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# REACTIONS AT 77°K IN PHOTOSYSTEM 2 OF GREEN PLANTS

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SUMMARY

1. Light-induced changes of absorbance in the region 520–580 nm have been studied in chloroplast preparations and leaves from higher plants at the temperature of liquid nitrogen (77°K). Reactions occurring at this temperature are close to a primary photochemical process.

2. Two absorbance changes were observed that represent respectively the photo-oxidation of cytochrome *b*-559HP (high-potential component, $E'_0 = +0.37$ V) and the photoreduction of a new pigment, P546. Both reactions have action spectra that show a "red drop" and are therefore associated with Photosystem 2.

3. P546 is revealed by absorption of the oxidized form at 546 nm at 77°K in a spectrum of the difference: oxidized *minus* reduced. The pigment has no Soret band and is thus neither a cytochrome nor a chlorophyll.

4. We suggest that cytochrome *b*-559HP may be involved in the photochemical oxidation of water by Photosystem 2 and show how this might occur in terms of a linear four-step mechanism for oxygen evolution.

5. Photooxidation of cytochrome *f* at 77°K could not be detected.

## INTRODUCTION

The primary photochemical process in photosynthesis is widely held to be an oxidation–reduction reaction. In organisms in which water acts as the hydrogen donor, two primary reactions are thought to be arranged in series according to the Z-scheme of HILL AND BENDALL[1]. The precise chemical nature of the primary reactions remains elusive, especially in the case of Photosystem 2 on which evolution of oxygen depends. In the present paper we report observations on chloroplast reactions occurring at the temperature of liquid nitrogen that are likely to be closely connected with the primary process of Photosystem 2 and may throw some light on the mechanism of oxygen evolution.

A considerable body of evidence supports the view that the primary processes of Photosystem 1 of green plants and bacterial photosynthesis involves the 1-equivalent oxidation of a special chlorophyll (P700) or bacteriochlorophyll (P870) molecule.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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The occurrence of the photooxidation of P700 and P870 at very low temperatures, as low as 1°K in the case of *Rhodopseudomonas spheroides*[2,3], is consistent with their postulated participation in a primary photochemical reaction. At the temperature of liquid nitrogen (77°K) secondary chemical reactions may also occur at a significant rate provided the activation energy is small. DEVAULT AND CHANCE[4] described a temperature-insensitive photooxidation of cytochrome in Chromatium which they ascribed to quantum mechanical tunnelling of the electron. PARSON[5] clearly demonstrated the secondary nature of cytochrome oxidation in Chromatium chromatophores. However, transfer of reducing equivalents through a chain of carriers is a thermal process that is immeasurably slow at 77°K and we may conclude that light-induced reactions occurring at this temperature are likely to be very close to a primary photochemical reaction.

In the case of green plants the photooxidation of cytochrome *f* at low temperatures has been reported by WITT *et al.*[6] and by CHANCE AND BONNER[7]. Contrary to our expectations we have been unable to observe the photooxidation of cytochrome *f* at 77°K. However, we have identified two distinct low-temperature changes of absorbance that represent the photooxidation of cytochrome *b*-559HP (high-potential component $E'_0 = +0.37$ V)[8] and the photoreduction of a new pigment, P546, the chemical nature of which is unknown. Action spectra show that both reactions are connected with Photosystem 2.

KNAFF AND ARNON[9–11] have reported similar low-temperature photoreactions and the observations reported here extend their results. P546 is probably identical with the C550 of KNAFF AND ARNON[10], who showed that the pigment has an absorption peak in the difference spectrum at 550 nm at room temperature and at 546–548 nm at 84°K. (We have preferred to use the prefix "P", an abbreviation for "pigment", rather than "C" to avoid any possible confusion with a cytochrome). However, we offer a different interpretation and suggest that cytochrome *b*-559HP may be intimately concerned with the mechanism for production of oxygen.

