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LIGHT DRIVEN SCATTERING CHANGES AND INCREASED 515 nm ABSORBANCE CHANGES ASSOCIATED WITH FATTY ACID INHIBITION OF PHOTOSYNTHESIS IN CHLORELLA

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SUMMARY

1. The size and time of half decay of the 515 nm positive and 475 nm negative light driven absorbance changes in Chlorella was considerably increased by $1 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M lipoic, caprylic and iodoacetic acids and by $1 \cdot 10^{-5}$–$8 \cdot 10^{-5}$ M nitrous acid. Salts of these acids at neutral pH, iodoacetamide and HCl did not affect the absorbance changes.

2. The absorbance changes were restored to their original size and photosynthetic CO_2 fixation partially restored on washing the cells free of lipoic acid.

3. Light driven absorbance changes at 350 nm (positive), 405 nm, 425 nm, 450 nm and 648 nm (all negative) were unchanged in magnitude on addition of acid but their decays were slowed. The greater part of the 515–475 nm changes is therefore not due to Chlorophyll *b*.

4. Addition of 10^{-3} M caprylic, lipoic, iodoacetic acids, methyl octonoate and hexyl resorcinol caused slow reversible light driven scattering changes which were not associated with volume changes of the whole cell.

INTRODUCTION

Photosynthesis in Chlorella is rapidly inhibited by acids such as lipoic[1], caprylic[2] or nitrous[3], the inhibition being readily reversed by washing the cells or raising the pH. Transients produced by these acids in the levels of ^{14}C and ^{32}P in intermediates of the photosynthetic carbon reduction cycle suggest the main site of inhibition to be the hexose and heptose diphosphatases (EC 3.1.3.11). Carboxydismutase (EC 4.1.1.39) and photophosphorylation are also affected. Similar photosynthetic inhibition is produced by addition of *n*-hexyl resorcinol and methyl octonoate[4], thus eliminating acidity and gross chemical properties as a common cause. A search was therefore begun to see if these diverse chemical substances produced a common physical change. During the initial experiments enlarged light driven 515 nm absorbance changes and

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

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reversible light scattering changes were produced on addition of some of these inhibitors and these observations form the basis of this paper.

MATERIALS AND METHODS

Chlorella pyrenoidosa (Emerson Strain) was grown in continuous culture. The cells were harvested by centrifugation at 4° (3000 × *g* for 10 min), washed once and resuspended in 10^{-3} M KH_2PO_4. The cell concentrations used ranged from 0.2 to 0.5 % v/v. Absorbance changes were measured (1) using a Cary 14M spectrophotometer modified for sample illumination with monochromatic light[5] and (2) a sensitive single beam spectrophotometer worked by a computer of average transients to allow repetitive flash experiments[6].

CO_2 fixation was measured in the "steady state apparatus"[7]. 3-(3,4-Dichlorophenyl)-1,1-dimethyl urea (DCMU), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), methyl octonoate, hexyl resorcinol (1,3-dihydroxy-4-*n*-hexylbenzene), lipoic, caprylic and valeric acids were made up in alcohol, all other reagents in water. Acids were added as the free acid unless otherwise stated.

RESULTS

When a suspension of Chlorella is illuminated there is an increase in absorbance at 515 nm and a corresponding although smaller decrease at 475 nm. Under the conditions of the present experiments this 515 nm positive light driven absorbance change is just visible when using the 0.0–0.1 *A* scale on the Cary spectrophotometer. On addition of an appropriate acid photosynthetic inhibitor the amplitude of this change

