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TITLE OF THESIS/TITRE DE LA THÈSE

Studies on the Mechanism
of Photosynthetic Oxygen
Evolution

UNIVERSITY/UNIVERSITÉ

Carleton

DEGREE FOR WHICH THESIS WAS PRESENTED/
GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE

Ph.D.

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE DEGRÉ

1976

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**Studies on the Mechanism of Photosynthetic Oxygen
Evolution**

by

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**A thesis submitted to the Faculty
of Graduate Studies in partial
fulfilment of the requirements
for the degree of Doctor of Philosophy**

**Department of Biology
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April , 1976

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ABSTRACT

The dark reactions associated with photosynthetic oxygen evolution were studied with the modulated polarograph and in flashing light. The polarographic study revealed that the rate constant, k , of the rate-determining step of oxygen evolution at 23°C had a value of 305 ± 20 (S.E.) sec^{-1} for Chlorella. When the algae were successively studied in solutions of D_2O and H_2O , a kinetic isotope effect, $k_{\text{H}}/k_{\text{D}} = 1.29 \pm 0.05$ (S.E.) was found. This suggested that the rate-limiting step did not involve the breaking of an OH bond in water. A temperature study of the rate constant indicated an activation energy of 5.9 ± 0.5 (S.E.) kcal.mole^{-1} , and an entropy of activation of $-25 \text{ cal.degree}^{-1}\text{mole}^{-1}$. The linearity of the Arrhenius plot between 8 and 42°C demonstrated that only one reaction was limiting over this temperature range. Work with spinach chloroplasts yielded results of a similar nature to those found with Chlorella but with minor differences in the values of the parameters measured. Removal of salts from the medium reduced the rate constant in spinach chloroplasts from 215 sec^{-1} to 110 sec^{-1} . However, the reaction in chloroplasts was insensitive to decarbonation, consistent with a site for bicarbonate action on the reducing side of photosystem II. It was also unaffected by the addition of $1\text{M NH}_4\text{Cl}$ to the medium or changes in the oxygen concentration and pH of the medium. This evidence suggested that the reaction was irreversible. The experiments in flashing light indicated that D_2O reduced only slightly the rate of the four dark reactions of the Kok scheme of oxygen

which was evidence that these processes did not involve the breaking of OH bonds. A model to describe these results is presented.

ACKNOWLEDGMENTS

I would like to thank Dr. John Sinclair for his guidance, particularly in the more complicated areas of this thesis, and for his time and assistance which were freely given throughout the course of this work. I also appreciate the help of my advisors, Dr. Ian C.P. Smith and Dr. John Webb.

Cliff Wilson provided invaluable technical assistance. Bill Ferguson and Don McIntosh designed and constructed the flash apparatus. The manuscript was typed by Mrs. Diane Labrèche.

My wife, Carol and my mother Dr. Elizabeth Arnason provided help and moral support during the preparation of the thesis.

Finally, I wish to acknowledge the financial support of an N.R.C. scholarship, an Ontario Graduate scholarship, and a research assistantship funded by Dr. Sinclair.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	amplitude of the modulated oxygen signal
CCCP	carbonyl cyanide m-chlorophenylhydrazone
C550	an absorbance change thought to be the primary electron acceptor of photosystem II
DCMU	2(3,4 dichlorophenyl)-1,1-dimethyl urea
DCPIP	2,6 dichlorophenyl indophenol
E_a	activation energy for the rate-limiting step of oxygen evolution
E_D	activation energy for the diffusion of oxygen in water
HEPES	N-2 hydroxyethyl piperazine N'-2-ethane sulfonic acid
k	the rate constant of the rate-limiting step of oxygen evolution
NADP	nicotinamide adenine dinucleotide phosphate
PC	plastocyanine
PQ	plastoquinone
P ₆₈₀	reaction centre of photosystem II
Q	primary electron acceptor of photosystem II
$S_0 \dots S_3$	the four states of the Kok model
$\Delta S^\ddagger$	entropy of activation
$Y_n(\Delta t)$	yield of oxygen from the n^{th} flash in a sequence when one of the flash intervals is Δt
Y	the water-splitting enzyme
Z	primary electron donor to photosystem II
α	number of misses of the Kok model
β	number of double hits of the Kok model

$\gamma_n(\Delta t)$	extent of reaction at time Δt for the n^{th} dark process of the Kok scheme
ξ	effective distance between the platinum electrode and the closest O_2 sources for a thick layer of cells
ϕ	phase of the modulated oxygen signal
ν	frequency of modulation
ω	angular frequency of modulation

I. INTRODUCTION

1. General Introduction

The way in which plants produce molecular oxygen is poorly understood compared to the rest of photosynthesis. However, in recent years progress in the elucidation of the mechanism of oxygen evolution has been made through kinetic studies. This was the approach taken in this work, in which rate processes associated with oxygen generation were studied by two different techniques. The first part of this work was an investigation of the rate-limiting step of oxygen production performed with the modulated electrode developed by Joliot, Hoffnung and Chabaud (1966). The second part involved experiments on several of the faster reactions leading to oxygen evolution, undertaken by means of a new flash technique (Kok, Forbush and McGloin, 1970). One of the main objectives of this study was an attempt to identify the reaction(s) in which water, the precursor of photosynthetic oxygen, was broken down.

2. A Review of Oxygen Evolution in Photosynthesis

2.1 Oxygen Evolution and Electron Transport

The modern idea of photosynthetic electron transport dates from 1939 when Hill separated the light reactions that led to oxygen evolution from the dark reactions of CO₂ fixation. Shortly thereafter Ruben et al. (1941) showed in their work with heavy isotopes that water was the precursor of photosynthetically produced oxygen. The series model of electron transport is now well established giving us the concept of electron transfer from water to NADP via several oxidation-reduction reactions and two

light-driven steps. The reactions of this scheme which were of most interest in this work are those in which water is oxidized to molecular oxygen with the transfer of electrons to the reaction center of photosystem II.

Photosynthesis is unusual in that it is probably the only biological process which evolves molecular oxygen, as opposed to the large number of biological reactions which consume oxygen, evidence enough of the remarkable nature of this reaction. Water is not a good reducing agent with a redox potential E_0' of +0.8 volts (Joliot and Kok, 1975) for the half reaction:

Thus, to remove electrons from water the primary electron donor to photosystem II must have an even more electropositive redox potential. Olson (1970) describes a logical scheme to explain how these reactions have evolved.

Our knowledge of the biochemistry of the oxygen producing reaction is relatively scanty but certain details are well established. For example, Cl and Mn have been shown to be crucial elements for the processes closely associated with oxygen generation (Bové et al. 1963; Heath and Hind, 1969a; Hind et al. 1969; Izawa et al. 1969; and Cheniae, 1970). Deficiency in either of these elements leads to a loss of oxygen evolving capacity but a substantial portion of electron transport can be restored by providing photosystem II with suitable artificial electron donors (Heath and Hind, 1969b).

There is evidence for the existence of a water-splitting enzyme which has been designated, Y_2 by Renger

(1970, 1972) and is thought to be the site of water oxidation and O_2 production. Braun and Govindjee (1972 and 1974) have reported the preparation of an antibody which specifically inhibits oxygen evolution. Heat treatments and extremes of pH rapidly cause chloroplasts to lose their ability to oxidize water while retaining their photosystem I activity (Good and Izawa, 1973). This evidence strongly suggests protein involvement in oxygen evolution. Some attempts have been made to fractionate and purify the polypeptides associated with photosystem II by gel electrophoresis (Levine, Burton and Durham, 1972). However, as yet it has not been possible to identify and characterize the water-splitting enzyme nor any of the other compounds associated with the oxidizing side of photosystem II.

The failure to extract and purify the water-splitting enzyme is perhaps the biggest problem in the study of O_2 evolution. By way of comparison, cytochrome oxidase, which catalyses the reduction of O_2 in the mitochondrion, was isolated as early as 1939 by Keilin and Hartree. Work on its structure is well advanced (Wikstrom, 1972) and classical enzyme kinetic studies have led to a fairly detailed understanding of its mechanism of action (Van Buren et al. 1971). Perhaps the failure to isolate the water-splitting enzyme stems from the absence of a characteristic absorption band or other characteristics to use as a marker during purification. Purification of the enzyme may lead to a better understanding of the active site of the enzyme where it is thought that Mn and perhaps

4.

Cl are cofactors. The multiple oxidation states of Mn are probably the key to the water-splitting act. The oxidation states observed in Mn compounds include: $\text{Mn}^{-\text{III}}$, $\text{Mn}^{-\text{I}}$, Mn^0 , Mn^{I} , Mn^{II} , Mn^{III} , Mn^{IV} , Mn^{V} , Mn^{VI} , and Mn^{VII} (Cotton and Wilkinson, 1962). Many of these states are represented by hydroxides or oxides of Mn. Evidence that multiple oxidation states of Mn exist in the photosynthetic apparatus comes from recent NMR studies by Wydrzynski et al. (1975).

Other compounds, besides the water-splitting enzyme, have been implicated in the reactions which link the water-splitting act and photosystem II. The reaction centre of photosystem II itself, designated P_{680} , has recently been identified on the basis of spectroscopic measurements (Döring et al. 1969). There probably also exists a primary electron donor to P_{680} , usually designated Z (Govindjee and Govindjee, 1975) which accepts electrons that have come from the water-splitting enzyme and donates them to P_{680} . The existence of this compound is suggested by work with inhibitors of O_2 evolution such as CCCP, salicylaldoxime, azide and antimycin A, as well as artificial electron donors such as diphenyl carbazide, hydroxylamine and ascorbate which bypass the water-splitting act and donate electrons directly to photosystem II (Kato et al. 1971). Cytochrome b_{599} has been identified as the primary donor Z, but recent studies indicate that these observations may be an artifact of work at low temperatures, (Bearden and Malkin, 1974).

Recently, Stemler and Govindjee (1973 and 1974) have

identified bicarbonate ion as a necessary cofactor for the evolution of oxygen. The removal of bicarbonate from chloroplasts is a complex process and perhaps this explains why this effect has been overlooked in the past. Work on chlorophyll fluorescence, delayed light emission and oxygen evolution in flashing light seemed to indicate that bicarbonate acts on the oxidizing side of photosystem II (Stemler, Babcock and Govindjee, 1974) but does not influence the oxygen evolving step. However, the most recent work indicates a site on the reducing side of photosystem II (Wydrzynski and Govindjee, 1975).

The redox reactions and the thermodynamic data described above are summarized as part of the energy diagram of the complete photosynthetic electron transport scheme (figure 1). A brief description of the scheme follows: with the energy provided by the light reaction, electrons produced in the oxidation of water are ultimately donated to the acceptor molecule of photosystem II. The light harvesting process itself will not be discussed here because it is complex and has been well treated in reviews (Sauer, 1975 and Knox, 1975). The primary electron acceptor of photosystem II, usually designated simply as Q, is probably identical to a compound named C₅₅₀ which has been identified by spectroscopy (Bearden and Malkin, 1974). Reducing power is passed from Q to the plastoquinone pool, and this step is thought to be the site of action of the classical inhibitor of photosystem II, DCMU. Finally, electrons are passed by a series of other redox reactions to photosystem I. Here, a second light reaction occurs,

Figure 1

The photosynthetic electron transport chain, showing electron transfer from water to NADP adapted from Bearden and Malkin (1974). Explanation of symbols not appearing in the text: plastocyanine, PC; plastoquinone, PQ; cytochrome *f*, cyt *f*.

which ultimately leads to the production of the strong reductant NADPH, used by the plant in the fixation of CO_2 .

Recently the assumptions on which the previous discussion is based have been challenged by Metzner (1975). In a critical reconsideration of water decomposition in photosynthesis, he concludes that water may not be the precursor for photosynthetic oxygen. His arguments are based mainly on isotope experiments and, although they may have some validity, for the purposes of this discussion, the classical water oxidation mechanism will be assumed until a more detailed alternative to our present view of electron transport is proposed.

2.2 Oxygen Evolution and Photosynthetic Production of ATP

The oxidation of water may be closely connected to the photosynthetic production of ATP.

Gould and Izawa (1974) have shown that one of the coupling sites for photophosphorylation of ADP is associated with the transfer of electrons from H_2O to photosystem II. According to the Mitchell (1969) hypothesis, the development of an electrochemical potential difference of hydrogen ions across the chloroplast membranes is an intermediate step leading to phosphorylation. A large amount of experimental evidence for this view has accumulated (Jagendorf, 1975). Gould and Izawa (1974) have suggested that the water-splitting act establishes the hydrogen ion gradient by releasing the products of water oxidation (O_2 and H^+) into the intrathylakoid space. There is some evidence for their proposal from studies with uncouplers at high pH (Cohn et al. 1975). Most convincing, however, are observations made

if the uncoupler gramicidin is added to make the thylakoid membrane permeable to H^+ . The close parallel between the complex kinetics of H^+ production and O_2 evolution leaves little doubt that the origin of the hydrogen ions is the water-splitting act, (Joliot and Kok, 1975). The fact that these kinetics are not observed for proton liberation when the uncoupler is absent, probably implies that the products of water-splitting are released inside the thylakoid membrane.

2.3 Correlation of Structure and Function

It has long been known that CO_2 fixation occurs in the stroma (unstructured interior) of the chloroplasts, while photosynthetic electron transport occurs in the grana and stromal lamellae (interior membrane structures) of the organelle (Boardman, 1968). Recently there have been great advances in the correlation of biochemical functions to the ultrastructure of these membranes (see Anderson (1975) and Arntzen and Briantais (1975) for reviews). In 1964, Anderson and Boardman were able to isolate fractions of the chloroplast membranes which were enriched in either photosystem I or photosystem II activity. At about the same time, freeze-etch microscopy revealed that particles of different sizes, in particular a $110\text{\AA}$ and a $175\text{\AA}$ particle, can be associated with different faces of the membranes (Park and Sane, 1971). Arntzen et al. (1972) brought these two studies together by demonstrating that the photosystem I fraction shows only the $110\text{\AA}$ particle while the photosystem II fractions reveal the $175\text{\AA}$ particles as well as some $110\text{\AA}$ particles when freeze-etched.

Thus, a model of the photosynthetic membrane has been constructed with photosystem II located in the inner half of the thylakoid and photosystem I situated in the outer half. Studies with a membrane marker, p-diazonium benzene sulphonic acid (DABS), (Giaquinta et al. 1973) and the enzyme lactoperoxidase (Arntzen et al. 1974) confirm this point of view. Obviously, these structural details correlate well with the proposal that the water-splitting act leads to the phosphorylation of ADP, by releasing H^+ into the interior of the thylakoid membrane.

It is possible that the particles are the functional site of Emerson and Arnold's (1932a) photosynthetic unit: the arrangement of a large number of light harvesting molecules around a single reaction center. However, since the premature proposal of the quantosome particle as the site of the photosynthetic unit (Park and Biggins, 1964) this subject has been treated with caution.

