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FURTHER STUDIES ON THE OXYGEN-REDUCING SYSTEM OF *ANABAENA VARIABILIS*

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SUMMARY

Photosynthetic lamellae from *Anabaena variabilis* accumulate a reductant when illuminated with an electron donor under anaerobic conditions. This reductant, the oxygen-reducing substance, is autooxidizable and both its formation and oxidation can be altered by substances which interact with Photosystem I. Cyclic electron flow can be made to compete with oxygen for the reducing power generated by the photoact. The specificity of the lamellae toward electron donors can be altered by the detergent Tween 20. Using the procedure of FUJITA AND MEYERS (*Plant Cell Physiol.*, 7 (1966) 599) for the isolation of the cytochrome-reducing substance, a substance can be reversibly removed from the lamellae which appears to be involved in oxygen reduction and in the oxidation of endogenous cytochromes by Photosystem I. This material appears to be similar to the cytochrome-reducing substance, to the ferredoxin-reducing substance of YOCUM AND SAN PIETRO (*Biochem. Biophys. Res. Commun.*, 36 (1969) 614) and the $S_{L\text{-eth}}$ factor of REGITZ *et al.* (*Planta Berlin*, 91 (1971) 8).

INTRODUCTION

We have previously described an oxygen-reducing system of illuminated photosynthetic lamellae from the blue-green alga *Anabaena variabilis*[1]. Electrons are transferred from reduced indophenol to oxygen *via* an autooxidizable component in Photosystem I. FUJITA AND MEYERS[2] had described a cytochrome-reducing substance in blue-green algae. YOCUM AND SAN PIETRO[3] have isolated a ferredoxin-reducing substance from higher plant chloroplasts. REGITZ *et al.*[4] have characterized a water-soluble factor from chloroplasts which neutralizes antibodies against the primary acceptor in Photosystem I. The antibody-neutralizing material is a redox agent with many similarities to cytochrome-reducing substance and ferredoxin-reducing substance. The inhibiting antibody reacts with spinach ferredoxin-reducing

Abbreviations: $S_{L\text{-eth}}$, a water-soluble factor released from lyophilised, ether-extracted chloroplasts; PMS, phenazine methyl sulfate; TCIP, 2,3′, 6-trichloroindophenol; $TCIPH_2$, reduced TCIP; MES, 2(*N*-morpholino)ethane sulfonic acid buffer; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea.

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substance and will block the oxygen-reducing system of *A. variabilis*. The antibody inhibition suggests a similar antigen is involved in Photosystem 1 of both blue-green algae and higher plants. This paper describes a solubilized component necessary for the oxygen-reducing system. In terms of its extractability, size, autooxidizability and absorption spectrum, the material to be described here appears to be identical to the cytochrome-reducing substance of FUJITA AND MEYERS[1], and is at least very similar to $S_{L\text{-}eth}$ (a water-soluble factor released from lyophilised ether-extracted chloroplasts) and to the ferredoxin-reducing substance.

METHODS

The methods for growth of algae, for isolation of photosynthetic lamellae and for measurement of chlorophyll, photophosphorylation and Hill reaction activities have all been described[5,6]. Measurements of oxygen consumption were made with a Yellow Springs oxygen electrode. The reaction chamber was illuminated with red light at an intensity of $5 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. The standard reaction mixture contained the following in μmoles in a total volume of 3 ml: Tris buffer (pH 7.6), 83; $MgCl_2$, 15; 2,3',6-trichloroindophenol (TCIP), either 0.09 or 0.36 as indicated; sodium ascorbate, 20; 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), $5 \cdot 10^{-3}$: and photosynthetic lamellae containing 100 μg chlorophyll. The photooxidation of cytochrome *c* was measured using a cross illuminated Eppendorf spectrophotometer. An actinic beam of $6 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1} was passed through a Baird Atomic narrow band pass filter (λ_{max} 685 nm) and focused on the reaction cuvette at 90° to the measuring beam which was filtered before and after passage through the cuvette so as to measure the change in absorbance at 547 nm. Cytochrome turnover was measured with an Aminco-Chance dual wavelength spectrophotometer made available to us through the generosity of Dr. W. Cramer. Spinach plastocyanin was prepared by the method of BÖGER *et al.*[7] and Anabaena plastocyanin by the method of LIGHTBODY AND KROGMANN[8]. Cytochrome c_{554} was isolated from *A. variabilis* by the method of SUSOR AND KROGMANN[5].