## MATERIALS AND METHODS

Peas (*Pisum sativum* var. Laxton's Superb) were soaked in water for 8 h and planted in moist, coarsely granular, vermiculite. They were usually grown for 2–4 weeks in a greenhouse at a minimum temperature of 18°, with additional artificial light (14-h day) in winter and shading in summer. Leaves of a pale variety of wych elm (*Ulmus montana* With.) were kindly provided by Dr. R. Hill. *Bergenia sp.* was garden grown. Pale tobacco was grown in the greenhouse and was the aurea mutant Su/su of the cigar variety John William Broadleaf (*Nicotiana tabacum* L.)[12]; seeds were kindly provided by Dr. H. Gaffron.

Chloroplasts were prepared by the method of COCKBURN *et al.*[13]. The grinding medium contained 0.33 M mannitol, 5 mM NaCl, 1 mM $MgCl_2$ and either 10 mM pyrophosphate (pH 6.5) or 10 mM phosphate buffer (pH 7.5). The pellet, after one wash by resedimentation in some cases, was resuspended either in grinding medium (pH 7.5) or in a medium containing 0.33 M mannitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 2.0 mM EDTA and 50 mM sodium HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate) buffer (pH 7.6). The suspension was stored at 0° in the dark.

For spectroscopic measurements the chloroplasts were usually submitted to a

hypotonic shock by an approx. 10-fold dilution in water. An equal volume of the HEPES medium at double the normal strength (see above) was then added; when hydroquinone was used as a reductant the HEPES buffer was replaced by phosphate buffer at pH 6.5. The final chlorophyll concentration was 150–180 $\mu$g chlorophyll per ml. Chlorophyll was measured by the method of ARNON[14].

Difference spectra were recorded with a Johnson Foundation split-beam spectrophotometer[15] and the low temperature cuvette of BONNER[16] was used, with an apparent path length of 2 mm. Samples were frozen in liquid nitrogen in strict darkness. The measuring beam used in these experiments (approx. 1 $\mu$W/cm$^2$) was too weak to cause a significant degree of photochemical action during the course of a single scan of the spectrum. Actinic illumination was obtained by opening the slits of the monochromator while the high-voltage supply to the photomultiplier was off. The positive and negative samples could be separately illuminated by this method.

For determination of action spectra, the difference spectra of the samples were recorded after each of several short periods of actinic illumination. The initial rate of change was calculated from the time courses and was corrected for the small effect of the periods of measurement with relatively weak light that affected positive and negative samples equally. The slit widths used for actinic illumination were adjusted to give equal quantum fluxes of the incident beams at each wavelength, as measured with a Hewlett-Packard PIN photodiode connected to a sensitive galvanometer.

Absolute light intensities were measured with a Hilger-Schwarz thermopile (FT23) connected to a microvoltmeter.

## RESULTS

The typical absorption spectra of chloroplasts in the $\alpha$-band region of the cytochromes have been reported by BOARDMAN AND ANDERSON[17]. The irreversible changes at 77°K induced by actinic light are illustrated in Figs. 1–4. The difference spectrum (untreated *minus* ferricyanide) of a chloroplast suspension frozen in liquid nitrogen in the dark (Fig. 1A) shows peaks at 552 and 548 nm due to cytochrome *f* and a peak at 556 nm due to cytochrome *b*-559HP. (The positions in liquid nitrogen are shifted 2–3 nm towards the violet compared with room temperature). Actinic illumination of the positive sample at 580 nm caused decreases in absorption in the region of 556 nm and 546 nm which resulted in the new difference spectrum shown in Fig. 1B. There appeared to be no change in the redox state of cytochrome *f*, and this is confirmed by the results in Fig. 2A which shows the difference spectrum caused by actinic illumination of the negative sample when both positive and negative samples were without added redox reagents. As would be expected from Fig. 1, Fig. 2A shows peaks at 557 and 547 nm and also a trough at 542 nm, but no change due to cytochrome *f* could be detected. Similar results were obtained when the chloroplast suspension contained hydroquinone or ascorbate, but in the presence of ferricyanide the peak at 546 nm and the trough at 542 nm were seen alone (Fig. 2B). The absorption changes in Fig. 2A can be ascribed to the photooxidation of a cytochrome *b*-559 and to a second pigment of unknown chemical nature which we refer to as P546. Chloroplasts contain two cytochrome *b*-559 components[8,18]. In these experiments only the high potential component would have been available for photooxidation.