2.4 Models of O_2 Evolution

Since we have limited direct information about the mechanism of oxygen evolution, one approach is to study other simpler and better understood reaction systems which produce molecular oxygen. These include electrolysis of water (Hoare, 1968; Wade and Hockerman, 1957; and Conway and Bougault, 1962) and ultraviolet photolysis of water (Chen and Taylor, 1957 and Boyle et al. 1969). In more sophisticated model systems, oxygen has been generated using some kind of catalytic agent and visible light (Metzner and Fischer, 1974, 1975; and Calvin, 1974).

Principally, the study of these model systems seems

to suggest that oxygen is usually generated through intermediate OH-radicals. Initially, it seems that a free radical mechanism is a poor model for the photosynthetic reactions since free radicals are rare in biological systems because of their uncontrolled reactivity. Vierke (1972) has also criticized the hydroxyl radical mechanism on the basis of kinetic experiments. However, some of the recent theoretical models have suggested stabilized radical mechanisms which overcome these objections (Olson, 1970 and Earley, 1973).

2.5 Kinetics

Another approach to the study of oxygen evolution is kinetics, the study of reaction rates. Chlorophyll a fluorescence, luminescence and direct measures of oxygen production are the parameters most commonly followed. Most in vivo fluorescence comes from chlorophyll a in photosystem II, (Papageorgiou, 1975) which fluoresces at 685 nm. This is more likely to occur if excitation energy arriving at the reaction centre can no longer be used in photochemical reactions. Thus, fluorescence can be correlated with the state of the primary electron acceptor of photosystem II, Q, as well as the electron donor to photosystem II. Luminescence or delayed light emission lasts up to several minutes after the light is turned off. Its origin appears to be a back reaction between the reduced primary acceptor, Q^- , and the oxidized primary donor Z^+ (Lavorel, 1975). Both of these methods have been useful in studying the reactions close to photosystem II. Direct measures of oxygen evolution, usually by polarography, yield more

about the reactions associated with the oxidation of water. Early work in this field has been reviewed by Kok and Cheniae, 1966 and Cheniae, 1970. Recently this work has been overshadowed by the studies leading to the Kok model of oxygen evolution.

2.6 The Kok Model

Perhaps the most significant advance of recent years has been the proposal of the four step Kok model, based on observations of oxygen yields from brief, high energy light flashes. Joliot et al. (1968) were the first to report these observations, later confirmed by Kok, Forbush, and McGloin (1970). When chloroplasts were subjected to a dark period of several minutes followed by a sequence of short (10 μ sec) saturating flashes of light at intervals of 0.1 sec., the following yields of oxygen were reported. Little or no oxygen yield from the first two flashes was observed. The largest yield of the entire sequence was obtained from the third flash. Thereafter, the flash yields oscillated with a period of four with the third, seventh, eleventh, etc. flashes yielding the largest amounts of oxygen and the sixth, tenth, fourteenth, etc. producing the minimum yields. However, these oscillations did not continue indefinitely, but were damped out, with the oxygen yields reaching a steady state level after five or six oscillations.

Kok, Forbush and McGloin (1970) have interpreted these results as evidence that four consecutive photoreactions occur before oxygen is produced. The reaction scheme, which is cyclical, is:

(2)

where S_0 , S_1 , S_2 , and S_3 , are the four states of the reaction center. After the absorption of a flash, the reaction center is thought to be promoted to the next state. The O_2 yields in a sequence of flashes can be predicted from this model if, in the dark, the reaction centers deactivate to the S_0 and S_1 states, with 25% in the S_0 state and 75% in the S_1 state. Thus no oxygen will be produced until the third flash and oxygen yields will be highest at every fourth flash thereafter. Furthermore, the damping of the oscillations can be predicted if there is a certain proportion of "misses", α , (the reaction does not proceed despite the absorption of a photon) and "double hits", β , (two photoreactions occur consecutively in one flash). Typical values for α and β which best predict the flash sequence for chloroplasts are 0.1 (i.e., 10% misses) and 0.05 respectively (Forbush *et al.* 1971).

Several other kinetic models have been proposed which also explain the flash sequences (Joliot *et al.* 1968; Mar and Govindjee, 1972; and Delrieu, 1974). The Kok model seems to be the simplest interpretation of the results. It has also become the companion to many of the theoretical molecular models of O_2 evolution (Earley, 1973; Olson, 1970; Mauzerall and Chivas, 1973; and Renger, 1970).

Basically the Kok model has six adjustable parameters (α , β and the dark values of S_0 , S_1 , S_2 , and S_3) to fit the observed flash sequence. Appendix IV describes a computer fit of the Kok model to an experimental flash yield sequence. The complexity of the flash sequence, involving damped oscillations justifies the large number of adjustable parameters to some extent. Several experimental studies show that the best fit values of the parameters are consistent with their proposed physical meaning. For example, the best fit of the starting values of S_0 and S_1 can be changed in a predictable way by the addition of redox compounds to the solution bathing the chloroplasts (Bouges-Bocquet, 1973). Furthermore, work with very short (3 nsec) laser pulses (Weiss et al. 1971) or very long (100 μ sec including tail) xenon flashes (Joliot et al. 1971) has confirmed the interpretation of β . It should be possible to increase the proportion of misses, α , by lowering the intensity of the flashes and Ley, Babcock, and Sauer (1975) have interpreted their results at non-saturating intensities in this way.

Parallel to the oxygen results is the observation of oscillations of flash yields in processes related to oxygen evolution. These include evolution of hydrogen ions (Joliot and Kok, 1975), luminescence (Zankel, 1971 and Joliot et al. 1971) and fluorescence (Deslome, 1971). Babcock and Sauer (1973) have demonstrated that signal II of E.P.R. studies varies in flashing light. They propose that signal II arises from an oxidized radical which is produced by a slow electron transfer to the S_2 and S_3

states of the Kok scheme.

It is interesting to note the discovery of a class of inhibitors whose action can be explained in terms of the Kok model. These compounds greatly accelerate the dark deactivation of the higher states, S_2 and S_3 to the ground states (Renger, 1971 and Renger et al. 1973) and for this reason are called ADRY reagents (acceleration of the deactivation reactions of the water-splitting enzyme, Y).

3. The Experiments Described in this Work

3.1 Introduction to this Work

The approach taken in this work has been to study the dark reactions leading to oxygen evolution, by measurements of oxygen. These reactions are very fast with half times of the order of 1 ms or less and appear to be very complex (Bouges-Bocquet, 1973). Thus, special kinetic techniques must be employed to study them. Conventionally fast reaction sequences in enzymology can be unravelled by such techniques as temperature jump kinetics (Hammes and Schlömmel, 1970) and transient phase kinetics (Laidler and Bunting, 1973). However, these techniques depend on rapid spectroscopic measurements of the concentration of the products and unfortunately, oxygen has no suitable magnetic resonance nor convenient absorption bands in the visible or near ultraviolet region where water is transparent. Perhaps the fastest method available for monitoring oxygen concentration is polarography. However, polarography necessitates the diffusion of oxygen molecules from their site of production within the organism to a detecting electrode (Pt), which leads to a significant

time lag in the measurements.

The two techniques used in the experiments described here manage to deal with these difficulties. The first technique, using the modulated electrode, is similar to the sector method (Caldin, 1964) which has been employed by chemists for some time in their studies of fast photochemical reactions. In their study of the modulated electrode, Joliot et al. (1966) have shown that the effect of diffusion on the oxygen measurements can be predicted and eliminated by use of an elegant mathematical model. In this way, an estimate of the slowest or rate-limiting step of the oxygen evolving sequence can be made. In the second technique, the flash procedure developed by Kok et al. (1970) and Bouges-Bocquet (1973), the organisms are subjected to a carefully timed sequence of brief ($10 \mu\text{sec}$) light flashes. The total yield of oxygen from each flash is measured and should be independent of diffusion. From these yields the time course of the four dark reactions leading to oxygen evolution can be constructed. Both of these techniques were used as tools for investigating the properties of the oxygen-generating reactions. They will be considered in detail below.

3.2 The Modulated Polarograph

The theory for the modulated polarograph, which made possible this study of the rate-limiting reaction of oxygen evolution, is described briefly here. The original polarograph was built in 1960 and is an adaptation of the simple O_2 polarograph of Haxo and Blinks (1950). The modulated polarograph has since become a major tool in

photosynthetic research, since it specifically measures photosynthetic rates of oxygen production with great sensitivity; as little as 10^{-16} moles of O_2 /sec can be detected (Joliot et al. 1966). The basis of this technique is the illumination of the photosynthetic organisms with a light beam, the intensity of which is varied in an approximately sinusoidal fashion. The biological material placed upon the detecting electrode produces waves of oxygen in response to this modulated light. The oxygen diffuses to the platinum electrode resulting in an oscillating flow of current in the polarographic circuit. The current is then amplified with a lock-in amplifier which selects only a narrow range of frequencies around that of the light modulation. In this way, the apparatus selects, amplifies and measures only the photosynthetically-generated oxygen current. Another feature of the apparatus is that the amplitude of the oxygen signal is proportional to the rate of photosynthetic generation of oxygen. Thus this technique yields a different result from that of the Clark electrode where only bulk oxygen concentration is measured.

The Joliot theory for the modulated polarograph takes into account both the diffusion of oxygen and the biological reactions limiting its production. The derivation of the diffusion terms involves the solution of a classical boundary value problem. For the biological reaction, a simple model was considered in which the reaction limiting the rate of oxygen evolution was assumed to be a first order with respect to some photochemically produced intermediate X^*

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$$\therefore \frac{d(O_2)}{dt} = k(X^*) \quad (4)$$

The final equations take the following form:

(a) For a thick (35μ) layer of cells on the electrode:

$$\phi = \frac{\pi}{4} + \frac{\xi\sqrt{\omega}}{\sqrt{2D}} + \tan^{-1}(\omega/k) \quad (5)$$

$$A = \frac{B\psi(o)}{\sqrt{\omega D}} \cdot \exp \frac{-\xi\sqrt{\omega}}{\sqrt{2D}} \cdot \frac{k}{\sqrt{(\omega^2 + k^2)}} \quad (6)$$

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

$$\phi = \frac{p\sqrt{\omega}}{\sqrt{2D}} + \tan^{-1}(\omega/k) \quad (7)$$

$$A = \frac{\psi(p)\Delta p}{D} \cdot \exp \frac{-p\sqrt{\omega}}{\sqrt{2D}} \cdot \frac{k}{\sqrt{(\omega^2 + k^2)}} \quad (8)$$

where ω = angular frequency of light modulation

B = proportionality constant

ξ = 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

$\psi(o)$ = 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
oxygen 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 equation (5) and the first in equation (7) 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 equations (6) and (8) describe the influence of diffusion on the amplitude of the signal while the final factors introduce the influence of a first order thermal reaction

Joliot et al. (1966) were able to demonstrate that their equations (5-8) adequately described the influence of diffusion in a variety of experiments with solutions of different viscosities. At low frequencies of modulation a rate constant of 820 sec^{-1} predicted their phase and amplitude results well. However, at higher frequencies there was some divergence of the theoretical and experimental curves and the value of 820 sec^{-1} represented a compromise fit between the phase and amplitude results. Evaluation of k at 3° and 27° allowed calculation of an activation energy of $7.7 \text{ kcal. mole}^{-1}$. Later, Sinclair (1969) made a second study which indicated that the rate constant was perhaps much higher than first indicated.

At the start of the work reported here, it was felt

that an attempt to estimate k more accurately was worthwhile. In particular, a great deal of additional information about the rate-limiting step could be obtained by making a thorough study of the properties of the rate constant. In early experiments, it was noted that the phase of the oxygen signal was considerably more stable during the course of an experiment than the amplitude, since it is not affected by the viability of the organisms.

For this reason, steps were undertaken to improve the resolution of the phase measurements and these alone were used to estimate k . A considerable amount of evidence was obtained which indicated that this procedure was valid. Thus a wide range of experiments on the rate-limiting step was undertaken with some confidence.

3.3 Temperature Experiments

Transition state theory allows the calculation of several useful parameters from the temperature dependence of a reaction. According to Eyring (1935), the potential energy barrier (or activation energy), E_a , that the reagents must cross in order to form products is related to the rate constant, k , by the equation:

$$k = \frac{kT}{h} \cdot e^{\Delta S^\ddagger / R} \cdot e^{-E_a / RT} \quad (9)$$

where k is the Boltzmann constant

h is Planck's constant

R is the gas constant

$\Delta S^\ddagger$ is the entropy of activation

Thus E_a is readily determined by a plot of $\log k$ vs $1/T$, which is the so-called Arrhenius plot. With values of k

and E_a , the change in entropy as the system proceeds from the initial to the activated state, $\Delta S^\ddagger$, can be calculated.

The Arrhenius plot, E_a and $\Delta S^\ddagger$ are important keys to the mechanism. According to Laidler (1958) the Arrhenius law has been found to be satisfactorily obeyed in all biological systems studied if properly applied to a simple rate constant describing one rate process. Curved Arrhenius plots can result, however, if an apparent rate constant, which describes the steady state behavior of a series of enzymatic steps is plotted. This behavior is seen in the cholinesterase system described by Wilson and Cabib (1956). Thus a plot of $\ln k$ versus $1/T$ for the rate-limiting reaction of oxygen evolution in photosynthesis at a variety of temperatures is an important test of the simple first order model of Joliot *et al.* (1966).

3.4 Deuterium Isotope Experiments

One of the purposes of this investigation was to study the participation of water in the rate-limiting step of oxygen evolution. Obviously, it is impossible to change the concentration of water in a biological system, but its replacement with deuterium oxide (heavy water) can yield information about the water term in the rate equation for the evolution of oxygen by the deuterium isotope effect. Basically, the deuterium isotope effect is a change in rate constant of a reaction when hydrogen is replaced by its heavy isotope, deuterium. In general, the reaction with the heavy isotope usually proceeds much more slowly if a chemical bond to the deuterium atom is broken.

The deuterium isotope effect can be accounted for by

looking at the potential energy surface of the reaction (Wiberg, 1955), in this case the breaking of an OH or OD bond (figure 2). The potential energy surface is the same for the two reactions, but the zero point energy for the OD bond, E_o^D , is lower than the zero point energy for the OH bond, E_o^H . The zero point energy represents the lowest and at room temperature the most heavily (99%) populated vibration energy level. Its value can be calculated for the simple model of bond vibration, the harmonic oscillator, as:

$$E_o = \frac{1}{2\pi c} \cdot \sqrt{\frac{k'}{\mu}} \quad (10)$$

where c is the speed of light

k' is the force constant of the bond

μ is the reduced mass of the bond

Although the force constant is the same for the OH and OD bond, the reduced mass is approximately 1 for the OH bond and 2 for the OD bond. This results in an energy difference of 1400 cal. between E_o^H and E_o^D . This difference in activation energy could produce a k_H/k_D as great as 10.6 at 25°C. However, in practice the ratio is usually in the range 3 to 7. The reduced isotope effect arises from weak bonding in the transition state, reflected in a small value for the force constant of the bond. Thus the equation for the harmonic oscillator indicates that a small difference in zero point energies also exists in the transition state, leading to a reduction of the difference between the activation energies for breaking an OH or OD bond, and a smaller isotope effect.

