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AN INVESTIGATION OF WATER SPLITTING IN THE KOK SCHEME OF PHOTOSYNTHETIC OXYGEN EVOLUTION

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SUMMARY

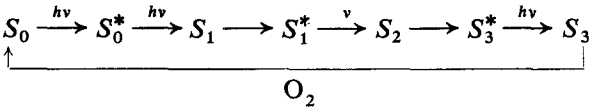
The involvement of OH bond breaking in the 4 dark reactions of the Kok scheme of photosynthetic oxygen evolution was investigated using *Chlorella* and spinach chloroplasts. When the photosynthetic material was suspended in a ^2H_2O based medium, the reaction rates in all 4 cases were only slightly reduced as compared to the rates observed in an H_2O based medium. This was evidence that these rate processes were probably not limited by the breaking of an OH bond. Observations were also made on the yields of O_2 from dark adapted *Chlorella* subjected to a sequence of brief saturating light flashes. The oscillating flash yield sequence observed with algae suspended in ^2H_2O showed greater damping of the oscillations than when the algae were suspended in H_2O. A computer fit of the Kok model to these results revealed a slightly higher proportion of misses, α (i.e. absorbed quanta that do not drive photochemistry) in the ^2H_2O case.

INTRODUCTION

In previous experiments with *Chlorella* [1] and spinach chloroplasts [2] we attempted to determine whether OH bond breaking was involved in the rate limiting step of oxygen evolution. Using the modulated polarographic technique developed by Joliot et al. [3] we examined the deuterium isotope effect and determined that the rate constant, k, of the rate limiting step of oxygen evolution was only slightly reduced when H_2O in the bathing medium was replaced with ^2H_2O. Since the ratio (k_H/k_{2H}) would probably have had a value between 3 and 7 if an OH bond had been cleaved, this was taken as evidence that the rate limiting step did not involve OH band cleavage.

In the experiments reported here we attempted to locate the water splitting act in the Kok model of oxygen evolution [5]. The Kok scheme is based on the observation of oscillating yields of oxygen produced by dark adapted photosynthetic organisms in response to a sequence of brief saturating light flashes. In this scheme 4 consecutive photoreactions are supposed to lead to the production of oxygen. After each very fast light step there follows a much slower dark process with half times that

have been estimated to range from 10^{-3}s to 10^{-4}s [6]. The scheme is as follows:



Although Kok et al. [5] made an initial study of some of the dark reactions, the most thorough examination of the time course and temperature dependance of these processes was made by Bouges-Bocquet [6, 7]. The latter studies were based on the observation of yields of oxygen produced in response to special sequences of flashes. Using her technique, we attempted to identify the OH bond breaking reactions of the Kok scheme by substituting ^2H_2O for H_2O.

MATERIALS AND METHODS

Chlorella vulgaris was obtained from the Carolina Biological Supply Co. and was grown under constant culture conditions as described by Myers [8, 9]. Preparation of chloroplasts and deuterated media has been described previously [1, 2].

Short (7 μs at $I_{max}/3$), saturating flashes of light were obtained from two E.G. and G. FX-127 flash tubes. The special sequences of flashes required for these experiments (after Bouges-Bocquet, ref. 6) were programmed by a flash controller designed and built by Carleton University Science workshops. Yields of oxygen from the flashes were measured by a procedure that was similar to that described by Babcock and Sauer [10].

The Kok model was fitted by a least squares procedure to the sequence of oxygen yields observed in flashing light. The parameters of the model which could be optimized in the computer were the proportion of misses, α, the proportion of double hits, β and the values of S_0, S_1, S_2 and S_3 after a dark period.

RESULTS

Our first observations involved the effect of ^2H_2O on the yields of oxygen produced by *Chlorella* in response to flashing light after a dark period of 5 min. In Fig. 1, the flash yield sequences are compared for one sample of algae, suspended first in an H_2O based medium and then in a ^2H_2O based medium. The oscillation of the flash yield sequence shows slightly greater damping in ^2H_2O than in H_2O. When the Kok model was fitted to the experimental results by the computer procedure, the increased damping in ^2H_2O was reflected in a higher value for the proportion of misses, (0.25 and 0.22 for ^2H_2O and H_2O respectively). This effect was consistently observed and was reversible even when the cells were held for 3 h in ^2H_2O. For five determinations the mean value of $(\alpha_{^2H_2O}/\alpha_{H_2O})$ was 1.10 with extremes of 1.19 and 1.07. As can be seen in Fig. 1 there were small changes in β but these were of little significance.

