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DIFFERENCE SPECTRA AND EXTINCTION COEFFICIENTS OF P700*

TETSUO HIYAMA** AND BACON KE

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387 (U.S.A.)

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SUMMARY

From the flash-induced absorption changes due to the redox reaction of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine coupled with P700, the difference extinction coefficients were determined to be 64 mequiv^{-1}·cm^{-1} at 700 nm for digitonin-treated Photosystem-I particles of spinach, and 70 mequiv^{-1}·cm^{-1} at 701 nm for Triton-treated Photosystem-I particles of *Anabaena variabilis*. The values were consistent with those obtained by the method using either cytochrome *c* or *Euglena* cytochrome 552 as direct electron donor after proper corrections. These extinction coefficients and the difference spectra constructed from the exponential recovery phases further strengthened the notion that P700 was a form of chlorophyll *a*.

INTRODUCTION

Discovered by Kok[1,2], P700, a speculative pigment represented by an absorption change widely observed in green-plant type photosynthetic organisms, seems to have established its position as the primary electron donor or the reaction center of Photosystem I*** (refs. 4–7).

It should be noted, however, that the role of P700 in the formation of NADPH, the ultimate product of photosynthetic electron transport, has not been confirmed yet, particularly in the *in vivo* systems, as some workers claim[8,9]. It seems that at least in some *in vitro* systems, $NADP^+$ as well as other artificial electron acceptors such as methyl viologen are reduced by Photosystem I with P700 as the reaction center. On this matter, among other evidences, the results of quantum efficiency measurement seem more convincing at the moment. An earlier work suggested[7] and a recent one confirmed the quantum yield of one for the photooxidation of P700 by far-red light in a certain cell-free system[10]. Though values reported earlier were somewhat lower[11–14], a recent measurement showed a quantum requirement of one both for $NADP^+$ and methyl viologen reduction by far-red light with artificial donor system in isolated chloroplasts[15]. These results indicate not only that in Photosystem I the

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $TMPD^+$, the oxidized TMPD (Wurster's Blue); PMS, *N*-methylphenazinium methylsulphate (phenazine methosulphate).

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** Present address: Carnegie Institution of Washington, Stanford, California 94305, U.S.A.

*** A photochemical pigment system activated mainly by far-red light, according to the definition of Duysens *et al.*[3].

absorbed light energy is extremely efficiently converted into chemical energy, but also that in certain systems there is little room for any reaction centers for $NADP^+$ photoreduction other than P700.

For the estimation of P700, its difference extinction coefficient ($\Delta\varepsilon$) is essential, since it can only be detected by its absorption change, induced either by light or by redox agents[4]. Several values for $\Delta\varepsilon$ of P700 are currently in use for the estimation of P700. Beinert and Kok[16] used tentative values for their calculation of ESR data based on the assumption that P700 was a specialized form of chlorophyll *a*. Witt *et al.*[18], on the other hand, used lower values which were reported to have been obtained from the reduction of either ferricyanide[17] or 2,6-dichlorophenolindophenol[18] coupled with P700.

Recently, another value of extinction coefficient was introduced which was based on the measurement of the oxidation of mammalian cytochrome *c* coupled with the re-reduction of P700 subsequent to an actinic flash in "P700-enriched particles" (HP700) isolated from *Anabaena variabilis*[19]. The value ($\Delta\varepsilon_{703\,nm} = 120$ mequiv$^{-1}\cdot$ cm^{-1})* was substantially higher than those used by others (80 mequiv$^{-1}\cdot$ cm^{-1} in ref. 16; 36 in ref. 17; 42 in ref. 18). A more recent study, however, favored a lower value ($\Delta\varepsilon_{700\,nm} = 64$ mequiv$^{-1}\cdot$cm^{-1}) to explain the observed stoichiometry in a cyclic electron flow involving P700, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and P430 in spinach particles[10]. From this value the quantum yield mentioned above was estimated[10].

The present paper first deals with the details of the method for obtaining $\Delta\varepsilon$ of P700 using TMPD as an indicator, and then with the apparent discrepancy between the previous[19] and the present values and its possible implications. Difference spectra of P700 in the Photosystem-I particles from spinach and *Anabaena variabilis* will also be presented

METHODS AND MATERIALS

D-144 particles were prepared from spinach according to Anderson and Boardman[20] and HP700 particles from *A. variabilis* grown in diphenylamine-supplemented medium according to Ogawa and Vernon[21]

The reaction with $TMPD^+$ was performed in a Thunberg-type cuvette (optical path, 10 mm) after evacuation. $TMPD^+$ was produced simply by aerating the reaction mixture for a second before evacuation. The concentration of $TMPD^+$ was determined by the absorption difference before and after adding excess ascorbate at the end of an experiment as described previously[10].

