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A LIGHT-DEPENDENT OXYGEN-REDUCING SYSTEM FROM *ANABAENA VARIABILIS*

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SUMMARY

Photosynthetic lamellae from *Anabaena variabilis* catalyze a vigorous photoreduction of O_2 to H_2O_2. The electrons come to O_2 from an artificial donor, reduced 2,3′,6-trichlorophenolindophenol, and the reaction does not require an exogenous autoxidizable substance. Evidence is presented to show that reduced 2,3′,6-trichlorophenolindophenol can donate electrons at two distinct sites. Photoreduction of O_2 is inhibited by antibodies which block the function of the ferredoxin-reducing substance. The O_2-reducing system may result from the formation of a reduced photoproduct which is more accessible to autoxidation than is the analogous product formed in higher plant chloroplasts.

INTRODUCTION

The reduction of O_2 by illuminated chloroplasts was observed and characterized by MEHLER[1] in 1950. This reaction involves the reduction by the Hill reaction of an autoxidizable electron acceptor such as FMN, and is the basis for pseudocyclic phosphorylation of chloroplasts[2]. There is, as yet, no evidence that the chloroplast electron donor which reduces FMN is itself autoxidizable.

This paper describes studies of an O_2-reducing system of the blue-green alga *Anabaena variabilis*. This O_2 reduction does not require an exogenous autoxidizable electron carrier although such carriers stimulate it. The O_2-reducing reaction is only observed when an artificial donor is the source of electrons. This reaction appears to be another manifestation of the cytochrome *c*-reducing substance described by FUJITA AND MEYERS[3] and of the ferredoxin-reducing substances described by YOCUM AND SAN PIETRO[4]. In *A. variabilis* this substance is evidently accessible to oxidation by O_2 or cytochrome *c* while in higher plant chloroplasts, these oxidations must be mediated by ferredoxin[5], or the chloroplast structure must be opened by sonication[6].

METHODS

The methods for cultivation of algae, for isolation of photosynthetic lamellae, and for measurement of chlorophyll, photophosphorylation and Hill reaction activity

Abbreviations: TCIP and $TCIPH_2$, oxidized and reduced 2,3′, 6-trichlorophenolindophenol; MES, 2-(*N*-morpholino)-ethane sulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

have all been described[7,8]. Unless otherwise noted, all measurements reported here were carried out in an illuminated Warburg apparatus at 25° with incandescent light filtered through a red cellophane layer to exclude wavelengths below 600 nm. The intensity of light at the surface of the reaction vessel was $8 \cdot 10^4$ ergs/cm^2 per sec. The standard reaction mixture contained the following components in μmoles in a total volume of 1 ml: 2-(*N*-morpholino)-ethane sulfonic acid (MES) buffer (pH 6.8), 25; $MgCl_2$, 5; sodium ascorbate, 10; oxidized 2,3',6-trichlorophenolindophenol (TCIP), 0.09; and photosynthetic lamellae containing 50 μg chlorophyll. Cytochrome c_{554} was prepared from *A. variabilis* by a published procedure[7]. Catalase was purchased from the Sigma Chemical Co. Spinach chloroplasts were prepared by the method of AVRON *et al.*[9]. The measurements in Table IV showing inhibition by antisera were made with a Yellow Springs O_2 electrode. The reaction conditions and control rate of O_2 consumption were nearly identical to those of the manometric measurements. The antisera and the autoxidizable compound isolated from spinach were the generous gift of Dr. A. Trebst[10–12].

RESULTS

Table I describes the general requirements for measuring light-dependent O_2 consumption by the photosynthetic lamellae of *A. variabilis*. The reaction requires light, reduced 2,3',6-trichlorophenolindophenol ($TCIPH_2$) and lamellae. Activity is destroyed by boiling and is inhibited by Tween 20 and by ethanol. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is not inhibitory. Addition of catalase decreases O_2 consumption by 30 %.

