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EVIDENCE FOR A TWO-DIRECTIONAL HYDROGEN ION TRANSPORT IN CHLOROPLASTS OF *EUGLENA GRACILIS**

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SUMMARY

The light-induced "pH rise" of Euglena chloroplasts is greatly enhanced by the addition of 2,4-dinitrophenol, and is reversed ("pH drop") by the addition of amine uncouplers. The pH rise observed without either addition equals the difference between the changes in the presence of dinitrophenol and that in the presence of amines. When chloroplasts are suspended in medium of low osmolarity (< 0.04 M), the pH drop in the presence of amines, as well as the stimulation by dinitrophenol, are lost.

The data suggest the existence of two pumps, transporting protons in opposite directions, possibly coupled to different internal compartments.

INTRODUCTION

Ever since the first measurements of pH changes coupled to electron transport were made, it was observed that while intact mitochondria actively excrete H^+ (refs. 1, 2), chloroplasts will actively accumulate them[3]. Our initial observations on H^+ uptake by Euglena chloroplasts showed this uptake to be much lower, as well as much slower, than that in spinach chloroplasts[4]. In fact, we often observed no pH change in response to illumination in chloroplast preparations which catalyzed photophosphorylation at a normal rate.

The data presented here suggest that Euglena chloroplasts can transport H^+ in two directions simultaneously—into the chloroplasts as well as out—and that possibly each component is associated with a different compartment within the chloroplast. The terms "H^+ pumping", "H^+ uptake" and "H^+ excretion" used in this paper are terms of convenience referring to pH changes in the external medium, and do not necessarily imply the active transport of H^+ *per se*.

MATERIALS AND METHODS

The culturing of *Euglena gracilis* and the isolation of the chloroplasts have been previously described[5]. The chloroplasts were washed once and suspended in a medium

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as described in the tables and figure to a chlorophyll concentration of 100–200 μg/ml. The suspension was adjusted to pH 6.8–6.9 with dilute HCl or NaOH.

The pH changes were followed with a Heath Model EUW-301 Recording Electrometer, equipped with an Instrumentation Laboratories No. 14043 combination electrode. The reaction was carried out in an 18 mm × 150 mm test tube immersed in a constant temperature water bath (10°). 2.5 ml of the chloroplasts suspension were used and were stirred with a 4-mm magnetic stirring bar. Illumination was provided by a 150-W G. E. photoflood lamp, which gave $1.5 \cdot 10^5$ erg/cm^2 per sec at the surface of the test tube. This light intensity was saturating for chlorophyll concentrations up to 250 μg/ml. All light-induced pH changes were monitored for at least three light-on, light-off cycles to assure reproducibility.

Photophosphorylation was determined as previously described[4]. Chlorophyll was measured according to ARNON[6].

RESULTS

When the isolated chloroplasts were washed and suspended in a medium containing 0.3 M mannitol, 0.35 M NaCl and $2 \cdot 10^{-5}$ M pyocyanin, the usual rise in pH upon illumination of the chloroplasts was slow and small (Fig. 1, No. I) as compared to that of spinach chloroplasts (refs. 7, 8 and 4, Table VII). The pH change was completely inhibited by $2 \cdot 10^{-5}$ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea, by $4 \cdot 10^{-5}$ M carbonylcyanide *m*-chlorophenyl hydrazone, by $2 \cdot 10^{-2}$ M methylamine, or by 0.1 %

