# Photoreactions of Chloroplasts and Chlorophyll *a* with Hydroquinones and Quinones: Coupled Photoreduction of Cytochrome *c**

Leo P. Vernon and Elwood R. Shaw

ABSTRACT: The photoreduction of cytochrome *c* by intact chloroplasts is stimulated by the addition of various quinones, hydroquinones, flavins, dyes such as phenazine methosulfate (PMS), and spinach ferredoxin. With reduced trimethyl-*p*-benzoquinone ($TMQH_2$) or reduced ubiquinone with two isoprene units in the side chain ($UQ_2H_2$) present, the photoreduction of cytochrome *c* is partially insensitive to the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, indicating that these hydroquinones can serve as electron donors for the long-wavelength system of chloroplasts. Purified chlorophyll *a*, either in aqueous solutions in the presence of Triton X-100 or in 95% ethanol, catalyzes cytochrome *c* photoreduction using $TMQH_2$ as the electron donor ($UQ_2H_2$, $UQ_6H_2$, and ascorbate are less effective). This reaction proceeds at a rate of 6100 μmoles cytochrome *c* reduced per hour per mg of chlorophyll *a* in aqueous media when the illuminating intensity is $3 \times 10^5$ ergs/cm²/sec. This intensity does not saturate the reaction.

Available data indicate the initial reaction is an electron transfer from the excited chlorophyll to TMQ (either present initially with the $TMQH_2$ or formed in a slow chemical reaction of $TMQH_2$ with cytochrome *c* in the dark). The semiquinone so produced reduces the cytochrome *c* and the oxidized chlorophyll regains an electron from $TMQH_2$. Thus, for solubilized chlorophyll *a* as well as chloroplasts, quinones serve as electron acceptors and hydroquinones serve as electron donors.

The participation of quinones in the electron-transfer reactions of photosynthesis was first shown by Warburg and Lüttgens (1946), who demonstrated that benzoquinone serves as an electron acceptor in water photolysis (Hill reaction). A requirement for endogenous chloroplast quinones for light-stimulated electron-transfer reactions in chloroplasts was shown by the work of Bishop (1959), and other investigations extended the initial observation (Krogmann and Olivero, 1962; Arnon and Horton, 1963; Whatley and Horton, 1963; Eck and Trebst, 1963; Henninger and Crane, 1963). Furthermore, spinach chloroplasts contain a high concentration of quinones (Lichtenthaler and Park, 1963), including four plastoquinones and four tocopherols in addition to vitamin $K_1$ (Henninger *et al.*, 1963; Dilley *et al.*, 1963).

Plastoquinone is generally considered to function as an electron acceptor for the light-activated chlorophyll in green plants, while bacteria employ ubiquinone for this role (Clayton, 1962; Bales and Vernon, 1963). Zaugg *et al.* (1964) demonstrated that bacterial chromatophore fragments catalyze the photoreduction of added $UQ_2$[1] or $UQ_6$ coupled to the photooxidation of added reduced cytochrome *c* under anaerobic conditions (Zaugg, 1963; Zaugg *et al.*, 1964), and similar reactions have been observed with spinach chloroplasts and solubilized chlorophyll (Vernon *et al.*, 1963). It had previously been shown that quinones function very efficiently as electron acceptors in photochemical reactions involving purified chlorophyll (Tollin and Green, 1962, 1963). The present investigation concerns the photoreduction of cytochrome *c* by chloroplasts or isolated chlorophyll *a* in the presence of quinones and hydroquinones. Both reactions appear to proceed via an initial photochemical reduction of quinone.

## Methods

The experimental procedures used were essentially those reported in a previous communication (Vernon *et al.*, 1963), which describes the preparation of chloroplasts and chlorophyll *a* as well as PMS, $UQ_2$, $UQ_6$, and the related hydroquinones. Anaerobic conditions were obtained by alternate evacuation and flushing with argon and were employed in all experiments involving purified chlorophyll *a*. Cytochrome *c* was purchased from Sigma Chemical Co., St. Louis, Mo. Ferredoxin was prepared from spinach chloroplasts and purified according to the directions of San Pietro and Lang (1958). Spectrophotometric measurements were made with a Beckman Model DB spectrophotometer as described previously (Vernon *et al.*, 1963). Illumination was accomplished with red light (tungsten lamp in conjunction with Corning filter No. 2403),

* From the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio. *Received August 7, 1964.* Contribution No. 162 from the Charles F. Kettering Research Laboratory.