Figure 2

The potential energy surface for the breaking of OH and OD bonds to illustrate the deuterium isotope effect (from Wiberg, 1955).

The interpretation of the isotope effect should be viewed with some caution since there are processes in which a bond to a deuterium or hydrogen atom is broken but no isotope effect is observed, e.g., the reaction of Grignard reagents with water, and other reactions where bond cleavage does not occur but an isotope effect is observed, eg., the decomposition of chlorosulfites (Wiberg, 1955). However these cases are rare and in general, the breaking of an OH bond leads to a deuterium isotope effect. Thus we might expect to see an isotope effect in the water-splitting reactions of photosynthesis.

3.5 Experiments in a Changed Chemical Environment

Since chloroplasts might be expected to be more responsive to changes in their environment than whole Chlorella cells, these organelles were investigated under a variety of conditions to see if any of these would have an effect on the rate constant. An investigation of the effect of uncouplers was suggested by the recent experiments on photophosphorylation discussed above. At Govindjee's suggestion, an attempt was made to see whether the bicarbonate effect was operating on the rate-limiting reaction, since its site of action is unclear. Sinclair's (1972) discovery of phase changes of the modulated oxygen signal in different ionic environments led to a more complete investigation of this phenomenon. Finally, the effect of oxygen concentration on k was monitored, since it is the reaction product, and may be involved in allosteric mechanisms.

3.6 The Effect of Deuterium Oxide on the Dark Reactions of the Kok Scheme of Oxygen Evolution

The objective of these experiments was to locate that part of the Kok model in which OH bonds were split. In the Kok scheme each light step occurs in about 10^{-15} sec, after which there follows a dark process (molecular rearrangement) with a half time which can range from 10^{-3} to 10^{-4} sec (Bouges-Bocquet, 1973). This more complex interpretation of the model may be written:

Although Kok *et al.* (1970) have made an initial study of some of the dark reactions, their work has been greatly extended by Bouges-Bocquet (1973) (see also Bouges, 1971). In the study presented here each of the four reactions was studied in H_2O and D_2O , since we might expect to see a slower rate in D_2O if OH bond breaking were involved.

In order to determine the time course of the dark reactions special sequences of flashes are required. Let us consider the generalized reaction $S_n^* \longrightarrow S_{n+1}$. We assume that S_n^* (the photolysed product of the preceding light reaction) is photochemically inactive until it has undergone dark rearrangement to S_{n+1} . Thus, if the $n + 1^{th}$ flash follows the n^{th} flash at a time interval Δt , we can

estimate the proportion of centres which have already converted to the S_{n+1} state, $\gamma_n(\Delta t)$, as a function of the flash yields of oxygen (see below). The plot of $\gamma_n(\Delta t)$ versus Δt , then, gives us the time course of the reaction

Bouges-Bocquet has derived approximate equations which relate $\gamma_n(\Delta t)$ to the flash yields of oxygen in special flash sequences. In this method, she considers a time interval of 0.3 sec sufficient for any of the dark reactions to reach completion and except where indicated otherwise, this is the interval between flashes. Also to estimate $\gamma_0(\Delta t)$ and $\gamma_3(\Delta t)$, the initial dark ratios of S_1/S_0 must be varied by the addition of redox reagents to the medium or by means of a special pre-illumination technique. $\gamma_n(\Delta t)$ indicates the yield of oxygen from the n^{th} flash, when one of the flash intervals is Δt . The first, second, third... flashes in a sequence are represented by the numbers 1, 2, 3... The Bouges-Bocquet scheme is summarized as follows:

Special conditions: 0.1 mM reduced DCPIP or pre-illumination:

5 min dark $\longrightarrow$ 1 $\longrightarrow$ 2 $\longrightarrow$ 3

Flash sequence: 5 min dark $\longrightarrow$ 1 $\xrightarrow{\Delta t}$ 2 $\longrightarrow$ 3 $\longrightarrow$ 4

$$\gamma_0(t) = Y_4(\Delta t)/Y_4(0.3 \text{ sec}) + (Y_3(\Delta t) - Y_3(0.3 \text{ sec}))/Y_4(0.3 \text{ sec}) \quad (12)$$

Special conditions: none

Flash sequence: 5 min dark $\longrightarrow$ 1 $\xrightarrow{\Delta t}$ 2 $\longrightarrow$ 3

$$Y_1(\Delta t) = Y_3(\Delta t) / Y_3(0.3 \text{ sec}) \quad (13)$$

Special conditions: none

Flash sequence: 5 min dark $\longrightarrow$ 1 $\longrightarrow$ 2 $\xrightarrow{\Delta t}$ 3

$$Y_2(\Delta t) = Y_3(\Delta t) / Y_3(0.3 \text{ sec}) \quad (14)$$

Special conditions: 0.1 mM ferricyanide

Flash sequence: 5 min dark $\longrightarrow$ 1 $\longrightarrow$ 2 $\longrightarrow$ 3 $\xrightarrow{\Delta t}$ 4 $\longrightarrow$ 5 $\longrightarrow$ 6 $\longrightarrow$ 7

$$Y_3(\Delta t) = a \cdot \frac{Y_7(\Delta t) - Y_7(0.3 \text{ sec})}{Y_7(0.3 \text{ sec}) - Y_7(0 \text{ sec})} + b Y_1(\Delta t) + c Y_2(\Delta t) \quad (15)$$

where a, b, c are constants

Bouges-Bocquet's experimental study has shown that the time course of these reactions is complex. Only the S_3^* S_0 step shows simple exponential kinetics and can be considered to be a first order reaction. The half time for this reaction is 1.2 msec at 20°C. The first two dark steps show similar non-exponential kinetics and Bouges-Bocquet has suggested that they share a common mechanism. On the basis of his spectroscopic studies, Döring (1975) has proposed that these steps are rate-limited by the reduction of the oxidized reaction centre (chl a ^{II} in his terminology). Bouges-Bocquet indicates that the first three dark reactions show complex temperature dependence

in addition to non-exponential kinetics and probably involve consecutive or parallel reaction schemes. These reactions are faster with half times of 0.2 to 0.5 μ sec.

In the experiments reported in this work the involvement of water in the dark reactions were studied. According to the model, only the $S_3^* \longrightarrow S_0$ reaction evolves oxygen. It has been proposed (Joliot and Kok, 1975) that this step is also the water-splitting reaction, since the oscillation of H^+ yields in flashing light strongly suggests the release of H^+ and O_2 in one final water-splitting act. Hence, this step should show a large deuterium isotope effect. On closer examination of the H^+ yields, an abnormally high yield is observed on the second flash. This is consistent with the release of one proton during the third dark process, $S_2^* \longrightarrow S_3$ and three times more during the final dark step. Thus the $S_2^* \longrightarrow S_3$ step may also show an isotope effect. Another possibility is that oscillation of the H^+ yields may have its origin in some other process than water-splitting. Thus, water-splitting could occur in any of the first three dark processes as long as oxygen is not evolved until the final dark reaction. For example:

where $n = 0, 1, 2$

Thus an investigation of the effect of deuterium oxide in all of the dark processes is an important test of the water-splitting mechanism.

11. MATERIALS AND METHODS

1. Culturing of Chlorella

Cultures of Chlorella vulgaris and Chlorella pyrenoidosa were obtained from the Carolina Biological Supply Co.. In early experiments the algae were grown in Warburg-Buck medium with constant aeration and natural lighting. However, since photosynthetic behavior can vary depending on the maturity of the culture and its previous growth conditions (e.g., light, CO₂ supply and temperature), a device was constructed to grow Chlorella under controlled conditions as described by Myers (1944). Three litres of Chlorella vulgaris culture were grown in a four litre bottle. The bottle was immersed in a water bath which was thermostated at $25^{\circ} \pm 0.5^{\circ}\text{C}$, the optimum for growth of Chlorella (Starr, 1971). Since the algae tended to settle, agitation was maintained by a stir-bar placed in the bottle with a magnetic stirrer under the water bath. Compressed air was used to aerate the culture but it was first passed through distilled water in a gas washing-bottle. The flow rate was monitored with a Brooks-Mite gas flow meter and maintained at a constant rate of 1.0 cubic foot per hour (28 l/hr.). The culture was illuminated by a Westinghouse 300 w floodlamp, which was placed 30 cm from the bottle and provided approximately 1200 foot-candles of illumination at the surface of the culture. Although Myers (Myers, 1944, and Phillips and Myers, 1954) suggests an automatic apparatus for diluting the culture, this method was found to be too complicated. Instead, the culture was diluted on a regular basis with fresh medium to maintain an

approximately constant population density (1.4×10^7 cells/ml, as measured with a hemocytometer). The optical density of the culture at 415 nm was chosen as a convenient index of population density for the dilution procedure. The specific growth rate of the culture, k^* , can be calculated according to the formula:

$$k^* = \frac{1}{N} \cdot \frac{dN}{dt} \quad (17)$$

where N is the number of cells in the culture and dN/dt is the daily increment in cell number (Phillips and Myers, 1954). The value of k^* for the culture was reasonably constant over a period of several months and had a value of 0.5 day^{-1} , indicating an actively reproducing culture which increased its population daily by 50%. This value of k^* is within the range reported in Myers' experiments (Myers and Graham, 1971; Phillips and Myers, 1954).

2. Chloroplast Preparation

Several methods of chloroplast preparation were used in this work. In some experiments, whole chloroplasts were prepared according to the method of Schwartz (1966) and broken chloroplasts were prepared according to MacSwain and Arnon's procedure (1968). However, greatest oxygen production was observed when a modified method of Cockburn et al. (1968) was used and this preparation became the standard one in most experiments.

In this procedure leaves of three-to-four-week-old spinach, Spinacia oleracea which had been grown in a controlled environment chamber were harvested, washed and had the midribs removed. The fresh material (25 gm) was

ground for 3-4 sec at high speed in a Waring blender. The grinding medium (100 mls) contained 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 0.33 M sorbitol, 5 mM MgCl_2 and 2 mM sodium isoascorbate. The latter compound was added after adjustment of the pH with HCl to 6.5 at 0°C. After grinding, the macerate was filtered through 8 layers of cheesecloth and in addition, one layer of fine mesh nylon, then centrifuged from rest to 4000 xg and to rest again in approximately 90 sec. The supernatant was discarded and the pellet was resuspended in a medium which contained 20 mM HEPES-NaOH buffer, pH 7.6, 0.33 M sorbitol, and 1.0 mM MgCl_2 . The chlorophyll concentration of the fresh preparation was assayed according to the method of Vernon (1960) and the preparation was diluted to the appropriate concentration (normally to a chlorophyll concentration of 0.5 mg/ml). The final chloroplast suspension was stored in the dark on ice until needed. All of the procedures with chloroplasts described above were performed at 0°C.

When the preparation was examined with a microscope under a 100x oil immersion objective, the chloroplasts appeared to be intact and free of large contaminating debris. The high activity of these chloroplasts is probably due to their rapid and relatively gentle isolation. It is entirely possible that preparation contained mitochondria which would have absorbed oxygen but the system used in these experiments for measuring photosynthetic O_2 evolution is very specific and is not influenced by respiratory O_2 uptake.

3. The Modulated Polarograph

3.1 Introduction

The modulated polarograph described here is similar to that of Joliot (Joliot et al. 1966; Joliot, 1972) but differs in details of the polarographic cell and instrumentation. The apparatus described below consisted of four parts: (a) a polarographic cell which held the photosynthetic material and detected oxygen production, (b) equipment for changing the medium which surrounded the preparation, (c) a modulated light source and associated optical equipment, and (d) a measuring circuit to amplify and analyse the modulated oxygen signal. Each of these is described in detail below, along with the procedure in a typical experiment.

3.2 The Polarographic Cell

In figure 3 a schematic cross-section of the polarographic cell is shown, of which more details of the assembly and exact dimensions are shown in appendix II. The cell was constructed principally of lucite plastic and had a window on top to admit the light beam. Internally, it was divided into three compartments by two dialysis membranes.

The lower compartment was the most important element of the polarographic cell. It held the photosynthetic material and contained the oxygen-detecting electrode (the cathode). This electrode was made of shiny platinum, which was machined to an even surface that covered most (2 x 10 mm) of the bottom of the chamber. The chloroplasts or Chlorella were injected into the lower compartment through

Figure 3

Schematic diagram of the polarographic cell.

fine holes drilled in the cell and were allowed to settle by gravity onto the platinum electrode. Since the oxygen signal was very sensitive to the thickness of the layer of Chlorella or chloroplasts, a reproducible method of depositing a given thickness of cells on the electrode was necessary. The biological material was centrifuged at 1000 xg for two minutes in a graduated centrifuge tube. Knowing the packed cell volume as a fraction of the total volume of the suspension and also the dimensions of the lower compartment of the polarographic cell, it was possible to calculate the thickness of the layer of biological material once it had settled by gravity onto the electrode. For example, a suspension whose volume was 10% cells settled in the lower compartment which was 350 μ deep to form a 35 μ layer of cells.

The middle compartment of the polarographic cell served as a conduit for fresh medium which diffused through the dialysis membrane to bathe the photosynthetic material in the lower chamber. The upper chamber contained the Ag/AgCl electrode, a coiled silver wire electrochemically coated with AgCl, which functioned as the current carrying anode. This compartment was bathed with solution from which was omitted compounds that adversely interacted with the electrode (especially ferricyanide).

3.3 Perfusion of the Photosynthetic Material in the Polarograph

As described previously, buffer flowing through the central compartment of the polarographic cell constantly supplied the material lying on the platinum electrode with fresh medium. This solution was held in a glass bottle and

was fed by gravity through tygon tubing to the polarographic cell. A special tap allowed the selection of several different media. Fine control of the flow rate was achieved by a second tap placed after the cell. Flow rates of about one ml/min were maintained to avoid disturbing the photosynthetic material on the electrode.

Chlorella were perfused with a medium containing 100 mM KCl and 50 mM potassium phosphate buffer, pH 6.4 (after Joliot et al. 1966). Chloroplasts which were not provided with an electron acceptor and which were presumably fixing CO_2 , were perfused with a medium that contained 20 mM HEPES-NaOH buffer, pH 7.6, 0.33 M sorbitol, 1 mM MgCl_2 , 5 mM NaHCO_3 , and 100 mM KCl (medium A). In experiments with chloroplasts where the signal was improved by providing NADP as an electron acceptor, the medium contained 20 mM HEPES-NaOH buffer, pH 7.6, 1 mM MgCl_2 , 1 mM NADP and 100 mM KCl (medium B). In the latter case the chloroplasts were enriched with ferredoxin (0.4 mg/mg of chlorophyll) before injection into the apparatus. The ferredoxin (type III) and HEPES were obtained from the Sigma Chemical Corporation (St. Louis, Mo.).

