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WATER-SOLUBLE CYTOCHROMES FROM A BLUE-GREEN ALGA

II. PHYSICOCHEMICAL PROPERTIES AND QUANTITATIVE RELATIONSHIPS OF CYTOCHROMES C (549, 552, AND 554 *ANACYSTIS NIDULANS*)

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SUMMARY

1. Properties of the purified cytochrome C (549) include a mesoheme prosthetic group, E_0' of -0.26 V, an acid isoelectric point, and a mol. wt. of about 20000 with 1 heme per molecule. This cytochrome forms a CO addition compound with a broad peak at 525–530 mμ with a shoulder at about 560 mμ and a sharp peak at 414 mμ but it does not react with KCN or KF.

2. Cytochrome C (554) has a mesoheme prosthetic group, an E_0' of $+0.35$ V, an acid isoelectric point, and a mol. wt. of about 23000 per heme and appears to be very similar to photosynthetic cytochrome *f* isolated from algae.

3. Cytochrome C (552) has been only partially characterized; it also has a mesoheme prosthetic group but has a basic isoelectric point like mammalian cytochromes *c*.

4. Quantitative analyses show the presence of 1 molecule of cytochrome C (549) per 220 molecules of chlorophyll, 1 molecule of cytochrome C (554) per 1550 molecules of chlorophyll, and 1 molecule of cytochrome C (552) per 21500 molecules of chlorophyll. Ferredoxin is present in roughly the same molar concentration as is cytochrome C (549).

INTRODUCTION

In the accompanying paper[1], we have described the isolation, separation and purification of 3 water-soluble cytochromes from the blue-green alga, *Anacystis nidulans*. Spectral evidence suggested that these cytochromes were all of the *c* type although cytochrome C (549) had a usually low α peak absorption band at 549 mμ and was very autoxidizable. We present here data on the physicochemical properties

Abbreviations: cytochrome C (549), cytochrome C (549, *Anacystis nidulans*); cytochrome C (552), cytochrome C (552, *Anacystis nidulans*); cytochrome C (554), cytochrome C (554, *Anacystis nidulans*).

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that demonstrate that these are *c*-type cytochromes. The relative molar concentrations of the cytochromes C (549 and 554) in relation to the molar concentration of chlorophyll present suggest a possible presence in the photosynthetic unit. The comparative biochemistry of these isolated cytochromes and those from other organisms will be discussed in an effort to understand the possible role of them in blue-green algal metabolism.

MATERIALS AND METHODS

The methods used were described earlier[1] with the addition of those given below.

Heme properties

Pyridine hemochromogens were prepared following the procedure of VERNON AND KAMEN[2]. The CO adduct was obtained by bubbling CO through the cytochrome in pH 7.0 phosphate buffer for 10 min.

Attempts to dissociate the heme from the protein of the cytochromes were made using the acid–methyl ethyl ketone procedure[3].

Oxidation–reduction potential

Cytochrome C (554) came off the DEAE-cellulose column in a reduced form[1] indicating its lack of autoxidizability. Ferricyanide oxidized the reduced form; the oxidized form could be readily reduced by ascorbate, cysteine, hydrosulfite, and borohydride. The potential was determined in phosphate buffer at pH 7.0 against the ferricyanide/ferrocyanide couple (E_0', 0.43 V) following in principle the procedure of DAVENPORT AND HILL[4]. A preliminary experiment using this procedure with horse-heart cytochrome *c* (Sigma) gave the expected E_0' +0.26 V.

