BBA 45962

THE REDOX POTENTIAL OF CYTOCHROMES *b*-559 AND *b*-563 IN SPINACH CHLOROPLASTS

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(Received February 10th, 1970)

SUMMARY

The redox potentials of the chloroplast cytochromes *b*-559 and *b*-563 have been measured *in situ* using a dual wavelength spectrophotometer. Titrations were performed anaerobically with the absorbance and potential changes being recorded simultaneously. The oxidation–reduction of the *b* cytochromes is described by a one-electron transition for each component, with the midpoint potentials being -180 ± 20 mV for the cytochrome *b*-563 and $+80 \pm 20$ mV for the cytochrome *b*-559 at pH 7.0. The redox potential of the cytochrome *b*-559, but not the cytochrome *b*-563, seems to be pH dependent, with the midpoint of the cytochrome *b*-559 being $+40$ mV at pH 8.0 and approx. $+120$ mV at pH 6.0. The redox potential of cytochrome *f in situ* is found to be $+340 \pm 10$ mV at pH 8.0.

INTRODUCTION

The position of the two *b*-type cytochromes in the electron transport chain of spinach chloroplasts is not accurately known, partly because of uncertainty regarding their redox potentials. Cytochrome *b*-563 belongs to detergent-separated Photosystem I chloroplast fractions[1], is reduced photochemically by Photosystem I (ref. 2, 3), and is reduced in the dark by NADPH, but not by ascorbate[3]. The midpoint potential of the cytochrome *b*-563, or cytochrome b_6, in etiolated barley chloroplasts was first determined to be -60 mV at pH 7.0 (ref. 4) and recalculated to be 0.0 V (ref. 5). Cytochrome *b*-559, previously called cytochrome *b*-560 (ref. 3), is found in the detergent fraction associated with Photosystem II (ref. 1) and can be reduced by ascorbate in spinach chloroplasts[1,3] and chloroplast fragments of *Chlamydomonas reinhardi*[6]. It has been reported that the midpoint potential of the cytochrome *b*-558 of Euglena chloroplasts is $+320$ mV between pH 6 and 9 (ref. 7). The latter measurement would indicate that the high-potential *b* cytochrome in chloroplasts is much closer to Photosystem I than to Photosystem II, and that there isn't enough, if any, of a potential drop between this cytochrome and cytochrome *f* for a coupled phosphorylation. Using dual wavelength spectrophotometry and anaerobic conditions we have determined the redox potentials of cytochrome *b*-559 and cytochrome *b*-563

Abbreviations: E_{m7}, midpoint potential at pH 7; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

in spinach chloroplasts through titrations in which absorbance change and potential were simultaneously measured. The midpoint potential of cytochrome *b*-563 was found to be -180 ± 20 mV; the midpoint of cytochrome *b*-559 is $+80 \pm 20$ mV at pH 7.0.

METHODS

Chloroplasts were prepared from store-bought or greenhouse-grown spinach. Depetioled leaves were ground in 0.4 M sucrose, 0.02 M Tricine–KOH, 0.01 M NaCl (pH 7.8), in a Waring blendor whose speed was controlled with a variac. The chloroplast pellet was defined by a low speed–high speed centrifugation cycle of $300 \times g$ (1 min), $3000 \times g$ (5 min), $300 \times g$ (1 min), and $3000 \times g$ (10 min). Freshly prepared chloroplasts resuspended in this sucrose-Tricine–KOH–NaCl buffer medium were stored in liquid N_2 until used. The redox potentials of the *b*-type cytochromes in chloroplasts stored in liquid N_2 were the same, within the experimental error, as in freshly prepared chloroplasts. Chlorophyll concentrations were determined in 80% acetone using the equations developed by VERNON[8].