3.4 The Optical Arrangement

The diagram in figure 4 shows the arrangement of the optical equipment used in these experiments. The light source for the modulated polarograph was a 500 W tungsten-halogen lamp (model Q/CL, Westinghouse Electric Corp., Bloomfield, N.J.) which was provided with a variable AC voltage from a variac. Since the lamp generated a considerable amount of heat, it was cooled by a flow of water passed

Figure 4

Schematic diagram of the optical arrangement of the modulated polarograph.

through copper coils wrapped around the lamp base. To ensure diffuse, even illumination, frosted glass was placed in front of the window of the lamp housing. The spectral quality of the light used in these experiments was determined with a spectroradiometer (model S.R., Instrumentation Specialties Company, Lincoln, Nebr.) and is shown in figure 5. Most of the radiation falls in the red end of the spectrum which is the region most efficiently used in photosynthesis. Routine measurements of global light intensity were made with a Yellow Springs Instruments Corp. radiometer (Yellow Springs, Ohio).

Modulation of the light beam was achieved by passing it through a light chopper (model 222, Princeton Applied Research, Princeton, N.J.). The design of the optical arrangement was such that the intensity of the light beam increased and decreased in an approximately sinusoidal fashion. A large lens ($f = 17.5$ cm) was used to focus a parallel beam of light through the light chopper and optical filters. These included a Corning infra-red filter (no. 4602) which was routinely used in these experiments. The intensity of the light could be attenuated with Kodak neutral density filters and in some experiments monochromatic radiation was obtained with Corning interference filters. A second lens ($f = 30.5$ cm) was used to bring the image of the filament of the lamp into focus at the platinum electrode of the polarographic cell, providing illumination of high intensity at this point.

3.5 The Measuring Circuit

The measuring circuit of the modulated oxygen

Figure 5

The relative intensity of the radiation used in the modulated polarograph versus wavelength. The light source was a Westinghouse 500 W lamp (model O/CL) provided with an AC voltage of 95 V. The spectrum shown here is that of the light which had passed through a Corning infra-red filter.

polarograph is shown in figure 6. Routinely the platinum electrode was polarized to -0.70 volts with respect to a calomel electrode placed in the bathing medium. When the platinum electrode was maintained at this potential the cell acted as an oxygen polarograph, i.e., the current which flowed through the cell was proportional to the concentration of O_2 in the compartment containing the photosynthetic organisms. The latter responded to the modulated light by producing waves of O_2 which in turn caused a fluctuating current to flow in the measuring circuit. This was preamplified by a transformer (Princeton Applied Instruments, Princeton, N.J., model 190 low noise transformer) before the signal arrived at the vector-voltmeter (Princeton Applied Instruments, model 129 lock-in amplifier and vector-voltmeter). This instrument selectively amplified and measured the amplitude of the modulated current in a narrow band of frequencies around the same reference frequency used to drive the light chopper (generated by a model F33 function generator, I.E.C., Anaheim, Ca.). Simultaneous with the amplitude measurements, the vector-voltmeter determined the phase lag of the modulated current behind the reference frequency. The output of the phase and amplitude channels of the vector-voltmeter were fed into an Omniscribe chart recorder (Houston Instruments, Bellaire, Texas), allowing long term monitoring of photosynthetic activity.

A final aspect of the design of this circuit was the elimination of 60 Hz noise, which was readily picked up by the high impedance polarographic cell. With careful

Figure 6

Schematic diagram of the modulated polarograph, described
in detail in the text.

shielding and grounding throughout the circuit, the noise could be kept to acceptable levels.

Accurate measurement of the phase and amplitude of the modulated oxygen current was the objective in the design of this circuit. The phase measurements at different frequencies of modulation were the basis of the determination of k and explain the choice of the PAR model 129 vector voltmeter/lock-in amplifier and the PAR model 222 light chopper. Other lock-in amplifiers, such as the PAR model HR-8 used by Sinclair (1969), have a tuned amplifier which introduces considerable phase error if the frequency trim is not accurately adjusted before each measurement. In addition, the model 222 light chopper has exceptional stability of operation achieved through the feedback circuitry, so that phase measurements with the vector-voltmeter are not limited by slight fluctuations in the frequency of light modulation.

Reactive elements in the measuring circuit introduce an additional phase lag and amplitude attenuation of the signal which is not described in equations of the Joliot model. To estimate these, phase and amplitude measurements were made with a photodiode replacing the polarographic cell. The photodiode responded with a 20 μ sec rise-time to a xenon flash of 7 μ sec duration. This can be considered an indicator of a virtually instantaneous response to the frequencies used in these experiments. To make measurements with the photodiode, the photoactive element was placed as close as possible to the normal position of the platinum electrode. Electrical connections were made in parallel

to the polarographic cell, in order to add the electrochemical capacitance of the electrodes to the circuit. Phase and amplitude measurements made using the rapidly responding photodiode showed all the influences of the reactive elements of the normal measuring circuit except those due to the biological production of O_2 and diffusion of O_2 . The phase measurements made with the photodiode were subtracted from the phase measurements made using the polarographic cell and Chlorella or chloroplasts to yield the phase of the O_2 signal as described by equations (5) and (7). Similarly, the true amplitude of the O_2 signal was determined by dividing amplitude measurements made with the polarographic cell, by the amplitude of the photodiode signal.

It was estimated that a phase measurement could be repeated with a precision of $\pm 1^\circ$ with Chlorella or $\pm 2^\circ$ with chloroplasts, which generally produced a smaller, noisier O_2 signal. However, because of difficulties in positioning the photodiode, measurements of this device introduced a constant error which in any given experiment could have been as high as $\pm 5^\circ$. Allowance for this was made by having a floating intercept at zero frequency, ϕ_0 , in the computer fit of the phase result.

3.6 Typical Experimental Procedure

After injection of the organism into the polarographic cell, the phase and amplitude of the modulated oxygen signal were monitored at 25 Hz until a steady signal indicated that the organisms had settled on the platinum electrode (about 10 - 20 min.). With some practice one

set of phase measurements at 15 to 20 different frequencies could be made in about 30 minutes. These formed the basis for one computer determination of k . With one sample of Chlorella it was possible to make five or six determinations of k interspersed with changes of solutions or temperatures in between. With chloroplasts only two to three determinations were attempted with any one sample because of declining viability of the organelles. At the end of the biological experiments, phase and amplitude readings were taken with the photodiode. Plots of ϕ versus $\sqrt{\nu}$ were prepared and the rate constant was estimated from these as described below.

Estimate of Rate Constant by Computer

The model based on oxygen 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 $\frac{\xi}{\sqrt{2D}}$ or $\frac{p}{\sqrt{2D}}$ and the phase at zero frequency, ϕ_0 , to calculate phase values according to equations (5) or (7) and then calculated the sum of squares of the deviations, between the theoretical and experimental values. This sum was minimized by an interactive procedure in which k , $\frac{\xi}{\sqrt{2D}}$ 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 program also provided the standard deviation of the phase results from the best fit values. A sample program is given in appendix II.

Temperature Experiments

Most early experiments were performed at room temperature (22°C) but in the ~~temperature~~ study of 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, Brinkman Instruments, Westbury, N.Y.) 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 $\pm 0.2^{\circ}\text{C}$. Once the temperature had stabilized a normal survey of the effect of modulation frequency on current amplitude and phase was performed. Such experiments were carried out at various temperatures between 8°C and 42°C .

6. Kinetic Isotope Experiments

For the kinetic isotope experiments, a deuterated perfusion medium was prepared by freeze-drying the basic water based medium. The dried chemicals were redissolved in D_2O and freeze-dried a second time, before final dissolution in D_2O . The organisms were perfused for about an hour in D_2O before measurements of the O_2 signal were taken. They were then perfused with an H_2O based medium for an hour before measurements in H_2O were made. The D_2O contained 99.8% of the heavy isotope and was obtained from I.C.N. (Irvine, Ca.) and Stohler Isotope Corporation (Rutherford, N.J.).

7. Diffusion Experiments

An original procedure for a physical measurement of the temperature dependence of the diffusion coefficient of oxygen was devised and is described here. (These independent measurements were undertaken for the purpose of comparison with the values obtained with Chlorella.) The method was based on the diffusion of oxygen from the central polarographic cell to the platinum electrode. This is essentially a one-dimensional diffusion problem, for which Crank (1956) has derived the equations. The circuit for the oxygen measurements is shown in fig. 7.

Here the cell was used as a simple oxygen polarograph, where the current flowing through the circuit was proportional to the oxygen concentration in the vicinity of the platinum electrode. The oxygen current was monitored with an oscilloscope (model R5031, Tektronix Instruments, Beaverton, Ore.) as the potential difference across the $2000\ \Omega$ resistor. To ensure that this relation was valid, a simple electronic feedback device was used to maintain the polarization of the Pt electrode at -0.7 V . At the start of an experiment, the cell was perfused rapidly ($\sim 20\text{ ml/min}$) with a buffer solution which had been depleted of O_2 by vigorous bubbling with N_2 . After a stable recording of current flowing through the cell was obtained, a tap was turned and the anaerobic buffer was replaced with a well aerated one. The time course of build up of current flowing through the cell was recorded on the oscilloscope as O_2 diffused from the middle compartment, through the lower compartment to the platinum electrode.

Figure 7

The simple resistive circuit for making measurements of oxygen concentration in the polarographic cell. When the platinum electrode was polarized to -0.7 V with respect to a standard calomel electrode the voltage across the $2\text{ K}\Omega$ resistor was proportional to the oxygen concentration in the vicinity of the platinum electrode.

The exact time of changeover was marked by introducing a small air bubble between the anaerobic and aerobic media, which caused a slight electrical disturbance in the O_2 current recording at time zero. Finally the diffusion coefficient of O_2 in buffer was computed from the time course of the oxygen current recording. The experiment was repeated at several temperatures.

8. Effect of Salts, Uncoupler, pH, O_2 and CO_2 Concentration

on the Rate Constant

The effect of salts, uncouplers, pH, CO_2 and O_2 concentration were monitored by perfusing one sample of chloroplasts with one of the standard medium and then with an altered medium. Special compressed oxygen/nitrogen mixtures were obtained from Linde for the O_2 experiments. As CO_2 is tightly bound to the chloroplasts, special CO_2 -free chloroplast preparations were made as described by Stemler and Govindjee (1973, 1974). Their procedure involved suspending chloroplasts in a decarbonating medium containing 250 mM NaCl, 40 mM sodium acetate, and 50 mM sodium phosphate buffer, pH 5.8. The suspension was maintained in the dark at $17^\circ C$ and was bubbled with CO_2 -free air for 20 minutes. Decarbonated chloroplasts were stored on ice until needed in screw cap tubes. CO_2 -free medium for the experiment was prepared by bubbling ordinary medium B with CO_2 -free air.

9. Flash Experiments

Flash polarography seeks to measure the yield of O_2 produced by photosynthetic organisms in response to short saturating flashes of light. Besides the two fundamental

articles on the subject, Joliot et al. (1968) and Kok, Forbush and McCloin (1970) a more recent review is given by Joliot and Kok (1975).

9.1 The Flash Lamp

A considerable amount of material has been written about the choice and design of flash lamps and associated circuitry (Markiewicz and Emmett, 1966 and Porter, 1963), the problem being to combine a short flash duration with maximum light output. In these experiments, short saturating flashes were obtained by discharging a 4 μ F capacitor charged to 1000 V through an FX-127 flash tube (EG & G, Salem, Mass.). The time course of the flash is shown in fig. 8 which demonstrates a 7. μ sec flash duration at $I_{\max}/3$. This duration was judged short enough to separate the photo-reactions leading to photosynthetic oxygen evolution (Weiss et al. 1971). The trace was obtained by using a fast-responding photovoltaic device in parallel to a 15 Ω resistor placed across the inputs of a storage oscilloscope (141 A oscilloscope with 1402 amplifier, Hewlett-Packard, Loveland, Colorado) with high frequency response (20 MHz). Triggering of the flash tube was provided by an FY-5G "lite-pak module" (EG & G., Salem, Mass.).

9.2 Flash Controller

A special flash controller was designed and built by Carleton Science Workshops to produce the sequence of flashes required in these experiments. The flash controller could initiate up to 99 flashes at intervals of 0.1 sec to 9.9 sec. One of the first ten flashes could be made to

Figure 8

The relative intensity of the xenon flash used in these experiments versus time. The flash was obtained when a 4 μ F capacitor charged to 1000 V was discharged through an EG & G. FX-127 flash tube.

follow the preceding flash by a time interval Δt . This parameter could be varied from 0.1 msec to 9999.9 msec in steps of 0.1 msec. Since the condenser of the flash lamp cannot recharge in the shorter intervals, the special flash which follows Δt was produced by a second flash lamp and condensor. In appendix III a block diagram and a circuit diagram for the flash controller are given.

9.3 Optical Arrangement

The two flash lamps were mounted on either side of the O_2 cell and large, powerful ($f = 10.5$ cm) lenses were used to focus the lamps on the platinum electrode. As before, the polarographic cell could be kept in total darkness (prior to the flash sequence) by closing the shuttered opening to the metal housing which surrounded the cell.

9.4 Measurement of O_2 Flash Yields

Both Kok's group (Kok, Forbush and McGloin, 1970) and Joliot's group (Joliot et al. 1971) have developed a special technique using the modulated polarograph to measure the yield of photosynthetic O_2 produced by Chlorella or chloroplasts in response to a brief flash of light. This technique has been further refined by Delrieu (1974). However the procedure used in these experiments was a simpler one, similar to that developed by Babcock and Sauer (1973). The measuring circuit was essentially the simple one shown in fig. 6. A fast responding chart recorder, the Techni-rite model 722 (East Greenwich, R.I.), was used to record the sharp O_2 spikes produced in response to the light flashes. As suggested by Duysens (1972), the height of each spike was used as a relative measure of the O_2

produced by each flash.

9.5 Computer Fit of the Kok Model to Flash Yield Sequence

The Kok model was fitted to the experimentally determined O_2 flash yield sequence in a least squares technique similar to that used to fit the equations for the modulated polarograph to the phase results. The parameters of the Kok model which were minimized were α , β , and the values of S_0 , S_1 , after dark deactivation. Appendix IV contains a sample program.

