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STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION

III. THE DIFFERENT EFFECTS OF METHYLAMINE AND ADP *PLUS* PHOSPHATE ON ELECTRON TRANSPORT THROUGH COUPLING SITES I AND II IN ISOLATED CHLOROPLASTS

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SUMMARY

1. The reduction of lipophilic (Class III) oxidants such as oxidized *p*-phenylenediamine consists of two components. One component requires both Photosystem II and Photosystem I and includes both sites of energy coupling associated with non-cyclic electron transport. The second component requires only Photosystem II and includes only the site of energy coupling located before plastoquinone (Site II). When oxidized *p*-phenylenediamine is being reduced by both pathways, the overall rate of electron transport is stimulated by the addition of ADP *plus* phosphate or the uncoupler methylamine. However, if the Photosystem I component of oxidized *p*-phenylenediamine reduction is eliminated by a low concentration of the plastoquinone-antagonist dibromothymoquinone, the stimulation of electron transport by ADP *plus* phosphate or methylamine is also abolished, although the remaining Photosystem II-dependent electron transport remains firmly coupled to phosphorylation (*via* coupling Site II). These results indicate that coupling Site II, unlike the well-known rate-limiting coupling site between plastoquinone and cytochrome *f* (Site I), does not exert any control over the rate of associated electron transport.

2. When substituted *p*-benzoquinones (*e.g.* 2,5-dimethyl-*p*-benzoquinone) or quinonediimides (*e.g.* *p*-phenylenediimine) are used as Class III acceptors in conjunction with dibromothymoquinone, a small but significant stimultation of electron transport by ADP *plus* phosphate is observed. However, it can be shown that this stimulation does not arise from coupling Site II but rather is due to a low rate of electron flux through coupling Site I even in the presence of dibromothymoquinone. Apparently the *p*-benzoquinones can catalyze an electron "bypass" around the dibromothymoquinone-induced block at plastoquinone, possibly by substituting partially for the natural electron carrier. If this bypass electron flow is blocked at plastocyanin by KCN treatment, the stimulation of electron transport by ADP *plus* phosphate is eliminated, although a high rate of phosphorylation (from Site II only) remains.

Abbreviations: P/e_2 ratio, the ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; dimethylquinone (DMQ), 2,5-dimethyl-*p*-benzoquinone.

3. These results provide strong evidence that a profound difference exists between the two sites of energy coupling associated with non-cyclic electron transport in isolated chloroplasts. That is, the rate of electron flow through coupling Site I, which is the rate-determining step in the Hill reaction, is strictly regulated by phosphorylating conditions, whereas the rate of electron flux through coupling Site II is independent of phosphorylating conditions.

4. A model is presented which accounts for the lack of control over electron transport exhibited by coupling Site II. It is postulated that Site II is coupled to an essentially irreversible electron transport step, so that conditions which affect the phosphorylation reaction would have no effect on the rate of electron transport through the coupling site. Two essentially irreversible reactions, closely associated with Photosystem II –the water-splitting reaction and the System II photoact itself— are discussed as possible locations for coupling Site II.

INTRODUCTION

Saha *et al.*[1] were the first to point out that lipophilic strong oxidants such as oxidized *p*-phenylenediamines could intercept electrons from the chloroplast electron transport chain primarily at a point between the two photosystems. Subsequent work has shown that the preponderant portion of the electron transport to these "Class III" oxidants is insensitive to the plastocyanin inhibitors KCN (ref. 2) and poly(L)-lysine (ref. 3) and the plastoquinone antagonist dibromothymoquinone[4,5]. Thus, when electron flow to Photosystem I is blocked by one of these inhibitors, Class III acceptors are reduced by an electron pathway which includes only Photosystem II. Recently our laboratory[4,5] and Trebst's laboratory[6] have shown that there is a site of energy conservation closely associated with Photosystem II-driven photoreduction of Class III acceptors.