The anaerobic titrations of redox potential were generally performed as described previously[9,10]. Anaerobic conditions were established by bubbling scrubbed prepurified Ar gas continuously through the cuvette. The higher chloroplast concentrations (100 μg chlorophyll per ml) used here under anaerobic conditions seemed to buffer the potential fairly effectively between -50 and -200 mV (on the hydrogen scale) without any further addition of redox buffers. The redox buffers used, whose potentials except where noted are given in ref. 11, were: 10 μM anthraquinone-1,5-disulfonate (midpoint potential at pH 7 (E_{m7}) $= -170$ mV); 5 μM 1,4-napthoquinone ($E_{m7} = +60$ mV); 5 mM potassium oxalate–50 μM $FeCl_3$, whose midpoint potential at pH 7 under our experimental conditions is somewhat greater than +60 mV (ref. 5); 5 mM EDTA–50 μM $FeCl_3$ ($E_{m7} = +120$ mV); and 100 μM 2,5-dimethylbenzoquinone ($E_{m7} = +180$ mV), at 25° in 0.1 M phosphate buffer at pH 6.0 and 7.0, and in 0.1 M Tricine at pH 8.0. The midpoint potentials may not be completely precise for our experimental conditions, but this does not matter since the potential was measured continuously, and not calculated, with a combination platinum electrode (Instrumentation Lab. 15020) inserted into the chloroplast suspension.

The titrations of the cytochrome absorbance changes were performed in a 1 cm $\times$ 2 cm anaerobic cuvette using an Aminco–Chance dual wavelength spectrophotometer. The reference wavelength was set at 540 nm. The half band width of the measuring beams was 2.8 nm except where indicated. The intensity of the measuring beams was approx. 2 ergs/cm^2·sec^{-1} for the 2.8-nm band width. The signal detection utilized an RCA 5819 photomultiplier tube blocked by a Balzers DT-G wide-band interference filter to eliminate any chloroplast fluorescence. The photomultiplier tube signal was fed to a PAR HR-8 Lock-In Amplifier fixed to the frequency of the light chopper, with the time constant of the output stage routinely set at 10 sec to minimize noise due to the Ar gas bubbled continuously through the cuvette and the light beam. However, the noise introduced by the gas stream was still somewhat excessive with the 60 cycles/sec chopping frequency of the spectrophotometer optics. The signal-to-noise ratio was much improved by replacing the chopping wheel with one cut to chop at 240 cycles/sec. The reference signal for the amplifier

was provided by a Monsanto M120C light emitting diode and a Texas Instrument H-11 silicon photodiode bracketed about the chopping wheel.

RESULTS

An anaerobic titration of the absorbance changes at 561 nm shows two components at pH 7.0 (Fig. 1). Fitting the Nernst equation to each component results in the dashed curve shown in Fig. 1, showing that the oxidation–reduction of each is best described by a one-electron transition, with the midpoint of the low- and high-potential components approx. −190 and +90 mV. After doing this titration many times we feel that the most accurate values for the midpoint potentials of the two components are −180 ±20 and +80 ±20 mV, respectively, at pH 7.0. Difference spectra for each of the two components shown in Fig. 1 were obtained by changing the potential back and forth over the appropriate potential range indicated by Fig. 1. For the component with the midpoint at −190 mV the potential was initially set at −320 mV and then quickly changed to −50 mV through ferricyanide addition. The potential was then changed back to −320 mV with dithionite. The peak of the difference spectra obtained in this way is at 563 nm (Fig. 2). Similarly, difference spectra for the high-potential (+90 mV) component of Fig. 1 are obtained by changing the potential from −50 to +240 mV with the addition of ferricyanide, and from +240 back to −50 mV with dithionite. The peak of the difference spectra for the −50–+240-mV transition at pH 7.0 is at 559 nm (Fig. 3). The half-band widths of the difference spectra of Figs. 2 and 3 are both about 8 nm, but there may be a small