The persistence of the absorbance change of P546 in the presence of ferricyanide

suggested that the 546 nm absorption was due to the oxidized form of a pigment that undergoes photoreduction (see ref. 11). This conclusion was confirmed by the fact that dithionite was found to cause reduction of P546 in the dark (Fig. 3). The spectrum of P546 was examined in more detail with chloroplasts isolated from the pale tobacco

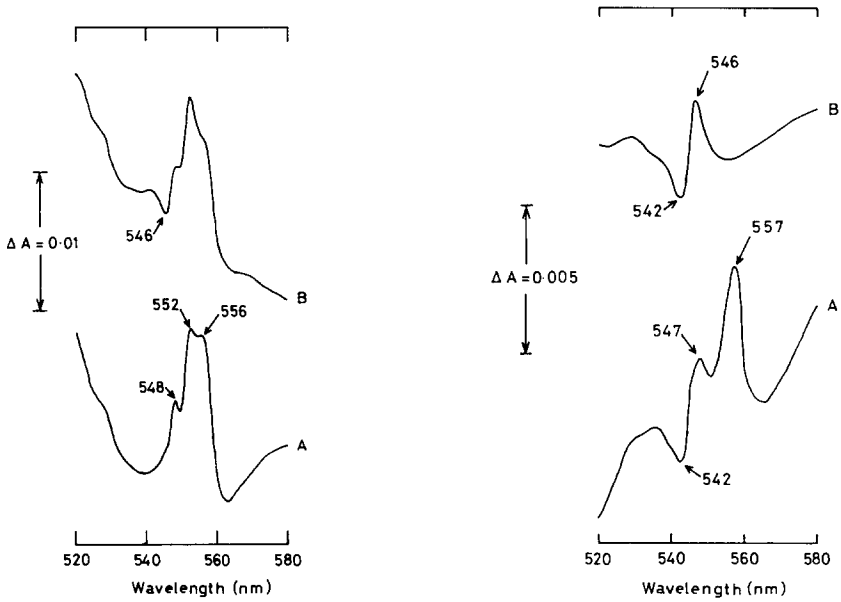


Fig. 1. Effect of actinic illumination on the difference spectrum (oxidized *minus* reduced) of spinach chloroplasts at 77°K. A sample of chloroplasts was hypotonically shocked and resuspended in the HEPES medium to give 143 $\mu$g chlorophyll per ml. The suspension was divided into two halves and to the negative (or reference) portion was added 2.5 mM $K_3Fe(CN)_6$. The cuvette was frozen in darkness. A, the difference spectrum (untreated *minus* ferricyanide oxidized) of this preparation; B, the spectrum of the same preparation after actinic illumination of the positive sample (100 $\mu$W/cm$^2$ at 580 nm for 2 min).

Fig. 2. Difference spectra of pea chloroplasts at 77°K induced by actinic illumination of the negative sample. Samples of chloroplasts were hypotonically shocked and resuspended in the grinding medium (pH 7.5) to give 145 $\mu$g chlorophyll per ml. The cuvette was frozen in darkness, the negative sample illuminated for 2 min at 580 nm (25 $\mu$W/cm$^2$) and then the difference spectrum recorded. A, no addition; B, in presence of 0.25 mM $K_3Fe(CN)_6$.

mutant Su/su of SCHMID[12]. Some evidence was obtained, in difference spectra that showed a peak at 546 nm, for weak, broad peaks at 515 and 490 nm but these were at the limit of sensitivity of the instrument. No Soret band was detectable. The red region was dominated by fluorescence changes of chlorophyll.