The instrument set-up for measuring the time course of absorption change induced by a flash has been described earlier[10,22]. A short flash (20 μsec duration) from a xenon lamp was passed through filters: a Corning 4-96 and a broad-band interference filter (400–500 nm, Baird Atomic) to obtain the blue actinic light; a Corning 2-58 and a broad-band interference filter (650–730 nm, Baird Atomic) to obtain the red actinic light. The intensities were $1.8\cdot10^{-3}$ J$\cdot$cm^{-2} for the red and $2.2\cdot10^{-4}$ J$\cdot$cm^{-2} for the blue at the surface of the cuvette. Corning 4-96 and 4-76 filters were placed before the phototube (EMI 9558) when the red actinic light was used; in case of

* Since neither the molecular structure nor the molecular weight of P700 is known, its extinction coefficient was expressed on a one-electron equivalent basis throughout the present paper.

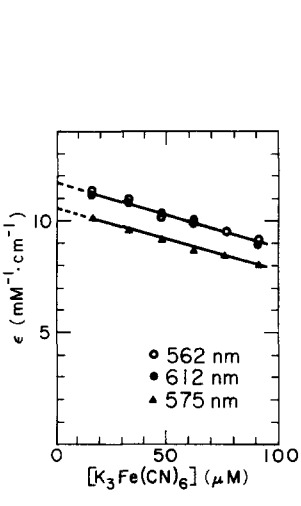
the blue flash a Bausch and Lomb monochromator was used to minimize the interference from fluorescence. All traces were the average of several measurements treated by a signal averager (Fabri-Tek Model 1062 Instrument Computer). Precautions to minimize the errors derived from the measuring light intensity and flash interval were described previously[10]. All the flash experiments were carried out in a cuvette with an optical path of 10.0 mm and at room temperature (22 °C).

Digitonin, Tricine, and ascorbic acid were products of Sigma and used without further treatment. Methyl viologen and TMPD (HCl salt) were from Eastman Kodak. Cytochrome *c* (horse heart) was a product of Sigma and reduced by H_2 with palladium charcoal as catalyst. *Euglena* cytochrome 552 was purified by Dr Sakae Katoh according to the method of Perini *et al.*[23].

RESULTS AND DISCUSSION

Extinction coefficient of TMPD+

TMPD is oxidized by a number of oxidants to form a stable radical known as Wurster's blue. The absorption spectrum and extinction coefficients of Wurster's blue ($TMPD^+$) have been reported in the literature, which were obtained either by bromine titration of TMPD[24] or by direct spectrophotometric observation of the perchlorate salt[25]. Since the two values reported in the literature[24,25] are slightly different from each other, the re-examination of the extinction coefficient of TMPD was performed by ferricyanide titration. Since the autooxidation of TMPD was impractically rapid



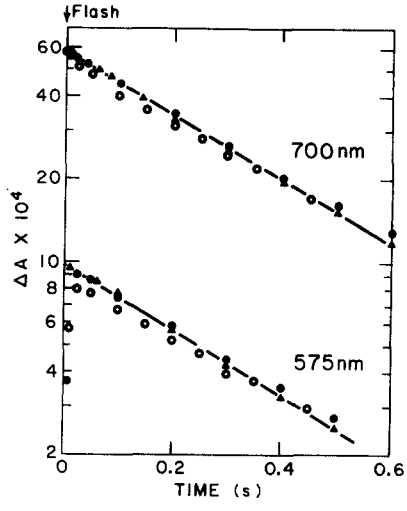


Fig. 1. Titration of TMPD by $K_3Fe(CN)_6$. Freshly dissolved TMPD in water (1 mM) was partially oxidized by $K_3Fe(CN)_6$ of indicated concentrations in an open cuvette at room temperature. Extinction coefficients (ε) were calculated by assuming that $K_3Fe(CN)_6$ could fully oxidize TMPD as a one-electron carrier.