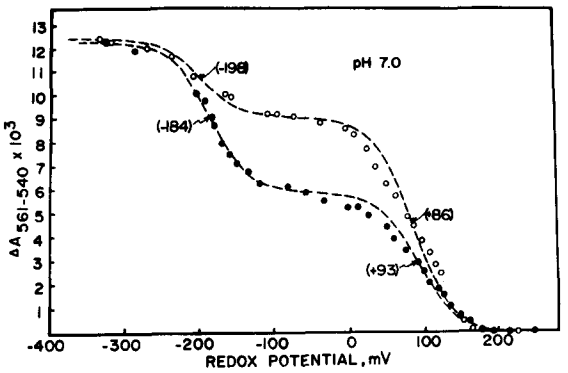

Fig. 1. Absorbance change at 561 *vs.* 540 nm of chloroplasts at pH 7.0 as a function of redox potential measured simultaneously with a platinum electrode. Chlorophyll concentration, 100 μg/ml. Anaerobic conditions. Closed circles: Reductive titration started at high potential with 1–5 μl of approx. 0.01 M dithionite solution as reductant added at each point. Open circles: Subsequent oxidative titration with successive addition of 1–5 μl of 0.01–0.1 M ferricyanide. Dashed curves: Hypothetical titration curves based on the Nernst equation for two one-electron transitions with different midpoint potentials. The low-potential component (E_m = −198 mV) of the oxidative titration is assumed to contribute 27.5% and the high-potential component (E_m = +86 mV) 72.5% to the total oxidative change. The weighting of the reductive titration is 52 and 48%, respectively, for the low-potential (E_m = −184 mV) and high-potential (E_m = +93 mV) components. The total absorbance change in the oxidative titration was within a few per cent of that in the reductive titration.

amount of non-specific absorbance in the cytochrome *b*-559 difference spectrum (Fig. 3). The 540–540 nm absorbance change is zero for the spectrum of Fig. 3. It is concluded that the midpoint potentials of cytochromes *b*-559 and *b*-563 in spinach chloroplasts are approx. +80 ±20 and −180 ±20 mV, respectively, at pH 7.0.

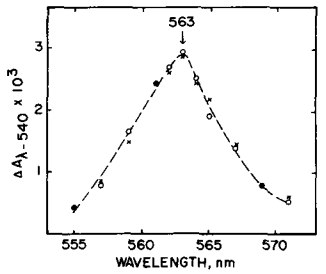

Fig. 2. Difference spectra for the low-potential component shown in Fig. 1. ×, oxidative change from −320 to −60 mV using ferricyanide as oxidant; O, reductive change from −60 to −320 mV using sodium dithionite as reductant. Chlorophyll concentration, 100 μg/ml; anaerobic conditions.

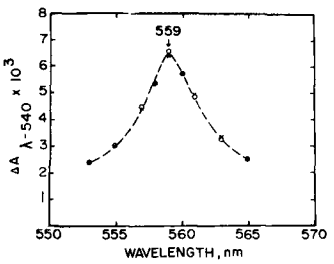

Fig. 3. Difference spectra for the high-potential component shown in Fig. 1. ×, oxidative change from −50 to +240 mV; O, reductive change from +240 to −50 mV. Chlorophyll concentration, 100 μg/ml; anaerobic conditions.

The cytochrome *b*-559 in the anaerobic chloroplast suspension is reducible by 0.1 mM NADPH, and by 0.1 mM NADH at a rate one third that of the NADPH. Succinate has no effect on the reduction of oxidized cytochrome *b*-559. NADPH and NADH both reduce cytochrome *b*-563 very slowly (data not shown).

The stoichimetry of cytochrome *b*-559 is about 1:350 chlorophylls using the data of Fig. 3 and an oxidized–reduced extinction coefficient of roughly $2 \cdot 10^4$ (ref. 12). Calculated on the same basis, cytochrome *b*-563 sometimes seems to be present at the same concentration as the cytochrome *b*-559 (as in the reductive titration of Fig. 1 and in Fig. 6) and sometimes seems to be present at lower concentrations (as in the oxidative titration of Fig. 1 and in Fig. 2).

The midpoint potential of cytochrome *b*-558 in Euglena chloroplasts, from which the soluble cytochrome *f* is missing, has been reported to be +320 mV[7]. We have measured the difference spectrum for the reversible absorbance changes in spinach chloroplasts occurring between +260 and +400 mV (Fig. 4). Ascorbate and ferricyanide were used as reductant and oxidant under anaerobic conditions. The difference spectra obtained by changing the potential from +260 to +400 mV and back again, in oxidative and reductive transitions, peak at 554.5 nm (Fig. 4). There is no indication of a *b*-type cytochrome in this potential range in spinach chloroplasts. Reversible titration of the absorbance changes at 554 nm gives a one-electron transition with a midpoint potential of +330 mV at pH 8.0 (Fig. 5). Several titrations indicate that the best value is 340 ±10 mV. This value obtained *in situ* is very close to the midpoint potential of purified cytochrome *f* ($E_{m\,7.7} = +365$ mV)[13]. Cytochrome *f* appears to be the only cytochrome in spinach chloroplasts with a midpoint potential between +260 and +400 mV.