TABLE I

REQUIREMENTS FOR LIGHT-DRIVEN O_2 CONSUMPTION

The reactions were measured in an illuminated Warburg apparatus over a 15-min illumination period. The standard reaction conditions are described under METHODS.

	μl O_2 uptake
Control	81
Boiled particles	0
DCMU, 5 μM	82
Tween 20, 0.1 %	29
Ethanol, 5 %	34
Minus ascorbate	0
Minus TCIP	0
Minus light	17
Catalase 0.01 mg	56

O_2 consumption by the lamellae preparation is dependent on chlorophyll concentration within the usual saturation limits. Carefully washed or osmotically shocked preparations failed to indicate a soluble rate-limiting participant.

We had previously noted that TCIP would elicit ATP synthesis by illuminated lamellae[8]. The relation of TCIP concentration to both O_2 consumption and ATP synthesis activity is described in Fig. 1. O_2 consumption is maximal at 0.4 mM TCIP and is slightly inhibited at higher concentrations. The figure shows that the

phosphorylation reaction reaches its maximum at about one-tenth the TCIP concentration needed for maximum O_2 consumption.

Table II shows the effect of exogenous cytochrome c_{554} on the O_2 consumption activity. In this experiment, cytochrome c_{554} stimulated the reaction 100% at a limiting TCIP concentration. The omission of TCIP eliminates all activity even in

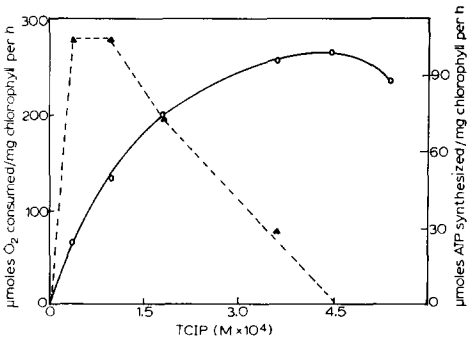

Fig. 1. The relation of TCIP concentration to O_2 consumption and ATP synthesis. The reaction conditions are the same as those described in METHODS except for the inclusion of 5 μmoles ADP; 3 μmoles phosphate buffer (pH 6.8) and 5 mμmoles DCMU. Illumination time was 10 min. The dashed line describes ATP synthesis while the solid line describes O_2 consumption.

TABLE II

EFFECT OF CYTOCHROME c_{554} ON THE O_2-REDUCING SYSTEM

The reaction conditions are the same as those described in METHODS. The same reaction mixture components were used except as noted. Washed particles had been diluted with 3 volumes of 2 mM EDTA, pH adjusted to 6.8, then centrifuged and resuspended in the sucrose–NaCl isolation medium.

TCIP	*μmoles O_2 consumed/mg chlorophyll per h*
Control particles	
0.09 μmole	97
0.09 μmole + 1.37 mμmoles cytochrome c_{554}	120
0.09 μmole + 2.74 mμmoles cytochrome c_{554}	179
0.00 μmole + 2.74 mμmoles cytochrome c_{554}	0
Washed particles	
0.09 μmole	54
0.09 μmole + 2.74 mμmoles cytochrome c_{554}	178
0.36 μmole	162
0.36 μmole + 2.74 mμmoles cytochrome c_{554}	161

the presence of high concentrations of cytochrome c_{554}. When the lamellae are washed at low ionic strength, a treatment which helps to solubilize endogenous cytochrome c_{554}, there is a diminution in O_2 consumption at low TCIP concentration. The diminution in activity that results from washing can be fully reversed by addition of cytochrome c_{554}. At a higher TCIP concentration, the O_2 consumption reaction proceeds at a maximum rate despite the washing treatment and this reaction is unaffected by exogenous cytochrome c_{554}.

Efforts were made to find a substitute electron donor for TCIP in supporting

the O_2-reducing reaction. Only ferrocyanide in the presence of excess ascorbate permitted appreciable O_2 consumption. Excess ferrocyanide alone was ineffective as were glutathione, dithiothreitol and hydroxylamine.