Fig. 2. Kinetics of absorption changes in spinach D-144 particles at different $TMPD^+$ concentrations. The reaction mixture (anaerobic) contained: D-144 particles (chlorophyll, 11 μg/ml); 50 mM Tricine–NaOH (pH 7.4); 67 μM TMPD; $TMPD^+$, 0.2 μM (○), 0.6 μM (●) and 1.2 μM (▲). Four and sixteen blue flashes were applied for the absorption changes at 700 nm and 575 nm, respectively. All experiments at room temperature.

at pH 8 where all the flash experiments were performed, the titration was carried out in water (pH approx. 5–6) in an open cuvette. A separate experiment with a Thunberg-type cuvette revealed that under anaerobic conditions in Tricine buffer (pH 8), a given amount of ferricyanide added from the side arm gave practically the same absorption change as observed in water. Thus, the effect of pH on the extinction coefficient seemed negligible in the pH range of 5–8. Since it was found that the absorption change due to the oxidation by ferricyanide was not proportional to the amount of the oxidant added to the TMPD solution, the extinction coefficient (ε) calculated from the absorption at a given ferricyanide concentration was plotted against the concentration of the oxidant and extrapolated to the zero concentration (Fig. 1). The ε values thus obtained were 10.6, 11.7 and 11.7 $mM^{-1} \cdot cm^{-1}$ (or $mequiv^{-1} \cdot cm^{-1}$) for 575, 562 and 612 nm, respectively. These values were slightly smaller than those reported earlier: $\varepsilon_{565\,nm} = 12.47$ (ref. 25) or 12.08 $mM^{-1} \cdot cm^{-1}$ (ref. 24). We have found that even the location of the absorption maxima seemed to have shifted by a few nm under the present experimental conditions. Nevertheless, ε value of 10.7 $mequiv^{-1} \cdot cm^{-1}$ at 575 nm, where an isosbestic point of P700 was located[10], was used in the present paper.

It should be noted that TMPD (the reduced form) had practically no absorption in the spectral region ranging from 400 to 700 nm. The values obtained above, therefore, would automatically be equal to the $\Delta\varepsilon$ values. It is also to be noted that the absorption of either TMPD or $TMPD^+$ at 700 nm was negligible, thus not interfering with the observation of P700 changes at 700 nm.

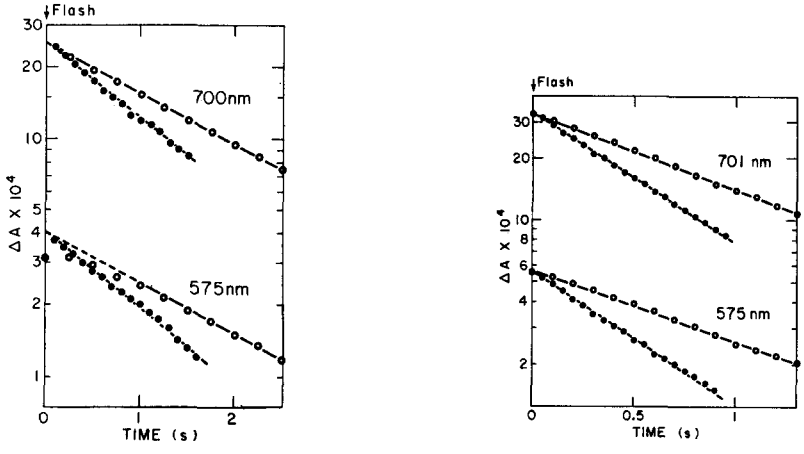


Fig. 3. Kinetics of absorption changes in spinach D-144 particles at different TMPD concentrations. The reaction mixture (anaerobic) contained: D-144 particles (chlorophyll, 8.0 μg/ml); 50 mM Tricine–NaOH (pH 8.0); 17 μM TMPD/0.1 μM $TMPD^+$ (○); 33 μM TMPD/1.0 μM $TMPD^+$ (●).

Fig. 4. Kinetics of absorption changes in *Anabaena* HP700 particles. The reaction mixture (anaerobic) contained: HP700 particles (chlorophyll, 3 μg/ml); 50 mM Tricine–NaOH (pH 8.0); 6 μM $TMPD^+$; TMPD, 33 μM (○) and 67 μM (●).

Kinetic basis and the estimation of $\Delta\varepsilon$ of P700

The principle of the present method for estimating the $\Delta\varepsilon$ of P700 has briefly been described earlier[10]. With TMPD/$TMPD^+$ as an electron carrier, spinach Photo-

system-I particles can perform a simple cyclic electron flow under anaerobic conditions:

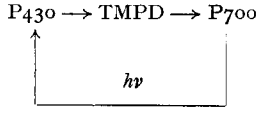


Fig. 2 demonstrates an exponential time course of P700 recovery measured at 701 nm, which was kinetically identical to that of TMPD re-oxidation following the rapid $TMPD^+$ reduction. To further confirm this coupling of TMPD and P700, two other combinations of TMPD/$TMPD^+$ concentrations were used in Fig. 3, where the decay kinetics of P700 and TMPD again agree with each other.