The midpoint potentials of the cytochromes *b*-563 and *b*-559 at pH 8.0 are approx. −185 and +40 mV (Fig. 6). We have also measured the 561–540 nm absorbance change at pH 6.0, where the titrations are somewhat less reversible, and

the data are summarized in Table I. The uncertainty in the midpoint potentials is estimated to be ± 20 mV. The potential of cytochrome *b*-563 is pH independent, but the variation in the potential of cytochrome *b*-559 between pH 6 and 8 appears significant.

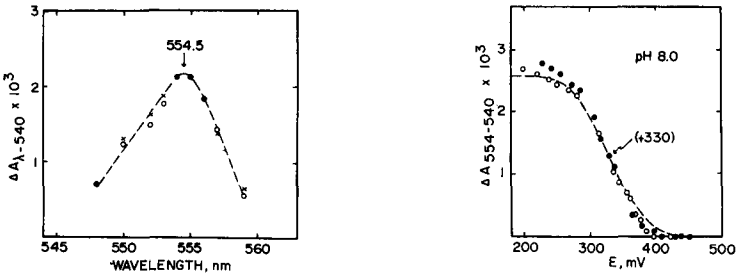

Fig. 4. Difference spectra for the absorbance change occurring between +260 and +400 mV at pH 8.0. ×, oxidative change from +260 to +400 mV; O, reductive change from +400 to +260 mV; measuring light band width, 2.1 nm; ascorbate used as reductant; chlorophyll concentration, 100 μg/ml; anaerobic conditions.

Fig. 5. Absorbance change at 554 *vs.* 540 nm of chloroplasts at pH 8.0 as a function of redox potential. Ascorbate used as reductant; chlorophyll concentration, 100 μg/ml; anaerobic conditions.

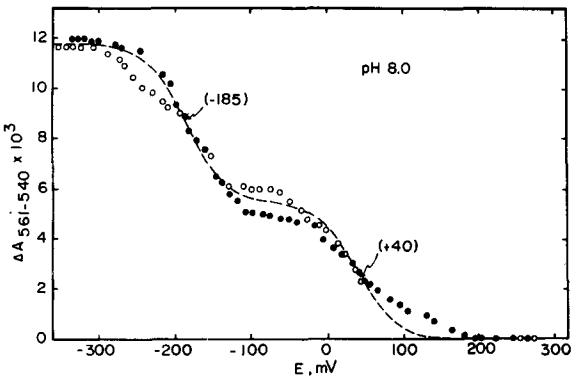

Fig. 6. Absorbance change at 561 *vs.* 540 nm of chloroplasts at pH 8.0 as a function of redox potential. O, oxidative titration; ●, reductive titration. ---, hypothetical titration curves with low-potential ($E_m = -185$ mV) and high-potential ($E_m = +40$ mV) components contributing 53 and 47 %, respectively, to the titrations. Experimental conditions otherwise as in Fig. 1.

TABLE I

MIDPOINT POTENTIALS OF CYTOCHROMES *b*-559 AND *b*-563 AS A FUNCTION OF pH

pH	*Midpoint potential (mV)*	
	Cytochrome b-559	*Cytochrome b-563*
6.0	+120	−190
7.0	+ 80	−180
8.0	+ 40	−180

DISCUSSION

Our determination of the potential of the cytochrome *b*-559 in spinach chloroplasts does not agree with the midpoint of +320 mV reported by IKEGAMI *et al.*[7] for the cytochrome *b*-558 in Euglena chloroplasts. The Euglena cytochrome *b*-558 potential also contrasts with that of the spinach cytochrome *b*-559 in being constant between pH 6 and 9 (ref. 7). The electron transport chain of Euglena chloroplasts differs from spinach chloroplasts in not having plastocyanin and having a soluble cytochrome *f*, which was intentionally left out of the chloroplasts used by IKEGAMI *et al.*[7]. The loss of these components in the Euglena chloroplasts may modify the redox properties of the cytochrome *b* in the main electron transport chain, as it is known that the redox potential of *b* cytochrome in mitochondria and bacteria is very sensitive to environment[14,15]. One important difference in experimental procedure is that IKEGAMI *et al.*[7] did not measure the redox potential of the chloroplast suspension, but calculated it from the ratio of added ferro- to ferricyanide. Because chloroplasts apparently contain a large amount of endogenous reductant[9], it is not possible to assume that they are passive in a redox experiment at positive potentials. If Euglena chloroplasts also have such a reductant, neglecting it could lead to a calculated potential which is somewhat too positive.