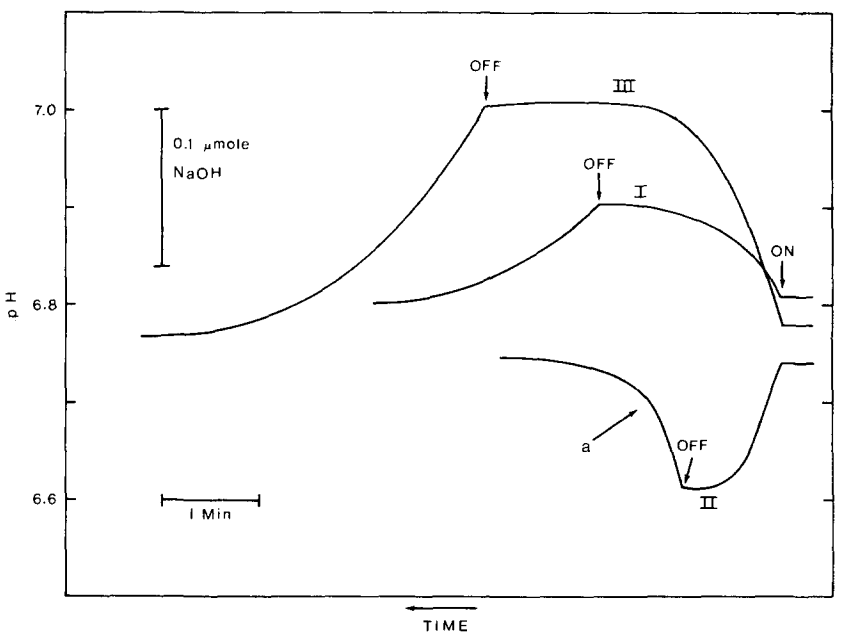

Fig. 1. The light-induced pH change in suspensions of chloroplasts of *E. gracilis*. Chloroplasts were washed and suspended in a medium containing 0.3 M mannitol *plus* 0.35 M NaCl. I, control, containing 180 μg/ml chlorophyll in 0.3 M mannitol, 0.35 M NaCl, and $2 \cdot 10^{-5}$ M pyocyanin; II, the same as in I *plus* $1 \cdot 10^{-2}$ M NH_4Cl; III, the same as in I *plus* $2 \cdot 10^{-4}$ M 2,4-dinitrophenol. For details, see text. Arrows point to light-on, light-off times.

Triton X-100. NH_4Cl, an uncoupler of photophosphorylation which inhibits the pH rise in spinach chloroplasts[9], inhibited the pH rise in Euglena chloroplasts at low concentrations ($< 1 \cdot 10^{-3}$ M). When higher concentrations were used, however, the direction of the pH change was reversed (pH drop), and appreciable excretion of H^+ into the medium was observed (Fig. 1, No. II). The maximum drop was obtained with $1 \cdot 10^{-2}$ M NH_4Cl. Butylamine at $5 \cdot 10^{-3}$ M and quinacrine at $1 \cdot 10^{-4}$ M showed a similar effect, but methylamine did not reverse the pH change even at $2 \cdot 10^{-2}$ M (Table I).

TABLE I

THE EFFECT OF UNCOUPLERS ON THE RATE OF PHOTOPHOSPHORYLATION AND LIGHT-INDUCED STEADY-STATE pH CHANGE IN SUSPENSIONS OF CHLOROPLASTS OF *E. gracilis*

Conditions as in Fig. 1. 200 μg chlorophyll per ml.

	ΔpH	*Photophosphorylation (μmoles ATP/mg chlorophyll per h)*
Control	+0.08	148
+ $1 \cdot 10^{-3}$ M NH_4Cl	−0.01	35
+ $4 \cdot 10^{-3}$ M NH_4Cl	0.05	15
+ $1 \cdot 10^{-2}$ M NH_4Cl	−0.08	4
+ $1.5 \cdot 10^{-2}$ M NH_4Cl	−0.08	—
+ $3 \cdot 10^{-5}$ M quinacrine	+0.04	—
+ $1 \cdot 10^{-4}$ M quinacrine	−0.04	—
+ $4 \cdot 10^{-3}$ M methylamine	+0.05	73
+ $2 \cdot 10^{-2}$ M methylamine	+0.01	17
+ $5 \cdot 10^{-3}$ M butylamine	−0.10	4
+ $1 \cdot 10^{-2}$ M butylamine	−0.10	< 1
+ $5 \cdot 10^{-5}$ M dinitrophenol	+0.12	—
+ $1 \cdot 10^{-4}$ M dinitrophenol	+0.15	151
+ $2 \cdot 10^{-4}$ M dinitrophenol	+0.17	—
+ $3 \cdot 10^{-4}$ M dinitrophenol	+0.18	141