Since these observations were made under identical conditions both at 700 nm and 575 nm, *i.e.* with the same reaction mixture and the same blue actinic flash, a stoichiometric relationship can be expected between the amplitudes of the absorption changes at 700 and 575 nm at any given time. Indeed, the ratio remained constant throughout the above experiments, supporting a stoichiometric relationship between the two components. Thus from the ratio of the initial absorption changes at 700 and 575 nm obtained by the extrapolation of the exponential time courses in Figs. 2 and 3, $\Delta\varepsilon$ of P700 was calculated using the $\Delta\varepsilon$ value of $TMPD^+$ discussed in the preceding section. A typical $\Delta\varepsilon$ value (the average of several measurements) for P700 was 64 $mequiv^{-1}\cdot cm^{-1}$ at 700 nm in D-144 particles. A similar experiment was carried out with the *Anabaena* HP700 particles[21]. The result, shown in Fig. 4, gave a typical value of 70 $mequiv^{-1}\cdot cm^{-1}$ at 700 nm.

Re-examination of the cytochrome method

The values obtained above are much smaller than those estimated earlier[19] in HP700 and spinach particles with cytochrome *c* and cytochrome 552, respectively. To clarify possible causes of this discrepancy, the cytochrome method was re-examined.

It appears that one of the most crucial points in estimating $\Delta\varepsilon$ from the flash-induced absorption changes in the P700-cytochrome system was the treatment of a rapid initial absorption increase at 550 nm preceding the major slower absorption decrease (*cf.* Fig. 1 of ref. 19 and Fig. 8 of the present paper). As was shown previously, the absorption decrease phase was monophasically exponential and corresponded to the decay of P700 measured at 703 nm (ref. 19). The onset of the absorption increase, on the other hand, was as fast as that of P700. This type of rapid absorption increase also took place with *N*-methylphenazinium methylsulphate (PMS)–ascorbate as a donor in place of the cytochrome. From the experiment with different concentrations of PMS, it was also confirmed that the recovery phase kinetically corresponded to those of P700 observed at 703 and 435 nm. It was therefore concluded that the initial absorption increase at 550 nm in the presence of either cytochrome or other donors was due to P700 (*cf.* the P700 spectra in refs. 26 and 27 and Figs. 10 and 11 of this paper). And simply because P700 was directly coupled to the cytochrome, the observed kinetics was the sum of the decay phase of P700 and the rise phase of cytochrome *c* which happened to be kinetically identical but originated from different species. Based on the above reasoning, the absorption change counted from the baseline to the point of maximal absorption decrease had been taken as the absorption

change due to the cytochrome oxidation corresponding to the P700 reduction in the previous work[19]. In other words, the total decay phase *minus* the initial increase was used for the calculation of $\Delta\varepsilon$ of P700.

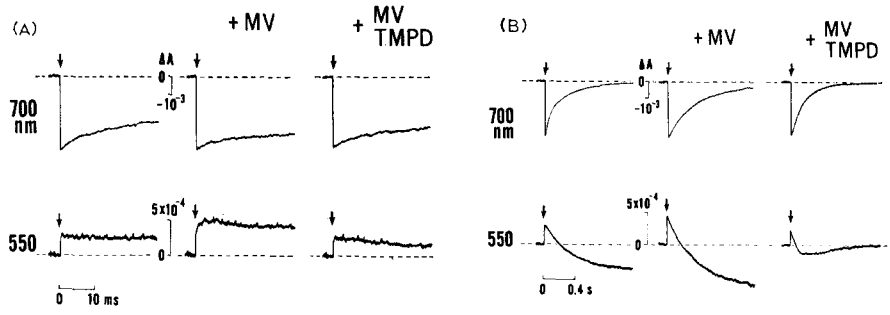


Fig. 5. Absorption changes in *Anabaena* HP700 particles. The reaction mixture in an open cuvette contained: HP700 particles (chlorophyll, 5.5 μg/ml); cytochrome *c*, 0.5 μM (approx. 50% reduced); 670 μM ascorbate, 50 mM Tricine–NaOH (pH 8.0); 67 μM methyl viologen (MV) and 67 μM TMPD where indicated. Short vertical arrows indicate when flash was fired. Eight and thirty-two blue flashes were applied for each trace at 701 and 550 nm, respectively. Traces in A and B are for the same experiments measured at different time scales.