Ascorbate at 5 mM saturates the O_2-consuming reaction as seen in Fig. 2. The ascorbate saturation characteristics are not significantly influenced by the TCIP concentration.

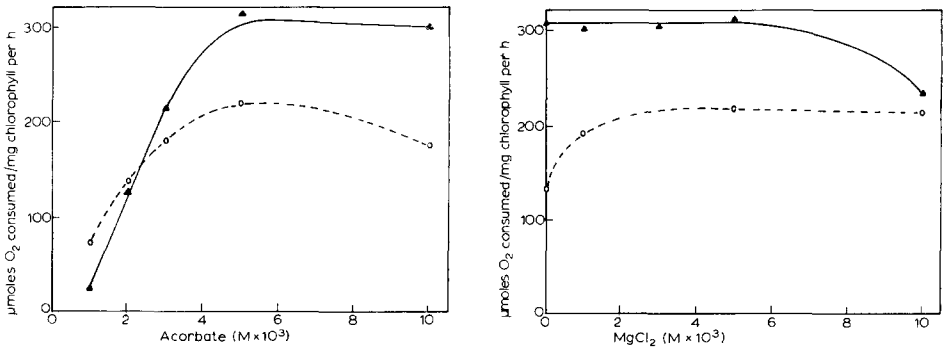

Fig. 2. The effect of ascorbate concentration on the O_2 reducing system. The reaction conditions and reagents are the same as those described in METHODS. The solid line refers to measurements with 0.36 μmole TCIP and the broken line to measurements with 0.09 μmole TCIP.

Fig. 3. The effect of Mg^{2+} concentration on the O_2-reducing system. The reaction conditions are the same as those described in METHODS. The solid line refers to measurements where 0.36 μmole TCIP was used while the dashed line refers to measurements with 0.09 μmole TCIP.

The effect of $MgCl_2$ on the rate of O_2 consumption is shown in Fig. 3. At a saturating TCIP concentration, the presence of $MgCl_2$ in the reaction mixture is without effect except at high concentration (100 mM) there is some inhibition. At a lower TCIP concentration, $MgCl_2$ (4 mM) gives a 60% increase in the rate of O_2 consumption.

Fig. 4 describes the effect of varying light intensity on O_2 consumption by *A. variabilis* lamellae. With a saturating concentration of TCIP, the reaction is

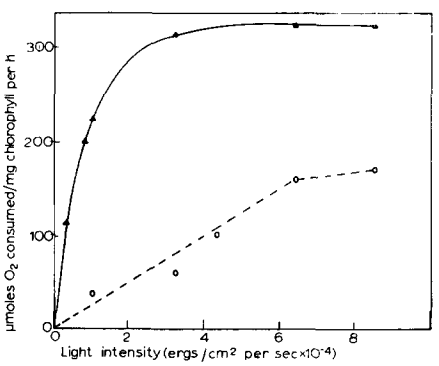

Fig. 4. The effect of varying light intensities on the O_2-reducing system. The reaction conditions are the same as described in METHODS. The solid line describes experiments done with 0.36 μmole TCIP and the dashed line with 0.09 μmole TCIP. Variation in light intensity was achieved with neutral density filters.

saturated at a low intensity, $4 \cdot 10^4$ ergs/cm^2 per sec. With a limiting concentration of TCIP, the light intensity for saturation is perceptibly raised to $6 \cdot 10^4$ ergs/cm^2 per sec.

The effects of variation in the temperature of the reaction are reported in Fig. 5. At a saturating TCIP concentration, the rate of reaction increases up to 40°, with a Q_{10} equal to 1.87.

Fig. 6 shows the response in lamellae O_2 consumption as a function of H^+ concentration. There is a general tendency to higher rates at a higher pH as is seen in many autoxidations. The reaction at limiting TCIP shows a slightly greater sensitivity to extremes of pH than does the reaction at saturating TCIP.

