*Biochimica et Biophysica Acta*, 325 (1973) 275–283


BBA 46634

# UNCOUPLER-DEPENDENT DECREASE IN MIDPOINT POTENTIAL OF THE CHLOROPLAST CYTOCHROME $b_6$*

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(Received May 29th, 1973)

## SUMMARY

1. The midpoint potential of cytochrome $b_6$ in freshly prepared coupled chloroplasts is approximately +5 mV and the slope of the titration curve is characteristic of a two-electron transition. Subsequent titrations performed one-half hour or more after chloroplast preparation show a slight negative shift of the midpoint potential and a one-electron slope.

2. Titrations of cytochrome $b_6$ in the presence of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) or $NH_4Cl$ show the midpoint potential of approximately half the $b_6$ complement negatively shifted to about −140 mV.

3. Brief exposure of the chloroplasts to $NH_4Cl$ and actinic illumination under typical uncoupling conditions with controlled potential conditions causes a shift in oxidation state consistent with a lowering of the average midpoint potential to about −100 mV.

It is concluded that the midpoint potential of at least half the cytochrome $b_6$ undergoes a negatively directed shift of 100–150 mV under uncoupling conditions in the presence of $NH_4Cl$ and FCCP.

## INTRODUCTION

In recent work of ours devoted principally to the pathway of cytochrome $b_6$ oxidation in chloroplasts[1], two facts emerged which were not easily explained by a previous finding in our laboratory that the midpoint potential of cytochrome $b_6$ was around −180 mV[2]: (1) Approximately one-fourth of the total cytochrome $b_6$ of coupled chloroplasts could be reduced by diaminodurol (midpoint potential at pH 7 $E_{m7} \approx +230$ mV, $E_{m8} \approx +175$ mV, slope of titration curve in electron equivalents ($n$) between 1 and 2, Cramer, W. A., unpublished data). A partial reduction of cytochrome $b_6$ by an excess of reduced diaminodurol would be more easily explainable on an energetic basis if the $b_6$ midpoint were closer to the value of 0.0 V

Abbreviations: $n$, slope of titration curve in electron equivalents; $E_{m7}$, midpoint potential at pH 7; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

* A preliminary version of this work has been presented: *Abstr. 9th Int. Congr. Biochem.* (1973), Stockholm, p. 217.

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determined by Hill[3] and Hill and Bendall[4]. (2) Action spectra for photoreduction of cytochrome $b_6$ in coupled chloroplasts do not rule out the possibility that cytochrome $b_6$ can be reduced by Photosystem II. This would not be expected if the midpoint potential of the primary acceptor of System II at the pH of the experiment was between $-50$ and $-100$ mV[5,6] and that of cytochrome $b_6$ is $-180$ mV. In addition, Knaff[7] has observed that the increase in amplitude of $b_6$ photooxidation obtained upon decreasing the ambient potential from $-75$ to $-205$ mV is much less than expected if the $E_m$ were $-180$ mV.

The explanation of these inconsistencies involving the $b_6$ midpoint potential now appears to be that the midpoint potential of the $b_6$ depends on the energy state and possibly the structural integrity of the chloroplasts. In our original titrations of the potentials of the two chloroplast *b* cytochromes[2], it was thought that the midpoint potentials would be unaltered as long as the cytochromes were membrane-bound. However, the chloroplasts were uncoupled in these experiments by the length (2–3 h) of the titrations and perhaps by the degassing technique. We report in this work titrations of cytochrome $b_6$ in chloroplasts coupled for phosphorylation and the effect on the titration parameters of defined uncoupling conditions. The midpoint potential of cytochrome $b_6$ in coupled chloroplasts is approximately zero volts. Uncoupling with $NH_4Cl$ or trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP) causes the midpoint of at least half of the $b_6$ complement to shift negatively by 100–150 mV. In addition, we note that the first titration curve of cytochrome $b_6$ obtained in freshly prepared chloroplasts generally has a two-electron slope.

