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# THE REDOX POTENTIALS OF THE *b*-TYPE CYTOCHROMES OF HIGHER PLANT CHLOROPLASTS

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## Summary

1. In fresh chloroplasts, three *b*-type cytochromes exist. These are *b*-559$_{HP}$ ($\lambda_{max}$, 559 nm; $E_m$ at pH 7, +370 mV; pH-independent $E_m$), *b*-559$_{LP}$ ($\lambda_{max}$, 559 nm; $E_m$ at pH 7, +20 mV; pH-independent $E_m$) and *b*-563 ($\lambda_{max}$, 563 nm; $E_m$ at pH 7, —110 mV; pH-independent $E_m$). *b*-559$_{HP}$ may be converted to a lower potential form ($\lambda_{max}$, 559 nm; $E_m$ at pH 7, +110 mV; pH-independent $E_m$).

2. In catalytically active *b-f* particle preparations, three cytochromes exist. These are cytochrome *f* ($\lambda_{max}$, 554 nm; $E_m$ at pH 7, +375 mV, p*K* on oxidised cytochrome at pH 9), *b*-563 ($\lambda_{max}$, 563 nm; $E_m$ at pH 7, —90 mV, small pH-dependence of $E_m$) and a *b*-559 species ($\lambda_{max}$, 559 nm, $E_m$ at pH 7, +85 mV; pH-independent $E_m$).

3. A positive method of demonstration and estimation of *b*-559$_{LP}$ in fresh chloroplasts is described which involves the use of menadiol as a selective reductant of *b*-559$_{LP}$.

## Introduction

There is some discussion in the literature as to the number and midpoint potentials of the *b*-type cytochromes, which are present in fresh chloroplasts isolated from higher plant tissues. Although workers are agreed upon the presence and midpoint potential of cytochrome *b*-559$_{HP}$ ($\alpha$-band maximum of reduced form at 559 nm; at pH 7 $E_m \approx +370$ mV [1]), the numbers and properties of the *b*-type cytochromes which are distinct from this species are

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate; $E_m$, midpoint potential of the species in question relative to a standard hydrogen electrode; $E_h$, ambient measured potential of the redox system in question.

contested. Some maintain that the remaining cytochrome represents a single species, termed cytochrome $b_6$, with an $\alpha$-band maximum in the reduced state at 563 nm, but with $E_m$ values at pH 7 estimated to be anywhere between 0 and −180 mV under appropriate conditions and with an $n$-value of either 1 or 2 [2—5]. Others have reported that this cytochrome $b_6$ is composed of two distinct species, cytochrome $b$-563 which is the major component, and cytochrome $b$-$559_{LP}$ ($\alpha$-band maximum of reduced form at 559 nm; $E_m$ at pH 7 in the range of 0 to +100 mV) [2,6—8], although the point has been raised that this latter species may merely be an altered form of $b$-$559_{HP}$ [6,9].

The purpose of this report is to define the numbers and thermodynamic properties of the $b$-type cytochromes which are present in our preparations of well-coupled lettuce chloroplasts, and of $b$-$f$ particles active in the catalysis of electron transport between plastoquinol-1 and plastocyanin [10]. This provides a thermodynamic basis on which to interpret the results of future experiments carried out with these systems.

## Materials and Methods

### *Biological materials*

Chloroplasts were prepared from fresh lettuce. Washed leaves were homogenised in 0.4 M sucrose, 10 mM sodium chloride, 10 mM Tris-HCl buffer (pH 8.0) and 10 mM sodium iso-ascorbate. After filtration through muslin to remove large debris, the chloroplasts were pelleted by centrifugation at $1500 \times g_{av}$ for 5 min. The chloroplasts were washed once by resuspension and recentrifugation in homogenisation medium without sodium iso-ascorbate. All operations were performed at 4°C as quickly as possible.