Both photoreactions were reversed when the preparation was thawed and could then be repeated after a second freezing. Dark reduction of P546 by dithionite was also reversed when the suspension was thawed and shaken with air. Thus a turnover of these components could be observed. Photooxidation of cytochrome *b*-559$_{HP}$ could not be observed in chloroplast suspensions that were incubated with dithionite for 10 min before being frozen in liquid nitrogen, presumably because the electron acceptor for cytochrome *b*-559$_{HP}$ has already been reduced by the dithionite.

The ability to photooxidize cytochrome *b*-559$_{HP}$ at 77°K shows that the reaction is close to a primary photochemical process and the mid-point potential of the cyto-

chrome suggested that it might be an electron donor to Photosystem 1. However, the action spectra for this reaction and for photoreduction of P546 showed a "red drop" when compared with the absorption spectrum (Fig. 4). Both photoreactions are therefore associated with Photosystem 2. KNAFF AND ARNON[9-11] reached a similar

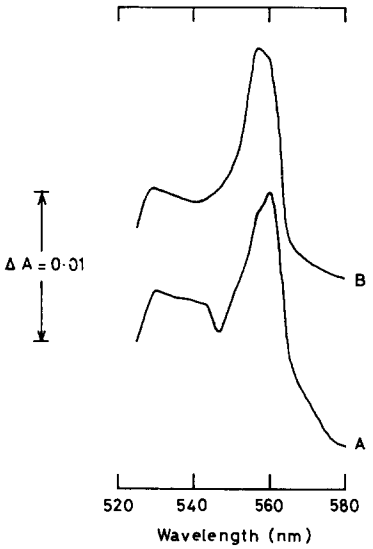


Fig. 3. Difference spectra (dithionite reduced *minus* ascorbate reduced) of pea chloroplasts at 77° K. A sample of chloroplasts was hypotonically shocked and resuspended in the grinding medium (pH 7.5) to give 163 $\mu$g chlorophyll per ml. A. The negative sample contained 5 mM ascorbate. The positive sample contained 5 mM ascorbate and 1 mg/ml sodium dithionite. The cuvette was incubated in darkness at room temperature for 20 min before it was cooled in liquid nitrogen. B. The difference spectrum of the same preparation after actinic illumination of the negative sample (25 $\mu$W/cm$^2$ at 580 nm for 5 min).

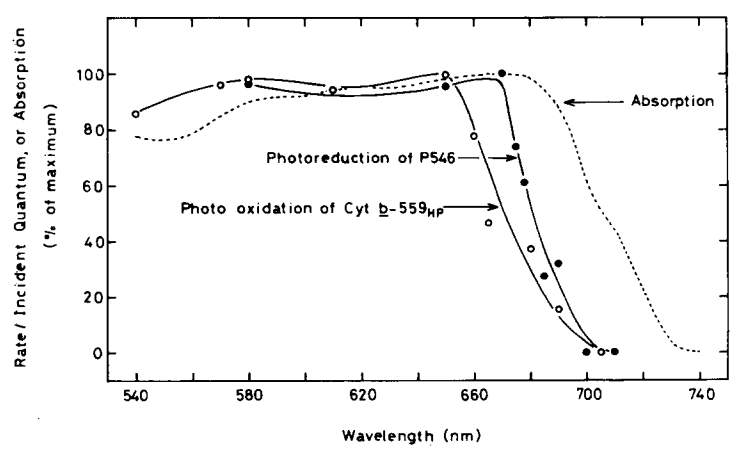


Fig. 4. Action spectra for photoreduction of P546 and photooxidation of cytochrome *b*-559$_{HP}$ in pea chloroplasts at 77° K compared with the absorption spectrum of the same preparations. P546. Chloroplasts were suspended in the HEPES medium and difference spectra (dithionite *minus* ascorbate, see legend to Fig. 3) were recorded after successive periods of actinic illumination of the negative sample. Cytochrome *b*-559$_{HP}$. Chloroplasts were suspended in grinding medium (pH 7.5) containing 2.5 mM hydroquinone and difference spectra were recorded after successive periods of actinic illumination of the negative sample.