## METHODS

### *(1) Chloroplast preparation*

The procedure for a rapid isolation of coupled chloroplasts from spinach leaves, grown in a controlled climate facility (22 °C, 8 h-light cycle), has been described previously[8].

### *(2) Anaerobic redox titrations*

All experiments were carried out at room temperature (approx. 22 °C). A lucite cuvette was used (1-cm pathlength, 3.5 ml volume) with provisions to hold a combination platinum electrode (Radiometer, PK149) and septa through which microliter syringe needles could be introduced anaerobically. The electrode was calibrated in saturated quinhydrone assuming that the reference potential is 699 mV[9]. Because it was found that the technique of bubbling argon through the chloroplast suspension to obtain anaerobic conditions tended to uncouple the chloroplast and reduce System II activity, the chloroplasts were stirred magnetically and argon gas blown over the top of the suspension. The general procedure was to flush the assembled cuvette for 15 min through all-gass connections with purified argon (Matheson prepurified argon passed through a heated column filled with copper precipitated on infusorial earth). The previously deaerated reaction mixture containing the redox buffers was injected through a septum, keeping the cuvette under positive argon pressure. Dithionite and ferricyanide were deaerated dissolved, and kept under anaerobic conditions as 10 mM stock solutions in 10 mM Tricine–NaOH, pH 8.0. After test titrations under these conditions, it was found that the following combination of

redox dyes would act as mediators in the region from +180 to −150 mV: 10 $\mu$M 1,2-naphthoquinone ($E_{m7}$=135 mV, $n$=2)[10], 20 $\mu$M 1,4-naphthoquinone ($E_{m7}$= 65 mV, $n$=2)[10]; 10 $\mu$M 2,5-dihydroxy-1,4-benzoquinone (recrystallized, $E_{m7}$= −60 mV, $n$=2); 10 $\mu$M 2-hydroxy-1,4-naphthoquinone ($E_{m7}$=−137 mV, $n$=2). At the above concentrations the absorbance changes of the redox dyes at the wavelengths of interest were found to be negligible. The accuracy of the method and the anaerobic conditions were checked by titrating FMN ($E_{m7}$=−205 mV, $n$=2). The above mentioned midpoints and slopes of 2,5-dihydroxy-1,4-benzoquinone and 2-hydroxy-1,4-naphthoquinone were also obtained from titrations performed in our laboratory. The deaerated chloroplast reaction mixture at pH 8.0 contained 25 mM Tricine–NaOH, 5 mM $MgCl_2$, 5 mM $K_2HPO_4$ and redox dyes at the concentrations indicated above. Titrations were otherwise performed as described previously[2]. The time required for the absorbance and potential values to reach a new equilibrium after addition of ferricyanide and dithionite (in steps of approx. 3 $\mu$M) was 1–3 min. A complete reductive or oxidative titration required about 30 min. The spectrophotometric parameters are: measuring beam half bandwidth, 1.1 nm; reference wavelength, 570 nm; the light intensity of 702 nm light defined by an interference filter and a Corning 2-62 filter was $8 \cdot 10^4$ ergs/cm$^2$ per s (Figs 3–5).