With the autoxidizable cytochrome C (549) measurements had to be made anaerobically in a Thunberg-type cuvette. Ferrous oxalate failed to bring about any reduction while a slight reduction of the cytochrome was detected when reduced riboflavin was added. Benzylviologen (E_0', −0.36 V) brought about rapid reduction and reduced Janus green (E_0', −0.23 V) caused slower reduction but could not be used for a potentiometric titration because of its overlapping absorption spectra with the cytochrome. It was possible to couple the reduction of the cytochrome C (549) with 2-anthraquinone sulfonate (Eastman Organic Chemicals, recrystallized from water before use). The oxidized form of the dye did not absorb at the 549-mμ peak that was used to determine the relative amount of reduced and oxidized forms of the cytochrome C (549). The reduced dye absorbed slightly at this wavelength but a correction could be made for its absorption on the basis of experimentally derived values of 0.0030 absorbance units/0.1 μmole of reduced dye per ml. The ratio of the oxidized and reduced forms of the dye was calculated from the absorbance at 506 mμ, an isobestic point in the cytochrome C (549) absorption spectrum.

Because of the extreme ease of autoxidation of the system, it was necessary to use a system completely free of traces of oxygen but which still would permit additions of a reducing agent. We followed the method of VELICK AND STRITTMATTER[5] using a Thunberg-type cuvette which had a sidearm and an opening on which a

rubber injection port was placed. A long syringe needle served as a gas entrance and a short one as an exit, and the solution was flushed with purified hydrogen. The absorption spectrum of the oxidized cytochrome C (549) (0.15 ml cytochrome C (549), 0.285 ml of 1.0 M phosphate buffer (pH 7.0), 2.415 ml of water) was run, and then 0.15 ml (0.363 μM) of a solution of sodium 2-anthroquinone sulfonic acid was tipped in from the sidearm. This system was poised by the step-wise addition of a sodium dithionite solution (5 mg/ml) (ref. 6) through a syringe through the injection port, the dithionite solution having been purged with hydrogen before use. The E_0' for 2-anthraquinone sulfonate at pH 7.0 was taken as -0.226 V (ref. 7).

QUANTITATIVE ANALYSES

Quantitative determinations of the pigments of Anacystis used the following protocol. Cells equivalent to about 10 g dry wt. were harvested by centrifugation and the resulting cell paste diluted to 200 ml. Three 2-ml aliquots were used for estimations of dry weight, chlorophyll and carotene, and phycocyanin. The remaining 194 ml were immediately lyophilized for cytochrome and ferredoxin determinations.

Chlorophyll and carotenes were extracted from an appropriate fraction of the diluted aliquot and estimated spectrophotometrically[8]. For the phycocyanin determination, an aliquot of cells was sonicated with a Branson sonifer for 6 periods of 30 sec with ice-bath cooling between treatments. After a low-speed centrifugation, the green residue was sonicated again and the entire procedure also repeated a third time. The method was generally that of MYERS AND KRATZ[8] and a mol. wt. of 138000 (ref. 9) was used in the calculations of phycocyanin content.

The cytochromes and ferredoxin analyses were accomplished essentially by quantifying the separation procedure described earlier[1]. The lyophilized cells were added to 160 ml water and stirred for 18 h at 4°, conditions shown earlier to bring about effective extraction of cytochromes and ferredoxin. After centrifugation the blue supernatant was saved and the green sediment resuspended in water and re-extracted for 3.5 h. After centrifugation the second supernatant was green, indicating that most of the phycocyanin had been removed during the first extraction. The sediment was extracted a third time, and the combined supernatants were dialyzed overnight with several changes of water. The blue solution containing the cytochromes and the ferredoxin was applied to a DEAE-cellulose column, and the column was washed with water until the eluant showed no absorption at 280 mμ. The eluant, which contained unadsorbed proteins including cytochrome C (552), was concentrated by lyophilization. This lyophilized material was added to water, and the small amount of insoluble material was removed by filtration through a millipore filter (0.45 μ pore size).

Pigments adsorbed to the column were eluted using a concentration gradient obtained by adding 300 ml of 0.5 M phosphate buffer (pH 7.0) to 300 ml water. The absorption spectrum of each 15-ml fraction coming off the column was scanned in the spectrophotometer, and cytochromes C (554 and 549) were contained in the first 24 fractions before any ferredoxin was observed. After removal of the cytochromes, the ferredoxin was eluted using first 1.0 M phosphate buffer (pH 7.0) and finally 1.0 M phosphate buffer containing 0.5 M NaCl. The 13 fractions containing ferredoxin were pooled, dialyzed, and lyophilized.