[1] The following abbreviations are used: TMQ ($TMQH_2$), trimethyl-*p*-benzoquinone; $UQ_2$ ($UQ_2H_2$), ubiquinone with two isoprene units in the side chain; $UQ_6$ ($UQ_6H_2$), ubiquinone with six isoprene units in the side chain; PMS ($PMSH_2$), phenazine methosulfate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The abbreviations in parentheses refer to the reduced form of the compound.

the intensity being $3 \times 10^5$ ergs/cm²/sec at the reaction tube. Cytochrome *c* reduction was calculated by using 21,000 for the change in the molar absorbancy index at 550 mμ upon reduction. Chlorophyll *a* was added to the reaction mixture from methanol, and its concentration in methanol was determined using a value of 74.5 for the specific absorption coefficient (g/liter).

## Results

*Chloroplast Reactions.* The photoreduction of cytochrome *c* by chloroplasts was reported by Holt (1950). The stimulation of this reaction by ferredoxin (called "methemoglobin reducing factor") was reported by Davenport and Hill (1960), and further properties of this reaction were described by Keister and San Pietro (1963). Whereas cytochrome *c* is only slowly photoreduced by the chloroplast, the reaction is markedly stimulated by the addition of a number of compounds which couple the chloroplast to cytochrome *c*, including ferredoxin, pyocyanines PMS, viologens, FMN, menadione, ferrocyanide, methyl red, and indophenol dyes.

Stimulation of cytochrome *c* photoreduction by TMQ is shown by the data given in Table I. This stimulation results from an initial photoreduction of the quinone by the chloroplasts in a regular Hill reaction followed by a chemical reduction of cytochrome *c* by $TMQH_2$ (or more probably the semireduced form). As expected, DCMU inhibits these reactions almost completely. The participation of TMQ as a Hill reagent in chloroplast reactions was previously reported (Trebst and Eck, 1963).

With $TMQH_2$ there is only a slow reduction of cytochrome *c* by chloroplasts in the dark, and illumination increases this rate markedly. This activity is probably due to the ability of $TMQH_2$ to serve as an electron donor for the long-wavelength pigment system. This would cause the $TMQH_2$ to be partially oxidized in the light, and the TMQ so formed stimulates cytochrome *c* reduction. Further evidence that $TMQH_2$ acts as an electron donor for the long-wavelength system of chloroplasts is shown by the DCMU insensitivity of this reaction. Some reaction occurs with TMQ in the presence of DCMU, probably because in the presence of chloroplasts some $TMQH_2$ would be formed, which could then sustain the reaction (it is not possible to maintain TMQ in the completely oxidized or reduced form in the presence of chloroplasts). It is not known why $UQ_6$ and $UQ_6H_2$ are less active than $UQ_2$ and $UQ_2H_2$, but it is logical to consider solubility as a factor, since $UQ_6$ is less soluble in aqueous systems. The stimulations shown by $UQ_2H_2$ and $UQ_6H_2$ are also resistant to the action of DCMU, indicating that they also can donate electrons to the long-wavelength system of the chloroplasts. Indeed, the addition of DCMU consistently produces a small stimulation of the rates observed with these hydroquinones.

Since $TMQH_2$ does not reduce cytochrome *c* in the dark at a significant rate, the data presented in Table I suggest that the reduction of cytochrome *c* is mediated by the semiquinone form of TMQ which would be formed initially by reaction with the chloroplast. A similar mechanism is proposed for the photoreduction of cytochrome *c* by $TMQH_2$ in the presence of chlorophyll *a*, as shown later.

TABLE I: Effect of DCMU on the Rates of Photoreduction of Cytochrome *c* by Spinach Chloroplasts in the Presence of Quinones and Hydroquinones.[a]

| Addition | Concentration (μmoles/3 ml) | Cytochrome Photoreduction (μmoles/hr/mg chlorophyll) Control | + DCMU ($10^{-5}$ M) |
|---|---|---|---|
| Chloroplasts | | 27 | 0 |
| + TMQ | 0.2 | 95 | 14 |
| | 0.4 | 85 | |
| | 1.0 | 81 | |
| + $TMQH_2$ | 0.2 | 76 | 52 |
| | 0.4 | 85 | |
| | 1.0 | 52 | |
| + $UQ_2$ | 0.2 | 37 | 0 |
| + $UQ_2H_2$ | 0.2 | 43 | 55 |
| + $UQ_6$ | 0.2 | 28 | 0 |
| + $UQ_6H_2$ | 0.2 | 29 | 36 |
| + *p*-Chloranil | 0.2 | 70 | 0 |

[a] The reaction mixture (3.0 ml) contained 0.05 M phosphate buffer at *p*H 7.0, 0.25 M sucrose, chloroplasts equivalent to 20 μg chlorophyll, 0.15 μmole cytochrome *c*, and the quinones as indicated.