## RESULTS

Dark titrations of absorbance changes associated with cytochrome $b_6$ in coupled chloroplast preparations are shown in Fig. 1 to occur in a potential region more positive than found previously[2]. Titrations performed immediately after the chloroplasts were prepared show a midpoint at approx. 0 V (+5 mV in Fig. 1), and a steeper slope than that associated with subsequent reductive and oxidative titrations. The first titration, which is reductive, is fit best by a two-electron transition, and the later titrations by a one-electron titration curve with a midpoint shifted slightly negatively to −16 mV (Fig. 1). A two-electron slope and similar midpoint were also obtained when the first titration was oxidative (data not shown). It should be noted that the reason that there are relatively few titration points included in the first two-electron titration is because the two-electron slope is lost if the time of incubation of the chloroplasts is excessive (>0.5 h). Since each titration point requires 2–3 min, it is not possible to include more than about 10 points in this titration. The steeper slope seen in this first titration which is most easily fit by a two-electron titration curve was most commonly, but not always (*e.g.* control titration of Fig. 3) observed. Because the two-electron slope is also lost as function of time and in the presence of uncouplers, it is believed that in the few cases where a two-electron slope was not observed in the control it was because of poorly coupled chloroplasts. The uncertainty in the midpoints in a given experiment is ±10 mV. However, we would estimate the overall uncertainly in the absolute value of the midpoint for different chloroplast preparations as +40 and −20 mV. The larger uncertainty on the positive side may be a consequence of oxygen introduced with the chloroplasts. Another point about the titrations is that there were a few occasions when the absorbance decrease caused by ferricyanide addition to reduced $b_6$ occurred at potentials 50 to 100 mV lower than shown in Fig. 1. The explanation of this inconsistency is not known.

The difference spectrum of Fig. 2 obtained by adding single aliquots of dithio-

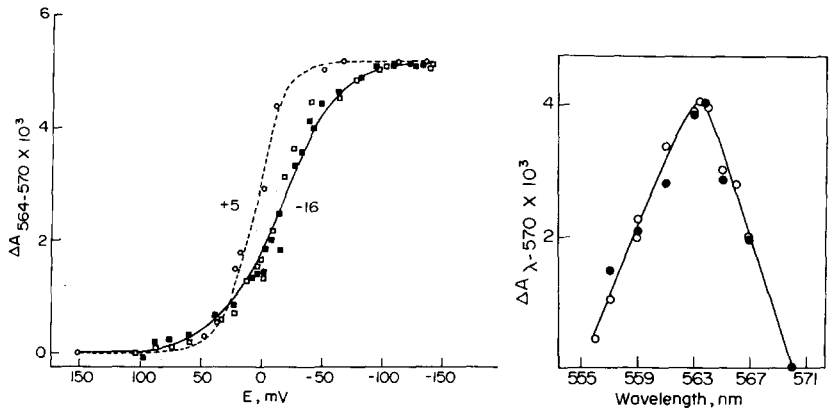


Fig. 1. Anaerobic dark titration of the cytochrome $b_6$ oxidation state as a function of the externally controlled oxidation-reduction potential. Oxidation changes of cytochrome $b_6$ were measured at 564 nm with 570 nm as the reference wavelength. Control of the oxidation potential was as described in Methods. The first titration (○) which was reductive, was done within 0.5 h after chloroplast preparation; the calculated titration curve which was fit to the data (- - - -) assumes a two-electron transition and a midpoint ($E_m$) of +5 mV. Data points from two different preparations with three subsequent oxidative (■) and reductive (□) titrations are shown. The calculated titration curve (—) assumes a midpoint of −16 mV and a one-electron transition. The titrations were all completed within 2–3 h after preparation of the chloroplasts. Chlorophyll concentration, 100 μg/ml.

Fig. 2. Difference spectrum for the absorbance change titrated in Fig. 1. The absorbance change obtained by changing the redox potential from −90 mV to about +130 mV by addition of a single aliquot of ferricyanide (○), and from +130 mV to about −90 mV by the addition of a single aliquot of dithionite (●), is plotted as a function of wavelength. The only redox buffer present was 1,4-naphthoquinone (20 μM). Chlorophyll concentration, 75 μg/ml. Conditions otherwise as in Fig. 1.

nite and ferricyanide to shift the potential from −90 to +130 mV demonstrates that the absorbance change titrated in Fig. 1 belongs to cytochrome $b_6$.