Active $b$-$f$ preparations from lettuce chloroplasts were obtained following approximately the initial steps of Nelson and Neumann [11]. Unwashed chloroplasts were resuspended in a medium containing 0.4 M sucrose, 10 mM Tris-HCl and 10 mM NaCl (pH 8.0) to a final chlorophyll $a + b$ concentration of 1 mg/ml, together with 0.1 M NaCl and 1.25% (w/v) digitonin. The mixture was thoroughly homogenised with a hand homogeniser and left overnight at 4°C. A solution of 2 mg/ml protamine sulphate was added, such that chlorophyll $a + b$/protamine sulphate was 2.04 : 1, and the whole was left for 60 min at 4°C followed by centrifugation at $100\,000 \times g_{av}$ for 30 min. The resulting clear yellow-green supernatant contained the $b$-$f$ particles and was used without further purification. This supernatant contained approx. 0.08 mg/ml chlorophyll $a + b$, 1nmol/ml cytochrome $f$ and 2.25 nmol/ml cytochromes $b$. Assay for catalytic activity in the reduction of plastocyanin by plastoquinol-1 as described in [10] showed that the particles at this stage of purification retained full catalytic activity.

Chlorophyll was determined by the method of Arnon [12].

### *Redox potentiometry*

Redox potentiometry was by standard methods [13,14]. The sample was kept anaerobic with a flow of oxygen-free nitrogen and potential was monitored with a platinum electrode against a saturated-KCl calomel reference. Buffers used were 50 mM Mes (pH 4—6.5), 50 mM potassium phosphate

(pH 6.6—7.5), 50 mM glycylglycine (pH 7.6—8.5) or 50 mM glycine (pH 8.6—11.2). The following mediators for a full titration were generally included: 20 μM benzoquinone; 20 μM 2-methylbenzoquinone; 20 μM 2,3-dimethylbenzoquinone; 25 μM duroquinone; 25 μM diaminodurene; 12.5 μM phenazine methosulphate; 12.5 μM phenazine ethosulphate; 25 μM 2-hydroxy-1,4-naphthoquinone; 10 μM 5-hydroxy-1,4-naphthoquinone; 20 μM 2-methyl-1,4-naphthoquinone; 5 μM anthroquinone; and 20 μM anthroquinone-2,6-disulphonate. Approx. 400 U/ml catalase (1 unit (U) decomposes 1 μmol $H_2O_2$ per min at pH 7 and 25°C). Potential was adjusted by additions of aliquots of sodium dithionite (reductive) or potassium ferricyanide (oxidative).

Redox states of cytochromes were determined spectrophotometrically with a Johnson Research Foundation split-beam spectrophotometer. The reference cuvette contained an identical sample of chloroplasts or *b-f* particles plus mediators, but which had generally been reduced with hydroquinone such that cytochromes *f* and *b*-559$_{HP}$ were reduced. A spectrum from 580 to 540 nm was generally scanned so that any baseline changes might be noted. In the case of chloroplasts, significant baseline changes were often observed throughout the titration (cf. Fig. 1). The contributions of these to the observed spectral changes were estimated by construction of a straight line between 547 and 575 nm. This line was then used as the baseline for estimation of the true spectral change at the appropriate wavelength.

*Oxygen evolution assays*

Oxygen evolution was monitored with a Clark-type oxygen electrode. Chloroplasts (approx. 30 μg chlorophyll) were firstly swollen by adding them to 1 ml of distilled water in the oxygen electrode chamber. 1 ml of anaerobic 200 mM mannitol/100 mM Hepes/10 mM $MgCl_2$/40 mM NaCl/20 mM potassium phosphate at pH 7.5 was added to this swollen chloroplast suspension, followed by 1.5 mM potassium ferricyanide. Oxygen evolution could then be observed on illumination with strong red/far-red light. Coupling was tested by addition of aliquots of 50 μM ADP, or by addition of 2 mM $NH_4Cl$ as an uncoupler.

## Results

Since it is known that the high potential form of cytochrome *b*-559 can denature into lower potential forms when the chloroplasts become damaged, care was taken to ensure that the chloroplast preparations used for the experiments were well coupled. The criterion used was that of phosphate potential control of the rate of oxygen evolution in the presence of potassium ferricyanide [15]. A typical ratio of 'State 3' (low phosphate potential) to 'State 4' (high phosphate potential) rates was between 3 and 4. Rates of oxygen evolution in the presence of potassium ferricyanide with ammonium chloride as uncoupler were approx. 4000 nmol oxygen evolved per min per mg chlorophyll *a* + *b* at 20°C.

*Redox titration of chloroplast b-type cytochromes*

Of the bioenergetic organelles which are generally studied, chloroplasts

perhaps present the most difficulties for titrations of cytochromes because of their extremely high background optical densities and low cytochrome concentrations. For this reason a split-beam spectrophotometer with a modified cuvette holder was used so that complete spectra of changes being monitored might be obtained. In this way, we were able to rule out any baseline artifacts which may be large in this system and may be particularly misleading when working with only two wavelengths (double beam mode).