III. RESULTS

1. The Modulated Polarogram

A preliminary study of the response of the modulated O_2 signal from Chlorella to the voltage applied to the platinum electrode was undertaken for two reasons: first, to determine a suitable working voltage and second, to determine the dependence of the phase of the signal on the applied voltage which had not been described previously. The results of this study are shown in figure 9a and 9b, where the amplitude and phase of the O_2 signal are plotted against the voltage applied to the Pt electrode. The amplitude results are of the characteristic polarographic form described by Joliot et al. (1966) and reach a plateau in the -0.6 V to -0.9 V range. The phase graph shows that in the same voltage region, the phase reaches a limiting value and becomes independent of the biasing voltage. Results of a similar form to Fig. 9a and 9b were found at a variety of temperatures and frequencies of modulation. Because of the variable onset of H_2 evolution (at -0.8 V to -1.0 V), a biasing voltage of -0.7 V was chosen for the experiments which follow.

2. Effect of Light Intensity

The response of the phase of the modulated oxygen signal to light intensity is an important indication of the reaction order of the rate-limiting step of oxygen evolution. The Joliot model predicts that the phase is independent of intensity in a first order reaction mechanism but varies with intensity in higher order mechanisms. Figure 10a demonstrates that the phase shows little change even at

Figure 9

The modulated polarogram. The phase and amplitude of the modulated O_2 signal from a thick layer of Chlorella was plotted against the voltage applied to the platinum electrode (measured with respect to a standard calomel electrode). The modulation frequency was 16 Hz and the temperature $30^\circ C$. The dotted line in b describes a region of unstable amplitude due to H_2 evolution.

Figure 10

The phase and the amplitude of the O_2 signal at 16 Hz obtained with a monolayer of chloroplasts plotted against the intensity of the modulated 650 nm light. The dotted line indicated the region where high light intensity was inhibitory to photosynthesis.

high intensities where the light begins to inhibit photosynthesis.

Figure 10b demonstrates the response of the amplitude of the modulated oxygen signal to the light intensity. Since a signal as large as possible is desirable and the Joliot theory is applicable only to the range where amplitude is directly proportional to intensity, a working intensity in the upper part of this range was selected. This calibration procedure was repeated routinely for Chlorella and chloroplasts, different thicknesses of biological material and light of different spectral quality.

3. The Value of k at 23°C

The results of a typical experiment with the modulated polarograph which made possible the determination of k is shown in figure 11. Here the phase lag of the oxygen signal with respect to the modulated light source was plotted against the square root of the frequency of modulation for a thick (35μ) layer of Chlorella. The line in figure 11 represents the best fit of the Joliot equation (1) to the experimental points. For 22 experiments at 23°C, a mean value for k of 305 ± 20 (S.E.) sec^{-1} was obtained, with extreme values of 160 sec^{-1} and 510 sec^{-1} . The results obtained with Chlorella vulgaris and Chlorella pyrenoidosa were similar.

Since much of the work which follows was based on the determination of k by the curve-fitting method, it is important to examine the validity of this procedure. As illustrated in figure 11, the best fit of equation (5) gave a good description of the experimental results at all

Figure 11

- (a) The phase lag between the current and the light modulation plotted against the square root of the modulation frequency (closed circles), obtained with a 35 μ layer of Chlorella. The solid line represents the curve generated by the equation

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

$\xi = 3.0 \times 10^{-4}$ cm and $\phi_0 = 50^\circ$. 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 $x = A\sqrt{v}$ and for the closed symbols $x = \frac{A\sqrt{v}\sqrt{k^2 + \omega^2}}{k}$ with $k = 330 \text{ sec}^{-1}$; both are plotted against $\sqrt{v}$. 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 ξ .

frequencies, with a standard deviation (S.D.) between the theoretical and experimental points of 2.3° . All the experiments reported here had an S.D. of 3° or less, and frequently very good fits were obtained as in figure 13 where an S.D. of 0.8° was calculated. The value of ϕ_0 (the phase intercept at zero frequency) computed from the results in figure 11 was 50° , which is close to the theoretically predicted value of 45° . The computer fit yielded a value of ξ (the distance from the Pt electrode to the closest oxygen sources) of 3.0μ with $D = 2.0 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$. This distance is about half the average cell diameter (6μ) which might be considered an approximation to ξ . Finally, the value of k obtained by computer also gave a good description of the amplitude results obtained with the same sample of Chlorella. Equation (6) predicts the linear relationship between $A \cdot \sqrt{\nu} \cdot \sqrt{k^2 + \omega^2} / k$ and observed in figure 11 even at low frequencies. The value of ξ could again be calculated from the slope of the upper line in figure 11. The figure obtained is close to the value determined from the phase results, 3.0μ . A similar analysis could be successfully applied to the thin layer results (see, for example, figure 13). In conclusion, the preceding observations indicated that the computer fit of the phase equations to the experimental results yielded meaningful data.

4. Experiments in D_2O

In figure 12, the dependence of ϕ on $\sqrt{\nu}$ for a 6μ layer of Chlorella at 22°C is shown in deuterium oxide and water based media. The rate constant in D_2O was calculated

initially from the results in Figure 2 as 232 sec^{-1} with an S.D. between the experimental and theoretical values of 1.3° . After $1\frac{1}{2}$ hours in D_2O another experiment was undertaken with nearly identical results. This time k was 230 sec^{-1} and the S.D. was 1.2° . After perfusion with H_2O buffer for one hour, the rate constant, as calculated from the results in figure 12 was 289 sec^{-1} with an S.D. of 0.8° . The results with four different samples of algae gave $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 1.29 \pm 0.05$ (S.E.M.). This ratio is much lower than would be expected for OH bond breaking.

Because D_2O is slightly more viscous than H_2O , diffusion is slower and the phase of the oxygen signal for a particular frequency has a higher value. A calculation based on the assumption that ξ has the same value in H_2O and D_2O gave 1.3 ± 0.1 (S.E.M.) for the ratio of the diffusion coefficients in H_2O and D_2O based buffers. This figure can be compared with that obtained by independent physical measurements on water and deuterium oxide. (There is probably little difference in the ratio $D_{\text{H}_2\text{O}}/D_{\text{D}_2\text{O}}$ for buffer or pure solvent.) Taking values of the viscosity of water and deuterium oxide from Katz and Crespi (1970), 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 $D_{\text{H}_2\text{O}}/D_{\text{D}_2\text{O}}$.

5. 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 figure 13a. A least squares line is

Figure 12

The polarographic results when D_2O is substituted for water in the experimental medium, for a 6μ layer of Chlorella vulgaris at $22^\circ C$. The open circles are the phase values obtained shortly after suspension of the algae in D_2O based buffer. Virtually the same results were obtained $1\frac{1}{2}$ hours later. After one hour in water based solution, the phase values represented by the closed circles were obtained. The upper line is the best fit curve for D_2O , generated for $k = 232 \text{ sec}^{-1}$, $p/\sqrt{D} = .136 \text{ sec}^{-\frac{1}{2}}$, and $\phi_0 = 0^\circ$. 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 $k = 289 \text{ sec}^{-1}$, $p/\sqrt{D} = .129 \text{ sec}^{-\frac{1}{2}}$, $\phi_0 = 0^\circ$ and the S.D. = $.8^\circ$.

drawn through the points, from which an activation energy, E_a of $5.2 \text{ kcal. mole}^{-1}$ was calculated for this experiment. The mean of the activation energies for 14 experiments was 5.9 ± 0.5 (S.E.M.) kcal. mole^{-1} . In the temperature range studied (8°C to 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 figure 13b, a plot of $\ln D/\xi^2$ vs. $1/T$ is shown, which was computed from the phase results of the experiment described in figure 13a. The results are again fairly linear. Eyring and Olander have developed an activated rate process analysis of diffusion, although the concept is not as well developed as for chemical rate processes. This, combined with the experimental observation of linear Arrhenius plots for many diffusion processes over a modest temperature range allows us to talk of an activation energy for diffusion (Himmelblau, 1964). For the Arrhenius plot in figure 13b, the activation energy for diffusion of oxygen in buffer (E_D) is $6.0 \text{ kcal. mole}^{-1}$. The mean for fourteen experiments was 5.7 ± 0.3 (S.E.M.) kcal. mole^{-1} .

Using values of k from the phase results, estimates of D/ξ^2 could also be derived from the amplitude results. Reasonably linear Arrhenius plots were obtained from these data as demonstrated in figure 14a. The analysis of three experiments yielded a mean value of E_D of $5.2 \text{ kcal. mole}^{-1}$.

The estimates of E_D assumed that ξ does not change with temperature. Since ξ is a function of cell size, the average cell diameter was examined in a microscope at 20°C and 40°C . Such difference as there was in the two

Figure 13

- (a) The natural logarithm of the rate constant, k , derived from the phase data, versus $1/T$, obtained for a 35μ layer of Chlorella. A least squares line is drawn through the points from which an activation energy of $5.2 \text{ kcal. mole}^{-1}$ was calculated.
- (b) The natural logarithm of D/ξ^2 from the same phase data, also plotted against $1/T$. The least squares line through these results corresponds to an activation energy of $6.0 \text{ kcal. mole}^{-1}$.

Figure 14

- (a) The natural logarithm of the diffusion coefficient of oxygen in water plotted against the reciprocal of temperature, calculated from the amplitude results. The slope of the line yields an E_D of 4.4 kcal. mole⁻¹.
- (b) The natural logarithm of the diffusion coefficient of oxygen in water plotted against the reciprocal of temperature determined by the independent physical measurements. The slope of the line yields an activation energy for diffusion of oxygen of 3.7 kcal. mole⁻¹.

populations was not significant to these results.

6. An Independent Physical Measurement of D

For purpose of comparison with the diffusion data arising from the experiments with Chlorella, independent physical measurements of oxygen diffusion were made by an original procedure. The typical experimental result used to calculate D is represented in figure 15a. Here the time course of diffusion of oxygen to the platinum electrode is shown after an anaerobic buffer flowing through the polarograph had been replaced with an oxygenated one. The total quantity of oxygen, Q_{O_2} which had reached the electrode at time t was computed from figure 15a and plotted in figure 15b. Extrapolation of the linear portion of the Q_{O_2} plot to the intercept on the time axis (L), allowed the calculation of D according to Crank's (1956) formula $D = \lambda^2/6L$, where λ is the length of the diffusion path. Using an estimate of the pore length in the dialysis membrane from Ginsburg and Katchalsky (1963) and the depth of the lower compartment of the polarograph, λ was evaluated to be 500 μ . Hence D was computed from the results in figure 15b to be $1.7 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$. Considering the variability of the results of diffusion measurements reported by Himmelblau (1964), this figure compares well with the usual literature value of $2.0 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$. The procedure described above was repeated at several different temperatures, which yielded reasonably linear Arrhenius plots such as the one shown in figure 14b. Three experiments gave an average value for E_D of 4.3 kcal. mole⁻¹. Another value for E_D was computed as 6.0 kcal.

Figure 15

- (a) The time course of oxygen diffusion to the platinum electrode when an anaerobic medium in the experimental cell was replaced by an oxygenated one. The concentration of oxygen recorded at the electrode is plotted against time.
- (b) The Q_{O_2} plot. The total amount of oxygen which has diffused to the platinum electrode in time t is plotted against t , for the same experiment as in a.

mole⁻¹ from figures quoted in Himmelblau's review (1964). These results are compared with the two values of E_D obtained with Chlorella in table I. Obviously the temperature dependence of D obtained from the computer fitting procedure compares favourably with the results obtained by independent methods.

7. Experiments with Spinach Chloroplasts

In figure 16 the dependence of the phase and amplitude of the oxygen signal on the square root of modulation frequency is shown, from which the rate constant for chloroplasts was determined. This experiment was performed at 23°C using basic medium B, with a bilayer of chloroplasts, which approximates the "thin layer" of cells of Joliot's theory. The solid lines were generated using the theoretical equations and show that values of k , ϕ_0 and $p\sqrt{\pi/D}$ could be obtained which gave excellent agreement between theory and experiment.

The best fit parameters for chloroplasts in medium A or in medium B are shown in table II. Each value is an average of seven and all experiments had a standard deviation between experimental and theoretical values of less than 3°. The differences in the best fit parameters for the two situations shown in table I probably result from the change in osmolarity of the medium. The chloroplasts which were provided with bicarbonate and suspended in the sorbitol-containing medium (medium A) were smaller than those in the sorbitol-free buffer (medium B). This was reflected in the smaller intercept in the sorbitol medium. Also with viscosity measurements it was possible

Table IActivation Energy for the Diffusion of Oxygen in Water

E_D (kcal. mole ⁻¹)	Source
5.7	phase results, <u>Chlorella</u>
5.2	amplitude results, <u>Chlorella</u>
4.3	independent physical measurement
6.0	Himmelblau (1964)

Figure 16

- (a) The phase lag between the oxygen signal and the light modulation plotted against the square root of frequency (closed symbols) obtained with the modulated polarograph using a sample of spinach chloroplasts. The curve is generated by the best fit of the equation:

$$\phi = \phi_0 + p \cdot \sqrt{\frac{\pi}{D}} \cdot \sqrt{\nu} + \tan^{-1} (2 \pi \nu / k)$$

Best fit values were 200 sec^{-1} for k , $9.82^\circ \text{ sec}^{-1/2}$ for p .

$\sqrt{\frac{\pi}{D}}$ and 13° for ϕ_0 . The standard deviation between the experimental and calculated points was 2.2° .

- (b) The amplitude results of the O_2 signal for the same sample as in figure 1a. For the closed symbols $x = 1/n A$ and for the open symbols $x = 1/n \left[A \times \frac{(k^2 + 4 \pi^2 \nu^2)^{1/2}}{k} \right]$, where A is the amplitude of the O_2 signal. The open circles show only the influence of diffusion, while the closed circles also reflect the effect of the rate-limiting reaction.

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Table IIBest Fit Parameters for Experiments with Chloroplasts at 23°C

	k sec^{-1}	$p\sqrt{H/D}$ $^{\circ}\text{sec}^{-\frac{1}{2}}$	ϕ_0 $^{\circ}$
medium A*	215 ± 9 (S.E.)	$10.3 \pm .5$ (S.E.)	3 ± 5 (S.E.)
medium B**	218 ± 10 (S.E.)	$9.7 \pm .2$ (S.E.)	13 ± 7 (S.E.)

*medium A contained 20 mM HEPES NaOH buffer pH 7.6, 0.33 M sorbitol, 100 mM KCl, 1 mM MgCl_2 , and 5 mM NaHCO_3

**medium B contained 20 mM HEPES NaOH buffer pH 7.6, 100 mM KCl, 1 mM MgCl_2 and 1 mM NADP

to estimate from the best fit values of $p\sqrt{\pi/D}$, that the average chloroplast volume was about 10% smaller in medium A.

The value of k was determined at two temperatures with seven different samples of chloroplasts yielding an average value of E_a of 7 ± 2 (S.E.) kcal. mole⁻¹. The average value of E_D was $6.1 \pm .9$ (S.E.) kcal. mole⁻¹, which is close to an independent physical measurement of 6.0 kcal. mole⁻¹ (1).