The pooled cytochrome fractions were dialyzed and added to another DEAE-cellulose column. Careful step-wise elution using increasing concentrations of phosphate buffer and scanning of the absorption spectrum of each fraction as it came off the column permitted separation of the 2 cytochromes. The absorption was followed in the Soret region since the phycocyanin present made it impossible to follow the α absorption bands. With 0.020 M followed by 0.035 M phosphate buffer (pH 7.0), it was possible to elute the cytochrome C (554) in 14 fractions of 15 ml each while the cytochrome C (549) was eluted with higher concentrations of buffer into 26 fractions. Very little phycocyanin was removed in the cytochrome C (554) fractions, but the cytochrome C (549) fractions contained much phycocyanin. Each group of fractions were pooled, lyophilized, and dialyzed, and any insoluble material removed by filtration through a millipore filter.

The cytochrome analyses depended upon the formation of the pyridine hemochromogens using the method described above. While this formation is specific for the heme proteins in these solutions, other pigments were present in these extracts and corrections had to be made for their absorption on the α band of the spectra where the absorbance was read. Phycocyanin was the only other pigment absorbing significantly in the 550-mμ region, and although the absorption of the phycocyanin was decreased under the conditions of the pyridine hemochromogen preparation, it was deemed necessary to make appropriate correction.

Pure phycocyanin was obtained from a water extract as used in the cytochrome preparation and precipitated with $(NH_4)_2SO_4$ at 70% saturation. The precipitate was dissolved in water, dialyzed and adsorbed on aged calcium phosphate gel (26.5 mg/ml). The pigments were eluted from the calcium phosphate gel by 20-ml step-wise increments in concentration of KH_2PO_4. Fractions eluted by 0.004–0.047 M buffer contained cytochromes and were discarded and most of the phycocyanin was removed with 20 ml 0.188 M K_2HPO_4. The absorption spectrum of this purified preparation has been published[10], and it appears to be free of chlorophyll, cytochromes and other pigments. This purified phycocyanin was treated with reagents used in the pyridine hemochromogen preparations, *e.g.*, 0.45 ml pyridine, 0.05 ml 6 M NaOH, and 0.75 ml of the phycocyanin solution. The absorbance of treated phycocyanin at 550 mμ was approx. 2% of the native phycocyanin absorbance in water at its 620-mμ peak and was linearly proportional to the concentration. This correction factor was applied to cytochrome solutions that contained phycocyanin by estimating the content of the latter by its absorbance at 620 mμ where the cytochrome does not absorb. In dilute solutions of cytochrome containing much phycocyanin the correction becomes significant.

The concentration of the cytochromes was calculated using an ε_{mM} of 29.10 (ref. 11).

The pooled ferredoxin fractions were purified by addition of $(NH_4)_2SO_4$ to 42% of saturation, centrifugation to remove the small amount of phycocyanin precipitate, and addition of $(NH_4)_2SO_4$ to 92% of saturation to precipitate the ferredoxin. The ferredoxin was dissolved in water, dialyzed, and insoluble substances removed by filtration. From the absorbance at 420 mμ, the concentration of ferredoxin was calculated using the provisional ε_{mM} of 10.32 of the spectrally similar ferredoxin[12].

RESULTS AND DISCUSSION

Physicochemical properties of cytochrome C (549)

Prosthetic group

A solution of cytochrome C (549) brought to pH 2.0 with 0.1 M HCl was unaffected by shaking with equal volume of methyl ethyl ketone indicating that the prosthetic group was not a protoheme[3]. When a cytochrome solution was treated with pyridine and NaOH and reduced with dithionite the resulting pyridine hemochromogen had an absorption spectrum identical to that of the analogous compound formed by a similar treatment of horse-heart cytochrome *c*. Maxima were obtained at 550, 520, and 414 mμ suggesting that the prosthetic group in cytochrome C (549) was a mesoheme and bound covalently to the protein.