It can be demonstrated that part of the cytochrome $b_6$ complement can be converted from the relatively high potential form shown in Fig. 1 to a lower potential form under defined uncoupling conditions. After a control titration which showed a midpoint of approx. −4 mV (Fig. 3), FCCP was added to the suspension with cytochrome $b_6$ in the reduced state. The chloroplasts were then illuminated with far-red actinic light for about 2 min. The resulting oxidative and reductive titrations performed in the dark show two one-electron components of approximately equal weight with midpoints of −25 and −140 mV (Fig. 3). An absorbance change was seen at positive potentials (dashed line in Fig. 3), which is attributed to a low potential form of cytochrome *b*-559[2,8,11,12]. This experiment shows that after illumination in the presence of FCCP approximately half of the cytochrome $b_6$ complement is shifted to a midpoint potential about 150 mV more negative than the control value, while the midpoint of the rest of the cytochrome $b_6$ is negatively shifted by only 20–30 mV. Similar results were obtained in dark titrations performed after illumination in the presence of $NH_4Cl$.

Consecutive cycles of cytochrome $b_6$ photooxidation under controlled redox conditions are compared, in the absence (Fig. 4A) and in the presence (Fig. 4B)

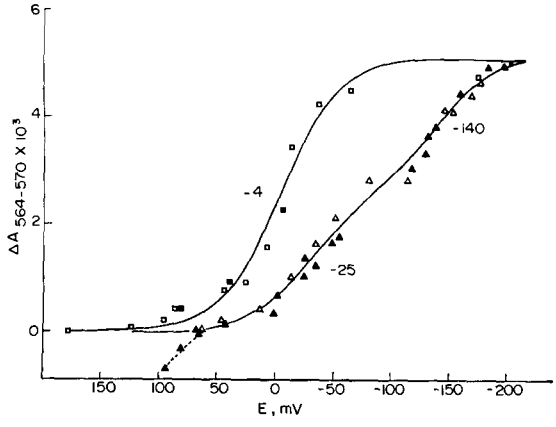


Fig. 3. The effect of FCCP on the titration of cytochrome $b_6$. Conditions are the same as in Fig. 1 except that FCCP (0.1 nmole FCCP/$\mu$g chlorophyll) was added to the redox mixture after the control titration, and the chloroplasts were then illuminated for 2 min with far-red actinic light. The oxidative (■) and reductive (□) control titrations in the absence of FCCP are fit to a one-electron titration curve calculated for $E_m = -4$ mV. The curve calculated for the oxidative (▲) and reductive (△) transitions obtained after FCCP addition assumes that a relatively high potential ($E_m = -25$ mV, $n = 1$) and a low potential component ($E_m = -140$ mV $n = 1$) contribute equally to the absorbance change measured at 564 nm.

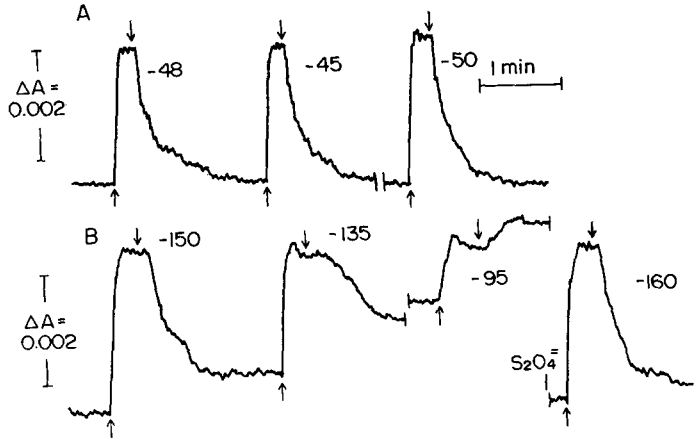


Fig. 4. Dependence of the amplitude of cytochrome $b_6$ photooxidation and subsequent dark reduction on $NH_4Cl$ under controlled potential conditions. The photooxidation of cytochrome $b_6$ and subsequent dark reduction were measured at the potentials indicated, in the absence of $NH_4Cl$ (A), and with a separate sample in the presence of 50 nmoles $NH_4Cl$/$\mu$g chlorophyll (B). In A and B three consecutive cycles of photooxidation and dark reduction are measured. After the third cycle in B, dithionite was added to lower the potential to $-160$ mV. Upward, 702 nm light on; downward arrows, light off. Upward deflection, absorbance decrease at 564 nm relative to a 570-nm reference. Conditions otherwise as in Fig. 1.