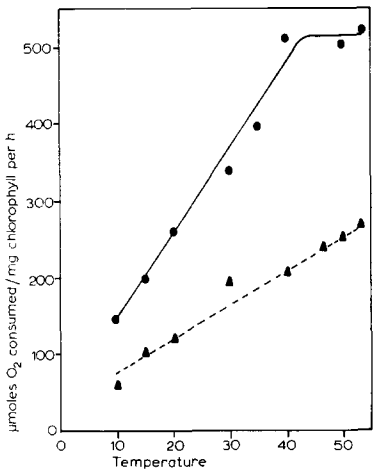

Fig. 5. The effect of temperature on the O_2-reducing system. The reaction conditions are the same as detailed in METHODS. ——, experiments with 0.32 µmole TICP; - - - - -, experiments with 0.08 µmole TCIP.

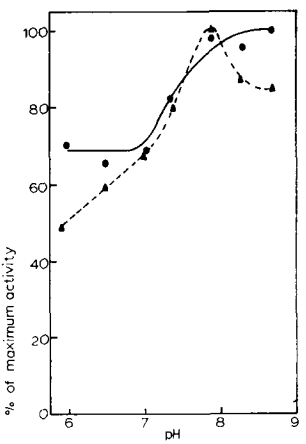

Fig. 6. The effect of pH variation on the O_2-reducing system. The pH was established by using 25 µmoles of Tricine–MES buffer adjusted with HCl or NaOH. The pH was measured before and after illumination and found to be the same. Otherwise the reaction conditions are the same as detailed in METHODS. ——, experiments with 0.32 µmole TCIP; - - - - -, experiments with 0.08 µmole TCIP.

TABLE III

COMPARISON OF PHOTOREDUCTION ACTIVITIES OF *A. variabilis* AND *S. oleracea* PREPARATIONS

The procedure for measurement of TCIP reduction has been described. The same conditions were used to measure cytochrome *c* reduction substituting 4 mg crystalline horse-heart cytochrome *c* for TCIP. O_2-reduction measurements were performed as described in METHODS.

Electron acceptor	*Rate as µmoles electrons transferred per mg chlorophyll per h*	
	A. variabilis	*S. oleracea*
TCIP	390	620
Cytochrome *c*	300	0
O_2	391	40
O_2 *via* benzyl viologen (0.4 mM)	520	460

Table III presents a comparison of *A. variabilis* lamellae to spinach chloroplasts with respect to photoreduction activities. Both preparations will reduce TCIP at a rapid rate. The *A. variabilis* preparation readily reduces horse-heart cytochrome *c* but the spinach chloroplasts do not.

LAZZARINI AND SAN PIETRO[5] showed a ferredoxin requirement in the reduction of cytochrome *c* by spinach chloroplasts. The *A. variabilis* lamellae used in this experiment contained no detectable ferredoxin nor was cytochrome *c* reduction stimulated by adding ferredoxin. The algal photosynthetic structures readily reduce O_2 when supplied with ascorbate and TCIP while washed spinach chloroplasts show negligibel O_2 consumption under these conditions. However, when the autoxidizable electron acceptor, benzyl viologen is added to the spinach chloroplast system, there is rapid O_2 consumption. Benzyl viologen also stimulates the O_2-consuming reaction of *A. variabilis* lamellae. REGITZ *et al.*[12] have prepared antibodies against chloroplast components and have been able to recognize two different antibodies which inhibit the primary electron acceptor in photosynthetic electron transport of chloroplasts. The antiserum $11C_{10}$ appears to block the function of a heat-stable prosthetic group on a chloroplast component which reduced ferredoxin. The inhibitory effect of $11C_{10}$ can be prevented by preincubation with an autoxidizable, heat-stable material isolated from chloroplasts and designated $S_{L\text{-}eth}$. The antibody $6C_{14}$ inhibits a protein component in the chloroplast which appears to be the apoenzyme of the ferredoxin-reducing system. Dr. Trebst had generously provided us with samples of these materials and their effects on the *A. variabilis* O_2-reducing system are outlined in Table IV. The antiserum $11C_{10}$, prepared by immunizing rabbits with higher plant chloroplasts, inhibits the O_2-consuming activity of *A. variabilis* lamellae. Inhibition of O_2 consumption by this antiserum is prevented by the $S_{L\text{-}eth}$ fraction which prevents inhibition of chloroplast reactions. The antiserum $6C_{14}$ also inhibits the *A. variabilis* O_2-reducing reaction. The chloroplast material, designated $S_{L\text{-}eth}$, is seen to stimulate *A. variabilis* O_2 consumption, as would be expected of an autoxidizable substance which will accept electrons from the photosystem as benzyl viologen does.