RESULTS

Fig. 1 illustrates an attempt to determine the amount of autooxidizable material which is generated by light in photosynthetic lamellae. The upper curve represents a preparation which had been illuminated for 5 min under anaerobic conditions. The oxygen electrode reaction chamber was bubbled with nitrogen for 5 min with vigorous stirring prior to illumination and DCMU was present to prevent photosynthetic O_2 production. The lower curve is a control measurement in which the illumination step was omitted. After the 5-min preincubation, water saturated with oxygen was injected into the measuring chamber and oxygen uptake was measured. Large amounts of autooxidizable material were generated during the preillumination. Thus it was necessary to inject repeatedly water saturated with oxygen to oxidize the photoproduct. These injections were continued until the rate of oxygen uptake by the preilluminated sample became equal to that of the dark control. By subtracting the dark control value, a light-induced oxygen uptake value of 0.26 μmole O_2 was obtained.

In separate experiments, the effect of oxygen concentration on the rate of oxygen

photoreduction was measured. When lamellae were incubated with either 0.08 or 0.3 μmole TCIP and excess ascorbate at varying oxygen tensions, the rate of photoreduction of oxygen increased in a linear fashion with increasing oxygen concentrations. No saturation of the reaction rate with oxygen was achieved as would be expected from an autooxidation reaction.

While the photoreduction of O_2 by *A. variabilis* lamellae proceeds rapidly without added autooxidizable substances[4], this reaction is inhibited by cofactors of

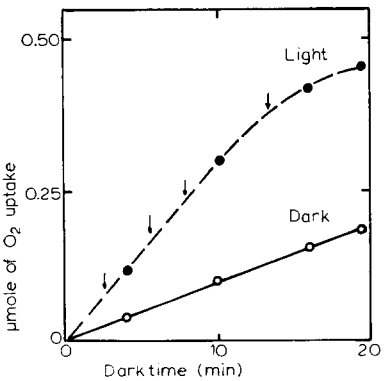


Fig. 1. Titration of photoreduced autooxidizable material in *A. variabilis* lamellae. Lamellae containing 100 μg chlorophyll were incubated in an O_2 electrode measuring chamber in a reaction mixture which contained in μmoles: 2(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 6.8), 116; $MgCl_2$, 15; TCIP, 0.3; sodium ascorbate, 20; and DCMU, 0.005 in a total volume of 3 ml. The reaction mixture was flushed with nitrogen for 5 min. ●----● describes a mixture which was then illuminated for 5 min with red light of an intensity of $5 \cdot 10^5$ ergs·cm^{-2}·sec^{-1}. After the illumination period, a 0.1-ml aliquot of water saturated with O_2 was injected into the reaction mixture (0 time on the abscissa). Subsequent additions of oxygen are indicated by the arrows and the measurement was continued until the rate of oxygen consumption became equal to the rate achieved with a control sample which had not been illuminated (○—○).