Evidence is now accumulating that this newly discovered coupling site close to Photosystem II differs in several fundamental aspects from the well-known coupling site located after plastoquinone and before cytochrome *f*[7,8]. When the two coupling sites are functionally isolated by partial reactions of the electron transport chain[9], the coupling site between plastoquinone and cytochrome *f* (Site I) exhibits a pH-dependent phosphorylation efficiency (P/e_2 ratio) (optimal $P/e_2=0.6$ at pH 8.0–8.5) whereas the coupling site located before plastoquinone (Site II) is less efficient, with a pH-independent P/e_2 ratio of 0.3–0.4. In addition, we have noted[9,10] that coupling Site II apparently exerts no control over the rate of coupled electron transport. That is, the rate of electron flux through coupling Site II is not stimulated by the presence of uncouplers or ADP *plus* phosphate (P_i). Conversely, Site I exerts tight control over the associated electron flow, responding sharply to the presence of phosphorylating or uncoupling conditions. From these results we have concluded that coupling Site I alone constitutes the rate-determining step in the reduction of conventional Hill oxidants such as ferricyanide or methylviologen[9].

However, Trebst and Reimer[6] have reported data which indicates that electron transport through coupling Site II is regulated by phosphorylating conditions. They reported that the reduction of substituted *p*-benzoquinones in the presence of dibromothymoquinone was stimulated by the addition of ADP *plus* P_i or amine uncouplers. In an effort to resolve the apparent discrepancy between their data and our own, we have re-examined this problem in considerable detail. In this paper we report conclusive evidence that substituted *p*-benzoquinones such as 2,5-dimethylquinone not only accept electrons at a point before the site of dibromothymoquinone inhibition[5] (*i.e.* before plastoquinone[11]) but also catalyze a "bypass" around the dibromothynoquinone block, allowing electrons to pass through coupling Site I to Photosystem I. When KCN[2] is used to block the bypass electron flow by inactivating plastocyanin[12], no stimulation of electron transport by ADP *plus* P_i is observed, indicating that Site II in fact does not exert control over coupled electron transport. This important difference in the properties of coupling Sites I and II may reflect a fundamental difference in the mode of energy transduction at the two sites.

EXPERIMENTAL METHODS

The techniques employed in this study were similar to those described in previous papers. Chloroplasts were isolated from fresh market spinach (*Spinacia oleracea* L.) as described earlier[9]. The photoreductions of 2,5-dimethylquinone and oxidized *p*-phenylenediamine were measured spectrophotometrically as the decrease in absorbance of the reaction mixture at 420 nm due to the reduction of excess ferricyanide[1]. Reactions (in a final volume of 2.0 ml) were run in thermostated cuvettes at 19 °C. Actinic light (>600 nm; 400 kergs$\cdot$s$^{-1}\cdot$cm^{-2}) was supplied by a 500-W slide projector and the appropiate colored glass filters.

ATP formation was determined for an aliquot of the reaction mixture as the residual radioactivity in the aqueous phase after extracting unreacted orthophosphate as phosphomolybdic acid into a butanol–toluene mixture (1:1, v/v) as described by Saha and Good[13]. Radioactivity in the final aqueous phase was determined using the Cerenkov technique of Gould *et al.*[14].

KCN-treated chloroplasts were prepared by incubating chloroplasts in 30 mM KCN (buffered at pH 7.8) at 0 °C for 90 min as described by Ouitrakul and Izawa[2].

Stock solutions of 2,5-dimethylquinone and dibromothymoquinone were prepared in ethanol–ethylene glycol (1:1, v/v) and diluted so that the final concentration of organic solvent in the reaction mixture never exceeded 2%.

RESULTS

We noted previously that dibromothymoquinone, which blocks electron flow at plastoquinone, strongly inhibits electron transport and ATP formation when ferricyanide is the electron acceptor, but only partially inhibits electron transport and phosphorylation when the lipophilic Class III oxidant *p*-phenylenediimine (oxidized *p*-phenylenediamine) serves as the electron acceptor[5]. Since the reduction of Class III acceptors is known to contain two components, one solely dependent on Photosystem II and one requiring both Photosystem II and Photosystem I (ref. 2), it was concluded that dibromothymoquinone was blocking the Photosystem I component of oxidized *p*-phenylenediamine reduction. This conclusion was confirmed by the observation that the reduction of Class III acceptors in the presence of dibromothymoquinone is completely insensitive to plastocyanin inhibition by KCN[10].