of $NH_4Cl$. In the presence of $NH_4Cl$ there is a decrease in the amplitude of the photooxidation with successive cycles, a decrease in the rate of the dark reduction, and an increase in the level of steady state oxidation in the dark. The cause of the potential increase in Fig. 4B during the first three light cycles is not known but may have been caused by $O_2$ evolved during illumination. The ambient potential is, in any case, more negative in Fig. 4B than in Fig. 4A, and would be negative enough to reduce cytochrome $b_6$ with an $E_m$ around zero volts. In the absence of $NH_4Cl$ (Fig. 4A), there is no decrease in amplitude of the photooxidation or the rate of dark reduction over the three cycles of actinic illumination, even though the ambient potential is substantially more positive. It is concluded that cytochrome $b_6$ assumes a lower potential after illumination in the presence of $NH_4Cl$. The effective midpoint of the cytochrome $b_6$ after the second cycle of illumination in the experiment of Fig. 4B must be about $-100$ mV since (a) the cytochrome is approximately half oxidized in the dark at $-95$ mV after two cycles of illumination, and (b) the amplitude of the photooxidation is restored to the control level after dithionite addition lowers the potential to $-160$ mV (last trace in Fig. 4B). It should be noted that if the negative shift in potential affects half the $b_6$ complement, as in Figs 3 and 5, an average $E_m$ of $-100$ mV at the end of the experiment of Fig. 4B means that half the $b_6$ has an $E_m$ much below $-100$ mV. This experiment shows that the negative shifts in cytochrome $b_6$ midpoint potential do not require unusual treatment to the chloroplasts. Fig. 4 demonstrates that a brief exposure to typical uncoupling conditions is sufficient to cause the changes in midpoint potential titrated more quantitatively and over a longer period of time in Fig. 3. It has not been possible to demonstrate a shift in midpoint of $b_6$ with ADP using the procedure of Fig. 4. It is possible that any effects of ADP are reversible in the dark.

It is known that under different conditions cytochrome $b_6$ can be either photoreduced or photooxidized. The $b_6$ tends to be photoreduced in chloroplasts under aerobic conditions where it is found oxidized in the dark[13,14], as well as in untreated whole cells of the red alga *Porphyridium aerugineum*[15]. Photooxidation of $b_6$ is the dominant reaction in whole cells of the green alga *Chlamydomonas reinhardi*[16] and in anaerobic chloroplast suspensions to which dithionite is added[1,7]. The transition between photooxidation and photoreduction is measured as a function of redox potential in the presence and absence of $NH_4Cl$ in Fig. 5. There are not enough points to measure accurately the slope and midpoint in the absence of $NH_4Cl$, but there are enough to show consistency with Figs 1 and 3, and also to estimate the transition between oxidation and reduction. The crossover between photooxidation and photoreduction is at approximately $-40$ mV in the presence of $NH_4Cl$, and 60 mV more positive in the absence of $NH_4Cl$.

The shape of the titration curves of the light-induced changes in the presence of $NH_4Cl$ in Fig. 5 is otherwise similar to the titration curves measured with chemical reduction and oxidation in the presence of FCCP in Fig. 3. The slope of the titration curve measured for light-induced changes in the presence of $NH_4Cl$ in Fig. 5 is not steep enough to be fit by a single component and is better fit by two one-electron components, with midpoints of approx. $-13$ and $-130$ mV contributing equally to the titration curve. The difference spectrum for photooxidation of cytochrome $b_6$ kept reduced at $-195$ mV shows a peak at 563–564 nm in the presence of 4 mM $NH_4Cl$ (data not shown; see Fig. 12 of ref. 1).