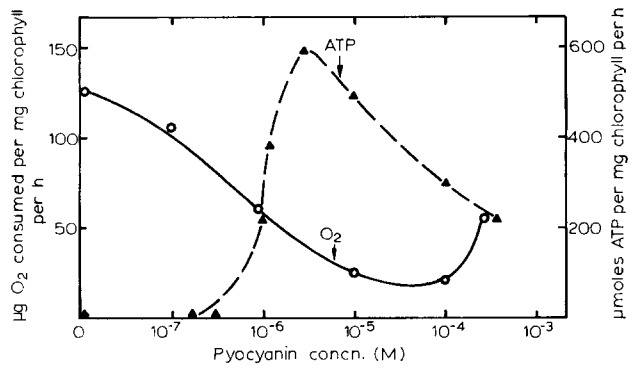


Fig. 2. The effect of varying pyocyanin concentrations on photophosphorylation and on oxygen consumption. For measurement of cyclic phosphorylation, the reaction mixture contained the following in μmoles: MES buffer (pH 6.8), 25; sodium phosphate buffer (pH 6.8), 3; $MgCl_2$, 5; ADP, 5; 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 0.005; and photosynthetic lamellae containing 100 μg chlorophyll in a total volume of 1 ml. Illumination was at 25° in a Warburg bath at a light intensity of $5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}. The same reaction mixtures with the addition of 0.09 μmole TCIP and 5 μmoles of sodium ascorbate were used to measure oxygen uptake with an oxygen electrode as described in METHODS. ▲--▲, phosphorylation experiments; ○—○, oxygen consumption measurements.

cyclic phosphorylation. The data described in Fig. 2 reveal a reciprocal relation between inhibition of oxygen photoreduction and support of cyclic phosphorylation by pyocyanin. A similar result was obtained with phenazine methyl sulfate (PMS). Thus it appears that catalysts of cyclic electron transport can compete very effectively with oxygen for the reducing power generated by the photoact. One might hope to reverse the inhibition by pyocyanin of the photoreduction of oxygen by finding some specific inhibitor of cyclic electron transport. A variety of inhibitors and treatments were tested, but most were unable to reverse the pyocyanin inhibition. Raising the pH of the reaction mixture did reverse pyocyanin inhibition of oxygen reduction (Fig. 3) and this reversal of inhibition correlates with the base catalysed autooxidation of pyocyanin.

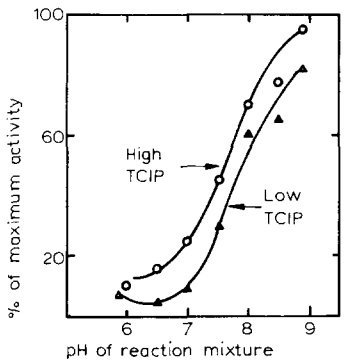


Fig. 3. The effect of H^+ concentration on pyocyanin inhibition of oxygen photoreduction. The conditions for measurement in this experiment are the same as those described in METHODS. The pyocyanin concentration was $1 \cdot 10^{-4}$ M. ○—○, oxygen uptake using 0.32 μmoles TCIP; ▲—▲, experiments with 0.09 μmole TCIP. The pH variation was achieved by using mixtures of Tris and MES buffers.

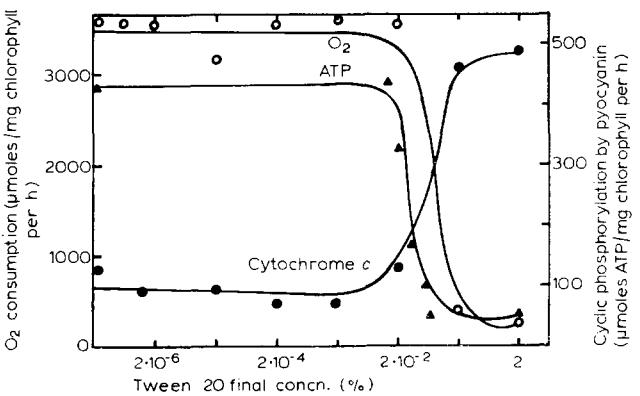


Fig. 4. The effect of Tween 20 on photosynthetic activities of *A. variabilis* lamellae. Cyclic photophosphorylation activity (▲—▲) was measured as described in Fig. 2 with 5 μmoles of pyocyanin. Oxygen uptake was measured as described in METHODS with the inclusion of 0.5 μmole of methyl viologen and 0.6 μmole TCIP. Cytochrome *c* photooxidation was measured as described in METHODS with the same reaction mixture used for oxygen uptake except that 12 mg of reduced horse heart cytochrome *c* replaced the TCIP and sodium ascorbate. Left axis: ○—○, oxygen uptake; ●—●, photooxidation of reduced cytochrome *c*.