The addition of 2,4-dinitrophenol to the chloroplasts caused a marked increase in the rate and magnitude of the pH rise (Fig. 1, No. III). The effect reached a maximum at 2.10^{-4}–$3 \cdot 10^{-4}$ M dinitrophenol (Table I). The same concentration of dinitrophenol had no effect on the rate of photophosphorylation by the same chloroplast preparations (Table I). In light of the higher initial rate of H^+ uptake in the presence of dinitrophenol, the rate of photophosphorylation as a function of the duration of illumination was investigated. No effect of dinitrophenol on ATP synthesis could be observed even over the initial 3 sec of illumination.

When both ammonia and dinitrophenol were added, the pH change depended on the concentration of each additive; and when both were saturating ($1 \cdot 10^{-2}$ M and $2 \cdot 10^{-4}$ M, respectively), no significant pH change could be observed (Table II).

The steady-state pH rise in the absence of ammonia or dinitrophenol (*i.e.* the control) approximated in every experiment the sum of the pH change in the presence

TABLE II

EFFECT OF BOTH AMMONIA AND DINITROPHENOL ON THE LIGHT-INDUCED pH CHANGE IN SUSPENSIONS OF CHLOROPLASTS OF EUGLENA

For conditions, see Fig. 1 and text.

	ΔpH	*μmole H^+/mg chlorophyll*
Control	+0.065	0.113
+ $2 \cdot 10^{-4}$ M dinitrophenol	+0.170	0.305
+ $2 \cdot 10^{-4}$ M dinitrophenol + $1 \cdot 10^{-3}$ M NH_4Cl	+0.070	0.129
+ $2 \cdot 10^{-4}$ M dinitrophenol + $2 \cdot 10^{-3}$ M NH_4Cl	+0.020	0.038
+ $2 \cdot 10^{-4}$ M dinitrophenol + $1 \cdot 10^{-2}$ M NH_4Cl	< −0.010	< −0.022
Control	+0.090	0.156
+ $1 \cdot 10^{-2}$ M NH_4Cl	−0.130	−0.290
+ $1 \cdot 10^{-2}$ M NH_4Cl + $1 \cdot 10^{-4}$ M dinitrophenol	−0.050	−0.112
+ $1 \cdot 10^{-2}$ M NH_4Cl + $2 \cdot 10^{-4}$ M dinitrophenol	−0.025	−0.056
+ $1 \cdot 10^{-2}$ M NH_4Cl + $3 \cdot 10^{-4}$ M dinitrophenol	< −0.010	< −0.022

TABLE III

THE EFFECT OF AMMONIA AND DINITROPHENOL ON THE MAGNITUDE OF THE LIGHT-INDUCED pH CHANGE IN SUSPENSIONS OF CHLOROPLASTS OF EUGLENA

For conditions, see Fig. 1 and text.

Expt. No.		*ΔpH*	*μmole H^+/mg chlorophyll*
6–12	Control	+0.15	0.208
	+ dinitrophenol	+0.25	0.434
	+ NH_4Cl	−0.095	−0.212
8–3	Control	+0.10	0.154
	+ dinitrophenol	+0.23	0.355
	+ NH_4Cl	−0.12	−0.238
9–16	Control	+0.12	0.185
	+ dinitrophenol	+0.18	0.278
	+ NH_4Cl	−0.07	−0.129
9–22	Control	+0.06	0.103
	+ dinitrophenol	+0.17	0.295
	+ NH_4Cl	−0.09	−0.202
	Control − pyocyanin	+0.015	0.026
	+ dinitrophenol − pyocyanin	+0.095	0.165
	+ NH_4Cl − pyocyanin	−0.065	−0.145

of dinitrophenol and the change in the presence of ammonia (Table III). These effects of ammonia and dinitrophenol, which differ markedly from those reported for spinach chloroplasts, prompted us to investigate whether the isotonic medium used (0.3 M mannitol *plus* 0.35 M NaCl) could account for the discrepancy.