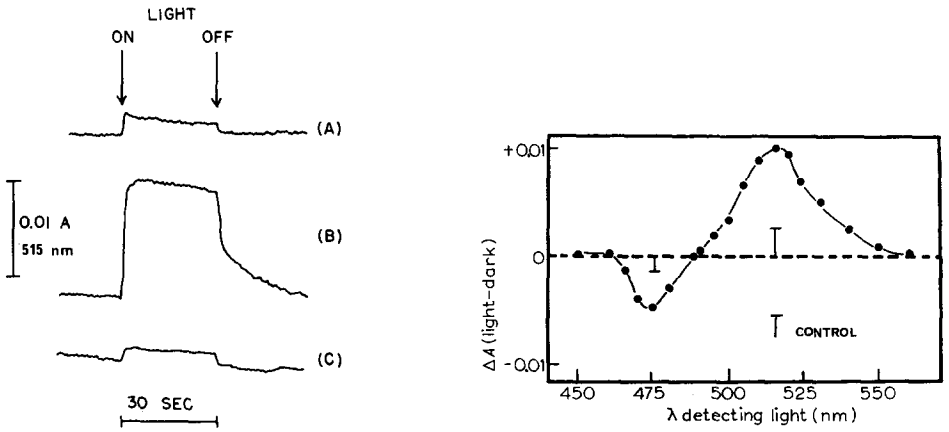

Fig. 1. Light induced absorbance changes at 515 nm. Chlorella cells (0.33 %, v/v, in 10^{-3} M KH_2PO_4) were illuminated with light of 680 nm (6000 erg·cm^{-2}·sec^{-1}). The results are tracings from a Cary 14M spectrophotometer of an experimental cycle of 30 sec light followed by 60 sec dark. A, control; B, *plus* $5 \cdot 10^{-4}$ M lipoic acid; C, as B but after washing twice with 10^{-3} M KH_2PO_4. Photosynthesis measured as CO_2 uptake, corresponding to A, B and C respectively was 17.3, 2.0 and 11.2 μmoles CO_2/ml packed algae per min.

Fig. 2. Light–dark difference spectrum for Chlorella poisoned with $5 \cdot 10^{-4}$ M lipoic acid. Cells treated as in Fig. 1. The points plotted are the difference between steady state levels obtained from cycles of 60 sec darkness and 30 sec illumination at each wavelength.

is greatly increased (up to 5 times the magnitude of the control) (Fig. 1). After washing the cells by twice centrifuging and resuspending them in phosphate buffer, the signal returns to the control level. The presence of the acid also inhibits CO_2 fixation but this can be partially restored by the washing. The spectrum of the acid increased absorbance changes is shown in Fig. 2 and is similar to that previously reported for untreated Chlorella cells[8]. The following acids have been found to increase the size of 515 nm absorbance change: $1–5 \cdot 10^{-4}$ M iodoacetic (but not iodoacetamide), caprylic, valeric, lipoic (but not their salts at pH 6.0) and $2–5 \cdot 10^{-5}$ M nitrous acid (added as KNO_2 at an appropriate pH).

Actinic light of 695, 700 and 705 nm (*i.e.* of photosystem I) was approximately twice as effective per absorbed quantum in driving the acid increased 515 nm signal as was light of 680 nm or shorter wavelengths.

Kinetic data were obtained from repetitive experiments in which the total running time of the CAT was approx. 100 sec. Thus the number of repetitions is inversely proportional to the time of each individual experiment. As the main effect of lipoic and similar acids is a slowing the dark decay rates it has been necessary to carry out experiments on a variety of time scales. Table I shows that the acid increased 515 nm signal can be satisfactorily observed with a regime of 2.0 sec light and 6.0 sec dark.

TABLE I

EFFECT OF LIPOIC ACID ON THE SIZE OF 515-nm ABSORBANCE CHANGE

Chlorella cells 0.2 % (v/v) were exposed to $4 \cdot 10^{-4}$ M lipoic acid for 10 min before making the measurements. The cuvette was shaken between each experiment to avoid anaerobicity. Actinic illumination 675 nm — 5000 erg·cm^{-2}·sec^{-1}.

Number of repetitions	*Time (sec)*		*515 nm light–dark* ($A \times 10^{-4}$)	*Time of half decay* (sec^{-1})
	Light	*Dark*		
100	0.5	0.5	Control 14	22
			Lipoic 18	17
25	0.5	3.5	Control 12	25
			Lipoic 25	4
6	0.5	15.5	Control 11	13
			Lipoic 23	3
12	2.0	6.0	Control 12	13
			Lipoic 35	3
6	2.0	14.0	Control 12	13
			Lipoic 37	2

The effect of lipoic acid on the kinetics of all the major light driven absorbance changes (except p 700) is shown in Table II. When the reaction schedule is 0.25 sec light and 0.75 sec dark lipoic acid causes a small increase in the rise rates compared to those in the control whilst the magnitudes of all signals are not greatly affected. Longer experiments (2 sec light, 6 sec dark) show that lipoic acid causes a threefold increase in the size of 515 nm and 475 nm signals, the size of the others being unchanged. The time of half decay of these signals is little affected but those of 515 nm and 475 nm approach 1 sec. Similar effects have been observed with caprylic, iodoacetic and nitrous acids.