The stimulation of cytochrome *c* reduction by other redox compounds is shown in Table II. These data are consistent with those previously reported by Keister and San Pietro (1963) and show that those compounds which are capable of functioning as Hill reagents with chloroplasts stimulate the photoreduction of cytochrome *c*, serving to couple cytochrome *c* to the reducing equivalents produced by the chloroplasts. The most active compound is PMS. The lower activity observed for ferredoxin, by comparison with the data of Keister and San Pietro, is owing to the absence of phosphorylating conditions in the present investigation. DCMU inhibits cytochrome *c* reduction in the presence of all compounds listed in Table II.

*Chlorophyll a as Photocatalyst.* AQUEOUS SYSTEMS WITH DETERGENT PRESENT. Chlorophyll *a* solubilized by Triton X-100 in aqueous solutions has the ability to catalyze a series of photoreactions between hydroquinones (quinones) and PMS ($PMSH_2$), as previously reported (Vernon *et al.*, 1963). Chlorophyll *a* solubilized in this fashion will also catalyze a rapid photoreduction of cytochrome *c* by $TMQH_2$ or reduced ubiquinones. Figure 1 presents typical reactions observed in the presence of chlorophyll *a* or chlorophyll solubilized from chloroplasts by the detergent. At the *p*H employed the dark chemical reaction between the hydro-

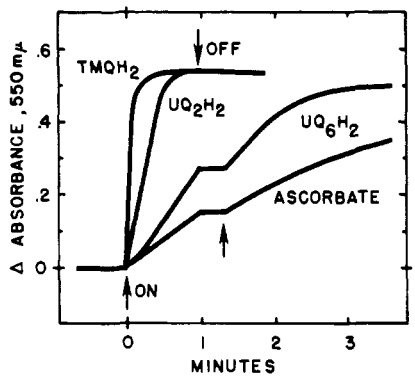


FIGURE 1: Photoreduction of cytochrome *c* by hydroquinones or ascorbate in the presence of chlorophyll *a* solubilized with the detergent Triton X-100. The reaction mixtures (3 ml) contained 0.05 M phosphate buffer at *p*H 6.7, 0.2 μmole hydroquinone or 1.2 μmoles ascorbate, 10 μg purified chlorophyll *a*, 0.14 μmole cytochrome *c*, and 0.2% Triton X-100. Anaerobic conditions were employed.

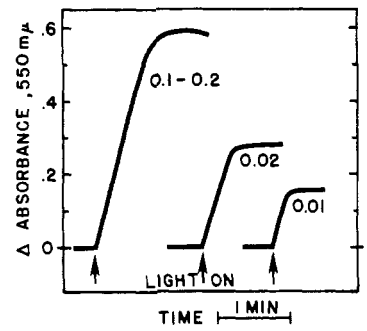


FIGURE 2: Stoichiometry of cytochrome *c* photoreduction: Variation of $TMQH_2$ concentration. The reaction mixture (3 ml) contained 0.05 M phosphate buffer, *p*H 6.7, 1 μg chlorophyll *a*, 0.14 μmole cytochrome *c*, and $TMQH_2$ present as indicated (μmoles) in 0.2% Triton X-100. Anaerobic conditions were employed.

quinones and cytochrome *c* is very slow, allowing a clear demonstration of the light-dependent reduction of cytochrome *c*. $TMQH_2$ is clearly the most active electron donor. The difference between $UQ_2H_2$ and $UQ_6H_2$ (as also observed with chloroplasts) probably reflects the difference in water solubility for these two hydroquinones, the former being more water soluble. It is interesting, however, that all these hydroquinones are more active than ascorbate. The rates of these reactions are given in Table III. The reaction is optimal at the *p*H employed in these reactions. With the light intensity available in this system, the reaction did not saturate as the light intensity was increased to a maximum ($5 \times 10^5$ ergs/cm²/sec).