Extensive experimentation subsequently revealed, however, that the addition of methyl viologen in order to block the back reaction and thus achieve a complete coupling of the cytochrome with P700, also resulted in a larger initial absorption increase at 550 nm than the control, while that of P700 measured at 700 nm remained unchanged (Fig. 5). The result indicates that some component other than P700 was also involved in the initial absorption increase. When other electron donors such as PMS were used instead of cytochrome *c*, this "extra" increase was not observed upon the addition of methyl viologen. The spectrum of the initial increase showed a prominent absorption maximum at 550 nm in the presence of methyl viologen (Fig. 6). It is, therefore, quite likely that the "extra" increase was due to the reduction of cytochrome *c*. Indeed, despite the prior reduction of cytochrome *c* by H_2 and the

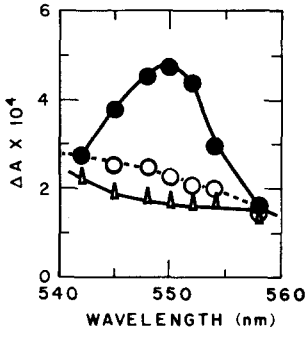


Fig. 6. Spectra of the initial absorption increase. Experimental conditions were the same as in Fig. 5. Maximal absorption changes within 5 ms (refer Fig. 5A) were plotted against wavelength. ○, no addition; ●, 67 μM methyl viologen added; △, 67 μM methyl viologen *plus* 67 μM TMPD. 0.5 μM cytochrome was present in all experiments.

presence of either ascorbate or dithiothreitol in the reaction mixture, nearly half of the cytochrome remained in the oxidized form in the reaction mixture. It is, therefore, reasonable to assume that part of the photo-reduced methyl viologen, a highly electronegative reductant, rapidly reduced cytochrome *c*, thus causing the "extra" initial absorption increase, which was kinetically hardly distinguishable with the present time resolution. This implication would further be supported by the results shown in the right column of Figs 5A and 5B, where the addition of TMPD, which reduced most of the cytochrome, virtually eliminated the "extra" increase. Thus, it became now obvious that a part of the initial increase should be taken into account to estimate the "real" amount of cytochrome *c* coupled to P700. Note that throughout Fig. 5, the magnitude of P700 change remained constant. It is, therefore, reasonable to assume that the "550-nm band" of P700 also stayed constant. Following this reasoning, only the "550-nm band" of P700 estimated from the TMPD experiment (Fig. 5, right) was subtracted from the maximum absorption change of the decay phase for estimating the amount of cytochrome *c* coupled to P700. The new $\Delta\varepsilon$ value of P700 in HP700 particles thus obtained was 75–80 mequiv$^{-1}\cdot$cm^{-1} at 701 nm, which was substantially smaller than the previous value of 120 mequiv$^{-1}\cdot$cm^{-1} and closer to the one estimated by the TMPD method described in the preceding section.

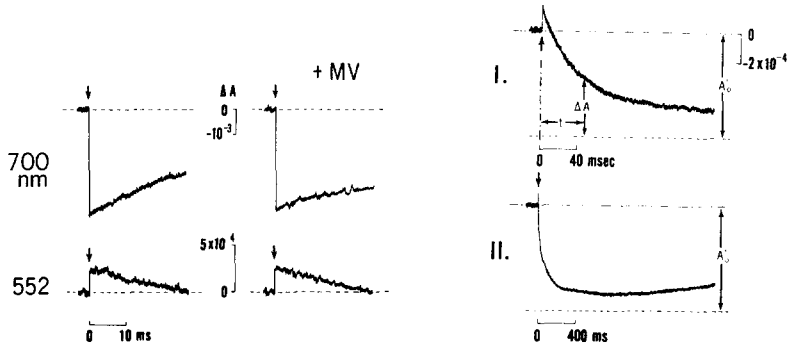


Fig. 7. Absorption changes in spinach D-144 particles. The reaction mixture contained: D-144 particles (chlorophyll, 6 μg/ml); cytochrome 552, 0.3 μM; 670 μM ascorbate; 67 μM methyl viologen (MV) where indicated. Sixteen and thirty-two blue flashes were used for the absorption changes at 700 and 552 nm, respectively.

Fig. 8. Typical traces of absorption changes at 552 nm in the spinach D-144/*Euglena* cytochrome 552 reaction. This figure illustrates the basis for the plot in Fig. 9. Experimental conditions were the same as in Fig. 7, except the chlorophyll concentration was 5 μg/ml. A'_0 was estimated from $\Delta A_{700\,nm}$, and the presumed value of $\Delta\varepsilon_{700\,nm}$, of P700 (64 mequiv$^{-1}\cdot$cm^{-1}) and $\Delta\varepsilon_{552\,nm}$ of cytochrome 552 (19.6 mM$^{-1}\cdot$cm^{-1}), assuming a complete coupling of P700 with the cytochrome. Trace I illustrates how ΔA at time t was estimated on the basis mentioned above.

