 in water and for the ratio of the diffusion coefficients of O_2 in water and ^2H_2O were both in good agreement with independent estimates Thus we have added to the credibility of Joliot's theory

The mean value of k obtained here was $305 \pm 20\ s^{-1}$ as compared with the value of 820 s^{-1} found by Joliot et al [1] The difference may in part be due to different methods of analysing the results and in part due to differences in biological material In other studies using different techniques, Etienne [6] obtained a limiting rate constant of 300 s^{-1} while Bouges-Bocquet [7] evaluated several rate constants associated with the oxidizing side of Photosystem II, the smallest of which was 580 s^{-1} Thus if we assume that Bouges-Bocquet [7] did observe the rate limiting reaction, there is not a very close consensus on the value of k

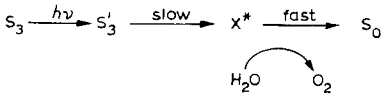
Our temperature experiments yielded a value for the activation energy (5 9 kcal mole^{-1}) which was similar to that obtained by Etienne (6 6 kcal mole^{-1}) but somewhat smaller than that of Joliot et al [1] (7 7 kcal mole^{-1}) One new result from these experiments was that only one reaction appears to be rate-limiting within the 8–42 °C temperature range This conclusion arises from the linearity of the Arrhenius plots

The entropy of activation can be calculated when the rate constant and activation energy are substituted in the formula below [8],

$$k = \frac{e\mathbf{k}T}{h} \exp\left(\frac{\Delta S}{R}\right) \exp\left(\frac{-Ea}{RT}\right) \qquad (5)$$

where $\mathbf{k}$ = Boltzmann constant, R = gas constant, h = Planck's constant For $k = 300\ s^{-1}$, $E_a = 5\ 9$ kcal mole^{-1} and $T = 295$ °K, the entropy of activation is -25 cal degree^{-1} mole^{-1} If Kok et al [9] are correct in proposing a build-up of positive charge prior to O_2 evolution, this negative entropy of activation might reflect an ordering of water molecules around the reaction centre

In our kinetic isotope experiments we obtained a value of 1 29 for the ratio of the rate constant in water to that in ^2H_2O This is much lower than would have been expected if the rate limiting step involved the rupture of an OH bond where typical values range between 3 and 7 [10] The work of Melville [11] on the mercury photosensitized decomposition of water offers a reasonable analogue of photosynthetic evolution of O_2 and the value of the kinetic isotope effect in this case was 3 There are several possibilities to explain the low value obtained here In rare cases reactions involving the breaking of an OH bond give small kinetic isotope effects because the bonding of the hydrogen in the transition state is similar to that in the initial state [10] A second possibility is that there exists a special pool of water molecules which is the source of the evolved O_2 and which was never significantly filled with ^2H_2O in our experiments If this were so, no kinetic isotope effect would be observed However, since we were able to obtain consistent results in ^2H_2O in two experiments separated by 2 h and since Ruben et al [12] were able to demonstrate the evolution of $^{18}O_2$ by *Chlorella* immersed in a $H_2{}^{18}O$ solution in a time which was comparable to our experiments, this possibility would seem rather remote The simplest explanation of our results is that the rate limiting dark reaction does not involve the rupture of an OH bond In fact, the small kinetic isotope effect appears to be that expected for a change in enzyme conformation [13] Referring to Kok's model of Photosystem II this might mean that one of the dark steps following a light reaction was the slow build-up of a charged complex or activated compound X* which, once formed, would react relatively rapidly with water to evolve O_2 If this occurred after the fourth photoreaction the scheme might be



$$S_3 \xrightarrow{h\nu} S_3' \xrightarrow{\text{slow}} X^* \xrightarrow{\text{fast}} S_0 \qquad H_2O \rightarrow O_2$$

In conclusion, our present study confirms the theory of the modulated O_2 polarograph and indicates that the photosynthetic O_2 evolution process has the following characteristics

(1) There is only one rate-limiting reaction between 8 and 42 °C (2) This reaction has a rate constant of about 300 s^{-1} at 22 °C (3) It has an activation energy of 5 9 kcal mole^{-1} (4) It probably does not involve the rupture of an OH bond

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