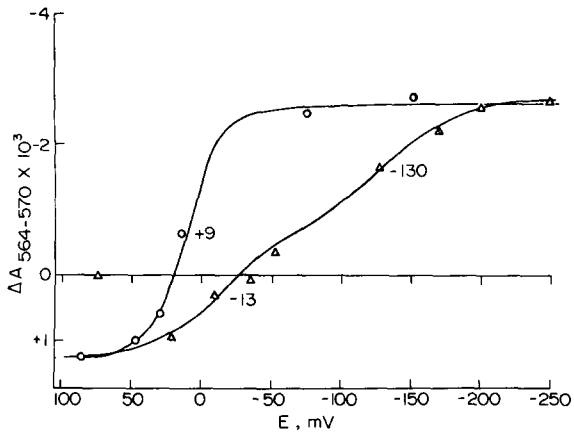


Fig. 5. Light-induced absorbance changes of cytochrome $b_6$ as a function of redox potential. Conditions as in Methods and Fig. 4. The absorbance change at 564 nm induced by 702-nm light was measured at defined redox potentials adjusted by either dithionite or ferricyanide additions. The curve drawn through the data points obtained in the absence of uncouplers (o) assumes a midpoint of +9 mV and a two-electron transition. The titration done after addition of 5 mM $NH_4Cl$ (△) is fit to a curve calculated assuming the existence of a high potential ($E_m = -13$ mV) component and a low potential ($E_m = -130$ mV) component, both with $n=1$, contributing equally to the absorbance change at 564 nm.

## DISCUSSION

The above experiments show that the midpoint potential of cytochrome $b_6$ is approx. 0.0 V in fresh coupled chloroplast preparations, and that the midpoint of at least half the cytochrome $b_6$ undergoes a negatively directed change in $E_m$ of 100–150 mV in the presence of uncouplers. It is concluded that the midpoint of −180 mV previously measured for cytochrome $b_6$[2] is a value which, within experimental errors, is characteristic of at least half the $b_6$ under systematically uncoupled conditions. The question of why only half the $b_6$ complement undergoes a significant shift in $E_m$ in the presence of the uncoupling treatment is not resolved. This phenomenon indicates that after uncoupling treatment the cytochrome $b_6$ is divided into two approximately equal non-equivalent parts, with one part perhaps more exposed to solvent or a polar environment (see below). In this regard one explanation of the $n=2$ slope observed in titration of the $b_6$ in freshly prepared coupled chloroplasts is that under these conditions the $b_6$ cytochrome contains two strongly interacting hemes which transfer two electrons.

The ability of Photosystem II light to reduce cytochrome $b_6$[1] now seems explainable at least on a redox basis. The data presented here would predict that the efficiency of the Photosystem II reduction of $b_6$ should decrease in uncoupled chloroplasts as the $E_m$ of the $b_6$ decreases below that of the primary acceptor of Photosystem II[5,6]. It is possible that such a shift in $E_m$ is significant in controlling the function of cytochrome $b_6$ in cyclic and non-cyclic electron transport, but this depends on whether such shifts in cytochrome $b_6$ midpoint can be detected under more physiological conditions.