The results of one such typical experiment on lettuce chloroplasts is shown in Fig. 1. After potential had been adjusted, the sample was left to equilibrate for up to 10 min before a spectrum was taken. Several spectra at the same potential were taken to ensure that equilibrium had been reached. This was further checked by performing both oxidative and reductive titrations to ensure that hysteresis was not occuring to a significant extent. The reference cuvette contained 1 mM hydroquinone to keep cytochromes *f* and *b*-559$_{HP}$ in the reduced state. A consecutive series of traces are shown, beginning with a reductive titration (bottom trace to −303 mV trace) and followed by several oxidative titration spectra (−303 mV trace to top trace). It may be seen that as the potential is lowered below +60 mV a peak progressively appears at

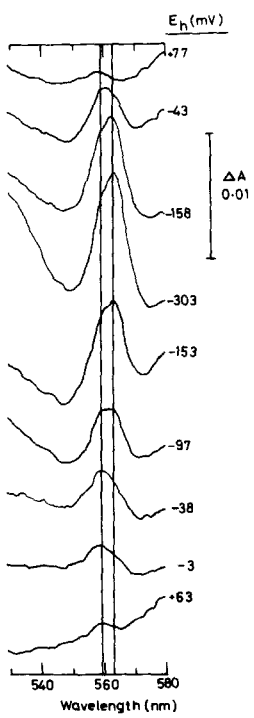


**Fig. 1. Redox titration of cytochromes *b*-559$_{LP}$ and *b*-563 in lettuce chloroplasts. Lettuce chloroplasts were resuspended to 104 μg chlorophyll/ml in 50 mM potassium phosphate at pH 7.2, to which had been added redox mediators and catalase and which had been made anaerobic with oxygen-free nitrogen. A part of this mixture was transferred to a reference cuvette and reduced with 1 mM hydroquinone. The remainder was kept anaerobic with a flow of nitrogen across the surface of the mixture in a redox cuvette. Difference spectra were taken after the potential had been poised at a suitable value and after sufficient time had been allowed for equilibration. The traces are a single sample beginning with the lower trace and continuing upwards.**

559 nm, corresponding to the reduction of $b$-$559_{LP}$. Further lowering of the potential below around −50 mV causes a progressive shift in the wavelength maximum of the peak to 563 nm and an increase in its height, corresponding to the reduction of $b$-563. Below −200 mV, no further changes in the spectrum are detectable. Raising the potential after complete reduction of the system reversed these effects and no significant hysteresis was observed, provided that enough time was allowed for equilibration.

Similar experiments were also performed to determine the potentials of the higher potential species. In these cases a reference cuvette in which the chloroplasts had been fully oxidised with 1 mM potassium ferricyanide was used, and the potential range covered was from around +450—0 mV.

To determine $E_m$ values of components from these data, plots of absorbance change vs. $E_h$ were constructed, at appropriate wavelength pairs and theoretical $n = 1$ Nernst curves were drawn through the data points. For the two overlapping species depicted in Fig. 1, which are interpreted to be $b$-$559_{LP}$ and $b$-563, it was necessary to estimate their relative contributions to total absorbance change between +100 and −300 mV. We used relative contributions of $b$-$559_{LP}$ : $b$-563 to be 0.2 : 0.8 at 563—548 nm and 0.4 : 0.6 at 558—548 nm. These ratios were arrived at from data available in the literature [16] and from our experiments with menadiol which are described below. Fig. 2 illustrates some typical data for estimation of the $E_m$ values at pH 7.0 of $b$-$559_{LP}$ and $b$-563 which gave $E_m$ values of +20 ± 20 mV and −110 ± 20 mV respectively.