On the other hand, in case of *Euglena* cytochrome 552 coupled with P700 in spinach particles, the photoreduction of the cytochrome through methyl viologen seemed insignificant as shown in Fig. 7. The result could be explained in part by the difference of the two cytochromes, as cytochrome 552 was mostly in the reduced form in the presence of ascorbate alone. Yet the $\Delta\varepsilon$ value obtained previously in Triton-fractionated spinach particles by the cytochrome method was 122 mequiv$^{-1}\cdot$cm^{-1} (ref. 19). It was noted, however, from the trace shown in Fig. 3 of the previous

paper[19] that the re-reduction of the cytochrome seemed fast enough to interfere with the rather slow oxidation kinetics, thus resulting in the underestimation of the cytochrome change. Unlike the P700-cytochrome *c* reaction in HP700 particles[19], the analysis of the recovery kinetics of P700 measured at 700 nm revealed that the P700 re-reduction with *Euglena* cytochrome 552 as a donor was not pseudo-first order but a special case of second-order reaction in D-144 particles (see footnote 6 of ref. 10): plotting the reciprocal of the absorption change against time gave a straight line (see Fig. 9 below).

For 552 nm, on the other hand, it was necessary to set up a presumed absorption change due to the initial concentration of reduced cytochrome which coupled to P700 and oxidized completely after infinite time under ideal conditions, *i.e.* without any re-reduction, shown in Fig. 8 as A'_0. To test the validity of the Δ_t value obtained by the TMPD method, an assumed value of A'_0 was calculated from the values of $\Delta\varepsilon_{700\,nm}$ of P700 (64 mequiv$^{-1}\cdot$cm^{-1}), $\Delta\varepsilon_{552\,nm}$ of cytochrome 552 (19.6 mM$^{-1}\cdot$cm^{-1}, ref. 23) and the observed absorption change at 700 nm in the same reaction system.

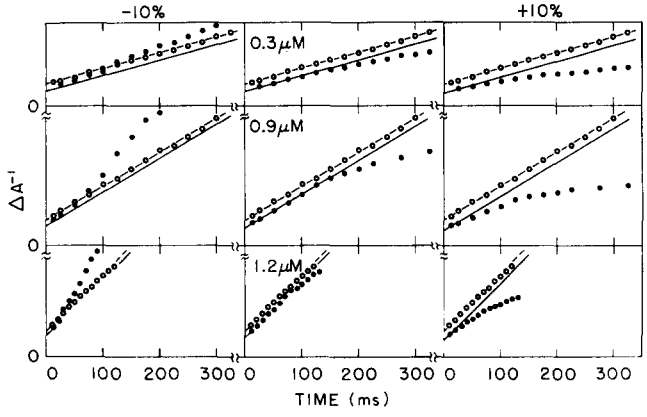


Fig. 9. Plot of the reciprocal of the absorption changes at 700 nm and 552 nm in the spinach D-144/*Euglena* cytochrome 552 reaction. Experimental conditions were the same as in Fig. 8. The scale of ΔA^{-1} was arbitrary. ○, $\Delta A^{-1}{}_{700nm}$,; ●, $\Delta A^{-1}{}_{552nm}$. In the middle column, the values of $\Delta A^{-1}{}_{552nm}$, were estimated by the procedure illustrated in Fig. 8 and plotted for the three separate experiments with different cytochrome concentrations indicated. In the left column, A'_0 was set as 90% of that in the middle column (*i.e.* $\Delta\varepsilon$ of P700 was set as 111% of the presumed value of 64 mequiv$^{-1}\cdot$cm^{-1}); in the right, A'_0 was set as 110% (*i.e.* $\Delta\varepsilon_{700nm}$, was set as 91%). ----, theoretical curves for the second-order decay at 700 nm based on the observed initial absorption change and half decay time; ———, theoretical curves at 552 nm calculated from the theoretical curve at 700 nm and $\Delta\varepsilon_{700nm}$ (64 mequiv$^{-1}\cdot$cm^{-1}) and $\Delta\varepsilon_{552nm}$ (19.6 mM$^{-1}\cdot$cm^{-1}) (see text for more details).

Thus ΔA was determined at a given time, t, and $1/\Delta A$ was plotted against t (Fig. 9, middle column). The results indicate a rather excellent agreement between kinetics of the two components, particularly at higher concentration of cytochrome 552. It would be reasonable to assume that the effect of re-reduction was larger at lower cytochrome concentrations where its oxidation was also slower, thus causing a gradually increasing discrepancy with time.

In order to test the feasibility of the above assumption for A'_0, it was deliberately shifted by *minus* and *plus* 10% in the left and right columns of Fig. 9, respec-

tively. In both cases, an obvious discrepancy resulted, thus supporting the above assumption. It was now evident from Trace II in Fig. 8 that even with a much higher rate of oxidation than in the previous case with Triton-fractionated particles[19], the observed maximal absorption change of the cytochrome was substantially lower than the calculated A'_0. Consequently, a direct reading of the trace could lead to the underestimation of the amount of cytochrome coupled, thus giving a higher $\Delta\varepsilon$ for P700. This may also explain the somewhat higher value obtained above by the cytochrome method in HP700 particles than that by the TMPD method.