Figs. 2 to 5 illustrate the effect of ^2H_2O on the four dark reactions of the Kok scheme. Here the time course of the nth dark reaction is represented by the plot of the extent of the reaction after a time interval Δt, γ_n (Δt), against Δt. Both the H_2O and ^2H_2O based curves were obtained with the same sample of chloroplasts or *Chlorella*. In all cases the reaction rates were slightly reduced in ^2H_2O based media and these

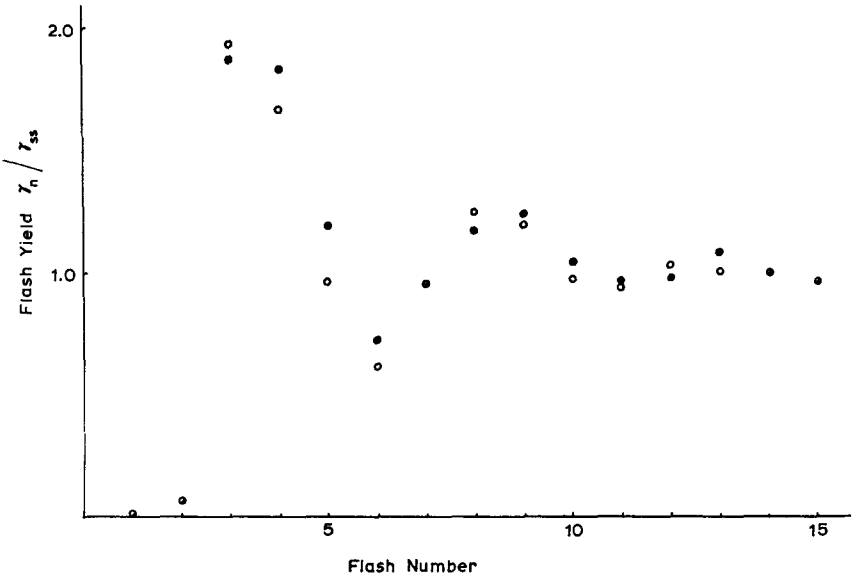


Fig. 1. The flash yield sequence in ^2H_2O and H_2O. *Chlorella* were kept in the dark for 5 min before being exposed to a sequence of 15 brief saturating flashes at intervals of 0.3 s. The yield of oxygen obtained from each flash was plotted against the number of the flash, after being normalized with respect to the mean yield of the last 5 flashes. The closed circles are the results obtained after 1 h in ^2H_2O and the open circles represent the values obtained later in H_2O. The values of α and β from the computer fit of the Kok model were 0.22 and 0.017 for the cells suspended in H_2O, and 0.25 and 0.008 for the cells suspended in ^2H_2O. The starting (dark) values of S_0, S_1, S_2, and S_3 were 25, 75, 0 and 0 % respectively.

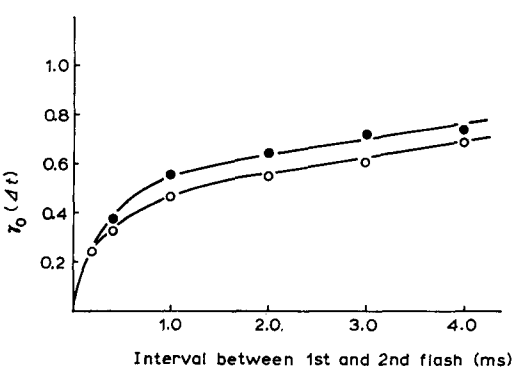


Fig. 2. Time course of the first dark reaction of the Kok scheme, $S_0^* \rightarrow S_1$ in H_2O (●) and ^2H_2O (○). *Chlorella* cells were kept in the dark for 5 min, then subjected to a sequence of three flashes at 0.3 s intervals (the pre-illumination sequence). This was followed by an additional 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 s. $\gamma_0(\Delta t) = [Y_4(\Delta t) + Y_3(\Delta t) - Y_3(0.3\text{ s})]/\gamma_4(0.3s)$ is plotted against Δt. $Y_n(\Delta t)$ represents the O_2 yield of the nth flash. The curves were fitted by eye.