Such experiments had to be performed within about 2 h at room temperature, since the $b$-$559_{HP}$ slowly converted to a form which was not reducible by hydroquinone and which therefore interfered with the baseline of the titration. Several experiments were performed to demonstrate this conversion, involving a comparison of titrations of fresh chloroplasts with those of chloroplasts which had been heated at 60°C for 10 min. The titrations were performed against a reference which had been oxidised by 1 mM potassium ferri-

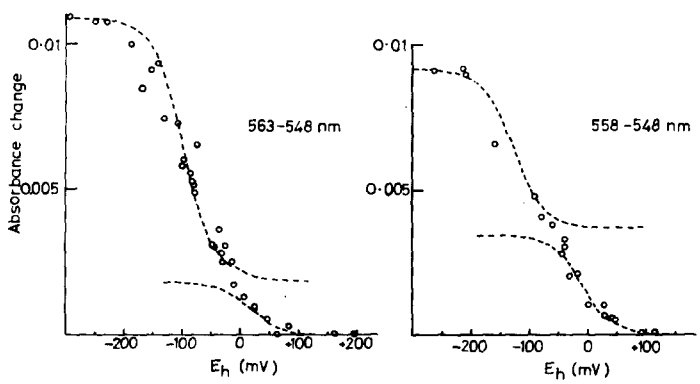


**Fig. 2. Graphical plots of the redox data on cytochromes $b$-$559_{LP}$ and $b$-563 of lettuce chloroplasts. The graphs represent the results of several experiments performed as in Fig. 1 at pH 7.2 and plotted as absorbance change versus potential. Both oxidative and reductive points are included. The curves are theoretical Nernst curves for $n = 1$ redox components. The relative contributions of the two species to the total absorbance change were estimated from the quantitative menadiol experiments discussed in the text.**

cyanide. It was found that the heat treatment caused a decrease in the $E_m$ at pH 7.0 of $b$-559$_{HP}$ from around +370 mV to a single component of $E_m$ of +110 mV. This form was the same as that which was produced by the slow natural decay of the chloroplasts. No evidence was found for more than one modified form of $b$-559$_{HP}$ and no effect of heat treatment on the properties of the other cytochromes was noted.

No significant variation of measured midpoint potentials of $b$-559$_{HP}$, heat-treated $b$-559$_{HP}$, $b$-559$_{LP}$ or $b$-563 was noted when the pH was changed between 6.0 and 8.0. Outside this pH range, serious baseline problems occurred and so experiments were confined to pH values within these limits.

Several experiments performed with chloroplasts isolated from barley shoots gave similar results.

*Redox titration of the cytochromes of b-f particles*

An identical series of experiments were performed with $b$-$f$ preparations derived from lettuce chloroplasts. The particles were rather more stable than chloroplasts and a wider pH range could be used. Since no $b$-559$_{HP}$ was present, it was possible to measure the $E_m$ of cytochrome $f$ in these preparations (this cytochrome is overlapped by the more dominant $b$-559$_{HP}$ in chloroplasts and is difficult to estimate during redox titrations). Besides cytochrome $f$, two $b$-type cytochromes are present, one with $E_m$ at pH 7 of +85 ± 20 mV and an $\alpha$-band wavelength maximum at 559 nm in the reduced state, and the other apparently identical to $b$-563 of chloroplasts with $E_m$ at pH 7 of −90 ± 20 mV. The accumulated results of these experiments at various pH values are illustrated in Fig. 3. It may be noted that the measured $E_m$ of cytochrome $f$ decreases measurably above pH 9, indicative of a p$K$ of an ionisable group on the oxidised protein in this pH range, as originally noted in Ref. 17. The 559 nm component had a pH-independent $E_m$, whereas cytochrome $b$-563 had a small pH-dependency of around −15 mV/pH unit over the entire pH range investigated. The ratio of the 559/563 nm components in $b$-$f$ particles (1 : 2.3 ± 0.2) was the same as the ratio of $b$-559$_{LP}$/$b$-563 in chloroplasts (1 : 2.4 ± 0.2). This ratio may be compared with a ratio of $b$-559$_{HP}$/$b$-563 of approx. 1 : 1 in intact chloroplasts.

*The effects of menadiol on chloroplast cytochromes*

Since the presence of a distinct $b$-559$_{LP}$ species in fresh chloroplasts is a somewhat controversial point, we designed an experiment to directly demonstrate its presence. The method involves the use of menadiol (2-methyl-1,4-naphthoquinol) as a selective reductant of $b$-559$_{LP}$ but not of $b$-563. The preparation and redox properties of this compound have already been described [18]. Its effects on lettuce chloroplasts were investigated with a split-beam spectrophotometer. Chloroplasts in 50 mM 2-(*N*-morpholino)ethanesulphonate at pH 6.0 were kept anaerobic and divided equally between two cuvettes. To both was added 1 mM hydroquinone so that cytochrome $f$ and $b$-559$_{HP}$ were reduced. 1 mM menadiol was then added to the sample cuvette and the difference spectrum was taken (Fig. 4, trace A). It can be seen that the 559 nm species became reduced but the $b$-563 remained almost fully oxidised. Prolonged incubation with the menadiol did not cause the $b$-563 to become