When chloroplasts were washed and assayed in a medium containing 0.1 M NaCl or KCl, the pH changes were the same as those above (Table IV). The addition

of $4 \cdot 10^{-6}$ M valinomycin had no effect, but it inhibited in part the dinitrophenol-stimulated H^+ uptake. The valinomycin had, however, an inhibitory effect on the rate of photophosphorylation. This small effect of valinomycin *plus* dinitrophenol is in contrast to their effect on spinach chloroplasts[10].

TABLE IV

THE EFFECT OF DINITROPHENOL, AMMONIA, AND VALINOMYCIN ON THE LIGHT-INDUCED pH CHANGE IN SUSPENSIONS OF CHLOROPLASTS OF EUGLENA

Chloroplasts washed and suspended in 0.1 M NaCl. Reaction mixture contained 0.1 M NaCl *plus* $2 \cdot 10^{-5}$ M pyocyanin and 100 μg chlorophyll per ml. For assay method, see text. Numbers in brackets represent rates of photophosphorylation (μmoles/mg chlorophyll per h).

		ΔpH	*μmole H^+/mg chlorophyll*
	Control	0.04	0.22
	+ $1 \cdot 10^{-4}$ M dinitrophenol	0.09	0.50
	+ $1 \cdot 10^{-2}$ M NH_4Cl	−0.05	−0.41
[64]	Control + 0.1 M KCl	0.04	0.22
[59]	+ $1 \cdot 10^{-4}$ M dinitrophenol	0.09	0.50
	+ $1 \cdot 10^{-2}$ M NH_4Cl	−0.04	−0.33
[30]	+ $4 \cdot 10^{-6}$ M valinomycin	0.04	0.22
[39]	+ dinitrophenol and valinomycin	0.045	0.25
	+ NH_4Cl and valinomycin	—	—

TABLE V

EFFECT OF SUSPENSION MEDIUM ON THE SENSITIVITY OF THE LIGHT-INDUCED pH CHANGE IN SUSPENSIONS OF CHLOROPLASTS OF EUGLENA TO DINITROPHENOL AND AMMONIA

All reaction media contained $2 \cdot 10^{-5}$ M pyocyanin. For method, see text. 80–120 μg chlorophyll per ml. Numbers in brackets represent rates of photophosphorylation (μmoles/mg chlorophyll per h).

	Suspension medium	*Reaction medium*	*ΔpH*	*μmole H^+/mg chlorophyll*
[32]	0.01 M NaCl	0.01 M NaCl	0.18	0.50
		+ $1 \cdot 10^{-4}$ M dinitrophenol	0.175	0.49
		+ $1 \cdot 10^{-2}$ M NH_4Cl	<0.01	<0.04
		0.05 M KCl	0.19	0.52
[14]		+ $4 \cdot 10^{-6}$ M valinomycin	0.19	0.52
[28]		+ dinitrophenol	0.18	0.50
[13]		+ dinitrophenol + valinomycin	0.17	0.47
		0.15 M NaCl	0.17	0.46
		+ dinitrophenol	0.16	0.43
		+ NH_4Cl	<0.01	<0.03
[39]	0.1 M NaCl	0.1 M NaCl	0.05	0.19
		+ dinitrophenol	0.14	0.53
		+ NH_4Cl	−0.10	−0.38
		0.04 M NaCl	0.11	0.42
		+ dinitrophenol	0.11	0.42
		+ NH_4Cl	<0.01	<0.04
[144]	0.01 M NaCl	0.055 M NaCl		
	+ 0.3 M mannitol	+ 0.15 M mannitol	0.08	0.43
		+ dinitrophenol	0.11	0.60
		+ NH_4Cl	−0.06	−0.53