The stoichiometry observed for the reaction (calculated from the extent of the reactions with limiting $TMQH_2$ present) is nearly that expected if it is assumed that $TMQH_2$ transfers two electrons to cytochrome *c*. Figure 2 presents the tracings observed for experiments in which the concentration of $TMQH_2$ was progressively decreased. The rate of the initial reaction does not decrease over the concentration range of $TMQH_2$ employed, while the extent of the reaction, which is a measure of the stoichiometry, varies in a regular manner, as expected. The rates of all reactions are identical and comparable to those given in Table III.

It is possible to carry out the photoreduction of cytochrome *c* by $TMQH_2$ in ethanol, since cytochrome *c* is soluble in 95% ethanol. Under these conditions, the reaction represented in Figure 3 was obtained, showing

TABLE II: Stimulation of Cytochrome *c* Photoreduction by Redox Agents Capable of Functioning as Hill Reagents with Spinach Chloroplasts.[a]

| Addition | Concentration (μmoles/3 ml) | Cytochrome Photoreduction (μmoles/hr/mg chlorophyll) |
|---|---|---|
| Chloroplasts | | 25 |
| + Purified spinach ferredoxin | (7.5 units)[b] | 150 |
| + PMS | 0.08 | 280 |
| + PMS | 0.04 | 220 |
| + PMS | 0.02 | 270 |
| + FMN | 0.2 | 109 |
| + Menadione | 0.2 | 105 |

[a] Reaction conditions are those given for Table I.
[b] As defined by San Pietro and Lang (1958).

TABLE III: Rates of Cytochrome *c* Photoreduction by Hydroquinones and Ascorbate with Chlorophyll *a* in the Presence of Triton X-100.[a]

| Addition | Rate of Photoreduction (μmoles Cyt *c*/hr/mg chlorophyll *a*) |
|---|---|
| Ascorbate | 155 |
| $UQ_6H_2$ | 265 |
| $UQ_2H_2$ | 960 |
| $TMQH_2$ | 6100 |

[a] The reaction conditions (anaerobic) are those of Figure 1.

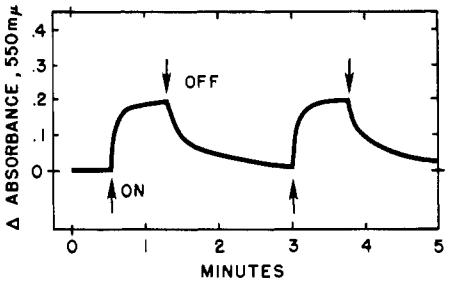


FIGURE 3: Photoreduction of cytochrome *c* with $TMQH_2$: Catalysis by chlorophyll *a* in ethanol. The reactions were performed under anaerobic conditions in 3.0 ml of 95% ethanol containing 5 μg of chlorophyll *a*, 0.14 μmole cytochrome *c*, and 0.2 μmole of $TMQH_2$.

that a fast photoreduction of cytochrome *c* occurs in the presence of $TMQH_2$. In ethanol a rapid and reproducible back reaction is observed when the light is turned off. No such back reaction is observed in aqueous media, since the redox potentials of the two compounds are such that the chemical reaction proceeds far in the direction of cytochrome *c* reduction by $TMQH_2$. It appears, therefore, that in ethanol the redox potential of one member of the couple is sufficiently changed so that the potentials of the two systems are closer together. Illumination apparently displaces the system from the equilibrium point, which allows the subsequent oxidation of reduced cytochrome *c* by TMQ in the dark.

Although not examined in detail, it has been found that chlorophyllin *a* also catalyzes the photoreduction of cytochrome *c* by $TMQH_2$. This chlorophyll derivative is water soluble, but it also gives a faster reaction when Triton X-100 is added to the chlorophyllin in an aqueous system.

## Discussion

Cytochrome *c* is not directly reduced by illuminated chloroplasts; its reduction requires the intervention of some intermediate electron carrier which couples cytochrome *c* to the reducing equivalents produced in the chloroplast system. Agents which are effective in this role are spinach ferredoxin, PMS, quinones, flavins, and redox dyes. Therefore the characteristics of cytochrome *c* photoreduction with spinach chloroplasts are those of the redox agent completing the couple.

Cytochrome *c* photoreduction can be supported by $TMQH_2$ (and to a lesser extent by $UQ_2H_2$) in the presence of DCMU, indicating that $TMQH_2$ serves as electron donor for the long-wavelength pigment system of chloroplasts. The compound directly reduced by the illuminated chloroplast would be TMQ (since there would be some of the oxidized form present). Since $TMQH_2$ reduces cytochrome *c* very slowly at the *p*H employed, the chemical reduction of cytochrome *c* is probably owing to semiquinone formed either from TMQ in the reductive reaction or from $TMQH_2$ in the oxidative reaction.