conclusion. Although in Fig. 4 there appears to be a small difference between the action spectra for P546 and cytochrome *b*-559HP, this may arise from the fact that they were determined with different chloroplast preparations by slightly different methods. The ratio of the extents of the two reactions appears to remain remarkably constant with different times of illumination under conditions that allow both reactions to be observed simultaneously.

Both photoreactions were observed in a wide variety of preparations and were not dependent on intact chloroplast structure. They were observed on the one hand in pieces of leaf from several higher plants (Fig. 5) and on the other hand in fragments of pea chloroplasts obtained by treatment with digitonin as described by ANDERSON AND BOARDMAN[19]. However, only about 70 % of the cytochrome *b*-559HP in normal chloroplast preparations could be photooxidized. This limitation is most likely determined by the amount of electron acceptor that is available, but other factors may also be involved.

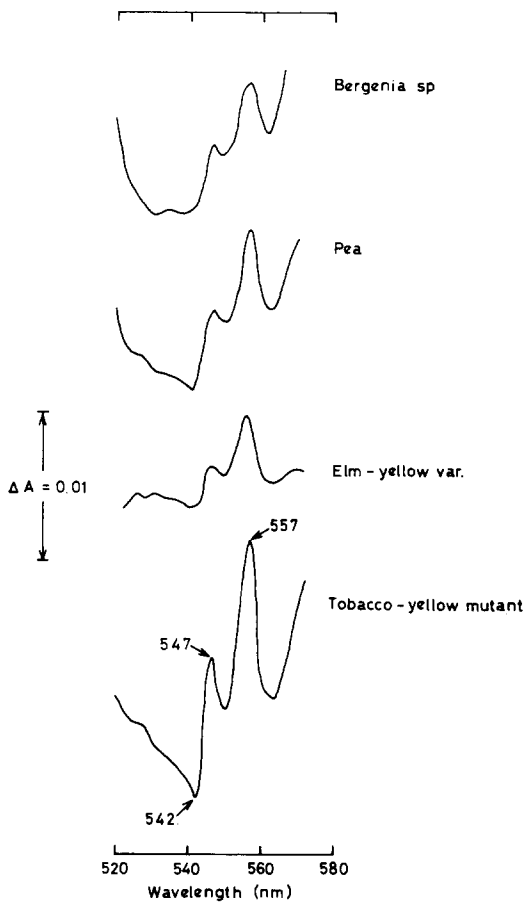


Fig. 5. Light-induced absorbance changes at 77°K of pieces of leaf from various higher plants. The spectra represent the irreversible changes induced by actinic illumination of the negative samples (25 $\mu$W/cm$^2$ at 580 nm for 2–4 min). Two thicknesses of leaf were used for peas and one thickness for the other plants.

Illumination of Chlorella cells at 77°K yielded spectral changes similar to those in Fig. 2A. These two photoreactions may therefore occur widely in organisms capable of the photochemical oxidation of water to molecular oxygen. The properties described above show that the reactions are intimately associated with the primary photochemical process of Photosystem 2 in green plants. The reactions in chloroplasts were insensitive to 1 $\mu$M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and this observation is consistent with the interpretation given above if we accept that DCMU acts between the primary and secondary acceptors for Photosystem 2. However, blue-green algae may be an exception as we were unable to observe the same spectral changes in *Plectonema boryanum*, and in cell-free preparations from this organism we could not obtain evidence for a cytochrome *b*-559 reducible by hydroquinone.