TABLE II

EFFECT OF LIPOIC ACID ON KINETICS OF ABSORBANCE CHANGES AT DIFFERENT WAVELENGTHS

The Chlorella cells were treated as in Table I. Size I and associated kinetic data represent the results from 100 repetitions of 0.25 sec light and 0.75 sec dark. Size II the results from 12 repetitions of 2 sec light and 6 sec dark. Values for the 648-nm absorbance changes were obtained using 480 nm actinic light; under these conditions the 515-nm change is still 3 × increased by lipoic acid.

Wavelength (nm)		*Polarity (+ or −)*	*Size I* ($A \times 10^{-4}$)	*Size II* ($A \times 10^{-4}$)	*Rise rate I* ($A \times 10^{-4} \cdot sec^{-1}$)	*Decay rate I* ($A \times 10^{-4} \cdot sec^{-1}$)	*Time of half decay I* (sec^{-1})	*Time of half decay II* (sec^{-1})
350	Control	+	—	4.0	—	—	—	4
	Lipoic			5.25				2
405	Control	−	2.0	2.6	23	23	25	>6
	Lipoic		1.8	2.8	38	20	17	>6
425	Control	−	2.3	3.5	36	43	33	>6
	Lipoic		2.8	3.85	56	30	13	>6
450	Control	−	1.5	2.8	10	14	20	>6
	Lipoic		1.6	2.8	21	9	11	>6
475	Control	−	4.5	5.4	40	30	25	>6
	Lipoic		5.3	14.7	60	20	10	2
515	Control	+	14.0	18.2	210	182	25	>6
	Lipoic		21.0	52.5	400	140	7	1
650	Control	−	4.8	5.1	40	42	13	>6
	Lipoic		4.5	5.2	51	39	13	>6

A comparison was made between the acid increased 515-nm signal and the control as affected by a number of well documented inhibitors. The photophosphorylation uncoupler CCCP completely abolishes the lipoic acid increased part of the signal at 10^{-6} M but has no effect on the control signal (Table III). DCMU (10^{-5} M) an inhibitor of electron flow from system II, does not affect the initial size of 515-nm signal but the steady state level is half that with lipoic acid alone. In the absence of lipoic acid DCMU reduces the signal by 50–70 %.

TABLE III

EFFECT OF CCCP ON 515-nm ABSORBANCE CHANGE ENLARGED BY $4 \cdot 10^{-4}$ M LIPOIC ACID

Conditions as for Table I. Each result is the average of 12 repetitions of 2 sec light and 6 sec dark. CCCP at the same concentrations in the absence of lipoic acid is without effect on any of the parameters of the signal.

	CCCP (M)						
	0	*0* *No lipoic*	10^{-7}	$3 \cdot 10^{-7}$	$5 \cdot 10^{-7}$	$7 \cdot 10^{-7}$	10^{-6}
515-nm signal ($A \times 10^{-4}$)	36	12	34	27	20	16	13
Time of half decay (sec^{-1})	2.0	25.0	3.2	4.2	6.2	10.0	12.5

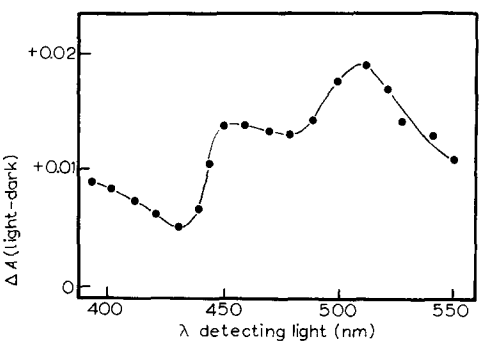

Fig. 3. Light–dark difference spectrum for slow absorbance (scattering) change induced by 10^{-3} M iodoacetic acid. The points are the difference between absorbance after 3 min illumination and 6 min subsequent darkness.