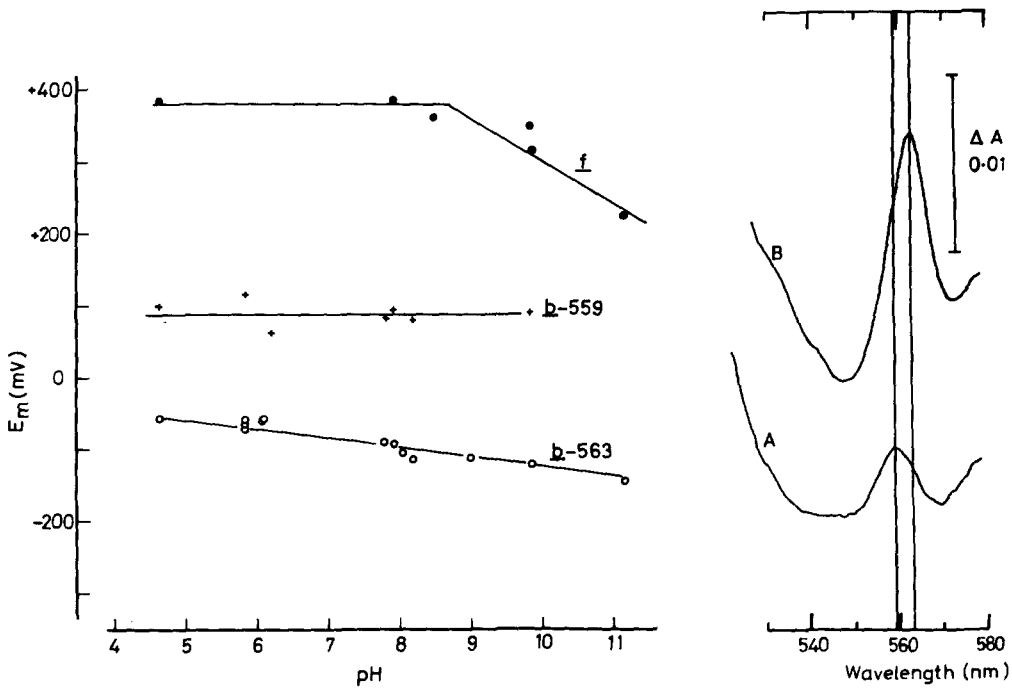


**Fig. 3. A summary of the redox data obtained on the cytochrome components of *b-f* particles at various pH values.**

**Fig. 4. The selective reduction of cytochrome *b*-559$_{LP}$ by menadiol in lettuce chloroplasts. Chloroplasts were suspended in anaerobic 50 mM Mes with 400 U/ml catalase at pH 6.0 to a chlorophyll concentration of 100 μg/ml. The mixture was divided equally between 2 cuvettes and 1 mM hydroquinone was added to both samples. 1 mM menadiol was then added to the sample cuvette and the difference spectrum was plotted (trace A). A few crystals of solid sodium dithionite were added to the sample cuvette. After 15 min, to ensure complete reduction of the cytochromes, 1 mM menadiol was added to the reference cuvette and the dithionite minus menadiol difference spectrum was plotted (trace B).**

reduced. A spectrum of the *b*-563 could therefore be obtained by taking a difference spectrum of dithionite-reduced chloroplasts minus chloroplasts treated with menadiol as described above. The result is shown in Fig. 4, trace B. In this spectrum the small trough caused by reduction of *c*-550 [19] is also clearly observable.