The results of four kinetic isotope experiments gave a ratio of k_{H_2O}/k_{D_2O} of 1.1 ± 0.1 (S.E.). Hence it does not appear likely that the rate-limiting step of oxygen evolution in spinach chloroplasts involves the breaking of an OH bond. The ratio of D_{H_2O}/D_{D_2O} was 1.2 which compares well with a value of 1.25 calculated from viscosity measurements.

The results of the experiments with different concentrations of inorganic ions is shown in table III. Each value is an average of four to six determinations. The effect of the salts on the value of k was reversible and did not depend on whether the chloroplasts were first perfused with the low salt medium (containing only 1 mM $MgCl_2$) on one of the high salt media (with 25 mM $MgCl_2$ or 100 mM KCl). In addition, some other salts were investigated. When ~~chloroplasts were perfused~~ with a medium containing 100 mM Na acetate or 100 mM $NaNO_3$, results similar to those with 100 mM KCl were obtained. Hence it does not appear that the presence of any one ion is responsible for the stimulation of k .

Table III

Effect of Inorganic Ions

Salts in medium*	k	p	$\sqrt{\pi/D}$	ϕ_0
	sec ⁻¹		°sec ^{-1/2}	°
1 mM MgCl ₂	110 ± 20 (S.E.)	10.9 ± 5 (S.E.)	11 ± 1 (S.E.)	
25 mM MgCl ₂	215 ± 16 (S.E.)	10.8 ± 5 (S.E.)	6 ± 3 (S.E.)	
1 mM MgCl ₂ 100 mM KCl	215 ± 11 (S.E.)	10.3 ± 5 (S.E.)	3 ± 5 (S.E.)	

*In addition the medium contained 20 mM HEPES NaOH buffer pH 7.6, 0.33 M sorbitol, and 5 mM NaHCO₃

Chloroplasts that had been decarbonated by the procedure of Stemler and Govindjee (1973, 1974) were perfused first with CO_2 free medium B, then with medium B to which was added 20 mM sodium bicarbonate. Within one minute of the change of solutions the amplitude of the oxygen signal at 20 Hz rose by 40% to a new steady state value, but the phase remained unchanged. Similar observations were made at a modulation frequency of 50 Hz. The rise of the amplitude is similar to Stemler and Govindjee's bicarbonate effect. If a change in k were responsible for the bicarbonate effect on the amplitude then a change in ϕ of the order of 20° could have been expected on the basis of equation (7). Instead the phase remained unchanged $\pm 2^\circ$ which is evidence that an altered value of k was probably not the origin of the bicarbonate effect. Since the rate-limiting step is thought to be on the oxidizing side of photosystem II, these experiments are consistent with Wydrzinski and Govindjee's suggestion (1975) that bicarbonate acts on the reducing side of photosystem II.

Many of the other situations that were investigated had little effect on the rate constant. In the pH range 5.8 - 8.2 where the signal size was large enough to make reliable phase measurements, there was no significant change in the value of k within the resolution of this technique. Similarly, when 1 mM NH_4Cl was added to the medium or when the medium was bubbled with 10%, 20% or 30% oxygen mixtures, no significant change in k was observed.

8. Involvement of Water-splitting in the Kok Scheme of Oxygen Evolution

The basic objective of these experiments was to study the involvement of water-splitting in the Kok scheme of oxygen evolution. However, before presenting these results, it is important to describe the flash yield sequence obtained with this experiment and demonstrate the computer fitting procedure for the Kok model. Figure 17 shows a typical record of the oxygen yields from a thin layer of Chlorella subjected to 5 minutes of darkness followed by a sequence of 15 flashes at 0.3 sec. intervals. The yield of oxygen from each flash was normalized with respect to the average yield of the last five flashes. These results show the characteristic damped oscillation with a period of four. The theoretical flash yields obtained from the best fit of the Kok model are shown in figure 18 as the closed circles. The standard deviation between the experimental and theoretical points was 0.04. Obviously the Kok model gives an excellent description of these results. The flash yields found here for Chlorella show more damping than found for chloroplasts (Kok, Forbush, and McGloin, 1970) and closely resemble the yields found by other workers for Chlorella (Ley, Babcock, and Sauer, 1975). Their values for α and β were 0.22 and 0.014 respectively which are comparable with the values of 0.25 and 0.015 obtained from the best fit of the results in figure 17.

The involvement of water-splitting in the Kok scheme was tested by suspending the organisms in deuterium oxide as in the previous experiments with the modulated polarograph.

Figure 17

The flash sequence from Chlorella. The cells were kept in darkness for 5 min. before a sequence of 15 brief saturating flashes at intervals of 0.3 sec. The open circles represent the yield of oxygen obtained from each flash plotted against the number of the flash. These yields were normalized with respect to the last 5 flashes. The closed circles represent the best fit of the Kok model obtained with $\alpha = 0.25$ and $\beta = 0.015$. The starting (dark) values of S_0 , S_1 , S_2 and S_3 were 25%, 75%, 0% and 0% respectively.

Figure 18

The flash yield sequence in H_2O and D_2O . The normalized yield of oxygen plotted against the flash number for a sample of Chlorella suspended for 1 hour, in D_2O (closed circles) and later in H_2O (open circles). The values of α and β from the computer fit of the Kok model were 0.22 and 0.017 respectively for cells in H_2O and 0.25 and 0.008 for cells in D_2O . Other conditions as in figure 17.

In figure 18, the normalized flash yield sequences are compared for a sample of Chlorella suspended first in an H_2O based medium and then in a D_2O based medium. The oscillation of the flash yields showed slightly greater damping in D_2O than in H_2O . This is reflected in the value of β found by the computer which was larger in D_2O than in H_2O (0.25 and 0.22 respectively). Thus the effect of D_2O was to increase the number of misses. This effect was consistently observed and was reversible even when the cells were held up to three hours in D_2O . For five determinations the value of $\alpha \cdot D_2O / H_2O$ was 1.1 with extremes of 1.19 and 1.07. Although there were small changes in β in the experiment shown in figure 18 as well as in other experiments, these were usually of minor significance to the interpretation of the changes in the flash yield oscillations.

Figures 19 to 22 illustrate the effect of D_2O on the four dark reactions of the Kok scheme. Both the H_2O and D_2O based curves were obtained with one sample of chloroplasts or Chlorella. The results were highly reproducible. D_2O had only a minor effect on the four reactions and this was to slow the reaction rate slightly. Table IV summarizes the effect of the heavy isotope on the half times of the reactions. D_2O did not significantly alter the shapes of the curves which in both media closely resembled those obtained by Bouges-Bocquet (1973). The small isotope effect observed was reversible and did not depend on whether the biological material was first suspended in H_2O or D_2O . The effect of the isotope was

Figure 19

Time course of the first dark reaction of the Kok scheme, $S_0^* \longrightarrow S_1$ in H_2O and D_2O . Chlorella cells were kept in the dark for 5 min., then subjected to a sequence of three flashes at 0.3 sec. intervals (the pre-illumination sequence). This was followed by an additional dark period of 3 min. before the second sequence of 25 flashes. In the second sequence the interval between the first and second flash was variable. All other intervals were 0.3 sec. $\gamma_0(\Delta t) = \frac{\gamma_4(\Delta t) + \gamma_3(\Delta t) - \gamma_3(0.3 \text{ sec.})}{\gamma_4(0.3 \text{ sec.})}$ is plotted against Δt .

The curves were fitted by eye.

Figure 20

The time course of the second dark reaction of the Kok scheme, $S_1^* \longrightarrow S_2$, in D_2O and H_2O . Chlorella cells which had been kept in the darkness for 5 min. were subjected to a flash sequence of 25 flashes in which the interval, Δt between the first and second flash were variable. The intervals between all other flashes was 0.3 sec. The variation of the yield of the third flash $Y_3(\Delta t)/Y_3(0.3 \text{ sec.}) = \gamma_1(\Delta t)$ is plotted against Δt . Curves fitted by eye.

Figure 21

The time course of the third dark reaction of the Kok scheme, $S_2^* \longrightarrow S_3$ in H_2O and D.O. Chlorella were left in the dark for 5 min. before the sequence of 25 flashes. Between the second and third flash a variable time interval Δt was used. The spacing between all other flashes was 0.3 sec. The variation of the yield of the third flash $Y_3(\Delta t)/Y_3(0.3 \text{ sec.}) = \gamma_2(\Delta t)$ is plotted against Δt . The curves were fitted by eye.

Figure 22

Time course of the fourth dark reaction of the Kok scheme $S_3^+ \longrightarrow S_0$ in D_2O and H_2O . Spinach chloroplasts, which were suspended in a medium containing 0.1 mM ferricyanide, were subjected to 5 min. darkness followed by a flash sequence of 35 flashes in which the interval between the third and fourth flash was variable. All other intervals between flashes were 0.3 sec.

$$\frac{Y_7(\Delta t) - Y_7(0.3 \text{ sec})}{Y_7(0.3 \text{ sec.}) - Y_6(0.3 \text{ sec.})} = x$$
 is plotted against Δt (circles). The triangles represent $\gamma_3(\Delta t) = 0.8 x(\Delta t) + 0.05 \gamma_0(\Delta t) + 0.18 \gamma_1(\Delta t)$. Curves were fitted by eye.

Table IV

Effect of D_2O on the Half Times of the Dark Reactions of
the Kok Scheme

reaction	number of determinations	$t_{\frac{1}{2}}^{D_2O} / t_{\frac{1}{2}}^{H_2O}$	extremes
$S_0^* \longrightarrow S_1$	3	1.3	1.0 and 1.6
$S_1^* \longrightarrow S_2$	3	1.0	0.95 and 1.1
$S_2^* \longrightarrow S_3$	7	1.2	1.0 and 1.4
$S_3^* \longrightarrow S_0$	3	1.1	1.0 and 1.2

quite stable; the initial reading in D_2O which was taken 1 hour after suspension in that medium could be repeated with some precision an hour later. Thus, no long term effect of D_2O was evident. As in the isotope experiments with the modulated polarograph the simplest interpretation of these results is that OH bond breaking is not involved in the rate processes studied here.

The calculation of γ_0 , γ_1 , and γ_2 for Chlorella was straightforward but the calculation of γ_3 was more complex involving measurements which could only be made with chloroplasts suspended in ferricyanide solution, plus terms in γ_0 and γ_1 . Although results from two organisms were used, the value of γ_3 is probably a good estimate for chloroplasts. This is because the contribution of the γ_0 and γ_1 terms from Chlorella is small (20%), and because Bouges-Bocquet (1973) has shown that the first three dark reactions are very similar in the two species.

IV. DISCUSSION

1. Confirmation of the Joliot Model

The study reported here clearly supports the Joliot theory for the modulated polarograph. In addition to a confirmation of many of the experimental results that Joliot et al. (1966) presented in favor of their theory, this work presents several new pieces of evidence. As mentioned in the results section, the derived values for the activation energy of diffusion of oxygen in water and values for the ratio of the diffusion coefficients of oxygen in water and deuterium oxide were both in good agreement with independent estimates. Thus this study has given further support to the Joliot theory.

When Joliot et al. attempted to fit the theoretical equations simultaneously to their phase and amplitude results, they achieved good fits only at low frequencies. This left some doubt as to whether an additional limiting phenomenon, perhaps related to the cell resistance, might be operating at high frequencies. In this study only the phase results were used in the curve fitting procedure because these could be measured with better resolution than the amplitude results. Not only were good fits found at all frequencies but the value of k determined in this manner led to an excellent description of the amplitude results, which yielded consistent values of ξ and E_p . It is concluded from this work that the original Joliot equations are an adequate description of the experimental results and no additional limiting phenomena need be proposed.

2. Value of the Rate Constant

The mean value of k obtained with Chlorella was $305 \pm 20 \text{ sec}^{-1}$ at 23°C . With their alternative curve fitting procedure Joliot et al. found a value of 820 sec^{-1} for k . The discrepancy may in part be due to the differences in analysis of data, since the computer fit of their data with the program used in this study yielded a somewhat lower rate constant of 660 sec^{-1} . The fit was relatively poor, however, with an S.D. of 5.2° , compared to the values of 3° or better reported in this work. Sinclair's (1969) much larger rate constant ($k \geq 5000 \text{ sec}^{-1}$) was estimated on the basis of the linearity of the ϕ versus $\sqrt{v}$ plots. This study has indicated that if Sinclair had examined higher frequencies, he would have found the non-linearities which are strong evidence of a lower rate constant.

The value of the rate constant can be compared with values obtained by other techniques. Most directly comparable to this work is a study of the rate-limiting step of oxygen evolution in Chlorella by Etienne (1968) who used a flow method to evaluate k . Her rate constant of 300 sec^{-1} for cells of a similar size (5μ) to those used in this study is in excellent agreement with the value of k reported here. Smaller cells (diameter less than 3.5μ) yielded a larger rate constant ($400\text{--}500 \text{ sec}^{-1}$). This size effect may be a second explanation for Joliot's high value for the rate constant, since the cells used in their study were between 3μ and 4μ in diameter. Using a standard kinetic analysis, Etienne demonstrated first order kinetics for the rate-limiting step, consistent with

this work. In another study, Vater et al. (1968) found a rate constant of 1150 sec^{-1} for electron flow between water and plastoquinone. This work was performed using the double flash technique of Emerson and Arnold (1932b) which may well measure a different rate constant from the one studied here.

It is of special interest to attempt to correlate one of the dark reactions of the Kok model with the rate-limiting step obtained with the modulated polarograph. Bouges-Bocquet's study (1973), confirmed by the flash experiments reported here, has revealed that only one of the dark steps of the Kok scheme resembles the rate-limiting step of the modulated polarograph study. This is the oxygen evolving step $S_3^* \longrightarrow S_0$, which is the rate-limiting step of the Kok scheme. It was found to be a first order reaction with a half time of 1.2 ~~msec~~ ^{msec} in spinach chloroplasts, which is somewhat smaller than the half time of 3.1 msec for the rate-limiting step of the Joliot model. The three other dark steps of the Kok scheme are still more rapid, with half times of 0.2 to 0.5 msec, and show much more complex kinetics.

At this point it should be emphasized that although both of these steps are presumed to be part of the same terminal sequence of reactions that lead to oxygen evolution, they probably represent different and consecutive processes. It must be remembered that the flash experiments are designed to follow the state of the reaction centre, whereas the modulated polarograph monitors the kinetics of oxygen production. In a later section of this thesis, a model

relating the two steps is presented.

A second significant difference that should be taken into account when comparing these two studies is the differences in experimental conditions. In the modulated polarograph, the biological material is observed under conditions that are close to a steady state. In the flash work, the chloroplasts or Chlorella are brought out of a long dark period with intense flashes of light. In this latter case, we can expect to see changes in pH, oxygen concentration and possibly other parameters which may effect the rate processes. It is entirely possible that the rate determining processes in the two studies were completely different.