On raising the level of lipoic acid to 10^{-3} M it was observed that an additional light driven apparent absorbance change was produced. This slow change (rise and decay times are of the order of 1 min) which was positive at all wavelengths, was superimposed on the usual fast absorbance changes. At higher concentrations only the slow changes remained. Their spectrum (Fig. 3) closely ressembles that of light scattering by Chlorella cells and studies with an Aminco spectrofluorimeter showed unequivocably that the reversible slow "absorbance" changes are in fact changes in light scattering. In addition to lipoic acid, caprylic, valeric and iodoacetic acids at 10^{-3} M or above induced the scattering changes as did hexyl resorcinol ($5 \cdot 10^{-4}$ M) and methyl octonoate ($5 \cdot 10^{-4}$ M). Measurements of cell volume made with a Coulter

counter indicated that these scattering changes did not result from large changes in cell volume. No real indication of the respective contributions of the two photosystems in driving the scattering changes can be given since the rate was not linear with light intensity but S-shaped at all wavelengths studied.

DISCUSSION

The light driven absorbance changes at 515 nm (positive) and 475 nm (negative) have been attributed to carotenoids[9], a compound related to plastoquinone[10] or to chlorophyll *b* (ref. 11). The last is based on the absence of the signals from plants lacking chlorophyll *b* and the exact correspondence of the 515–475-nm signals in all parameters with those of a further negative signal at 648 nm. In the present experiments the 515–475-nm signals were increased by poisoning the cells with a number of organic acids but there was no corresponding increase in the 648-nm signal. An investigation or organisms which lack chlorophyll *b* might well reveal acid enhanced absorbance changes which could be ascribed to particular compounds for example carotenoids. Preliminary experiments have indicated that increased 515-nm changes can be observed in Porphyridium.

In the present work the entry of undissociated acids couples in absorbance changes at 475 nm and 515 nm which are not usually visible and which have decay times which are much longer than those of absorbance changes at other wavelengths. This may indicate that the changes are associated with proton movement or represent an enhanced leakage of some component following structural damage. It is unlikely that the changes can represent oxidation–reduction changes directly on the electron transport pathway from H_2O to NADPH. The extreme sensitivity of these acid enlarged signals to the uncoupler CCCP suggests a connection with photosynthetic phosphorylation. However this result must be treated with caution since photoproduction of O_2 by Chlorella cells using nitrite as oxidant is also extremely CCCP sensitive and transient studies of intermediates of the photosynthetic carbon reduction cycle produced by CCCP cannot distinguish between an uncoupler or an inhibitor of cyclic electron flow[4]. It has previously been shown that the 515–475-nm signals are larger under anaerobic than aerobic conditions apparently due to the bleaching at 515 nm by oxygen[9]. The present results might have been due to a similar effect, either by acid induced rapid anaerobosis or by bleaching of 515 nm. As vigorous shaking in air failed to effect the results and in the presence of nitrite there was still some photosynthetic O_2 production the first explanation seems improbable. The second possibility was investigated by a survey of difference spectra between normal and inhibited cells in the dark. No consistent bleaching at 515 nm or increase in absorbance at 475 nm was observed although other unrelated changes in light scattering and absorbance may have obscured it.

Reversible light driven scattering changes with slow kinetics are well documented in isolated chloroplasts[12] as are volume and scattering changes of chloroplasts in leaves[13]. They are believed to result from ion movements across the membranes. Such changes do not seem to have been previously reported from whole algae cells with a fairly rigid cell wall although decreased chloroplast size has been reported in illuminated Nitella[14]. The slow kinetics, shape of rate *versus* light intensity curve and sensitivity to CCCP of the scattering changes in Chlorella observed in the presence

of the fatty acid inhibitors suggest they have the same origin as those previously reported. It is of interest that a large number of reagents will elicit these responses in Chlorella and that most of these also inhibit carbon fixation by inhibition of the hexose and heptose diphosphatases and photophosphorylation. This supports the view that structural integrity is essential for maximum activity of photosynthetic carbon reduction cycle.

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