A totally different picture emerged when chloroplasts were suspended in a medium containing only 0.01 M NaCl. Both the stimulation by dinitrophenol, as well as the pH drop in the presence of ammonia, were eliminated. Valinomycin had no effect on the pH change with or without dinitrophenol, but it continued to inhibit the rate of photophosphorylation (Table V). It appears that the component of the pH change, which is sensitive to dinitrophenol and responsible for the pH drop in the presence of ammonia, was lost when the chloroplasts were treated with the low NaCl medium. The loss of this component could not be reversed by raising the NaCl concentration back to $>$ 0.1 M. The effect is apparently an osmotic one, since the high NaCl could be substituted by 0.3 M mannitol (Table V). There was no correlation between the rates of H^+ uptake and the rates of photophosphorylation. Both preparations of NaCl-treated chloroplasts (0.1 and 0.01 M) showed a low rate of photophosphorylation, while the preparation treated with low NaCl *plus* 0.3 M mannitol had a much higher rate (Table V).

When ammonia was added during illumination in the steady-state stage, there was a sudden, rapid ($<$ 10 sec) pH drop to the steady-state pH observed when ammonia was added prior to illumination (below the initial pH, before the light was turned on). When dinitrophenol was added during illumination in the steady-state stage, there was no sudden pH rise, but a gradual one, with the shape of the curve similar to that observed in the presence of ammonia when the light was turned off (Fig. 1, a). These observations could be interpreted as indicating two separate compartments for the two pumps. Ammonia makes the compartment which accumulates H^+ leaky, causing the rapid pH drop, while dinitrophenol does not make the other compartment leaky but just inhibits the H^+ pump. In addition, when ammonia was added as the light was turned off, the rapid pH drop was followed by a slow pH rise, which again was similar to the rate shown in Fig. 1, Curve a.

The omission of pyocyanin reduced the rate, as well as the magnitude of the steady-state pH change, but the effect of ammonia and dinitrophenol remained qualitatively the same (Table III).

DISCUSSION

The data presented here shows that the light-induced pH changes in Euglena chloroplasts are more complex than those reported for spinach[11–13]. The additional component in Euglena chloroplasts which is responsible for the pH drop in the presence of amines is, however, readily lost by osmotic shock; and the question is posed whether spinach chloroplasts are really deficient in this component or whether it is lost during isolation. There has been a recent report on pH drop in spinach chloroplasts[14] under specialized conditions, indicating a possible transport of phenazine methosulfate and its reversible reduction. In the Euglena chloroplasts, however, the pH drop was obtained in the absence of any cofactor, and was lost irreversibly after osmotic shock. In all these experiments, no correlation between the rate of photophosphorylation and of the net or dinitrophenol-stimulated pH change could be observed. The presence of two opposite pH changes, possibly occurring in different compartments, would require reevaluation of data attempting to correlate pH changes with ATP synthesis[12–14]. Not only is the net rate and magnitude of such a pH change a false indicator of the inside–outside pH gradient, but the possibility exists that the gradient between

the two compartments is the critical one. Preliminary data on the effect of dinitrophenol on photophosphorylation as a function of pH provides support for this possibility.

Euglena chloroplasts did not show the synergistic sensitivity to valinomycin and dinitrophenol reported for chloroplasts of higher plants[10]. Valinomycin showed some inhibition of the pH rise in dinitrophenol-stimulated chloroplasts, but had no effect on chloroplasts which showed no dinitrophenol stimulation (low NaCl-treated). It also inhibited the pH drop in the presence of ammonia, which is difficult to reconcile with the suggestion that valinomycin accelerates the excretion of NH_4^+, thus causing a decrease in H^+ uptake[11]. Valinomycin uncoupling of photophosphorylation in Euglena chloroplasts was not affected by dinitrophenol, and was 90% complete at $7 \cdot 10^{-6}$ M.

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