When CO was bubbled through a pH 7.0 solution of cytochrome C (549), an addition compound was formed which had a broad peak at 525–530 mμ with a shoulder at about 560 mμ and a sharp band at 414 mμ (Fig. 1). White light did appear to bring about some reversal of this reaction but no quantitative data were obtained.

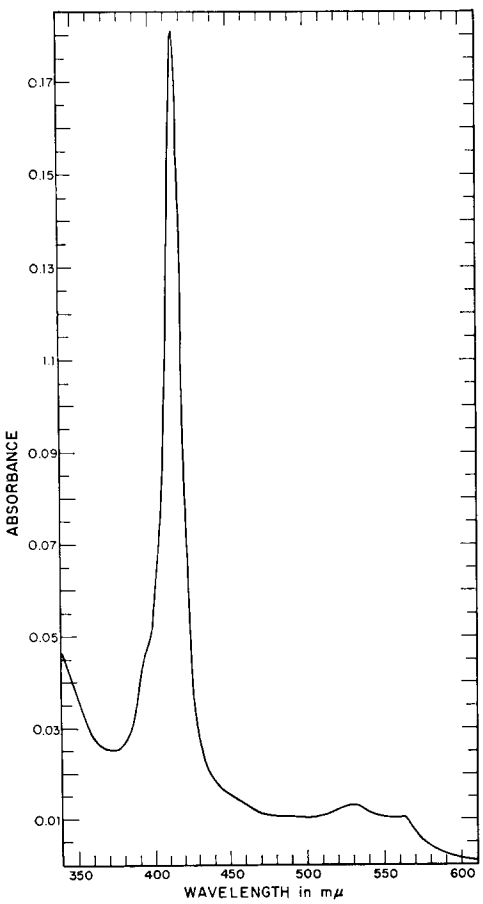

Fig. 1. Absorption spectrum of reduced CO–Anacystis cytochrome C (549) complex at pH 7.0.

Addition of either $5 \cdot 10^{-3}$ M KCN or $5 \cdot 10^{-3}$ M KF caused no spectroscopically detectable changes in the spectrum of the oxidized cytochrome C (549). These characteristics of cytochrome C (549) in the presence of the ligands mentioned above are nearly identical to those described for *Chromatium* cytochrome *c* (ref. 13).

Oxidation–reduction potential

The midpoint potential was −0.26 V using the 2-anthroquinone sulfonic acid couple in 0.1 M phosphate buffer (pH 7.0). Because of the experimental difficulties in determining the E_0' under completely anaerobic conditions, it was estimated that determination was accurate to ± 0.02 V. This strongly negative redox potential appears to be the lowest of any *c*-type cytochrome isolated. It is considerably more negative than that of *Desulfovibrio desulfuricans* cytochrome c_3 at −0.205 V (ref. 6), *D. gigas* cytochrome c_3 of −0.22 V (ref. 14), and cytochrome *c* (C (552)) of *Escherichia coli* of −0.20 V (ref. 15).

Isoelectric point

At pH 8.3 conditions used in starch-gel and polyacrylamide-gel electrophoresis and at pH 7.0 in a Tiselius apparatus, cytochrome C (549) moved towards the anode. Hence the isoelectric point is less than 7.0 like those of many other of the *c*-type cytochromes including *Chromatium* cytochrome *c* (ref. 13) and *D. gigas* cytochrome c_3 (ref. 14). *D. desulfuricans* cytochrome c_3 differs in that the isoelectric point is basic[6].

Molecular weight estimations

A single sedimentation in the analytical ultracentrifuge gave a sedimentation constant of $s_{20,w} = 2.0$ S in a 0.27 % cytochrome solution. No correction was made for radial dilution. Only 1 peak was noted in this dilute solution. A sedimentation equilibrium run was made in phosphate buffer (pH 7.10, I 0.1) with a more concentrated sample which contained several contaminants and using an assumed partial specific volume of 0.71; the best estimate of molecular weight from this experiment was between 17000 and 20000.