Difference spectra of P700

Summarizing the definitions proposed by workers since Kok, P700 would be defined as a component represented by an absorption change with a maximum around 700 nm induced either by actinic light or chemical redox agents[4–6]. Other properties which have been attributed to P700 are:

(1) The oxidation represented by an absorption decrease is induced mainly by far-red light and in intact cells or chloroplasts red light can cause the reduction[6,7,28,29]. Blue light is also effective for P700 oxidation[5,6,28,29].

(2) The redox potential is approx. 0.43 V (ref. 4).

(3) The onset time of flash-induced oxidation is less than 20 nsec (ref. 30)*.

(4) The rate of dark reduction by artificial electron donors is a function of the donor concentration and often follows a pseudo-first-order kinetics in certain subchloroplast particles[6,26].

(5) There is another absorption band with the maximum around 430 nm.

The blue band of P700 has been of particular importance, since such properties as the onset time** and effectiveness of actinic light of different wavelength were determined in this spectral region (refs 5, 10)***. An earlier suggestion by Kok[4] was confirmed by later works[5,6] which showed the parallelism of the re-reduction kinetics at 428 and 705 nm after flash excitation in the presence of PMS–ascorbate of different concentrations and at different pH values among other similarities. For the moment, it seems that the decay kinetics of P700 induced by a flash, which are exponential under certain conditions, would be the most reliable means of identifying P700 (ref. 26). The spectra reported by Rumberg and Witt[6], however, seem to have been oversimplified: the spectra measured by the same principle had more detailed structures[26,27].

In the present paper, the difference spectra of P700 in the two Photosystem-I particles used for the determination of $\Delta\varepsilon$ were examined in more detail. Details of method and precautions for extracting the absorption changes due to P700 from other changes have been described earlier[10]. In these particular preparations, with the exception of around 430 nm where rapid turn-over of P430 was also visible but readily discriminated from P700, no other complications were likely with proper selection

* A similar result was obtained by D. DeVault, T. Kihara and M. Seibert (personal communication).

** The difficulty in measuring the sub-microsecond absorption change at 700 nm might be the separation of actinic light produced by a Q-switched ruby laser (694 nm) from the measuring beam. Excitation at another wavelength, say, 530 nm, a second harmonic of a Nd-YAG laser[30] could not avoid interference from fluorescence.

*** By the technique of Kok and Gott[28] it was possible to obtain the action spectra by measuring the 700-nm response using red actinic light.

of dyes[10]. The recovery kinetics in these preparations were exponential virtually in the entire spectral region examined. Half decay times were also identical. Thus, the spectra were constructed from the initial absorption change *versus* wavelength except at those wavelengths where P430 also contributed to the initial absorption changes. The portion of the initial absorption changes derived from P700, in this case, was estimated by the extrapolation of the exponential phase (straight line on semi-log scale) to time zero. The results are shown in Figs 10 and 11.

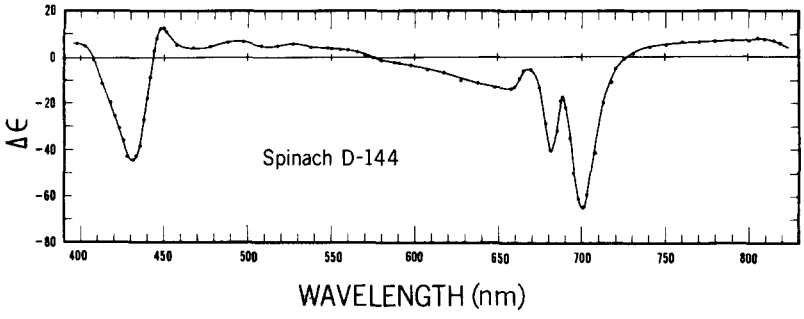


Fig. 10. Difference spectrum of P700 in spinach D-144 particles derived by flash excitation. The reaction mixture contained: D-144 particles (chlorophyll, 10 μg/ml); 67 μM methyl viologen, 50 mM Tricine–NaOH (pH 8.0); 670 μM ascorbate; 3.3 μM PMS, except for the wavelengths between 400 and 450 nm where 67 μM TMPD was used instead. All $\Delta\varepsilon$ values were calculated from ΔA based on $\Delta\varepsilon_{700\text{ nm}}$, = 64 mequiv$^{-1}\cdot$cm^{-1}.