TABLE IV

INHIBITION OF THE O_2-REDUCING SYSTEM BY ANTIBODIES TO CHLOROPLAST ANTIGENS AND STIMULATION OF O_2 CONSUMPTION BY A CHLOROPLAST FRACTION

The antibody preparations were unfractionated rabbit antisera containing 26 mg protein/ml.

	O_2 consumption (% of control)
0.08 ml of 1/10 $11C_{10}$	65
0.02 ml of conc. $11C_{10}$	26
0.03 ml of conc. $11C_{10}$ + 0.1 ml $S_{L\text{-}eth}$	84
0.04 ml of 1/10 $6C_{14}$	65
0.08 ml of conc. $6C_{14}$	37
0.2 ml $S_{L\text{-}eth}$	134
0.4 ml $S_{L\text{-}eth}$	154
0.6 ml $S_{L\text{-}eth}$	157

DISCUSSION

The reduction of O_2 by photosynthetic particles of *A. variabilis* appears to be an autoxidation of a photoreduced electron carrier. FUJITA AND MEYERS[3] have described a cytochrome *c*-reducing system in blue-green algae by which electrons are removed from reduced cytochrome *c* in a photoact and returned to cytochrome *c* in a dark reaction. This reaction system led to the isolation of a cytochrome-reducing substance, a compound whose reduction was driven by Photosystem I (refs. 13, 14). YOCUM AND SAN PIETRO[4] have reported the isolation of a heat-labile material from spinach chloroplasts which is reduced in the light and which in turn is able to reduce ferredoxin. This substance is postulated to be the primary electron acceptor for Photosystem I and has been called the ferredoxin-reducing substance. REGITZ *et al.*[12] have isolated a heat-stable material from higher plant chloroplasts which neutralizes an antibody capable of inhibiting Photosystem I. This antibody apparently combines with the primary electron acceptor of Photosystem I. The heat-stable material which neutralizes this antibody and prevents inhibition of Photosystem I is postulated to contain the prosthetic group of the primary electron acceptor. This material shares some physical characteristics with cytochrome-reducing substance and ferredoxin-reducing substance. The O_2 consumption by illuminated lamellae as described in this paper seems to be related to these reducing systems and in reference to the earlier designations of cytochrome- and ferredoxin-reducing substances, we shall refer to this reaction as the O_2-reducing system. The O_2-reducing system effects a DCMU-insensitive transfer of electrons from $TCIPH_2$ to O_2 analogous to the $TCIPH_2$ to $NADP^+$ reaction associated with Photosystem I. The antisera of REGITZ *et al.*[12] are the strongest link in associating cytochrome-reducing substance, ferredoxin-reducing substance and O_2-reducing system. The antiserum $6C_{10}$ inhibits the function of all three. If the same type of electron donor is operative in both higher plants and blue-green algae, this compound must be more accessible to O_2 in the algae than in the higher plant. FUJITA AND MEYERS[3,15] had noted that O_2 competes with cytochrome *c* for the reducing power in the cytochrome-reducing substance of *Anabaena cylindrica*. The reduction of cytochrome *c*, without ferredoxin as a necessary intermediate electron carrier, may be another indication of the greater accessibility of this electron donor in blue-green algae. Higher plant chloroplasts require ferredoxin for cytochrome *c* reduction[5].