From the heme content of a sample from which dry weight was also obtained, the molecular weight per heme was found to be 26100. While more accurate estimations of the molecular weight would be desirable, it seems evident that cytochrome C (549) is a low-molecular-weight hemoprotein of about 20000 with 1 heme per molecule.

Physicochemical properties of cytochrome C (554)

Prosthetic group

Treatment of cytochrome C (554) with 0.1 M HCl in methyl ethyl ketone did not split off the heme suggesting that it was not of the proto type[3]. The reduced pyridine hemochromogen had an absorption spectrum with peaks at 414, 520.5, and 550 mμ, identical to that obtained for horse-heart cytochrome *c* pyridine hemochromogen prepared in the same way suggesting that the heme was of the meso type. Treatment of a solution of the reduced cytochrome with CO did not alter the absorption spectrum.

Oxidation–reduction potential

The midpoint potential was determined with the ferro-/ferricyanide couple and found to be +0.35 V at pH 7.0 with an estimation of accuracy of ± 0.01 V. This E_0' is similar or identical to that of +0.34 V for cytochrome *c* (C (554)) from Navicula[16], +0.37 V for cytochrome C (552) from Euglena[17], +0.35 V for cytochrome *f* from

Chlorella[18], +0.365 V for cytochrome *f* from parsley[4], and +0.335 V for cytochrome *f* for *Porphyra tenera*[19].

Isoelectric point

Similar results from the starch-gel, polyacrylamide, and Tiselius electrophoretic experiments described for cytochrome C (549) suggest that the isoelectric point is less than pH 7.0 for cytochrome C (554). During starch-gel electrophoresis, cytochrome C (554) moves towards the anode somewhat more rapidly than does the cytochrome C (549). The isoelectric point of other high-potential photosynthetic cytochromes *f* is likewise acid for those from Euglena[17], parsley[4], and *Porphyra tenera*[19].

Molecular weight estimation

From the heme content of a highly purified sample, a mol. wt. of 23000 per heme was calculated. This value is regarded as tentative. However, the characteristics during electrophoresis and column chromatography are so similar to those of cytochrome C (549), it is presumed that the molecular weights are also similar and that the ratio of 1 heme per molecule also holds for cytochrome C (554).

Physicochemical properties of cytochrome C (552)

The very small amount of cytochrome C (552) available, precluded detailed study. The pyridine hemochromogen absorption spectrum is like that of horse-heart cytochrome *c*; hence it is presumed that the heme is of the meso type. For most samples autoxidation was slight or negligible. Because this cytochrome was not adsorbed onto DEAE-cellulose but was adsorbed on the cation-exchange resin, XE-64 (Amberlite), it was clear that the isoelectric point was on the basic side. During acrylamide-gel electrophoresis, cytochrome C (552) behaved like horse-heart cytochrome *c* suggesting the isoelectric point may be as high as the pH 10 of cytochrome *c*. During column chromatography there was some evidence of 2 fractions with similar absorption characteristics, suggesting that the partial autoxidation mentioned above may have been observed because of a mixture of cytochromes present. Such observations may also be explained by the presence of a mixture of native and partially denatured cytochrome C (552). Because the absorption spectra presented[1] show well-defined peaks it is suggested that this possibility may be the best explanation now available.

Properties of the 3 cytochromes are summarized in Table I.

Quantitative analyses

The results of 2 quantitative experiments are presented in Table II. The actual cytochrome analyses are given in terms of molecules of heme but in the calculations for the cytochrome to chlorophyll ratios it is assumed that there is only 1 heme per cytochrome molecule on the basis of data thus far obtained.