The mechanism of the *in vitro* chlorophyll-catalyzed photoreduction of cytochrome *c* in the presence of $TMQH_2$ is not definitely known. Similar reactions are observed for chlorophyll *a* in aqueous media (with detergent) or in ethanol. One can conceive of three means whereby this reaction could proceed: (1) direct reduction of cytochrome *c* by excited chlorophyll, followed by regeneration of chlorophyll by reaction with $TMQH_2$; (2) reaction of excited chlorophyll with $TMQH_2$ to produce reduced chlorophyll, which then reduces cytochrome *c;* or (3) reaction of excited chlorophyll with TMQ (either added in small amounts with the $TMQH_2$ or formed during the slow reaction of $TMQH_2$ with cytochrome *c* in the dark) to produce the semiquinone of TMQ, which then reduces cytochrome *c*. The third mechanism is favored for the reasons given below.

A direct reduction of cytochrome *c* by excited chlorophyll is not favored as a mechanism for two reasons. First, cytochrome *c* is not reduced directly by chloroplasts in a manner similar to Hill reagents (quinones, ferricyanide, redox dyes) but requires an intermediate compound to serve as a Hill reagent. Secondly, the accompanying paper (Ke *et al.*, 1965), dealing with rapid reactions of chlorophyll by the method of flash photolysis, presents evidence against a direct reduction of cytochrome *c* in ethanol. The kinetics of the cytochrome change are far slower than the kinetics of the chlorophyll reaction upon illumination with a flash of light.

A reaction mechanism involving a preliminary reaction of excited chlorophyll with $TMQH_2$ to produce reduced chlorophyll is not favored for the following reasons. First, quinones function as direct acceptors of electrons from illuminated chlorophyll *a* in organic solvents, as shown by electron spin resonance spectra (Tollin and Green, 1962, 1963) but hydroquinones have not been shown to function as electron donors to excited chlorophyll. $TMQH_2$ is inactive as an electron donor to chlorophyllin *a* in an aqueous system designed for NADP photoreduction (Vernon *et al.*, 1965). Second, a detailed study of a related reaction, chlorophyll-sensitized dye reduction in the presence of ascorbate, shows that the initial reaction is dye reduction by excited chlorophyll, not the reduction of excited chlorophyll by ascorbate. Chlorophyll reduction by ascorbate proceeds at a much slower rate and cannot account for the speed of dye reduction in the coupled system (Seely, 1965).

The mechanism favored in light of the available evidence is the third one listed above, which involves the reduction of quinone by excited chlorophyll as the initial reaction:

$$\mathrm{Chl} \xrightarrow{\text{light}} \mathrm{Chl}^* \qquad (1a)$$

$$\mathrm{Chl}^* + \mathrm{TMQ} \longrightarrow \mathrm{Chl}^+ + \mathrm{TMQ}^- \qquad (1b)$$

$$\mathrm{TMQ}^- + \mathrm{Fe}^{3+}(\mathrm{Cyto}) \longrightarrow \mathrm{TMQ} + \mathrm{Fe}^{2+}(\mathrm{Cyto}) \qquad (1c)$$

$$Chl^+ + TMQH_2 \longrightarrow Chl + TMQH\cdot + H^+ \quad (1d)$$

The TMQH· formed in reaction (1d) would also be available for cytochrome *c* reduction.

If the reaction mechanism given is valid, it should be possible to stimulate the reaction by adding TMQ to the system containing $TMQH_2$. The data of Figure 2 show that the rate of the reaction is not a function of $TMQH_2$ concentration within the concentration range employed. Furthermore, adding TMQ to any of the reaction systems described in Figure 2 has no influence upon the reaction. It is possible, however, to produce an effect of added TMQ upon the reaction by decreasing the $TMQH_2$ concentration to such a low level that the rate of the reaction is dependent upon $TMQH_2$ concentration. When this condition applies, added TMQ stimulates the reaction, as shown in Table IV. These data, coupled to the known ability of excited chlorophyll to react with TMQ (Tollin and Green, 1962, 1963), strongly support reactions (1a–d) as those responsible for cytochrome *c* photoreduction by $TMQH_2$ catalyzed by chlorophyll *a*. The addition of TMQ to a reaction system using ascorbate as the electron donor causes a marked stimulation (greater than 5-fold), which further supports the mechanism described above. There is no reason to believe the same reaction sequence does not apply to both aqueous and ethanolic solutions.