Autooxidizable compounds of a low redox potential were found to stimulate oxygen photoreduction, as would be expected in a conventional MEHLER[9] reaction, and representative data are presented in Table I. Pyocyanin is also an effective inhibitor of these reactions.

Detergents stimulate photooxidation of cytochrome *c* by *A. variabilis* lamellae and the rate of this reaction is especially high in the presence of methyl viologen[8]. However, we had reported earlier that 0.1 % Tween 20 inhibited the oxygen reducing system[4]. Fig. 4 describes the contrasting effects of Tween in causing inhibition of oxygen photoreduction with reduced TCIP as electron donor and in causing a stimulation of cytochrome *c* photooxidation. It appears that the concentration of detergent which inhibits the TCIP reaction stimulates the cytochrome *c* reaction. Washing the lamellae after treatment with Tween did not alter the effects of the detergent on

TABLE I

STIMULATION OF OXYGEN REDUCTION BY LOW-POTENTIAL ELECTRON ACCEPTORS

The reaction conditions are described in METHODS. The concentrations of the various electron acceptors are those which give the maximum rate of oxygen consumption. The control rate of oxygen consumption in this experiment was 300 μmoles electrons transferred per mg chlorophyll per h.

Electron acceptor	*Concn.*	*Rate (% of control)*	
	(M)	—	*+ pyocyanin*
O_2		100	14
Benzyl viologen	$1.6 \cdot 10^{-4}$	300	12
Methyl viologen	$1.6 \cdot 10^{-4}$	298	7
FMN	$1.6 \cdot 10^{-4}$	375	12
Menadione	$3.3 \cdot 10^{-5}$	385	36
Methyl red	$1.6 \cdot 10^{-4}$	208	11
Ferredoxin	$3.0 \cdot 10^{-6}$	136	12

TABLE II

TWEEN 20-MEDIATED OPENING OF A NON-SPECIFIC SITE FOR ENTRY OF ELECTRONS INTO PHOTOSYSTEM I

The reactions conditions are described in METHODS. The concentrations of the various electron donor proteins are those which give the maximum rate of oxygen consumption. In every case 20 μmoles of sodium ascorbate was added to keep the donor in the reduced state.

Additions	*O_2 consumption (μmoles electrons transferred per mg chlorophyll per h.)*	
	—	*+ 2 % Tween 20*
0.09 μmole TCIP	295	40
0.36 μmole TCIP	400	40
0.09 μmole TCIP + 1 nmole *A. variabilis* plastocyanin	385	—
0.09 μmole TCIP + 10 nmoles *S. oleracea* plastocyanin	255	—
0.0 μmole TCIP + 1 nmole *A. variabilis* plastocyanin	0	210
0.0 μmole TCIP + 10 nmoles *S. oleracea* plastocyanin	0	200
0.0 μmole TCIP + 3 nmoles *A. variabilis* cytochrome c_{554}	50	280
0.0 μmole TCIP + 0.7 nmole horse heart cytochrome *c*	66	214
0.0 μmole TCIP	0	0

these reactions. The detergent might stimulate cytochrome *c* photooxidation by increasing the accessability of cytochrome *c* to an electron acceptor site in the lamellae. Inhibition of the $TCIPH_2$ (reduced TCIP) oxidation might be due to a new rate limitation imposed by the detergent between the dye and the oxidant to which $TCIPH_2$ normally donates electrons. Previous work had indicated two sites for $TCIPH_2$ oxidation[1], but as seen in Table II, Tween is inhibitory regardless of the $TCIPH_2$ concentration. Plastocyanin stimulates the rate of oxygen reduction when a low concentration of $TCIPH_2$ is used. This is quite like the stimulation of this reaction by cytochrome c_{554} reported earlier[1]. This effect is not achieved with plastocyanin isolated from spinach. If TCIP is omitted, plastocyanin which is kept reduced by excess ascorbate will not serve as an electron donor for oxygen photoreduction. However, Tween allows the plastocyanin to support oxygen photoreduction. Spinach plastocyanin will work as an electron donor in the presence of Tween 20 as will *A. variabilis* cytochrome c_{554} and horse heart cytochrome *c*.