Inadvertently, the 2 batches of algae were grown under somewhat different conditions of illumination which affected the absolute amounts of pigment present. Thus while the absolute amounts of pigment have changed significantly, the ratio of the pigments to chlorophyll concentration has remained quite constant in accord with data presented previously[8]. It will be noted that the molar ratio of cytochrome C (549) to chlorophyll suggests a size of the photosynthetic unit similar to that indicated

TABLE I

SUMMARY OF PROPERTIES OF CYTOCHROMES C (549, 554 AND 552)

	Cytochrome C (549)	*Cytochrome C (554)*	*Cytochrome C (552)*
State after purification	Oxidized	Reduced	Reduced (mostly)
Autoxidizability	Rapid	None	Partial
Redox potential (pH 7.0, 27°)	−0.26 ± 0.02 V	+0.35 ± 0.01 V	—
Reaction with CO	+	—	—
Mol. wt. (heme content)	26 100	23 000	—
Mol. wt. (sedimentation equilibrium)	17 000–20 000	—	—
Estimated isoelectric point	≤ 7.0	≤ 7.0	≥ 7.0
Prosthetic group	Mesoheme	Mesoheme	Mesoheme

TABLE II

CONCENTRATIONS OF PHOTOSYNTHETIC PIGMENTS, WATER-SOLUBLE CYTOCHROMES AND FERREDOXIN

Pigment	*Concn. (μM/g dry wt.)*		*μM Chlorophyll/μM pigment*	
	Expt. 34	*Expt. 35*	*Expt. 34*	*Expt. 35*
Chlorophyll	20	13	—	—
Carotene	16	12	1.2	1.1
Phycocyanin	1.2	0.94	17	14
Cytochrome C (549)	0.080	0.068*	250	190
Cytochrome C (554)	0.013	0.0080	1 500	1 600
Cytochrome C (552)	0.00082	0.00069	24 000	19 000
Total cytochrome*	0.094	0.076	—	—
Ferredoxin	0.101	0.090	200	145

* In Expt. 34, no separate analysis was run on the total cytochrome fraction, and the figure given is the sum of the separate components. In Expt. 35, a separate analysis on the total cytochrome fraction was run, and when the cytochrome C (549) fraction was accidentally lost, the cytochrome C (549) content was estimated by difference.

by the 400:1 ratio of chlorophyll to cytochrome *f* found earlier in chloroplasts[4]. The ferredoxin is also present on a molar basis at about the same concentration in our extracts as that of cytochrome C (549), suggesting circumstantially a possible functional relationship for these 2 iron-containing compounds with low redox potentials.

DISCUSSION

Since our initial observation of these water-soluble cytochromes from Anacystis[20], similar cytochromes have been observed in extracts of *Anabaena cylindrica*[21], *A. variabilis*[22] and our observations on Anacystis have been confirmed[23]. The cytochrome C (554) appears to be clearly similar in nature to the photosynthetic *f*-type cytochromes isolated from many photosynthetic systems. The function of the cytochrome C (549) presents a more difficult problem to interpret. Its strongly negative E_0', autoxidizability, and reaction with carbon monoxide suggest that it may be isolated in a denatured form. However, mild conditions of nearly neutral pH at 4°

and the constant quantitative relationship of cytochromes C (554 and 549) during extractions do not support this point. The same experimental conditions allow isolation of cytochrome C (554) in an apparently undenatured form; fractionation on DEAE-cellulose of an extract obtained from fresh cells disrupted by ultrahigh-frequency sound in acetate buffer (pH 4.7) also separates cytochromes C (549 and 554) (ref. 1) and disruption in a French press is likewise effective[23].

A more likely point of activity involves the hydrogenase system. For example, GRAY AND GEST[24] point out that the presence of low-potential cytochromes in certain anaerobic bacteria may fulfill the electron-transfer functions in hydrogenase reactions attributed to ferredoxins in other anaerobic bacteria. However, ferredoxin does stimulate NADP reduction by the hydrogenase systems found in blue-green algae[21].