The O_2-reducing system appears to function only when $TCIPH_2$ is the electron donor. The lamellae preparations have the capacity to transfer electrons from water to a variety of Hill oxidants. Why electrons from water cannot be used in the reduction of O_2 is an important question which we cannot answer yet.

The O_2-reducing system generates H_2O_2 as a product and this, especially in an organism lacking catalase, militates against any physiological significance or occurrence *in vivo* of such a reaction. PATTERSON AND MEYERS[16] have sought catalase activity in blue-green algae and found it only in *Anacystis nidulans*. These investigators found that illumination of intact Anacystis cells caused the appearance of small but detectable amounts of H_2O_2. HOCH *et al.*[17] presented clear evidence for photorespiration in blue-green algae, so one might suppose a role for the O_2-reducing system in the intact cell if a suitable way to dispose of H_2O_2 exists. We found no detectable catalase activity in these preparations confirming the observations of

PATTERSON AND MEYERS[16]. Added catalase diminishes the rate of O_2 consumption suggesting that H_2O_2 is formed by the reduction of O_2. By accelerating the breakdown of H_2O_2 to water and O_2, catalase decreases net O_2 consumption. If H_2O_2 were the only product of the reaction, catalase should diminish the apparent O_2 consumption by half, but Table I shows a less than 50% decrease in O_2 consumption when catalase is added. In this experiment, 3.5 μmoles of O_2 were consumed in the control flask containing 5 μmoles of ascorbate. Ascorbate had been tipped in from a sidearm at the beginning of illumination and none could be detected at the end of the illumination period. Ideally 5 μmoles of ascorbate should permit 5 μmoles of O_2 consumption. It is likely that some of the ascorbate or $TCIPH_2$ was chemically oxidized by H_2O_2 in a reaction which produced no O_2. In the presence of catalase the stoichiometry was 2.5 μmoles of O_2 consumed per 5 μmoles ascorbate which indicates catalase splitting of 5 μmoles of H_2O_2. In this case, all of the ascorbate could be utilized for reduction of O_2 since it was protected by catalase from H_2O_2 oxidation.

The fact that O_2-reducing system operates at high rate in the absence of an autoxidizable carrier such as a flavin or menadione should be emphasized. Washing of the lamellar particles by centrifugation through a sucrose density gradient causes no loss of O_2-reducing system activity, yet it removes all the ferredoxin and other easily water-soluble material. In contrast, the MEHLER[1] reaction of higher plant chloroplasts is attributed to the reduction of an autoxidizable contaminant in chloroplasts and is diminished or eliminated by washing the chloroplasts.

The O_2-reducing system measurements described here indicate multiple sites for the oxidation of $TCIPH_2$. KATOH AND SAN PIETRO[18] have presented elegant evidence for two sites of oxidation of electron donors in Euglena chloroplasts. YAMASHITA AND BUTLER developed similar experiments indicating multiple oxidation sites in higher plant chloroplasts. Also the work of HIND AND IZAWA[21,22] indicates multiple entry of exogenous reducing power to the chloroplast electron transport chain. In all of the above instances, a DCMU-insensitive site near Photosystem I and a DCMU-sensitive site near Photosystem II were discerned. YAMASHITA AND BUTLER[19] recognized two DCMU-insensitive oxidation sites, one proximal to P700 and involving no coupled phosphorylation, the other distal to P700 and involving a phosphorylating step. The chloroplast oxidation site proximal to P700 appears to work best at low electron donor concentrations while the site distal to P700 is favored by high donor concentrations. In the *A. variabilis* O_2-reducing system experiments reported here, two DCMU-insensitive oxidation sites are suggested. The oxidation site close to P700 is served by high concentrations of $TCIPH_2$, is not stimulated by added cytochrome c_{554}, plastocyanin or $MgCl_2$, is less sensitive to high temperature and extremes of pH, and is saturated with light at lower intensities. The other oxidation site is served by a lower concentration of $TCIPH_2$ and is assumed to be more remote from P700 since its activity is stimulated by cytochrome c_{554} and $MgCl_2$; it is more sensitive to temperature and pH; and is saturated at a higher light intensity. Cytochrome c_{554} and plastocyanin did stimulate the transfer of electrons from $TCIPH_2$ to $NADP^+$ (ref. 23) at $TCIPH_2$ concentrations comparable to those which reduce the distal site of the O_2-reducing system. $MgCl_2$ stimulates the Hill reaction of *A. variabilis* and at lower concentrations the $TCIPH_2$ to $NADP^+$ reaction. The $MgCl_2$ saturation curves for electron transfer from $TCIPH_2$ to O_2 are similar, while the absence of a $MgCl_2$ stimulation at high dye concentration may mean that