The oxygen-reducing reaction studied here showed many similarities to the cytochrome-reducing system of FUJITA AND MEYERS[10,11]. Thus the procedure which those authors devised to extract the cytochrome-reducing substance was applied to the oxygen-reducing system[2]. Table III shows that the FUJITA AND MEYERS[2] extraction procedure does cause a decrease in the rate of oxygen reduction and that readdition of a heat stable, non-dialysable material from this extract restores the activity. The lamellae extract was exhaustively dialyzed to remove all traces of salts. It is important to note that NaCl also stimulates the extracted lamellae but not in a way that precludes further stimulation by the extracted material. There is no stimulatory effect of NaCl on lamellae prior to extraction. Fig. 5 shows that the stimulation of extracted lamellae can be made proportional to the amount of extract added back. Measurement of reduced cytochrome *c* oxidation by Photosystem I light gave results which parallel the oxygen photoreduction measurements in terms of removal and readdition of the extractable factor. Since the extracted lamellae were quite stable to storage at −15°, stimulation of oxygen-reducing activity by the

TABLE III

REVERSIBLE DEPLETION OF THE OXYGEN-REDUCING ACTIVITY

The reaction conditions are those described in METHODS. 0.5 μmole of methyl viologen was added as indicated. Extracted lamellae were obtained by applying the FUJITA AND MEYERS[2] procedure for the isolation of cytochrome-reducing substance to 30 g of fresh cells. The amount of dialysed extract added back is enough to give a maximum response and represents, in terms of the amount of chlorophyll in the lamellae used to prepare the extract, a 3-fold increase in concentration over the material removed from the depleted lamellae.

	μmoles electrons transferred per mg chlorophyll per h to	
	O_2	*Methyl viologen*
Control particles	300	1400
Extracted particles	120	870
Extracted particles + boiled, dialysed extract	235	1260
Extracted particles + 0.15 M NaCl	225	—
Extracted particles + 0.15 M NaCl + boiled, dialysed extract	333	—

extracted lamellae proved to be a very convenient and reproducible assay for locating the active material during fractionation.

The material active in restoring oxygen reduction could be purified by DEAE-cellulose and Sephadex chromatography using the procedures developed by FUJITA AND MEYERS[2,12]. When absorbed to a DEAE-cellulose column at pH 6, the active material was eluted by 0.3 M NaCl.

Repeated Sephadex chromatography and comparison to marker substances (insulin, mol. wt. 58000, cytochrome *c*, 13000) suggested that the active material has a molecular weight of 3000–4000. The ultraviolet absorption spectrum of the purified material showed a peak at 265 nm and there was no absorption of visible light.

Fig. 6 shows lamellar absorbance changes at 558 nm. This wavelength was shown to be the point of maximum light induced change in this region of the spectrum. Sodium ascorbate gives an increased absorbance at this wavelengths and an actinic beam of 700 nm red light gives the opposite change, presumably due to the chemical