Fig. 1 shows the effect of dibromothymoquinone on the reduction of ferricyanide and oxidized *p*-phenylenediamine in the presence and absence of a complete phosphorylating system. As the dibromothymoquinone concentration approaches $5 \cdot 10^{-7}$ M the rate of electron transport to oxidized *p*-phenylenediamine in the phosphorylating ($+P_i$) system falls to the level of the nonphosphorylating ($-P_i$) system. Nevertheless, this dibromothymoquinone-insensitive electron transport remains firmly coupled to ATP formation, even though the stimulation of electron transport by ADP *plus* P_i is no longer observed. Similar results can be seen when ferricyanide acts as the electron acceptor. This is because dibromothymoquinone, at concentrations greater than $2.5 \cdot 10^{-7}$ M, not only blocks at plastoquinone but also functions as a Class III electron acceptor[10], the reduced dibromothymoquinone being rapidly reoxidized by the excess ferricyanide present in the reaction mixture. In both systems the P/e_2 ratio falls to around 0.4, the characteristic efficiency of coupling Site II[2,4,5,9,10]. Since both systems (in the presence of $5 \cdot 10^{-7}$ M dibromothymoquinone) are insensitive to plastocyanin inhibition by KCN, we can conclude that only coupling Site II is involved.

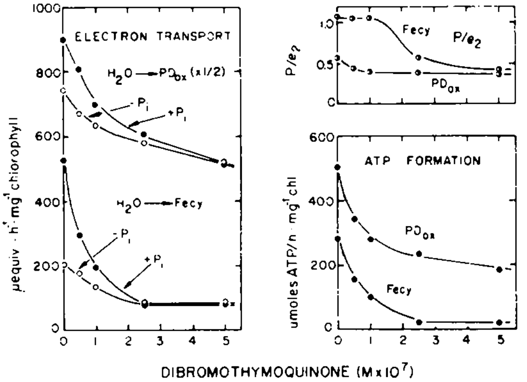

Fig. 1. Effect of the plastoquinone-antagonist dibromothymoquinone on electron transport and ATP formation associated with the photoreduction of ferricyanide and oxidized *p*-phenylenediamine by isolated chloroplasts. The reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM Tricine–NaOH buffer (pH 8.0), 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$ (when added), chloroplasts equivalent to 15 ug chlorophyll, and the indicated acceptor system. The acceptor systems were: Fecy, 0.4 mM potassium ferricyanide; PD_{ox}, 0.5 mM *p*-phenylenediamine *plus* 1.5 mM potassium ferricyanide. Note that as the Photosystem I component of PD_{ox} reduction is eliminated, the stimulation of electron transport by P_i is also eliminated, even though a high rate of ATP formation remains. Also note that at dibromothymoquinone concentrations $\geq 2.5 \cdot 10^{-7}$ M, where dibromothymoquinone itself functions as a Class III acceptor[10], the stimulation of electron transport by P_i is not observed, although phosphorylation, with the characteristic Site II P/e_2 ratio of 0.3–0.4, does occur.

While the data in Fig. 1 clearly show that coupling Site II exerts no control over Photosystem II electron transport, Trebst and Reimer[6], using substituted *p*-benzoquinones as Class III acceptors, have shown that electron transport to these compounds is stimulated by ADP *plus* P_i and by amine uncoupling, even in the presence of dibromothymoquinone concentrations which completely block electron transport to Photosystem I. From this data they concluded that coupling Site II does exert control over the rate of electron transport. To resolve this apparent discrepancy with our own results, we performed similar experiments using 2,5-dimethyl-*p*-benzoquinone as the Class III acceptor.

Fig. 2A shows that in the mixed system (*i.e.* dimethylquinone reduction by both Photosystem II alone and Photosystem II *plus* Photosystem I), considerable stimulation of electron transport by the complete phosphorylation system ($+P_i$) is observed. These data confirm the earlier results of Saha *et al.*[1]. If $5 \cdot 10^{-7}$ M dibromothymoquinone is added (Fig. 2B), however, the rate of electron transport is inhibited, indicating that the Photosystem I component of dimethylquinone reduction[2,5] has been largely eliminated. When no dimethylquinone is present in the reaction mixture (dimethylquinone = 0, Fig. 2B), the residual rate of electron transport, which is due to the Class III-type reduction of ferricyanide *via* dibromothymoquinone[10], shows no stimulation by ADP *plus* P_i. Nevertheless, when dimethylquinone is added, a small but significant stimulation of electron transport by ADP *plus* P_i is observed. This confirms the findings of Trebst and Reimer[6] that control