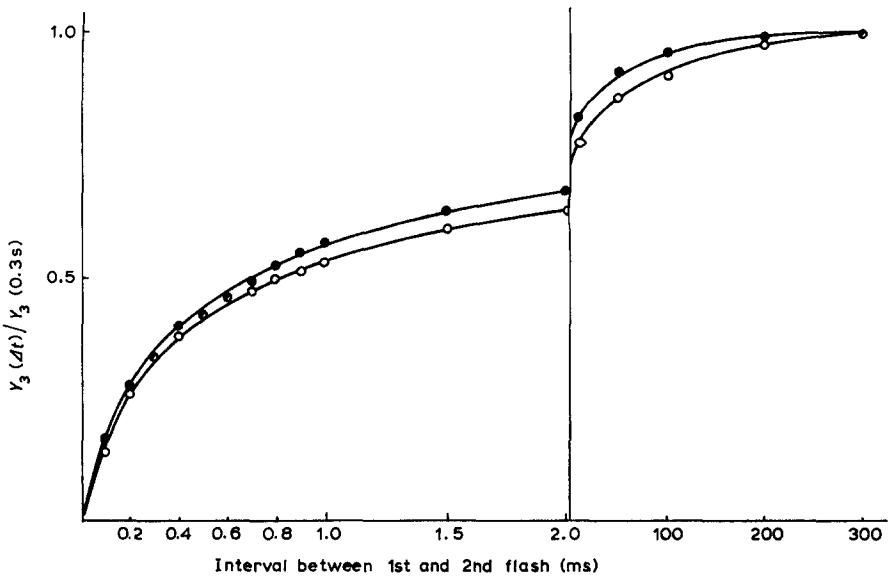


Fig. 3. The time course of the second dark reaction of the Kok scheme, $S_1^* \rightarrow S_2$, in ^2H_2O (○) and H_2O (●). *Chlorella* cells which had been kept in the darkness for 5 min, were subjected to a flash sequence of 25 flashes in which the interval, Δt, between the first and second flashes was variable. The intervals between all other flashes was 0.3 s. The variation of the yield of the third flash $Y_3(\Delta t)/Y_3(0.3\text{ s}) = \gamma_1(\Delta t)$ is plotted against Δt. Curves fitted by eye.

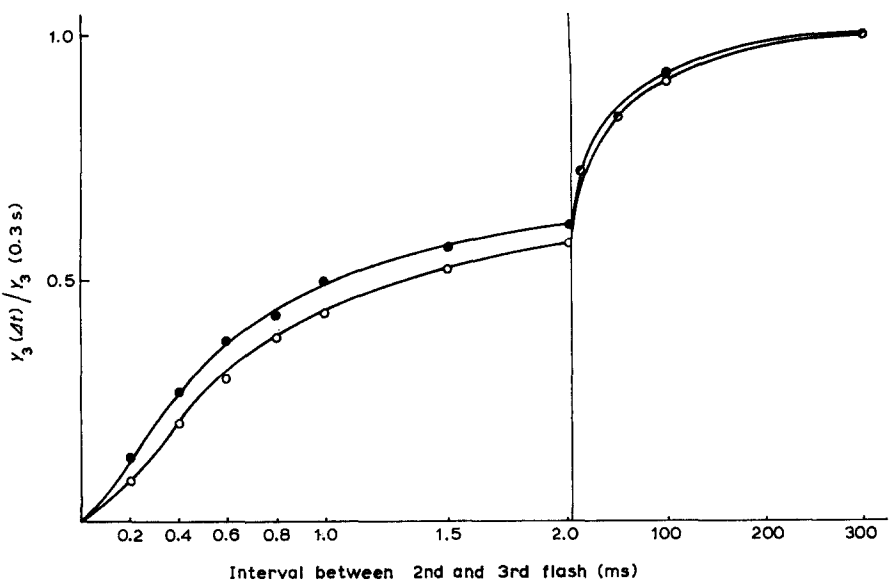


Fig. 4. The time course of the third dark reaction of the Kok scheme, $S_2^* \rightarrow S_3$ in H_2O (●) and ^2H_2O (○). *Chlorella* were left in the dark for 5 min before the sequence of 25 flashes. Between the second and third flash a variable time interval Δt was used. The spacing between all other flashes was 0.3 s. The variations of the yield of the third flash $Y_3(\Delta t)/Y_3(0.3\text{ s}) = \gamma_2(\Delta t)$ is plotted against Δt. The curves were fitted by eye.