the salt-stimulated step is by-passed at high dye concentration. A difference in light saturation value has been taken as a measure of proximity to the photoact and the lower light intensity required to saturate the reaction at high $TCIPH_2$ concentration is consistent with oxidation close to or at P700.

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REFERENCES

1 A. H. MEHLER, *Arch. Biochem. Biophys.*, 33 (1951) 65.
2 T. NAKAMOTO, D. W. KROGMANN AND B. MAYNE, *J. Biol. Chem.*, 235 (1960) 1843.
3 Y. FUJITA AND J. MEYERS, *Arch. Biochem. Biophys.*, 112 (1965) 519.
4 C. F. YOCUM AND A. SAN PIETRO, *Biochem. Biophys. Res. Commun.*, 36 (1969) 614.
5 R. A. LAZZARINI AND A. SAN PIETRO, *Biochim. Biophys. Acta*, 62 (1962) 417.
6 Y. FUJITA AND F. MURANO, *Plant and Cell Physiol.*, 8 (1967) 269.
7 W. A. SUSOR AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 120 (1966) 65.
8 W. C. DUANE, SR., M. C. HOHL AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 109 (1965) 108.
9 M. AVRON, A. T. JAGENDORF AND M. EVANS, *Biochim. Biophys. Acta*, 26 (1957) 262.
10 R. BERZBORN, W. MENKE, A. TREBST AND E. PISTORIUS, *Z. Naturforsch.*, 21b (1966) 1057.
11 R. BERZBORN, *Z. Naturforsch.*, 23b (1968) 1096.
12 G. REGITZ, R. BERZBORN AND A. TREBST, in the press.
13 Y. FUJITA AND J. MEYERS, *Arch. Biochem. Biophys.*, 113 (1966) 730.
14 Y. FUJITA AND J. MEYERS, *Plant and Cell Physiol.*, 7 (1966) 599.
15 Y. FUJITA AND J. MEYERS, *Arch. Biochem. Biophys.*, 119 (1967) 8.
16 P. PATTERSON AND J. MEYERS, *Plant. Physiol.*, 43 (1968) S-12.
17 G. HOCH, O. H. OWENS AND B. KOK, *Arch. Biochem. Biophys.*, 101 (1963) 171.
18 S. KATOH AND A. SAN PIETRO, *Arch. Biochem. Biophys.*, 122 (1967) 144.
19 T. YAMASHITA AND W. BUTLER, *Plant Physiol.*, 43 (1968) 1978.
20 T. YAMASHITA AND W. BUTLER, *Plant Physiol.*, 43 (1968) 2037.
21 G. HIND AND S. IZAWA, *Biochim. Biophys. Acta*, 172 (1969) 277.
22 G. HIND AND S. IZAWA, *Biochim. Biophys. Acta*, 172 (1969) 290.
23 J. J. LIGHTBODY AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 131 (1967) 508.