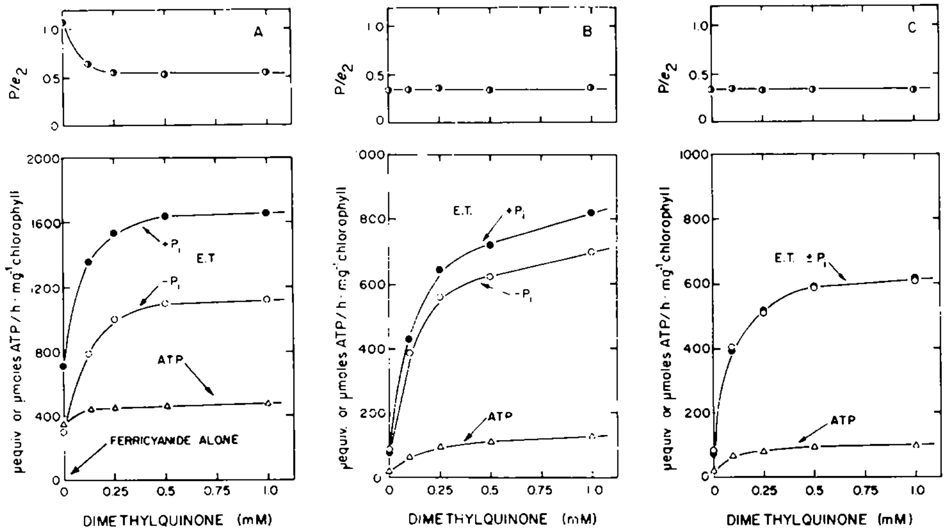

Fig. 2. Effect of the electron transport inhibitors dibromothymoquinone and KCN on electron transport (E.T.) and phosphorylation (ATP) when dimethylquinone is the electron acceptor. The basic reaction mixture is as described in Fig. 1 when ferricyanide was the electron acceptor. Specific conditions are as follows: (A) No inhibitor added. Note that when no dimethylquinone is present (*i.e.* ferricyanide is being reduced *via* the normal Hill reaction) there is a large stimulation of electron transport by the complete phosphorylating system ($+P_i$). When dimethylquinone is added, a large increase in the rates of electron transport is observed, although the absolute amount of stimulation by P_i remains about the same. (B) Dibromothymoquinone ($5 \cdot 10^{-7}$ M) was added. Here rates of electron transport are lower since dibromothymoquinone blocks the Photosystem I component of dimethylquinone reduction. Note that in the absence of dimethylquinone (*i.e.* when dibromothymoquinone serves as the electron acceptor) no stimulation by ADP *plus* P_i is observed. When increasing concentrations of dimethylquinone are added, however, a small but significant stimulation of electron transport by the complete ($+P_i$) system is observed. (C) Chloroplasts blocked at plastocyanin by KCN treatment (see Methods); $5 \cdot 10^{-7}$ M dibromothymoquinone added. Note that the stimulation of electron transport by the complete phosphorylating system (see B) is abolished by plastocyanin inhibition, indicating that electrons are bypassing the dibromothymoquinone-induced block. Nevertheless, a substantial rate of ATP formation remains even when this bypass is blocked by KCN.

or other, substituted quinonediimides are used as Class III acceptors, suggesting that the *p*-benzoquinones may be able to substitute partially for the natural electron carrier plastoquinone.

The effect of the uncoupler methylamine on electron transport and phosphorylation associated with the reduction of ferricyanide and oxidized *p*-phenylenediamine (in the presence of $5 \cdot 10^{-7}$ M dibromothymoquinone) is shown in Fig. 3. As is already widely known, methylamine stimulates the rate of electron transport when ferricyanide is the electron acceptor by uncoupling electron flow from a rate-limiting energy conservation reaction[15]. Since we have shown previously that coupling Site I is the rate-determining step for the Hill reaction[9], we can conclude that methylamine's preponderant effect on electron transport is by releasing the rate-limitation at Site I. Methylamine has an entirely different effect on a Photosystem II partial reaction (utilizing only Site II), however. Instead of stimulating the rate of electron transport as Site II becomes uncoupled, methylamine inhibits electron flow. Since this inhibition increases with increasing concentrations of methylamine, even after ATP formation has been completely abolished, it seems likely that the inhibition is actually due to a secondary effect of the amine on Photosystem II. Indeed, high concentrations of methylamine have been shown to inhibit the water-splitting reaction[16].

