PHOTOPHOSPHORYLATION BY ISOLATED CHLOROPLASTS OF EUGLENA GRACILIS [1]

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One of the difficulties encountered in the use of chloroplasts from Euglena has been the low and inconsistent rate of photophosphorylation obtained with the isolated chloroplasts. This has been particularly true of non-cyclic phosphorylation, when ferricyanide served as electron acceptor.

We have developed techniques with which we are able to obtain photophosphorylation coupled to cyclic (pyocyanin) as well as non-cyclic (ferricyanide) electron flow with isolated chloroplasts of Euglena gracilis.

MATERIALS AND METHODS. Euglena gracilis, strain Z, were grown heterotrophically on a rotary shaker in 2-liter Erlenmeyer flasks, containing 1 liter of modified Hutner's medium (Price and Vallee, 1962), with 1.5% ethanol as carbon source. The cells were grown at 23-25°C, and illuminated with 1000 lux of Sylvania's Gro-lux fluorescent lights.

The cells were harvested when a population density of 2-3X10^6 cells/ml was reached (towards the end of the log phase of growth). They were washed twice with 10^{-3}M NaCl, and suspended in 5 times their volume of a grinding medium made up of 0.5 M mannitol, 0.05 M tris buffer, pH 8.0, and 0.01 M sodium chloride (MTN). β -mercaptoethanol was added to a final concentration of 10^{-3}M. All steps of harvesting and washing were done at 0-2°. The cell suspension was rapidly frozen in a low temperature bath to facilitate breakage, thawed, homogenized with 1/8 the volume 3-5 mm polyethylene pellets in a chilled Waring Blendor for 2-3 minutes, and filtered through cheese cloth to remove the beads. The filtrate was centrifuged at 1600g for 1 minute to remove intact cells, cell debris, and paramylum granules from the mixture. The supernatant was then centrifuged at 3000g for 10 minutes to sediment whole chloroplasts

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which were resuspended in MTN and resedimented by centrifugation at 3000g for 10 minutes. The washed chloroplasts were finally suspended in MTN.

The reaction mixture for assay of photophosphorylation is described in each table. The samples were illuminated for 4 minutes at 17°C, with 20,000 lux of red light (610-780 mμ), and ATP formation measured with the aid of ^{32}Pi by a modification of the method of Lindbergh and Ernster (1956). Chlorophyll was determined by the method of Arnon (1949).

RESULTS. The effect of the grinding medium on the rate of photophosphorylation by the chloroplasts is given in Table I. The addition of β-mercaptoethanol to the grinding medium stimulated the rate of photophosphorylation of the isolated chloroplasts by 10-40%. In contrast to spinach chloroplasts, the presence of EDTA in the grinding medium had no stimulating effect.

Table I

Effect of Grinding Medium on the Rate of Photophosphorylation by Euglena Chloroplasts

Medium	Specific Activity (μmole Pi esterified/mg chlorophyll/hr)
MTN, pH 8.0	21.8
+$5X10^{-3}$M ascorbate	12.0
+$1X10^{-3}$M β-mercaptoethanol	25.9
+$2X10^{-3}$M EDTA	22.4
Lysed in $2X10^{-3}$M $MgCl_2$	5.1
MTN, pH 7.0	4.7

The reaction mixture, in a final volume of 1.5 ml, contained: 10 μmoles tris buffer, pH 7.8, 35 μmoles NaCl, 1 μmole ADP, 4 μmoles phosphate buffer containing 500,000 CPM ^{32}Pi, 0.05 μmole pyocyanin, 15 μmoles glucose, 5 μmoles $MgCl_2$, 1 μgm (0.15 units) crystalline hexokinase, 0.5 mg crystalline BSA and chloroplasts containing 20 μgm chlorophyll in 0.1 ml MTN.
MTN - 0.5 M mannitol, 0.01 M Na Cl, 0.05 M tris buffer

Lysing the isolated chloroplasts in dilute $MgCl_2$ solutions, which enhances the activity of spinach chloroplasts, strongly inactivated the chloroplasts from Euglena.

Since Euglena chloroplasts contain an active Mg^{++}-dependent ATPase, glucose and hexokinase were added to the reaction mixture. One μgm (0.15 units) of crystalline hexokinase per reaction mixture (1.5 ml) was sufficient to give maximal rates of ^{32}Pi esterification.

With both ferricyanide and pyocyanin, the rate of phosphorylation was linear for at least 8 minutes of illumination. The rate was also linear up to a chlorophyll concentration of 20 μgm/ml.