## DISCUSSION

The technique used in these experiments reveals irreversible changes of absorbance that represent reactions close to primary photochemical processes. The simplest interpretation of the results is that cytochrome *b*-559HP acts as a primary donor and P546 as a primary acceptor in Photosystem 2. Reduction of P546 by dithionite would then result in the observed inhibition of the cytochrome photooxidation.

The chemical nature of P546 is unknown. The peak at 546 nm in the difference spectra may represent either the disappearance of a closely similar absorption band in the absolute spectrum of the pigment or, taken together with the trough at 541 nm, a small shift in the position of a much larger absorption band. The lack of a Soret band shows that the pigment is neither a haem compound nor a chlorophyll. The known properties of the pigment may be summarized as follows. (i) The oxidation–reduction potential at pH 7 is less than or about equal to zero volts. This is shown by the fact that dithionite reduces P546 but not ascorbate. (ii) The pigment absorbs in the oxidized form at 546 nm. (iii) The pigment can be photoreduced by Photosystem 2 in a reaction that is insensitive to DCMU and to temperature. (iv) The reduced form can be reoxidized in a dark reaction. (v) Oxidation and reduction are reversible. P546 and the component C550 of KNAFF AND ARNON[10,11] seem to be identical.

Contrary to the observations of WITT *et al.*[6] and of CHANCE AND BONNER[7] we have been unable to detect the photooxidation of cytochrome *f* at 77°K either in chloroplast preparations or in whole leaves. Although we would not have detected a rapidly reversible change, CHANCE AND BONNER found that dark reduction did not occur at a significant rate. Structural dislocation of cytochrome *f* from the reaction centre, a possibility suggested by the work of HILDRETH[20], is unlikely in our experiments with leaves. We have excluded the possibility that the measuring beam was too intense or the actinic beam too weak, and in the case of chloroplasts cytochrome *f* was known to be initially in the reduced state and so available for photooxidation. Thus only a small proportion of the total cytochrome *f* can have been photooxidized and yet have escaped detection.

The action spectrum suggests that cytochrome *b*-559HP is concerned with the photooxidation of water to oxygen, yet the ferricytochrome is not a sufficiently strong oxidizing agent to oxidize water directly. KNAFF AND ARNON[11] suggested that the

reduction of cytochrome *b*-559$_{HP}$ by water is dependent on an additional light reaction which they refer to as IIb. An alternative solution to the problem lies in the proposal of KAMEN[21] that higher oxidation states of a cytochrome might be involved, by analogy with the mechanism of action of catalase and peroxidase[22,23]. KOK *et al.*[24] have interpreted their elegant measurements of the yield of oxygen from a series of flashes in terms of a carrier that undergoes a series of four 1-equivalent oxidation steps before reaction with water. The five states of this carrier are described as $S_0$, $S_1^+$, $S_2^{2+}$, $S_3^{3+}$ and $S_4^{4+}$. The possible identity of this carrier with cytochrome *b*-559$_{HP}$ is worthy of serious consideration.

The oxidation–reduction potential at pH 7 for the 4-equivalent reaction

$$S_0 \rightleftharpoons S_4^{4+} + 4e^-$$

may be assumed to be approximately the characteristic potential of the oxygen electrode (+0.81 V) since plants can develop a pressure of oxygen of one atmosphere at least[25]. The potentials of the four 1-equivalent intermediate reactions by which $S_0$ is oxidized to $S_4^{4+}$ are unlikely to be equal to that of the 4-equivalent process. The breakdown of the overall reaction into two 2-equivalent processes, which is conveniently considered first, may be described by the theory developed for the intermediate formation of semiquinones in the oxidation–reduction of organic dyes. Under any given set of conditions we may write, following CLARK[26];

$$E_h = E_1 + \frac{RT}{2F} \ln \frac{[I]}{[R]} = E_2 + \frac{RT}{2F} \ln \frac{[O]}{[I]}$$