Regarding the mechanism of the uncoupler-induced midpoint shifts observed here, we do not know whether they are primary consequences of uncoupling or secondary effects of changes in membrane structure or membrane properties which result from the uncoupling. It has been suggested[17] that a negative membrane potential caused by ATP addition in mitochondria is the cause of the negative shift in midpoint of cytochrome oxidase caused by ATP addition[17,18]. This mechanism does not explain, however, why ATP causes a positive change in the potential of mitochondrial *b*-type cytochromes[19,20]. The mechanism also does not predict why the chloroplast *b*-type cytochromes should show a negative change in redox potential when illuminated in the presence of FCCP or $NH_4Cl$, since the light-induced chloroplast membrane potential is thought to be positive. FCCP, at least, will tend to lower the magnitude of this positive potential, but since there is no evidence for a change of sign of the membrane potential in the presence of these uncouplers, the effect of the positive membrane potential should always be to make the cytochrome redox potential more positive than the value measured in the dark. An alternative explanation of the negatively directed changes in redox potential involving membrane properties might be to consider the possibility of a change in dielectric constant in the neighborhood of the heme groups of the chloroplast *b*-type cytochromes. If the ferric form of the cytochrome had a larger absolute charge in the neighborhood of the heme iron, the effect of an increase in dielectric constant will be to lower the cytochrome midpoint potential, since the ferric form would be stabilized[21]. Uncoupling conditions might conceivably cause an increase in the local proton concentration or of solvent accessibility to normally hydrophobic regions of the chloroplast membrane or heme environment. The presence of FCCP or $NH_3$ in the membrane might in itself perturb the local dielectric constant.

## ACKNOWLEDGMENTS

This research has been supported by National Science Foundation Grant No. GB-34169X, N.I.H. Research Career Development Award I KO4 GM 29735-10 from the Institute of General Medical Sciences (W.A.C.) and a travel grant from the Heinrich Hertz Stiftung (H.B.) We would like to thank Dr David Knaff and Dr Richard Kassner for communicating unpublished results to us, the machine shop of Mr Eric Henss, the technical assistance of Mr Ted Van Rijk, and Dr P. Heytler for a gift of FCCP.

## REFERENCES

1 Böhme, H. and Cramer, W. A. (1972) *Biochim. Biophys. Acta* 283, 302–315
2 Fan, H. N. and Cramer, W. A. (1970) *Biochim. Biophys. Acta* 216, 200–207
3 Hill, R. (1954) *Nature* 174, 501–503
4 Hill, R. and Bendall, D. S. (1967) in *Biochemistry of Chloroplasts* (Goodwin, T. W., ed.), Vol. 2, pp. 559–564, Academic press, London
5 Cramer, W. A. and Butler, W. L. (1969) *Biochim. Biophys. Acta* 172, 503–510
6 Erixon, K. and Butler, W. L. (1971) *Biochim. Biophys. Acta* 234, 381–389
7 Knaff, D. B. (1972) *FEBS Lett.* 23, 92–94
8 Cramer, W. A. and Böhme, H. (1972) *Biochim. Biophys. Acta* 256, 358–369
9 Hovorska, F. and Dearing, W. C. (1935) *J. Am. Chem. Soc.* 57, 446–453
10 Clark, W. M. (1960) *Oxidation–Reduction Potentials of Organic Systems*, p. 375, Williams and Wilkins, Baltimore

11 Böhme, H. and Cramer, W. A. (1971) *FEBS Lett.* 15, 349–351
12 Cramer, W. A., Fan, H. N. and Böhme, H. (1971) *J. Bioenerg.* 2, 289–303
13 Hind, G. and Olson, J. M. (1966) *Brookhaven Symp. Biol.* 19, 188–194
14 Cramer, W. A. and Butler, W. L. (1967) *Biochim. Biophys Acta* 143, 332–339
15 Amesz, J., Pulles, M. P. J., Visser, J. W. M. and Sibbing, F. A. (1972) *Biochim. Biophys. Acta* 275, 442–452
16 Levine, R. P. (1969) in *Progress Photosynthetic Research* (Metzner, H., ed.), pp. 971–977
17 Hinkle, P. and Mitchell, P. (1970) *Bioenergetics* 1, 45–60
18 Wilson, D. F. and Dutton, P. L. (1970) *Arch. Biochem. Biophys.* 136, 583–584
19 Wilson, D. F. and Dutton, P. L. (1970) *Biochem. Biophys. Res Commun.* 39, 59–64
20 Berden, J. A., Opperdoes, F. R. and Slater, E. C. (1972) *Biochim. Biophys. Acta* 256, 594–599
21 Kassner, R. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 2263–2267