The relative rates of photophosphorylation obtained with different electron acceptors is given in Table II. Pyocyanin gave consistently

much higher rates than phenazine methosulfate (PMS), even when the reaction mixture was preilluminated with white light, to convert the PMS to pyocyanin (Jagendorf and Margulies, 1960).

Table II

The Efficiency of Various Electron Acceptors in Photphosphorylation by Euglena Chloroplasts

Acceptor	Concentration	Specific Activity
None	----	0.1
Pyocyanin	$3.3X10^{-5}$	19.9
FMN	$5X10^{-4}$	9.3
PMS	$1.6X10^{-5}$	9.1
Menadione	$5X10^{-5}$	7.3
Ferricyanide	$5X10^{-4}$	7.4
2,6-dichlorophenol-indophenol	$1X10^{-4}$	6.2

Except for the electron acceptor, reaction mixture as in Table I. Cofactor concentrations are those giving optimal rates of phosphorylation. PMS - Phenazine Methosulfate

Optimal phosphate concentration was higher for pyocyanin-catalyzed than for ferricyanide-catalyzed phosphorylation (Table III). Since the rate of phosphorylation with pyocyanin is higher than with ferricyanide, this could mean that the rate-limiting step in ATP formation is the esterification of inorganic phosphates.

Table III

Effect of Phosphate Concentration on Photophosphorylation by Euglena Chloroplasts

Phosphate conc., M	Specific Activity	
	Pyocyanin	Ferricyanide
$6.7X10^{-4}$	11.1	3.0
$1.3X10^{-3}$	16.2	3.7
$2.7X10^{-3}$	19.4	3.5
$5.3X10^{-3}$	20.0	3.8

Reaction mixture as in Table I, except for the phosphate concentration and the substitution of 0.75 mole potassium ferricyanide for pyocyanin in column 3.

Table IV

Effect of pH of the Reaction Mixture on the Rate of Photophosphorylation by Euglena Chloroplasts

pH	Specific Activity	
	Pyocyanin	Ferricyanide
7.0	15.2	1.9
7.5	23.3	3.8
7.7	23.7	4.0
8.0	22.0	4.4
8.5	19.5	4.2

Reaction mixture as in Table I, except that 0.75 μmole potassium ferricyanide was substituted for pyocyanin in column 3.

The optimal pH of the reaction mixture was slightly higher for ferricyanide as compared to pyocyanin (Table IV). We found no difference between tris buffer and tricine buffer [tris (hydroxymethyl) methyl glycine], provided care was taken to use ammonia-free tris. (Final concentration of ammonia in the reaction mixture was less than 10^{-4}M).

The addition of 0.25-0.5 mg of crystalline bovine serum albumin per reaction mixture (1.5 ml) greatly stimulated the rate of ATP formation (Table V). The addition of larger amounts had no effect.

Table V

The Effect of Various Additions to the Reaction Mixture on the Rate of Photophosphorylation by Euglena Chloroplasts

	Specific Activity	
	Pyocyanin	Ferricyanide
Control	25.9	7.4
-BSA	14.0	3.6
+2X10^{-3}M NH_4Cl	9.1	4.9
+2X10^{-5}M Atebrin	0.0	0.0
+5X10^{-6}M CMU	15.7	0.0
+1X10^{-5}M CMU	11.1	0.0
+0.3M Sucrose	10.1	-

Reaction mixture of control as in Table I, except for the substitution of 0.75 μmole ferricyanide for pyocyanin in column 3. CMU - p-chlorophenyl-1, 1-dimethylurea. BSA - Bovine serum albumin.

Ammonium chloride and atebrin uncoupled the Euglena chloroplasts. p-Chlorophenyl-1, 1-dimethylurea (CMU) completely inhibited non-cyclic phosphorylation (with ferricyanide), but inhibited cyclic phosphorylation (with pyocyanin) only partially (Table V). These results with CMU are similar to those obtained with spinach chloroplasts by Jagendorf and Margulies (1960).

CONCLUSIONS. With the method described here, reasonable rates of photophosphorylation can be obtained with chloroplasts of Euglena gracilis.

The rate of ATP formation with these chloroplasts is much lower than with chloroplasts isolated from spinach (Jagendorf and Avron, 1958). It appears to be low due to a slow rate of electron transport, and not due to uncoupling, since the rate of ferricyanide reduction by these chloroplasts is only 20-25 μmole/mg chlorophyll/hr. Assuming a $P/2e^-$ of 1, this would give a maximal rate of 10-12 μmole ATP formed/mg chlorophyll/hr, which is not much higher than our optimal rate (with ferricyanide) of 7.4-7.9.

The low rates of phosphorylation are not due to the heterotrophic mode of growth, since chloroplasts from Euglena grown in autotrophic medium showed only a slightly higher rate of phosphorylation.

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