BENDALL[16] has reported in an abstract that the potential of cytochrome-559 in pea chloroplasts is +0.37 V at pH 6.5 and 7.5, though there also appears to be a 559-nm component of much lower potential which is not reducible by ascorbate. There are not sufficient details available at present to allow discussion of these experiments. HIND AND OLSON[17] refer to an experiment in which the midpoint potential of the cytochrome *b*-559 in spinach chloroplasts was found to be +55 mV at pH 7, in approximate agreement with our results, although KNAFF AND ARNON[18] state that the cytochrome *b*-559 midpoint is +0.33 V at pH 8.2. The supporting data is not yet available in either of these experiments.

The cytochrome *b*-559 potential which we have determined is consistent with this component being close to Photosystem II in the main electron transport chain of spinach chloroplasts[1,3,6], as shown in Fig. 7. Such a position for the cytochrome

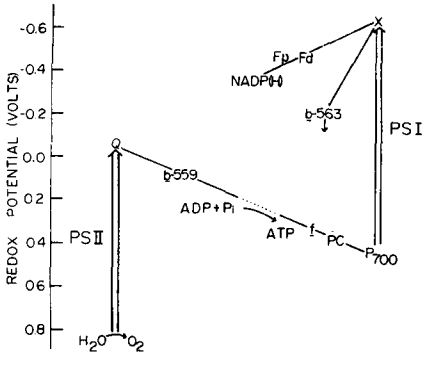

Fig. 7. The Z-scheme of photosynthetic electron transport in terms of those components with known redox potentials. The *b*-type cytochromes are arranged according to the potential values determined at pH 7.0 and existing data on their light-induced absorbance changes[2,3,6]. PS I, Photosystem I; PS II, Photosystem II; Fd, ferredoxin; PC, plastocyanin.

b-559 is supported by the System I light-induced oxidation and System II reduction of cytochrome *b*-559 in chloroplast fragments of *C. reinhardi*, from which the cytochrome *b*-563 is missing[6]. It has also been shown that the cytochrome *b*-559 is reducible by System II light in spinach chloroplasts and undergoes red, far-red reversible reduction and oxidation in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)[3]. System I light also oxidizes the cytochrome *b*-559 in spinach chloroplasts in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)[3] and antimycin A[19]. A problem with placing the cytochrome *b*-559 in a simple series chain (Fig. 7) is that in the absence of these particular inhibitors and uncouplers of electron transport, System I light oxidizes the cytochrome *b*-559 much more slowly than cytochrome *f* (refs. 3, 19). Fig. 7 is also unable to account for the recent finding of KNAFF AND ARNON[20] that System II light oxidizes cytochrome *b*-559 more efficiently than System I light at liquid N_2 temperature and in Tris-treated chloroplasts.

The midpoint potential and pH dependence of the chloroplast cytochrome *b*-559 appears to be similar to that of the cytochrome *b* in NO_2^--treated Keilin and Hartree preparations of mitochondria from heart muscle[21]. The midpoint potential of the mitochondrial cytochrome *b* determined anaerobically using calculated potentials from the succinate to fumarate ratio is between +55 and +75 mV at pH 7, and the potential varies by approx. 60 mV per pH unit between pH 6.4 and 8.2. Mitochondrial contamination of the chloroplast preparations used in our experiments was found to be negligible.

The original determination of −60 mV for the midpoint potential of cytochromes b_6 or *b*-563 by HILL[4] falls in between our measurements of the cytochromes *b*-563 and *b*-559 midpoints, as does the recalculation of 0.0 V by HILL AND BENDALL[5]. We believe that the difference between these determinations of the cytochrome *b*-563 potential and our own is most likely due to spectrophotometric separation of the cytochromes *b*-559 and *b*-563 in the latter. The cytochrome *b*-563 has a midpoint potential which is pH independent and more negative than Q (ref. 9). It seems likely that the cytochrome *b*-563 is not part of the chain connecting Photosystem II and Photosystem I, as originally inferred from its reduction by Photosystem I (refs. 2, 3). Its main function may be in a cyclic electron transport chain[22], and it is shown in Fig. 7 as a carrier between Photosystem I and the components of the main chain, all of which are energetically if not enzymatically reducible by the cytochrome *b*-563.