It is also interesting to note that in the colorless blue-green algae investigated by WEBSTER AND HACKETT[25], no evidence of a cytochrome oxidase was found. Instead a CO-binding pigment was observed which had absorption maxima *in vivo* at 417, 535, and 570 mμ. The Anacystis cytochrome C (549) CO adduct has peaks at 414, 525–530 and 560 mμ and these are similar even though Anacystis and the colorless blue-greens studied may be phylogenetically only distantly related. WEBSTER AND HACKETT suggest that the terminal oxidase is an *o*-type cytochrome but our knowledge of respiration generally of blue-green algae is still meager. The most difficult feature to reconcile of ascribing such a function to cytochrome C (549) is its relatively high concentration in Anacystis and the very low respiration rate of this organism.

It is likewise worth noting that in GOEDHEER's[26] experiments on Anacystis *in vivo* there was a strong cytochrome oxidation system 10 times more active than the similar system in the green alga, Chlorella. As is pointed out by STANIER AND VAN NIEL[27], there is suggestive evidence for a very close functional linkage between photosynthesis and respiration in procaryotic cells and the 2 electron-transport chains could conceivably contain enzymes and carriers common to both, *e.g.*, cytochrome C (549).

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REFERENCES

1 R. W. HOLTON AND J. MYERS, *Biochim. Biophys. Acta*, 131 (1967) 362.
2 L. P. VERNON AND M. D. KAMEN, *J. Biol. Chem.*, 211 (1954) 643.
3 F. W. J. TEALE, *Biochim. Biophys. Acta*, 35 (1959) 543.
4 H. E. DAVENPORT AND R. HILL, *Proc. Roy. Soc. London, Ser. B*, 139 (1952) 327.
5 S. F. VELICK AND P. STRITTMATTER, *J. Biol. Chem.*, 221 (1956) 265.
6 J. R. POSTGATE, *J. Gen. Microbiol.*, 14 (1956) 545.
7 H. W. MEYER AND W. D. TREADWELL, *Helv. Chim. Acta*, 35 (1952) 1444.
8 J. MYERS AND W. A. KRATZ, *J. Gen. Physiol.*, 39 (1955) 11.
9 A. HATTORI AND Y. FUJITA, *J. Biochem. Tokyo*, 46 (1959) 633.
10 L. W. JONES AND J. MYERS, *J. Phycol.*, 1 (1965) 7.
11 D. L. DRABKIN, *J. Biol. Chem.*, 146 (1942) 605.
12 F. R. WHATLEY, K. TAGAWA AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 266.
13 R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 235 (1960) 825.
14 J. LE GALL, G. MAZZA AND N. DRAGONI, *Biochim. Biophys. Acta*, 99 (1965) 385.

15 T. FUJITA AND R. SATO, *Biochim. Biophys. Acta*, 77 (1963) 690.
16 T. YAMANAKA AND M. D. KAMEN, *Biochim. Biophys. Acta*, 112 (1966) 436.
17 F. PERINI, M. D. KAMEN AND J. A. SCHIFF, *Biochim. Biophys. Acta*, 88 (1964) 74.
18 S. I. HONDA, J. E. BAKER AND A. M. MUENSTER, *Plant Cell Physiol. Tokyo*, 2 (1961) 151.
19 S. KATOH, *Plant Cell Physiol. Tokyo*, 1 (1960) 91.
20 R. W. HOLTON AND J. MYERS, *Science*, 142 (1963) 234.
21 Y. FUJITA AND J. MYERS, *Arch. Biochem. Biophys.*, 111 (1965) 619.
22 W. A. SUSOR AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 120 (1966) 65.
23 R. M. SMILLIE, *Plant Physiol.*, 40 (1965) 1124.
24 C. T. GRAY AND H. GEST, *Science*, 148 (1965) 186.
25 D. A. WEBSTER AND D. P. HACKETT, *Plant Physiol.*, 41 (1966) 599.
26 J. C. GOEDHEER, *Biochim. Biophys. Acta*, 66 (1963) 61.
27 R. Y. STANIER AND C. B. VAN NIEL, *Arch. Mikrobiol.*, 42 (1962) 17.