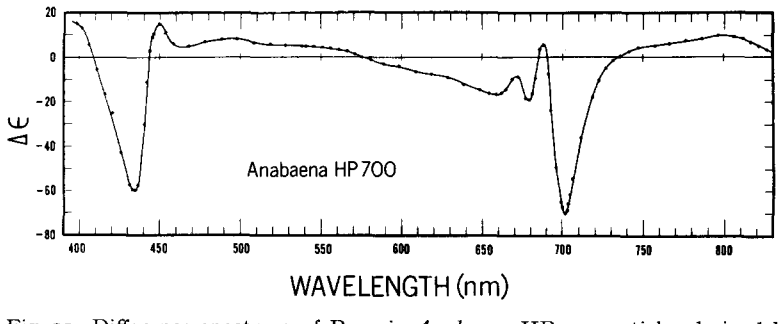


Fig. 11. Difference spectrum of P700 in *Anabaena* HP700 particles derived by flash excitation. The experimental conditions were similar to those in Fig. 10. All $\Delta\varepsilon$ values were calculated from ΔA based on $\Delta\varepsilon_{701\text{nm}}$, = 70 mequiv$^{-1}\cdot$cm^{-1}.

Although certain details are different, both difference spectra have common features. A minor peak around 680 nm was first noticed by Kok[2]. Later works of Döring *et al.*[31] and Murata and Takamiya[32] confirmed its nature as another absorption band of P700. The sizes of the peak, however, differed greatly from spectrum to spectrum. Even in the same D-144 particles, the ratio of $\Delta A_{682\text{nm}}/\Delta A_{700\text{nm}}$ in our previous reports[10,27] was different from our present one. The particular ratio of 0.4 has never been observed in any D-144 particles of subsequent batches. The present ratio of about 0.6 was quite reproducible, and agreed with the one reported by Döring *et al.*[38]. Preliminary results indicated that the increase of digitonin concentration in the reaction mixture had little effect on the ratio. Another preliminary experiment

with Photosystem-I particles prepared by the French press[33] showed a similar ratio of 0.6 which also agrees with the result of Fork and Murata[34].

Triton X-100, on the other hand, was found to cause a drastic reduction of this ratio to as low as 0.13. We are inclined to believe, therefore, that the smaller ratio observed in the previous reports[10,27] could have been caused by an accidental contamination of a small amount of detergent such as Triton. This would partly explain the notable difference of the 680-nm peak in HP700 particles (Fig. 11), which had been treated by Triton. It was observed that the detergent caused less significant deformation on the other parts of the spectrum, although both far-red and blue major bands shifted toward blue.

Although its identification was not clear, Ke[5] observed a broad positive band ranging from 450 to 570 nm, which was included in the P700 difference spectrum. As confirmed in subsequent papers[19,26,27] and the present one, the positive band was indeed due to P700. It should be noted that under certain conditions a part of the "515" change[35] could contribute to this broad band of P700 although the magnitude might be much smaller as was pointed out earlier[5].

It is of particular interest that the π-cation radical of chlorophyll *a* (chlorophyll $a^{+\cdot}$) prepared chemically by charge-transfer reaction showed a similar difference spectrum particularly in this green region[36]. In relation to this, a small positive peak around 800 nm is also of interest, since chlorophyll $a^{+\cdot}$ also had a peak at 820 nm (ref. 36). A small peak around 660 nm and a shoulder of the blue band around 420 nm would be another support for P700 as a form of chlorophyll *a*.

Table I summarizes $\Delta\varepsilon$ values at several peaks and troughs in the P700 spectrum. It should be pointed out that with the present value of $\Delta\varepsilon$ of P700 the discrepancy between the results obtained by ESR and optical measurement would

TABLE I

EXTINCTION COEFFICIENT OF P700

Parentheses represents a broad peak.

D-144 (spinach)		*HP700 (Anabaena)*	
λ (*nm*)	$-\Delta\varepsilon$ ($mequiv^{-1} \cdot cm^{-1}$)	λ (*nm*)	$-\Delta\varepsilon$ ($mequiv^{-1} \cdot cm^{-1}$)
420*	25	420*	25
430	44	434	60
449	−12	449	−15
(470)**	− 4	(468)**	− 4
(493)	− 7	(494)	− 8
(528)	− 7	(530)	− 6
(560)	− 3	(560)	− 4
(658)	14	(658)	17
668**	4	671**	8
682	40	679	20
689**	17	688**	− 7
700	64	701	70
(810)	8	(799)	10

* A shoulder.
** A trough.

become smaller; the ratio between P700 and spin concentrations would approach unity[14,16,37].

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