The representation of the electron transport chain between Photosystem II and Photosystem I shown in Fig. 7 includes only those components whose redox potential is known and it may not be complete. It does not explicitly include plastoquinone or quinone derivatives although reduced Q may be a plastochromanoxyl free radical.[23] There is also evidence for an electron transport factor of unknown identity between cytochrome *b*-559 and cytochrome *f* in chloroplast fragments of mutant ac-21 of *C. reinhardi*[6]. Although there appears to be enough of a potential drop between the cytochrome *b*-559 and cytochrome *f* to allow a coupled phosphorylation site, this cannot be decided until the ac-21 factor is identified and its potential measured. The redox potentials of Q and cytochrome *b*-559 are pH-dependent (ref. 9 and Table I above) at pH 7 and 8. The potential of purified cytochrome *f* is known to be strictly pH independent between pH 6.1 and 7.7, but above pH 8 the $\Delta E_m/pH = -60$ mV[13]. The potential of purified plastocyanin is constant between pH 5.4 and

9.9 (ref. 24). Assuming the P_{700} potential is pH independent, it would appear that those electron transport components which contain H^+ dissociating groups are concentrated near System II and those which just transport electrons near Photosystem I.

ACKNOWLEDGMENTS

We thank Drs. M. Stiller and D. Krogmann for helpful discussions.
This research was supported by National Science Foundation Grant GB-7863 and Health Sciences Advancement Award 5 s04 FR06013-03.
H.N.F. is a David Ross Predoctoral Fellow of Purdue University.

REFERENCES

1 N. K. BOARDMAN AND J. M. ANDERSON, *Biochim. Biophys. Acta*, 143 (1967) 187.
2 G. HIND AND J. OLSON, *Brookhaven Symp. Biol.*, 19 (1966) 188.
3 W. A. CRAMER AND W. L. BUTLER, *Biochim. Biophys. Acta*, 143 (1967) 332.
4 R. HILL, *Nature*, 174 (1954) 501.
5 R. HILL AND D. S. BENDALL, in T. W. GOODWIN, *Biochemistry of Chloroplasts*, Vol. 2, Academic Press, London, 1967, p. 559.
6 R. P. LEVINE AND D. S. GORMAN, *Plant Physiol.*, 41 (1966) 1293.
7 I. IKEGAMI, S. KATOH AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 162 (1968) 604.
8 L. P. VERNON, *Anal. Chem.*, 32 (1960) 1144.
9 W. A. CRAMER AND W. L. BUTLER, *Biochim. Biophys. Acta*, 172 (1969) 503.
10 W. A. CRAMER, *Biochim. Biophys. Acta*, 189 (1969) 54.
11 W. M. CLARK, *Oxidation–Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, 1960, Chapter 14.
12 R. GOLDBERGER, A. L. SMITH, H. TISDALE AND R. BOMSTEIN, *J. Biol. Chem.*, 236 (1961) 2788.
13 H. E. DAVENPORT AND R. HILL, *Proc. Roy. Soc. London, Ser. B*, 139 (1952) 327.
14 S. S. DEEB AND L. HAGER, *J. Biol. Chem.*, 239 (1964) 1024.
15 R. GOLDBERGER, A. PUMPHREY AND A. SMITH, *Biochim. Biophys. Acta*, 58 (1962) 307.
16 D. S. BENDALL, *Biochem. J.*, 109 (1968) 46P.
17 G. HIND AND J. OLSON, *Ann. Rev. Plant Physiol.*, 19 (1968) 249.
18 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 715.
19 G. HIND, *Photochem. Photobiol.*, 7 (1968) 369.
20 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 962.
21 J. P. STRAUB AND J. P. COLPA-BOONSTRA, *Biochim. Biophys. Acta*, 60 (1962) 650.
22 K. TAGAWA, H. Y. TSUJIMOTO AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 567.
23 D. H. KOHL, J. R. WRIGHT AND M. WEISSMAN, *Biochim. Biophys. Acta*, 180 (1969) 536.
24 S. KATOH, I. SHIRATORI AND A. TAKAMIYA, *J. Biochem.*, 51 (1962) 32.