In this experiment (Fig. 3) the rate of electron transport in the presence of $\geqslant 5$ mM methylamine is somewhat lower when oxidized *p*-phenylenediamine is the electron acceptor than when ferricyanide is the electron acceptor. This is due to the dibromothymoquinone present in the *p*-phenylenediamine system, since dibromothymoquinone has been shown to have a secondary effect on the quantum efficiency of Photosystem II[10]. In the absence of dibromothymoquinone (Fig. 4) similar results are obtained for the effect of methylamine on ferricyanide and oxidized *p*-phenylenediamine reduction. It is clear that, as the rate limitation at coupling Site I is released, the rate of ferricyanide reduction increases until the secondary inhibition of Photosystem II by methylamine becomes the rate-limiting factor.

DISCUSSION

There is an impressive amount of evidence accumulating which indicates that the mechanisms of energy conservation at Sites I and II are not identical. When these sites are isolated by partial reactions of the electron transport chain[9], it can be demonstrated that they differ in their coupling efficiencies (P/e_2 ratios) and in the effect of pH on these efficiencies. Site II exhibits a characteristic P/e_2 ratio of 0.3–0.4 which is practically pH-independent from pH 6 to 9, whereas Site I, which is the rate-limiting step for the Hill reaction, exhibits a strongly pH-dependent P/e_2 ratio having an optimal value of about 0.6 at pH 8.0–8.5. Furthermore, it has recently been shown that $HgCl_2$, which is an energy transfer inhibitor in chloroplasts[17], can preferentially inhibit the coupling reaction at Site I without affecting ATP formation supported by Site II[18]. In this paper we have reconfirmed that, unlike Site I, coupling Site II does not exert control over the rate of associated electron flux. That is, the rate of electron transport through coupling Site II is independent of the presence of ADP *plus* P_i or uncouplers. Nevertheless, under phosphorylating conditions ATP formation supported by Site II can occur at very high rates.

Furthermore, we have presented data which allows a reinterpretation of the findings of Trebst and Reimer[6], who concluded from experiments with dibromothymoquinone and 2,6-dimethyl-*p*-benzoquinone that coupling Site II does exert control over electron transport. Apparently certain *p*-benzoquinones, in the presence of dibromothymoquinone, can catalyze an electron bypass around the dibromothymoquinone-induced plastoquinone block, perhaps by substituting for plastoquinone. This does not seem unlikely in view of the structural similarities between these *p*-benzoquinones and plastoquinone. When KCN blocks this bypass, however, no control over electron transport by Site II is observed. Indeed, Trebst and Reimer[6] noted that when they used higher concentrations of dibromothymoquinone in the presence of ferricyanide (so that dibromothymoquinone itself functioned as a Class III acceptor) no effect of uncouplers on electron transport was observed. This observation is in agreement with our own results obtained in a more extensive study of dibromothymoquinone as an electron acceptor[10]. It is also important to note that oxidized *p*-phenylenediamines, when used as Class III acceptors, do not catalyze this bypass reaction around the dibromothymoquinone block.

It is possible to construct a model which explains the observed differences between Site II and Site I in their ability to regulate electron transport. In many respects coupling Site I resembles sites associated with the mitochondrial respiratory chain[9]. By analogy with mitochondria[19], the electron transport between adjacent electron carriers (A and B) at Site I can be viewed as an equilibrium system. Thus, in the light, when Photosystem I is rapidly draining electrons from B, the reaction ($A \rightarrow B$) would be pulled to the right. However, as the high energy state ($\sim$) generated in the coupled energy conservation reaction begins to accumulate, a back pressure is created against the flow of electrons from A to B by reversal of the energy conservation steps. When a complete phosphorylating system (*i.e.* ADP *plus* P_i) is present, however, the pool of high energy intermediate is much smaller since it is continually being utilized to drive ATP formation. Thus the back pressure exerted by this pool is diminished and electron transport from A to B is stimulated. Similarly, in the presence of uncouplers, the high energy state is rapidly dissipated and no significant back pressure is present, allowing very high rates of electron transport.

Coupling Site II does not exhibit the tight control over electron transport seen at Site I. This can be readily explained, however, if the oxidation–reduction reaction which gives rise to the energy conservation step at Site II is essentially an irreversible step. That is, the nature of the forward reaction ($A \rightarrow B$) is such that the reverse reaction is thermodynamically prevented. In this case, the accumulation of the high energy intermediate or state ($\sim$) would still exert a back pressure on the forward reaction, but this back pressure would have no effect on the rate of electron transport from A to B. This would account for the fact that conditions which drain or dissipate the high energy intermediate pool do not effect the rate of electron flow at Site II.