where [O], [R] and [I] are the concentrations of the fully oxidized, the fully reduced and the intermediate forms, respectively. These concentrations are related by the equation

$$\frac{[I]^2}{[O][R]} = K$$

where $K$ is the intermediate formation constant. Hence

$$E_2 - E_1 = \frac{RT}{2F} \ln K$$

and if we write

$$E_h = E_m + \frac{RT}{4F} \ln \frac{[O]}{[R]}$$

for the 4-equivalent process, it follows that

$$E_2 - E_m = E_m - E_1.$$

Thus the 2-equivalent reactions will have mid-point potentials displaced by an equal amount above and below the mid-point potential of the 4-equivalent process. The sign and size of the displacement depends on the value of $K$. If $K > 1$, then $E_2 > E_m > E_1$. Only when $K = 1$ is there no displacement.

The breakdown of the 2-equivalent process into 1-equivalent reactions can be treated in a similar manner, with suitable adjustment of the value of $n$, the number of electrons transferred, in the above equations.

KOK *et al.*[24] found it necessary to assume that $S_1$ is stable to explain the fact

that the yield from the third flash is high in their experiments and exceeds that of the fourth flash. This suggests that the intermediate formation constant is high for the reaction $S_0 \rightleftharpoons S_1 \rightleftharpoons S_2$. In this case $E_1 < E_m < E_2$ ($E_1$ applies to $S_0 \rightleftharpoons S_1$). A second necessary assumption was that $S_3$ and $S_2$ decay to $S_1$ and not to $S_0$. This requirement suggests that the intermediate formation constants for the reactions $S_0 \rightleftharpoons S_2 \rightleftharpoons S_4$ and $S_2 \rightleftharpoons S_3 \rightleftharpoons S_4$ are also large. If this is so the mid-point potentials for the various reactions are related in a qualitative manner as shown in Fig. 6.

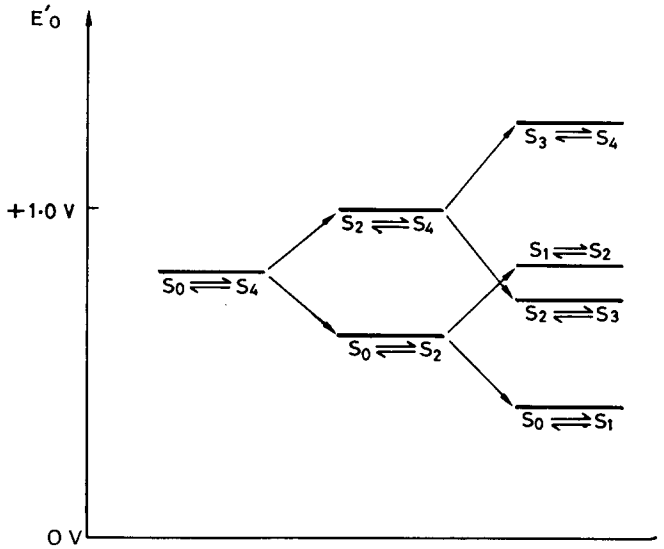


Fig. 6. Oxidation–reduction potentials for a 4-equivalent process that leads to the oxidation of water to molecular oxygen. See text for explanation.

The above considerations are consistent with a simple model of Photosystem 2 in which electrons are transferred through an irreversible light-driven process to a single acceptor which has an oxidation–reduction potential of about zero volts. The arguments developed above for the potentials of S apply only to the equilibria that might be established between the various states of S and other redox systems. For chemical or structural reasons S may not in reality form truly reversible systems. Nevertheless, in the present state of our knowledge, the postulated identity of cytochrome *b*-559HP and KOK's carrier S is clearly consistent with the measured potential of +0.37 V for the ferrous/ferric transition of the cytochrome, which would correspond to the reaction $S_0 \rightleftharpoons S_1$.

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