This experiment represents an experimental demonstration of the presence of a *b*-559$_{LP}$ species in fresh chloroplasts. The $E_m$ at pH 6 of the menadione system is around +60 mV [18] and we have measured a generated $E_h$ of around −20 mV when 1 mM menadiol is added to anaerobic, hydroquinone-reduced chloroplasts. Hence, given our $E_m$ values at pH 6 for *b*-559$_{LP}$ and *b*-563 of +20 an −110 mV, respectively, we may calculate that in a menadiol-reduced minus hydroquinone-reduced difference spectrum, *b*-559$_{LP}$ is approx. 80% reduced, whereas *b*-563 is only around 5% reduced. Hence this method may be used as a semiquantitative method of cytochrome estimation. If we approximate that total *b*-559$_{LP}$ = 1.25 × (peak in menadiol minus hydroquinone difference spectrum) and *b*-563 = (peak in dithionite minus menadiol difference spectrum), then the quantitative estimates of the amounts of the two cyto-

chromes obtained are virtually identical to those obtained by the standard technique [16].

## Discussion

The results of this investigation demonstrate conclusively the presence of three distinct *b*-type cytochromes in intact chloroplasts, in agreement with earlier findings from this laboratory [16]. The suggestion that the $b$-$559_{LP}$ is merely a denatured or modified form of $b$-$559_{HP}$ is not borne out by the experimental findings that, even in fresh chloroplasts, a 559 nm species exists, and this 559 nm species has a significantly lower midpoint potential than the altered form of $b$-$559_{HP}$.

The midpoint potential values that we have obtained are summarised in Table I. Our values for the midpoint potentials of cytochromes *f* and $b$-$559_{HP}$ are in good agreement with previously reported values (see references cited in Ref. 6). It may also be noted that cytochrome *f* has a p*K* on the oxidised protein at around pH 9, as originally found by Davenport and Hill [17] for the solubilised cytochrome. The value of +20 ± 20 mV that we have found for the midpoint potential of $b$-$559_{LP}$ is somewhat lower than several others in the literature, but these values may have resulted from a failure to distinguish between a converted form of $b$-$559_{HP}$ and the true $b$-$559_{LP}$ species.

The most serious discrepencies in the literature concern the values reported for the midpoint potential of cytochrome *b*-563 which tend to fall close to either −100 mV (and with which the values reported here agree) or 0 mV. There is the further complication that in one case [4], a two-equivalent slope was found for the first titration curve with freshly isolated chloroplasts, but this was not shown to be reversible and subsequent titrations, both oxidative and reductive, gave the expected one-equivalent slope. An explanation which was offered for the variations is that the midpoint potential and *n*-value of cytochrome *b*-563 is strongly dependent on the state of the chloroplast material being titrated. We have not been able to confirm this result, and in

TABLE I

A SUMMARY OF THE REDOX DATA MEASURED ON THE CHLOROPLAST AND *b*—*f* PARTICLE CYTOCHROMES

n.d., not determined.

| Component | Chloroplasts | | *b*—*f* Particles | |
|---|---|---|---|---|
| | $E_m$ (pH 7 )* (mV) | pH-Dependence (mV/pH) | $E_m$ (pH 7) * (mV) | pH-Dependence (mV/pH) |
| $b$-$559_{HP}$ | +370 | 0 | — | — |
| Heat-treated $b$-$559_{HP}$ | + 110 | 0 | — | — |
| *f* | n.d. | n.d. | +375 | 0 (below pH 9)<br>−60 (above pH 9) |
| $b$-$559_{LP}$ | +20 | 0 | +85 | 0 |
| *b*-563 | −110 | 0 | −90 | −15 |

* Accuracy of ±20 mV.

our hands $E_m$ is stable whether the chloroplasts are fresh or several hours old, and whether they are coupled or uncoupled. The only peculiarity we have found is the pH-dependence (in the *b-f* particles but not in chloroplasts) of −15 mV/pH unit, which we assume to be due to a secondary effect of pH on the heme environment.

The value of +85 mV obtained for the midpoint potential of the 559 nm component of *b-f* particles does not allow its positive identification as *b*-$559_{LP}$ or as the modified *b*-$559_{HP}$ species. However, since the *b*-$559_{HP}$ species is thought to be associated with Photosystem II [6] and since the ratio of *b*-559/*b*-563 is the same in *b-f* particles as is the ratio of *b*-$559_{LP}$/*b*-563 in intact chloroplasts, it is felt that the component is probably *b*-$559_{LP}$ with a slightly modified $E_m$ caused by the purification procedure.

The use of menadiol as a reductant has two advantages. It allows the positive identification of the *b*-$559_{LP}$ species in fresh intact chloroplasts and provides a reasonably quantitative procedure for the estimation of *b*-$599_{LP}$ and *b*-563 which may be used to confirm the normal method [16].

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