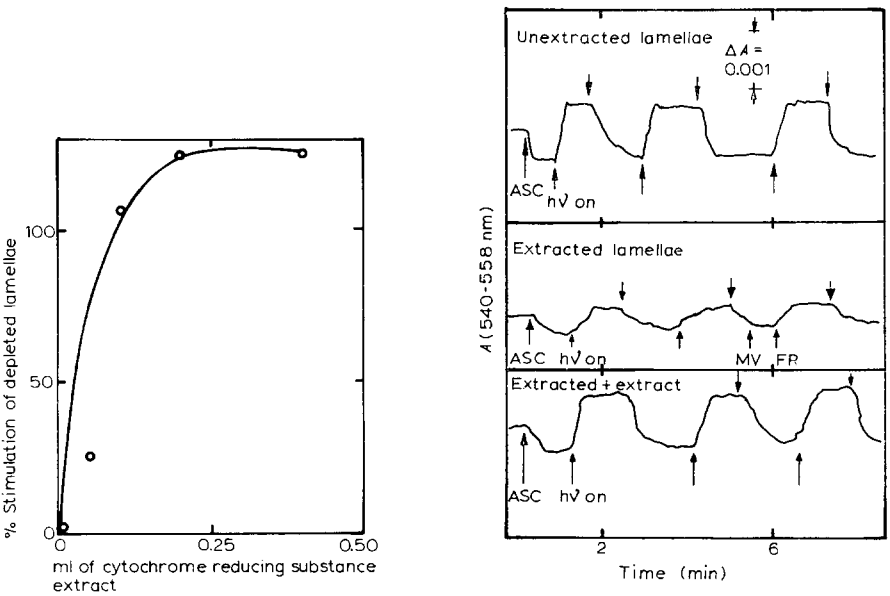


Fig. 5. Restoration of oxygen-reducing activity. Photosynthetic lamellae were subjected to the FUJITA AND MEYERS[2] extraction procedure and showed absolute rates nearly identical to those reported in Table III. The extracted material was concentrated so that at saturation a 3-fold excess of extract over lamellae was present on a chlorophyll basis. The assay conditions were as described in METHODS with a high concentration (0.36 μmole) of TCIP to avoid possible rate limitations due to loss of plastocyanin and cytochrome c_{554}.

Fig. 6. The effects of extraction and readdition of materials necessary for oxygen photoreduction on the photooxidation of endogenous cytochromes. Cytochrome photooxidation was measured with an Aminco-Chance dual wavelength spectrophotometer. The reaction mixtures contained the following in μmoles: Tricine buffer (pH 8), 25; $MgCl_2$, 15; methyl viologen, 2; sodium ascorbate where indicated, 5; and photosynthetic lamellae containing 250 μg chlorophyll. The extracted lamellae and saturating amounts of extract were as described in Fig. 6. The measuring light intensity was 1 erg·cm^{-2}·sec^{-1} and the actinic light was $7 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} for the "far red" beam of 713 nm. Unless marked upward arrows indicate turning on the actinic beam and downward arrows turning it off. ASC, ascorbate; MV, methyl viologen; FR, far red beam.

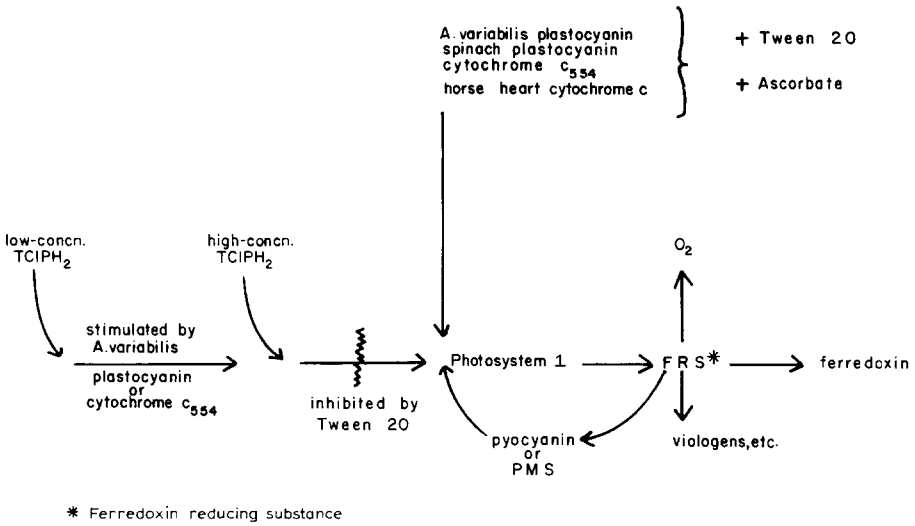
reduction and photooxidation of a cytochrome. Lamellae which have been depleted of the substance necessary for oxygen photoreduction show a diminished photooxidation of endogenous cytochrome. This photooxidation cannot be restored by methyl viologen. Addition of the purified material which stimulates oxygen reduction restores the photooxidation of the cytochrome.