3. Temperature Experiments

An important result of the temperature experiments was the linearity of the Arrhenius plots. This indicates that only one reaction is limiting in the 8°C to 42°C range. Thus Joliot's simple first order model of oxygen evolution appears to be an adequate description of the rate-limiting step.

The temperature experiments yielded a value for the activation energy (5.9 kcal. mole⁻¹ for Chlorella) which was similar to that obtained by Krienne (6.6 kcal. mole⁻¹) but somewhat smaller than that (7.7 kcal. mole⁻¹) of Joliot et al. (1966) which was estimated from work at only two temperatures. The size of the activation energy is a valuable indicator of the type of reaction mechanism. For example, experiments by Boyle et al. (1969) indicated that no activation energy should be expected if the rate-limiting

reaction was the recombination of OH radicals. Although this mechanism was strongly suggested by the modelling experiments (see introduction) the activation energy found here is large enough to rule out this mechanism as a possibility for the rate-limiting step. On the other hand, ordinary molecular reactions catalysed by enzymes often have much larger activation energies (Laidler, 1958) than that found for the rate-limiting step. There are several plausible mechanisms that have activation energies in the right range. Radical abstraction reactions, for example the reaction of OH radicals with molecular species, lead to values of E_a of 10 kcal. mole⁻¹ or less (Wilson, 1970). Some redox processes (Massey *et al.* 1965) have activation energies of about the same size as the rate-limiting step.

The entropy of activation indicates changes in order as the reaction system proceeds from the initial to transition states. For example, in most enzymatic reactions there is a slight unfolding of the enzyme as it accepts the substrate which contributes to a small positive $\Delta S^\ddagger$ of the order of 10 e.u. If charged groups of opposite sign are brought together in the transition state, a large positive $\Delta S^\ddagger$ may be observed, as in the formation of the myosin ATP complex (Ouellet, Laidler and Morales, 1952). In the latter reaction it is thought that the liberation of solvent molecules accounts for at least part of the 49 e.u. gain in entropy. In the experiments reported here a value of -25 cal. degree⁻¹ mole⁻¹ was calculated for the entropy of activation for the rate-limiting step in Chlorella. If Kok *et al.* (1970) are

correct in proposing the build up of positive charge prior to the evolution of oxygen, this negative entropy of activation may reflect an ordering of water molecules around these charges.

The size of the activation energy for the diffusion of oxygen derived from the biological study was close to that obtained by independent physical measurements. These results not only add credibility to the curve fitting procedure but also add to the known diffusion data for oxygen which is relatively sparse and imprecise, (Himmelblau, 1966). The linearity of the Arrhenius plots confirm the Eyring analysis for an activated rate process for diffusion. Here E_D must be considered to be the average energy barrier that an oxygen molecule must cross in a step of the random walk.

4. The Deuterium Isotope Effect in the Rate-limiting Reaction

In the kinetic isotope experiments a value of 1.29 for the ratio of the rate constant in water to that in deuterium oxide was found. 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 for chemical reactions (Wiberg, 1955) as well as enzymatic processes (Jencks, 1969; Laidler and Bunting, 1973). The work of Melville (1937) on the mercury photo-sensitized decomposition of water offers a reasonable analogue of photosynthetic evolution of oxygen. In this case, the value of the kinetic isotope effect 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. A second possibility is that there exists a special pool of water molecules which is the source of the evolved oxygen and which was never significantly filled with D_2O in these experiments. If this were so, no kinetic isotope effect would be observed. However, since it was possible to obtain consistent results in D_2O in two experiments separated by two hours and since Ruben et al. (1941) 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 these experiments, this possibility seems rather remote. The simplest explanation of these results is that the rate-limiting reaction does not involve the rupture of an OH bond. In fact, the small isotope effect appears to be that expected for a change in enzyme conformation caused by the weaker hydrogen bonding of the heavy isotope (Jencks, 1969).

Other workers (Rietz and Bonhoeffer, 1935; Curry and Trelease, 1935; Craig and Trelease, 1937; and Pratt and Trelease, 1938) have studied the effect of replacing H_2O with D_2O on photosynthesis. However, these early experiments were not undertaken under conditions where the rate processes associated with oxygen evolution could be studied and have little bearing on the experiments presented here.

5. Experiments with Chloroplasts

It would appear from this study that the rate-limiting thermal reaction associated with photosynthetic oxygen evolution in spinach chloroplasts is similar to that in

Chlorella. The rate constant is somewhat similar: 218 sec^{-1} for chloroplasts vs. 305 sec^{-1} for Chlorella, but the activation energy is slightly larger: $7 \text{ kcal. mole}^{-1}$ for chloroplasts compared with $5.9 \text{ kcal. mole}^{-1}$ for Chlorella. The deuterium isotope effect has a low value in both species indicating that this step probably does not involve the breaking of an OH bond in a water molecule. These similarities indicate that higher and lower plants share a common mechanism in the rate-determining step. This is consistent with the similarities in the flash yield sequence of the two species and the application of the Kok model for oxygen evolution to both (Joliot et al. 1971).

The effect of inorganic ions on k stresses the importance of these ions in photosynthetic electron transport, as already demonstrated in the effect of these ions on enhancement and the oxygen burst (Sinclair, 1972). This effect was not merely an osmotic one, since large changes in the sorbitol concentration of the medium had no effect on k . Although stimulation of oxygen evolution by chloride ion is well established (see introduction), the results of these experiments indicated that the increases in the rate constant cannot be attributed to any one ion but rather to the effect of inorganic ions in general. Salt-induced changes in the membrane structure or protein conformation may be responsible for these effects.

The possibility that the rate-limiting reaction of O_2 evolution was directly involved in phosphorylation was investigated in two ways. First, by altering the pH, an attempt was made to observe whether a change in the

electrochemical potential difference of hydrogen ions across the membrane could affect the value of k . Second, NH_4Cl was used in this study to see if uncoupling had any effect on k . Neither the pH changes nor the addition of NH_4Cl to the medium appeared to change the value of k . This seems to be consistent with the observations of Gould and Ort (1973) that the rate of electron transport through coupling site II is independent of phosphorylation and uncoupling. The results are perhaps best explained if the rate-limiting step of oxygen evolution is irreversible.

Oxygen concentration did not affect the rate constant in these experiments. As oxygen is the product of water-splitting, this observation suggests that the rate determining step is not affected by a feedback mechanism involving oxygen.

Although the rate-limiting reaction may not be controlled by oxygen concentration, oxygen can control other sites in photosynthesis. It has long been known that oxygen inhibits carbon fixation via the Warburg effect (Gibbs, 1970). Anaerobic conditions can also greatly increase the damping of the flash yield oscillations (Ley, Babcock and Sauer, 1975). This effect appears to be explained by co-operative feedback mechanism on the oxidizing side of photosystem II, which has been identified by Diner and Mauzerall (1973).

The bicarbonate experiments, which showed no effect of decarbonation on k , were consistent with Wydrzynski and Govindjee's recent (1975) hypothesis of a bicarbonate site on the reducing side of photosystem II. This hypothesis

is based on the observation of chlorophyll a fluorescence from tris-washed chloroplasts that are incapable of oxygen evolution. In the presence of artificial donors of photosystem II, the fluorescence transient resembles that of normal chloroplasts, but if bicarbonate is removed, the transient shows a rapid initial rise that is characteristic of a block in electron transport. Since only the reducing side of photosystem II is thought to be operating in this situation, this is most probably the site of bicarbonate action.

6. The Effect of Deuterium Oxide on the Dark Reactions of the Kok Scheme

None of the four dark reactions of the Kok scheme showed a deuterium isotope effect appreciably larger than observed in the rate-limiting step. Thus, the same arguments apply and it must be concluded that these rate-processes probably do not involve the breaking of an OH bond. The slightly slower reaction rates in D_2O can be accounted for by the poorer H-bonding of the heavy isotope and its effect on enzyme conformation.

This effect probably accounts for the slightly higher proportion of misses, α found in D_2O . A plausible water-splitting mechanism that predicts a small isotope effect in the reactions of the Kok scheme is discussed in a later section of this work.

These experiments have been interpreted in terms of the conventional electron transport model where H_2O is the precursor of the evolved O_2 . However, the absence of an isotope effect is also consistent with Metzner's hypothesis

(1975) that water is not the origin of photosynthetic oxygen. Thus, it is important to look at Metzner's arguments carefully. Evidence is cited that seems to indicate that CO_2 is the O_2 precursor. For example, the initial kinetics of CO_2 uptake and O_2 production after a dark period show close parallels, and $^{18}\text{O}/^{16}\text{O}$ ratios in photosynthetic oxygen are consistently higher than in cell water, more closely matching those found in CO_2 . Enzymatically enhanced exchange water and carbon dioxide, according to Metzner, invalidates Ruben's (1941) classic ^{18}O labelling experiment which claimed to demonstrate water as the precursor of molecular oxygen. Metzner concludes that the O_2 precursor must have at the minimum a higher $^{18}\text{O}/^{16}\text{O}$ ratio than water and the breaking of a CO or OO bond is postulated as an alternative to OH bond-breaking for the production of oxygen. In the experiments reported here no evidence of OH bond-breaking was observed. This result does not prove or disprove Metzner's hypothesis. Thus further work is necessary to establish the existence of an alternative to water as the oxygen precursor or to identify some other dark reaction associated with oxygen evolution as the water-splitting step.

7. A Model of Photosynthetic Oxygen Evolution

A more detailed model of the Kok scheme which has been adapted from Van Gorkom and Donze (1973) (reactions 1-8) can be used to explain these experimental results. The reaction centre P_{680} reacts with its donor, Z, and acceptor, Q, in much the same manner in each of the four photoacts.⁴ Charge accumulation occurs in the four states

of an Mn complex in the water-splitting enzyme (Y_0, Y_1, Y_2 , and Y_3). However, back reactions are possible and S_3 for example can be any mixture of Y_3ZP, Y_2Z^+P, Y_2ZP^+ , or $Y_1Z^+P^+$. After the fourth photoreaction, the water-splitting intermediate Y_3^* is formed in the rate-determining reaction of O_2 evolution. Once formed, Y_3^* rapidly and irreversibly oxidizes water. The scheme, then, is as follows:

Reactions 2-8 determine the time courses of the four dark reactions of the Kok model. If reactions 1-7 are fast compared to 8, the scheme reduces to the simple Joliot model on which the study with the modulated polarograph was based.

The reaction scheme shown above is consistent with many of the observations of this study. If the rate-limiting step (8) determined with the modulated polarograph is followed by a fast and irreversible water-splitting reaction (9), then oxygen concentration, uncouplers, pH and D_2O would have little effect on the rate-limiting step. As well, a kinetic isotope effect is not expected in the dark reactions of the Kok scheme. It is proposed

that the rate-limiting reaction is an electron transfer process between the water-splitting enzyme and the oxidized primary donor, Z^+ . The activation energy for k is in the right range for a redox reaction (Massey et al. 1965).^{*} The effect of bicarbonate on the dark reactions of the Kok model is explained if bicarbonate acts on the acceptor side of photosystem II to stimulate reoxidization of Q^- . As long as reactions 1 to 7 are faster than 8, decarbonation will have no effect on the rate-limiting step.

The need for a more elaborate model arises from the interpretation of fluorescence. Although fluorescence might be considered the reverse of reaction (1), interpretation of the oscillation of fluorescence yield in flashing light is much more difficult. Fluorescence yields are high when the concentration of $(S_0 + S_1)$ are high, and low when $(S_2 + S_3)$ are low (Delosme, 1972). Thus fluorescence yields oscillate inversely to the O_2 yields and cannot be correlated in a logical way to the state of the primary donor, Z . Joliot and Joliot (1973) have proposed the existence of two different quenchers with different efficiencies to explain these results. There may also be two sites for charge accumulation on the oxidizing side of photosystem II. In any case, elaboration of the model as described above is not incompatible with the features of the simpler model used to explain the experimental results of this thesis.

In order to test whether the water-splitting act occurs in a final rapid reaction, a powerful technique for separating the reactions must be found. Temperature jump

kinetics would most likely accomplish this. Perhaps with the advent of new types of spectroscopy such as ^{17}O NMR, a sensitive fast-responding method for detection of O_2 (or H^+) will become available. It will then be possible to repeat these isotope experiments in order to resolve the problem of water-splitting.

8. Summary

The following conclusions may be drawn from these experiments:

- (1) This study confirms the Joliot theory of the modulated O_2 polarograph and its simple first order model for the rate-limiting reaction of O_2 evolution.
- (2) There is only one rate-limiting reaction between 8° and 42°C in Chlorella.
- (3) At 23°C this reaction has a rate constant of 305 ± 20 (S.E.) sec^{-1} for Chlorella and 215 ± 11 (S.E.) sec^{-1} for spinach chloroplasts.
- (4) The reaction has an activation energy of 5.9 kcal. mole^{-1} for Chlorella and 7 kcal. mole^{-1} for spinach chloroplasts. The temperature dependence of the diffusion of oxygen in water can be described by an activation energy for diffusion, E_D of 4.3 to 5.7 kcal. mole^{-1} .
- (5) The reaction probably does not involve the rupture of the OH bond in either organism.
- (6) The reaction in chloroplasts is sensitive to low salt concentrations with a low rate constant of 110 sec^{-1} .

- (7) The reaction in chloroplasts is insensitive to decarbonation. This is further evidence of a site for bicarbonate action on the oxidizing side of PII.
- (8) The reaction in chloroplasts is unaffected by the addition of 1 mM, NH_4Cl to the medium or changes in the oxygen concentration and pH of the medium. This evidence suggests that the reaction is irreversible.
- (9) Deuterium oxide slightly increases the damping of the oscillation of the oxygen yields in flashing light, reflected in a higher value for the parameter α of the Kok model.
- (10) The four dark reactions of the Kok scheme of oxygen evolution proceed at a slightly lower rate in D_2O as compared to H_2O , indicating that these rate processes probably do not involve OH bond breaking.
- (11) A model is described to explain these results.

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VI. APPENDIX I.

The Polarographic Cell

The following figures are reproductions of the blueprints for the polarographic cell. The exact dimensions and assembly of the cell are shown.

Figure 23

Diagrams of the polarographic cell.

Figure 24

Diagrams and assembly of the polarographic cell.

APPENDIX II.

Computer Program to Fit the Joliot Equations to the PhaseResults

The computer program which follows fits the phase equation of the Joliot theory to the experimentally determined phase results by a least squares procedure. In this way the best fit values of ϕ_0 , k and $m(\xi\sqrt{\pi/D}$ for a thick layer or $p\sqrt{\pi/D}$ for a thin layer) are determined. A typical experiment with chloroplasts is used to demonstrate the curve fitting procedure.