TABLE IV: Stimulation of $TMQH_2$-supported Cytochrome *c* Photoreduction by Added TMQ.[a]

| Additions (μmole/3 ml) | | Rate of Photoreduction (μmoles Cyt *c*/hr/ mg chlorophyll *a*) |
|---|---|---|
| TMQ | $TMQH_2$ | |
| 0.5 | | 5300 |
| | 0.005 | 3600 |
| 0.5 | 0.005 | 6200 |
| 0.25 | | 3100 |
| 0.05 | | 180 |
| | 0.005 | 3600 |
| 0.05 | 0.005 | 6200 |
| 0.005 | | 0 |
| | 0.001 | 2700 |
| 0.005 | 0.001 | 3800 |

[a] The reaction system contained 5 μg chlorophyll *a* and cytochrome *c*, Triton X-100, and phosphate buffer as given for Figure 1.

Although many differences exist in the *in vivo* and *in vitro* systems investigated, the general similarity of quinone-chlorophyll interaction is apparent. With both chloroplasts and solubilized chlorophyll *a*, TMQ serves as an electron acceptor and $TMQH_2$ serves as an electron donor when the chlorophyll is illuminated. It is not known if the quinones and hydroquinones interact directly with chlorophyll on the chloroplast, but the present study shows that such direct reactions are possible.

Acknowledgments

The authors extend their appreciation to Dr. D. L. Keister for making available the purified spinach ferredoxin used in these experiments and to Dr. W. S. Zaugg for his assistance in preparing the hydroquinones.

References

Arnon, D. I., and Horton. A. A. (1963), *Acta Chem. Scand. 17*, S 135.

Bales, H., and Vernon, L. P. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro, A., and Vernon, L. P., eds., Yellow Springs, Ohio, Antioch Press, p. 269.

Bishop, N. I. (1959), *Proc. Natl. Acad. Sci. U.S. 45*, 1696.

Clayton, R. K. (1962), *Biochem. Biophys. Res. Commun. 9*, 49.

Davenport, H. E., and Hill, R. (1960), *Biochem. J. 74*, 493.

Dilley, R. A., Henninger, M. D., and Crane, F. L. (1963), *Natl. Acad. Sci.–Natl. Res. Council, Publ. 1145*, 273.

Eck, H., and Trebst, A. (1963), *Z. Naturforsch. 18b*, 446.

Henninger, M. D., and Crane, F. L. (1963), *Biochemistry 2*, 1168.

Henninger, M. D., Dilley, R. A., and Crane, F. L. (1963), *Biochem. Biophys. Res. Commun. 10*, 237.

Holt, A. S. (1950), *in* U.S. Atomic Energy Commission Document ORNL-752.

Ke, B., Vernon. L. P., and Shaw, E. R. (1965), *Biochemistry 4*, 137 (this issue; accompanying paper).

Keister, D. L., and San Pietro, A. (1963), *Arch. Biochem. Biophys. 103*, 45.

Krogmann, D. W., and Olivero, E. (1962), *J. Biol. Chem. 237*, 3292.

Lichtenthaler, H. K., and Park, R. B. (1963), *Nature 198*, 1070.

San Pietro, A., and Lang, H. M. (1958), *J. Biol. Chem. 231*, 211.

Seely, G. R. (1965), *J. Phys. Chem. 69* (in press).

Tollin, G., and Green, G. (1962), *Biochim. Biophys. Acta 60*, 524.

Tollin, G., and Green, G. (1963), *Biochim. Biophys. Acta 66*, 308.

Trebst, A., and Eck, H. (1963), *Z. Naturforsch. 18b*, 694.

Vernon, L. P., San Pietro, A., and Limbach, D. (1965), *Arch. Biochem. Biophys.* (in press).

Vernon, L. P., Zaugg, W. S., and Shaw, E. (1963), *Natl. Acad. Sci.–Natl. Res. Council, Publ. 1145*, 509.

Warburg, O., and Lüttgens, W. (1946), *Biokhimiya 11*, 303.

Whatley, F. R., and Horton, A. A. (1963), *Acta Chem. Scand. 17*, S 140.

Zaugg, W. S. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 100.

Zaugg, W. S., Vernon, L. P., and Tirpack, A. (1964), *Proc. Natl. Acad. Sci. U.S. 51*, 232.