DISCUSSION

The autooxidizable photoproduct in *A. variabilis* lamellae shows many properties that are common to the cytochrome reducing substance of FUJITA AND MEYERS[2] and the ferredoxing reducing substance of YOCUM AND SAN PIETRO[3]. Both the cytochrome-reducing substance and ferredoxin-reducing substance are autooxidizable when solubilized from the photosynthetic membranes. The measurements of autooxidizable material which accumulates in lamellae indicate 10 reducing equivalents generated per molecule of chlorophyll. FUJITA AND MEYERS[13] estimated a maximum of only one-fifth an equivalent of cytochrome-reducing substance per chlorophyll. The data of YOCUM AND SAN PIETRO[3] indicate about 2 reducing equivalents per chlorophyll ($a + b$) in the spinach ferredoxin-reducing substance assays. The measured pool of autooxidizable photoproduct seems too large in comparison with other photosynthetic electron carriers. Either our algae contain an exceptionally large pool of photoreductant or more autooxidizable material is generated by Photosystem 1 than is involved in the photosynthetic electron transport chain.

It is not surprising that a number of low-potential redox carriers will stimulate the oxygen photoreduction reaction. These reactions are useful in establishing the maximum rate of electron transport through the photosystem, and it is noteworthy, as shown in Table II, that the high rates seen in these reactions suffer the same diminution and restoration as the more sluggish oxygen-reducing system in response to procedures which accomplish the depletion and restoration of cytochrome-reducing substance. YOCUM AND SAN PIETRO[14] showed a similar dependence of methyl viologen photoreduction on ferredoxin-reducing substance. Inhibition of oxygen reduction by pyocyanin or PMS is most easily interpreted by assuming that these compounds have a higher affinity for the photoproduct than does oxygen. The parallel responses of inhibition of oxygen reduction and support of cyclic phosphorylation by PMS and pyocyanin suggest that the reducing power is being cycled through the photoact. Our inability to find any inhibitor which would reverse the pyocyanin effect suggests that there are few enzymatic steps unique to cyclic electron transport.

Our previous studies indicated that there were two sites of $TCIPH_2$ oxidation which served in oxygen reduction[1]. One site could operate at maximal activity at a low concentration of $TCIPH_2$ only when supplemental cytochrome c_{554} or plastocyanin was added to the lamellae. At higher dye concentration there was no stimulation by cytochrome c_{554} or plastocyanin suggesting that $TCIPH_2$ was reducing a carrier closer to P_{700} in the chain. FUJITA AND MURANO[15] obtained similar results with $NADP^+$ photoreduction by Anabaena lamellae using $DCIPH_2$ as a donor. Tween 20 allows plastocyanin or cytochromes to by-pass the dye-requiring reactive sites by donating electrons directly. Since plastocyanin from either *A. variabilis* or spinach (these proteins have opposite net charges[8,16]) or cytochromes from either *A. variabilis* or horse heart will serve serve as electron donors, Tween has opened up a new site for



reduction which has little specificity toward electron donors. The data of FUJITA AND MURANO[16] indicate a similar effect can be achieved with spinach chloroplast fragments prepared by sonication.

Recent experiments have suggested several candidates for the primary reducing substance in photosynthesis. MALKIN AND BEARDEN[17] have suggested that a bound form of ferredoxin is the primary electron acceptor of Photosystem I. Both CLAYTON AND STRALEY[18] and HIYAMA AND KE[19] have implicated an absorbance change centering on 430 nm as a property of the primary acceptor. All of these claims for a place on the reducing side of Photosystem I indicate that the enzymatic machinery there may be more complex than previously supposed.

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