A partial explanation of some of the fortran variables used in the program follows:

NCRV the number of experiments to be analysed

N the number of phase and frequency points

C the rate constant, k

A the slope, $\xi\sqrt{\pi/D}$ or $P\sqrt{\pi/D}$

B the phase intercept at zero frequency ϕ_0

D1 the increment of k

D3 the increment of the slope

D2 the increment of ϕ_0

P(I) the experimentally determined phase point

TP(I) the theoretical phase value calculated from the Joliot equation

V(I) the frequency of modulation

K the number of increments that have been performed on the variable in question

SQ(K) or SUMSQ(V,P,N,C,A,B) the value of the sum of the squares of the difference between the theoretical and experimental phase points

```

0001 DIMENSION V(50), P(50), SQ(1000), ICEM(20), TP(50), W(50)
0002 READ 33, NCRV
0003 33 FORMAT (G,C)
0004 DO 44 C = 1, NCRV
0005 READ 101, ICEM
0006 101 FORMAT (2CA4)
0007 PRINT 102, ICEM
0008 102 FORMAT (1F, 20A4)
0009 READ 1, N, C, A, B
0010 1 FORMAT (4G,0)
0011 OUTPUT N, C, A, B
0012 READ 2, (V(I), P(I), I=1,N)
0013 2 FORMAT (2G,0)
0014 NCTM = 1
0015 30 READ 40, D1, D2, D3
0016 40 FORMAT (3G,0)
0017 OUTPUT D1, D2, D3
0018 DO 31 L=1, 6
0019 GO 10 75
0020 CONTINUE
0021 K=1
0022 SQ(K) = SLPSQ(V,P,N,C,A,B)
0023 K = K + 1
0024 C = C + D1
0025 SQ(K) = SLPSQ(V,P,N,C,A,B)
0026 10 IF (SQ(K) - SQ(K-1)) 4, 5, 5
0027 4 C = C + D1
0028 K = K + 1
0029 SQ(K) = SLPSQ(V,P,N,C,A,B)
0030 GO 10 10

```

0031	5	C = C - C1
0032		IF (K-2) 6, 6, 7
0033	6	K=1
0034		SQ(K) = SLPSQ(V,P,N,C,A,B)
0035	61	K = K + 1
0036		C = C - C1
0037		SQ(K) = SLPSQ(V,P,N,C,A,B)
0038		IF (SQ(K) = SQ(K-1)) 61, 71, 71
0039	71	C = C + C1
0040	7	CONTINUE
0041		GO TO 53
0042	73	CONTINUE
0043		K=1
0044		SQ(K) = SLPSQ(V,P,N,C,A,B)
0045		A = A + C2
0046		K = K + 1
0047		SQ(K) = SLPSQ(V,P,N,C,A,B)
0048	20	IF (SQ(K) = SQ(K-1)) 8, 9, 9
0049	8	A = A + C2
0050		K = K + 1
0051		SQ(K) = SLPSQ(V,P,N,C,A,B)
0052		GO TO 20
0053	9	A = A - C2
0054		IF (K-2) 11, 11, 52
0055	11	K = 1
0056		SQ(K) = SLPSQ(V,P,N,C,A,B)
0057	81	K = K + 1
0058		A = A - C2
0059		SQ(K) = SLPSQ(V,P,N,C,A,B)
0060		IF (SQ(K) = SQ(K-1)) 81, 12, 12
0061	12	CONTINUE
0062		A = A + C2
0063	52	CONTINUE
0064		GO TO 43
0065	93	CONTINUE
0066		K=1

```

0067      SQ(K) = SLPSQ(V,P,N,C,A,B)
0068      K=K+1
0069      B=B+D3
0070      SQ(K) = SLPSQ(V,P,N,C,A,B)
0071      IF (SQ(K)-SQ(K-1)) 21, 22, 22
0072      21      B=B+D3
0073      K=K+1
0074      SQ(K) = SLPSQ(V,P,N,C,A,B)
0075      GE IC 23
0076      B = B - D3
0077      IF (K-2) 24,24,28
0078      K=1
0079      SQ(K) = SLPSQ(V,P,N,C,A,B)
0080      K = K + 1
0081      B=B-D3
0082      SQ(K) = SLPSQ(V,P,N,C,A,B)
0083      IF (SQ(K)-SQ(K-1)) 26, 25, 25
0084      B = B + D3
0085      CONTINUE
0086      CONTINUE
0087      IF (NCTR = 2) 14, 14, 15
0088      NCTR = NCTR + 1
0089      GE IC 3C
0090      CONTINUE
0091      PRINT 6C, C, A
0092      FORMAT (17F RATE CONSTANT IS , E10.3, 5X , 10H SLOPE IS , E10.4)
0093      PRINT 62, B, SQ(K)
0094      FORMAT (13F INTERCEPT IS , E10.3, 9X, 18H SUM OF SQUARES IS ,
0095      1      E10.3)
0096      PRINT 88
0097      88      FORMAT (3CF      V(I)      P(I)      TP(I)      )
0098      DE 55 I=1,N
0099      W(I) = 2.C*3.1415*V(I)/C
0100      TP(I) = 18C.0/3.14159*ATAN(W(I)) + A*(V(I)**(.5)) + B
0101      PRINT 55, (V(I), P(I), TP(I), I=1, N)
0102      FORMAT (3E10.3)
0103      CONTINUE
0104      END

```

0001	FUNCTION SUMSQ(V, P, N, C, A, B)
0002	DIMENSION V(50), P(50), W(50), TP(50)
0003	SUMSQ = C.
0004	DO J I = 1, N
0005	A(I) = 2.C*3.1415*V(I)/C
0006	TP(I) = 18C.C/3.14159*ATAN(W(I)) + A*(V(I)**(.5)) + B
0007	SUMSQ = SUMSQ + (P(I)-TP(I))**2
0008	RETURN
0009	END

EXPERIMENT WITH CHLOROPLASTS IN MEDIUM B

N = 15

C = 200.000

A = 10.0000

B = 10.0000

D1 = 5.00000

D2 = .500000

D3 = .500000

D1 = 1.00000

D2 = .100000

D3 = .100000

D1 = .200000

D2 = 2.000000E-02

D3 = 2.000000E-02

RATE CONSTANT IS .259E+03

INTERCEPT IS .151E+02

V(I) P(I) TP(I)

.600E+01 .40E+02 .47E+02

.900E+01 .550E+02 .564E+02

.120E+02 .670E+02 .647E+02

.160E+02 .780E+02 .745E+02

.250E+02 .950E+02 .946E+02

.300E+02 .104E+03 .104E+03

.400E+02 .120E+03 .120E+03

.490E+02 .132E+03 .133E+03

.640E+02 .148E+03 .149E+03

.810E+02 .163E+03 .165E+03

.100E+03 .179E+03 .175E+03

.121E+03 .193E+03 .192E+03

.144E+03 .205E+03 .205E+03

.169E+03 .217E+03 .217E+03

.196E+03 .229E+03 .228E+03

STOP

SLOPE IS .9640E+01
SUM OF SQUARES IS .325E+02

APPENDIX III.

The Flash Apparatus

The following figures show the flash lamp discharge circuit, the power supply circuit used to charge the flash lamp capacitor, and a block and circuit diagram for the flash controller.

Figure 25

Power supply circuit and flash lamp circuit.

Figure 26

Block diagram of the flash controller circuit.

Figure 27

Circuit diagram of the flash controller.

APPENDIX IV.

Computer Program to Fit the Kok Model to the Flash Yield
Sequence

This program fits the Kok model of oxygen evolution to the observed oxygen yields produced after brief flashes of light. The best fit values of the parameters α , β , and the value of S_1/S_0 after a dark period are determined by a least squares procedure. A list explaining some of the fortran variables used in the program follows:

NCRV the number of experiments to be analysed

P(I) the oxygen yield from the Ith flash

NF the total number of flashes in the sequence

A the proportion of misses, α

B the proportion of double hits, β

SR the ratio of S_1/S_0 after a dark period

S(1,1) the value of S_0 after a dark period

S(2,1) the value of S_1 after a dark period

S(3,1) the value of S_2 after a dark period

S(4,1) the value of S_3 after a dark period

S(1,2) the value of S_0 after the first flash

DA the increment of α

DB the increment of β

DSR the increment of SR

Y(I) the normalized flash yields

TY(I) the theoretical flash yields predicted by the Kok
 model


```

0001      DIMENSION S(4,100), P(100), Y(100), TY(100), SQ(1000), ICOM(20)
0002      READ 1, NCRV
0003      FORMAT (G.0)
0004      DO 12 J=1, NCRV
0005      READ 13, ICOM
0006      FORMAT (20A4)
0007      PRINT 14, ICOM
0008      FORMAT (1H, 20A)
0009      READ 15, NF, A, B, SR, S(3,1), S(4,1)
0010      FORMAT (6G.0)
0011      READ 17, (P(I), I=1, NF)
0012      FORMAT (G.0)
0013      S(1,1) = 4. / (SR + 1.)
0014      S(2,1) = 4.0 * SP/(SR + 1.)
0015      OUTPUT A, B, SR, S(1,1), S(2,1), S(3,1), S(4,1)
0016      J = 10
0017      SM = 0.
0018      SM = SM + P(J)
0019      I = I + 1
0020      IF (I-NF) 18, 18, 19
0021      I=1-1
0022      K=SM/(NF+9)
0023      DO 20 I=1,NF
0024      Y(I) = P(I)/K
0025      DO 66 J1 = 1, 2
0026      READ 47, DA, DB, DSR
0027      FORMAT (3G.0)
0028      OUTPUT DA, DB, DSR
0029      DO 66 J2 = 1, 2
0030      DO 50 L = 1, 3

```

```

0031      K=1
0032      SQ(K) = SUMSQ(Y, TY, A, B, S, NF)
0033      LDUM=0.
0034      K=K+1
0035      IF (L-1) 80, 80, 21
0036      A=A+DA
0037      GO TO 23
0038      IF (L-2) 22, 22, 24
0039      B=B+DB
0040      GO TO 23
0041      IF (L-3) 26, 26, 32
0042      SR = SR +DSR
0043      S(1,1) = 4. / (SR + 1.)
0044      S(2,1) = 4.0 * SR/(SR + 1.)
0045      GO TO 23
0046      STOP
0047      IF (LDUM-2) 91, 54, 54
0048      SQ(K) = SUMSQ(Y, TY, A, B, S, NF)
0049      IF (SQ(K)-SQ(K-1)) 33, 34, 34
0050      LDUM = 0
0051      IF (L-1) 35, 35, 36
0052      A=A-DA
0053      IF (A) 71, 71, 72
0054      A=0.
0055      CONTINUE
0056      GO TO 37
0057      IF (L-2) 55, 55, 38
0058      B=B-DB
0059      IF (B) 73, 73, 74
0060      B=0.
0061      CONTINUE
0062      GO TO 37
0063      IF (L-3) 39, 39, 40
0064      SR = SR -DSR
0065      IF (SR) 75, 75, 76

```

0066	75	PRINT 500
0067	500	FORMAT (5HSR=0)
0068		STOP
0069	76	CONTINUE
0070		S(1,1) = 4. / (SR + 1.)
0071		S(2,1) = 4.0 * SR / (SR + 1.)
0072		GO TO 37
0073	46	STOP
0074	37	CONTINUE
0075		IF (LDUM-1) 50, 51, 54
0076	50	IF (K-2) 48, 48, 49
0077	48	LDUM = 1
0078		K=1
0079		SQ(K) = SUMSQ(Y, TY, A, B, S, NF)
0080	52	K=K+1
0081		GO TO 47
0082	51	SQ(K) = SUMSQ(Y, TY, A, B, S, NF)
0083		IF (SQ(K)-SQ(K-1)) 52, 53, 53
0084	53	LDUM = 2
0085		GO TO 90
0086	49	CONTINUE
0087	54	CONTINUE
0088	66	CONTINUE
0089		OUTPUT A, P, S(1,1), S(2,1), S(3,1), S(4,1), SQ(K-1)
0090		PRINT 60
0091	60	FORMAT (25H, I, Y(I), TY(I))
0092		PRINT 61, (I, Y(I), TY(I), I=1,NF)
0093	61	FORMAT (1H, I2, 2(10.3)
0094	12	CONTINUE
0095		END

0001	FUNCTION SUMSQ(Y, TY, A, B, S, NF)
0002	DIMENSION S(4,100), Y(100), TY(100)
0003	N=1
0004	N=N+1
0005	S(1,N)=(1-A-B)*S(4,(N-1))+B*S(3,(N-1))+A*S(1,(N-1))
0006	S(2,N)=(1-A-B)*S(1,(N-1))+R*S(4,(N-1))+A*S(2,(N-1))
0007	S(3,N)=(1-A-B)*S(2,(N-1))+R*S(1,(N-1))+A*S(3,(N-1))
0008	S(4,N)=(1-A-B)*S(3,(N-1))+R*S(2,(N-1))+A*S(4,(N-1))
0009	TY(N)=((1-A-B)*S(4,(N-1))+R*S(3,(N-1)))/(1-A)
0010	IF (N-NF) 2,3,3
0011	SUMSQ = 0.
0012	TY(1) = 0.
0013	N=1
0014	N=N+1
0015	SUMSQ = SUMSQ + (TY(N)-Y(N))**2.
0016	IF (N-NF) 4, 5, 5
0017	CONTINUE
0018	RETURN
0019	END

FLASH EXPERIMENT WITH CHLORELLA

A = .300000

B = 5.000000E-02

SR = 3.00000

S(1,1) = 1.00000

S(2,1) = 3.00000

S(3,1) = .000000

S(4,1) = .000000

DA = 2.000000E-02

DB = 6.999999E-03

DSR = .100000

DA = 4.999999E-03

DB = 9.999999E-04

DSR = 1.000000E-02

A = .234999

B = 1.699999E-02

S(1,1) = .704237

S(2,1) = 3.29576

S(3,1) = .000000

S(4,1) = .000000

SR(K-1) = 5.235000E-02

I Y(I) TY(I)

1 .000E+00 .000E+00

2 .000E+00 .000E+00

3 .750E+01 .110E+00

4 .194E+01 .128E+01

5 .167E+01 .160E+01

6 .970E+00 .888E+00

7 .615E+00 .508E+00

8 .965E+00 .890E+00

9 .125E+01 .123E+01

10 .120E+01 .119E+01

11 .900E+00 .931E+00

12 .955E+00 .866E+00

13	.103E+01	.982E+00
14	.109E+01	.107E+01
15	.101E+01	.104E+01
16	.990E+00	.974E+00
17	.100E+01	.962E+00
18	.102E+01	.100E+01
19	.102E+01	.102E+01
20	.101E+01	.101E+01
21	.100E+01	.991E+00
22	.101E+01	.990E+00
23	.101E+01	.100E+01
24	.101E+01	.101E+01
25	.101E+01	.100E+01
26	.101E+01	.997E+00
STOP		

END

2

4

0

3

7

7

F

I

N