Several observations lead us to believe that this model does indeed provide a reasonable explanation for the differences between Site II and Site I discussed in this paper. We have shown previously that coupling Site II occurs at a point in the electron transport chain before the electrons from the independent Photosystem II units are pooled[10]. This indicates that coupling Site II is located prior to plastoquinone in the electron transport chain. The insensitivity of the phosphorylation associated with the reduction of Class III acceptors to the plastoquinone antagonist dibromothymoquinone lends strong support to this argument. Indeed, the existence of a Photosystem II-driven "proton pump" before plastoquinone has been demonstrated[9].

There are at least two reactions in this portion of the electron transport chain which could be considered essentially irreversible. One of these is the System II photoact itself. It has been suggested that a photochemical quantum conversion results in the formation of a reduced acceptor Q^- and an oxidized donor Z^+ on opposite sides of the thylakoid membrane (*e.g.* ref. 20). According to this model the resulting electrical field or membrane potential could serve as an energy reservoir to drive ATP formation as elaborated by Mitchell[21].

A second essentially irreversible reaction closely associated with Photosystem II electron transport is the water-splitting reaction. The existence of a coupling site on the water-oxidizing side of Photosystem II has already been considered by several authors[6,22–24]. It has been suggested that the protons from water oxidation are released to the inside of the thylakoid membrane, generating a transmembrane H^+ gradient which is capable of driving ATP formation[25].

Experiments are currently in progress in an attempt to further define the exact location of coupling Site II in chloroplasts.

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REFERENCES

1 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) *J. Biol. Chem.* 246, 3204–3209

2 Ouitrakul, R. and Izawa, S. (1973) *Biochim. Biophys. Acta* 305, 105–118

3 Ort, D. R., Izawa, S., Good, N. E. and Krogmann, D. W. (1973) *FEBS Lett.* 31, 119–122

4 Gould, J. M., Izawa, S. and Good, N. E. (1973) *Fed. Proc.* 32, 632

5 Izawa, S., Gould, J. M., Ort, D. R., Felker, P. and Good, N. E. (1973) *Biochim. Biophys. Acta* 305, 119–128

6 Trebst, A. and Reimer, S. (1973) *Biochim. Biophys. Acta* 305, 129–139

7 Avron, M. and Chance, B, (1966) *Brookhaven Symp. Biol.* 19, 149–160

8 Böhme, H. and Cramer, W. A. (1972) *Biochemistry* 11, 1155–1160

9 Gould, J. M. and Izawa, S. (1973) *Biochim. Biophys. Acta* 314, 211–223

10 Gould, J. M. and Izawa, S. (1973) *Eur. J. Biochem.* 37, 185–192

11 Böhme, H., Reimer, S. and Trebst, A. (1972) *Z. Naturforsch.* 26b, 341–352

12 Izawa, S., Kraayenhof, R., Ruuge, E. K. and DeVault, D. (1973) *Biochim. Biophys. Acta* 314, 328–339

13 Saha, S. and Good, N. E. (1970) *J. Biol. Chem.* 245, 5017–5021

14 Gould, J. M., Cather, R. and Winget, G. D. (1972) *Anal. Biochem.* 50, 540–548

15 Good, N. E. (1960) *Biochim. Biophys. Acta* 40, 502–517

16 Izawa, S., Heath, R. L. and Hind, G. (1969) *Biochim. Biophys. Acta* 180, 388–398

17 Izawa, S. and Good, N. E. (1969) *Prog. Photosynth. Res.* 3, 1288–1298

18 Bradeen, D. A., Gould, J. M., Ort, D. R. and Winget, G. D. (1973) *Plant Physiol.*, in the press

19 Chance, B. (1972) *FEBS Lett.* 23, 2-30
20 Witt, H. T. (1971) *Q. Rev. Biophys.* 4, 365-477
21 Mitchell, P. (1966) *Biol. Rev.* 41, 445-602
22 Schwartz, M. (1968) *Nature* 219, 915-919
23 Yamashita, T. and Butler, W. L. (1968) *Plant Physiol.* 43, 1978-1986
24 Böhme, H. and Trebst, A. (1969) *Biochim. Biophys. Acta* 180, 137-148
25 Junge, W., Rumberg, B. and Schröder, H. (1970) *Eur. J. Biochem.* 14, 575-581