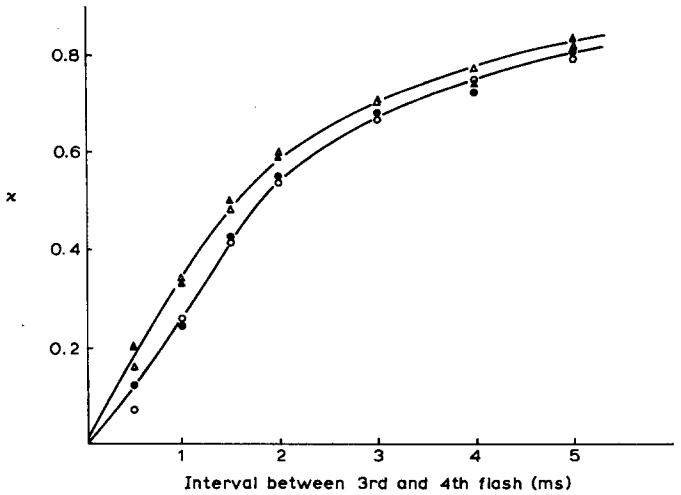


Fig. 5. Time course of the fourth dark reaction of the Kok scheme $S_3^* \rightarrow S_0$ in ^2H_2O (○, △) and H_2O (●, ▲). Spinach chloroplasts, which were suspended in a medium containing 0.1 mM ferricyanide, were subjected to 5 min darkness followed by a flash sequence of 35 flashes in which the interval between the third and fourth flash was variable. All other intervals between flashes were 0.3 s. $[Y_7(\Delta t)-Y_7(0.3\ s)]/[Y_7(0.3\ s)-Y_6(0.3\ s)] = x$ is plotted against Δt (circles). The triangles represent $\gamma_3(\Delta t) = 0.8x(\Delta t)+0.05\gamma_0(\Delta t)+0.18\gamma_1(\Delta t)$. Curves were fitted by eye.

TABLE I

EFFECT OF ^2H_2O ON THE HALF TIMES OF THE DARK REACTIONS OF THE KOK SCHEME

Reaction	Number of determinations	$t_{\frac{1}{2}}(^2H_2O)/t_{\frac{1}{2}}(H_2O)$	Extremes
$S_0^* \rightarrow S_1$	3	1.3	1.0 and 1.6
$S_1^* \rightarrow S_2$	3	1.0	0.95 and 1.1
$S_2^* \rightarrow S_3$	7	1.2	1.0 and 1.4
$S_3^* \rightarrow S_0$	3	1.1	1.0 and 1.2

results are summarized in Table I. ^2H_2O did not significantly alter the shapes of the curves which in both media closely resembled those obtained by Bouges-Bocquet [6]. The small isotope effect observed was reversible and did not depend on whether the biological material was first suspended in H_2O or ^2H_2O. The initial reading in ^2H_2O which was taken 1 h after suspension in that medium could be repeated with some precision an hour later and thus no long term effect of ^2H_2O was evident.

It was possible to use the results of the experiments with *Chlorella* to calculate $\gamma_0(\Delta t)$, $\gamma_1(\Delta t)$, and $\gamma_2(\Delta t)$ in a straightforward manner, but the calculation of $\gamma_3(\Delta t)$ could only be based on measurements made with chloroplasts suspended in a ferricyanide solution, plus terms in $\gamma_0(\Delta t)$ and $\gamma_1(\Delta t)$ derived from the *Chlorella* results. Obviously combining results from two organisms is less than ideal but since the contribution of the $\gamma_0(\Delta t)$ and $\gamma_1(\Delta t)$ terms from *Chlorella* is small (20 %) and also since Bouges-Bocquet has shown that the first three dark reactions are very similar in the two species, this value of $\gamma_3(\Delta t)$ is probably a good estimate for chloroplasts.

DISCUSSION

The release of oxygen during the reaction $S_3^* \rightarrow S_0$ in the Kok model is accompanied by a large release of hydrogen ions and it has been proposed [11] that this reaction involves the water splitting act. These observations led us to expect that there might be a significant reduction in the reaction rate in ^2H_2O. In addition there is a smaller but still appreciable release of hydrogen ions associated with the $S_2^* \rightarrow S_3$ step [11] and so we also examined this reaction with special care. The results showed that in none of the dark reactions was the rate affected to an extent which indicated that OH bond splitting was a rate-limiting factor.

A plausible water-splitting mechanism that predicts a small isotope effect in the reactions of the Kok scheme could involve the slow build up of some activated charge complex, X^* followed by its rapid and irreversible reaction with H_2O:

$$\text{e.g.} \quad S_3 \xrightarrow{h\nu} S_3^* \xrightarrow{\text{slow}} X^* \xrightarrow{\text{fast}} S_0$$

The slightly slower reaction rate in ^2H_2O can be accounted for by the poorer H bonding of the heavy isotope and its effect on enzyme confirmation. This effect may well also account for the slightly higher proportion of misses found in ^2H_2O.

These experiments have been interpreted in terms of the conventional electron transport model where H_2O is the precursor of the evolved O_2, but the absence of an isotope effect is also consistent with Metzner's hypothesis [12] that water is not the origin of photosynthetic oxygen. On the basis of isotope experiments, Metzner concluded that the O_2 precursor must at the minimum have a higher $^{18}O/^{16}O$ ratio than that found naturally occurring in water. The breaking of a CO or OO bond was postulated as an alternative to OH bond breaking for the production of oxygen. In these experiments no evidence of OH bond breaking was observed. So this work which might have cast great doubt on Metzner's hypothesis, leaves the plausibility of this hypothesis undiminished.

In order to test whether the water-splitting act occurs in a final rapid reaction as suggested above, 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 the detection of O_2 (or H^+) will become available. It will then be possible to repeat these experiments in order to resolve the problem of water splitting.

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