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EVIDENCE FOR THE OCCURRENCE OF CYCLIC PHOTOPHOSPHORYLATION *IN VIVO*

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SUMMARY

1. It is shown that the ATP level in leaves is increased many times upon illumination in the presence of *p*-chlorophenyldimethylurea (CMU), in spite of the fact that CMU inhibits completely O_2 evolution and CO_2 fixation.

2. The light effect, in the presence of CMU, is observed under strictly anaerobic conditions as well as in air, and is very rapid.

3. It is concluded that leaves synthesize ATP in the light by means of cyclic photophosphorylation.

INTRODUCTION

Cyclic photophosphorylation can be demonstrated in chloroplasts isolated from higher plants only when cofactors of electron transport are added in catalytic amounts. Among these cofactors, the non-physiological dye PMS promotes the highest rates of photophosphorylation[1]. Other redox substances, *e.g.* FMN and vitamins K_3 and K_5 (see refs. 2, 3), are efficient cofactors of photophosphorylation. However, it has been reported recently[4,5] that photophosphorylation catalyzed by these last cofactors requires the presence of O_2, and is inhibited by CMU[6], an inhibitor of the oxygen evolution reaction. It has been proposed[4-6] that in the presence of these cofactors a pseudocyclic process is operative, in which O_2 is evolved from water in the reduction of the cofactor and then utilized in its reoxidation. When no redox cofactors are added, the rates of photophosphorylation observed are very low[7], (about 1 % of the rates observed with the addition of PMS), and ATP synthesis depends on O_2 and is inhibited by CMU, which indicates that a true cyclic electron transport is not operating under these conditions. The available evidence from studies with chloroplast preparations cannot settle the question whether a cyclic photophosphorylation, coupled to the electron transport from the reductant to the oxidant photochemically generated without the participation of O_2, exists *in vivo*, in higher plants, as a physiological process. Such a question is of importance not only for the understanding of the mechanisms of electron transport involved in photophosphorylation, but possibly

Abbreviations: CMU, *p*-chlorophenyldimethylurea; PMS, methyl phenazonium methosulfate ("phenazine methosulfate").

also to permit a better evaluation of the quantum utilization efficiency in photosynthesis.

The demonstration of cyclic photophosphorylation in intact leaves was sought by means of experiments in which the light-dependent synthesis of ATP was studied in the presence of CMU, an inhibitor of photosynthetic O_2 evolution. The site of action of this inhibitor has been clearly established at the reaction(s) leading to O_2 evolution[6,8]: indeed, it inhibits all chloroplast reactions involving O_2 evolution, while it has no effect on cyclic photophosphorylation catalyzed by PMS. LOSADA *et al.*[8] have recently demonstrated that TPN photoreduction by chloroplasts, which is normally associated to O_2 evolution in a system depending on water as the terminal electron donor, can be made resistant to CMU if an artificial electron donor system is substituted for water. In a previous report it was shown that CMU inhibits completely photosynthesis even in intact leaves[9], and nevertheless favors ATP accumulation in the light. Such results have now been confirmed and the evidence of the occurrence in leaves of cyclic photophosphorylation under anaerobic conditions is given.

METHODS AND MATERIAL

Spinach leaves were purchased at the local market, and *Saxifraga crassifolia* was cultivated in an orchard.

All manometric experiments were run in an illuminated Warburg respirometer, light intensity being 10000 lux at the flask level. Dark control flasks were wrapped in 4 layers of aluminum foil. CO_2 partial pressure was kept constant at 1% by means of PARDEE buffer[10] present in the center well of the flasks. For CO_2 fixation experiments, the PARDEE buffer was prepared with $KH^{14}CO_3$, and its total radioactivity was measured. At the end of the experiment, the leaf discs were disintegrated in 2 N HCl, and the homogenate was hydrolyzed for 3 h at 100°. The hydrolyzate was made up to a known volume, centrifuged and its radioactivity was measured. All radioactivity measurements were done on 0.04-ml samples, dried on metal planchets, by means of a gas-flow counter. For ATP determinations, the leaves or leaf discs were incubated, in unbuffered distilled water, in 300 ml flasks immersed in a water bath at 20°. The flasks were flushed with the appropriate gas mixture, bubbling through the liquid. The illumination was provided by incandescent lamps, and light intensity was approx. 30 000 lux at flask level. At the end of the experiment water was sucked off the flasks by means of a water pump, and liquid air was poured on the leaves. The frozen leaves were then ground in a mortar to a fine powder, which was mixed with one volume of 7% perchloric acid. After allowing the mortar contents to thaw in the cold room, additional comminution was achieved with the aid of quartz sand. The perchloric extract combined with three washings of the mortar was centrifuged in the cold, and the precipitate was washed with cold 1% perchloric acid. The clear perchloric extract, containing all the acid-soluble components of the tissues, was then brought to pH 7.6–7.8 with KOH. The $KClO_4$ precipitate was allowed to settle for 20 min in an ice-bath; it was centrifuged in the cold and washed with cold 0.5 M Tris buffer of pH 7.6. The neutralized supernatant, combined with the washings of the perchlorate precipitate, was made up to a known volume and ATP was determined as described by MARRÈ AND FORTI[11], except that the hexokinase–glucose system was used to dephosphorylate ATP instead of the muscle ATPase previously used[12]. Duplicate samples

of each extract were analyzed, and always found to agree within 10%. The reproducible recovery of over 90% of the ATP added to the tissues in the mortar proves the reliability of the method.

Yeast hexokinase (type IV) was obtained from Sigma, St. Louis, Mo. (U.S.A.).

RESULTS

The complete inhibition of photosynthetic O_2 evolution by CMU has been shown to occur in intact pea and Elodea leaves[9] at the same concentration of the inhibitor required for 100% inhibition in chloroplast preparations[6]. This finding has been confirmed for spinach leaf discs. When the experiment is run in 0.05 M phosphate buffer (pH 5.9) instead of the carbonate–bicarbonate buffer[12], and in the presence of 1% CO_2, the results shown in Table I are obtained. As can be seen, CMU inhibits completely both O_2 evolution and $^{14}CO_2$ fixation at a concentration of $6 \cdot 10^{-5}$ M. The inhibitor has no significant effect on respiration in the dark.

TABLE I

EFFECT OF CMU ON PHOTOSYNTHESIS IN SPINACH LEAF DISCS

Each flask contained 8 discs (200 mg of fresh weight) in phosphate buffer 0.05 M (pH 5.9), 1% CO_2 in the gas phase, provided by PARDEE buffer containing $2 \cdot 10^6$ counts/min of ^{14}C, temperature, 25°C.

Conditions	*CMU concentration (M)*	Q_{O_2} *(μmoles)*	*Net photosynthesis (μmoles O_2)*	CO_2 *fixation (counts/min)*
Dark	None	−6.35	—	198
Dark	10^{-4}	−6.42	—	185
Light	None	2.00	8.35	6670
Light	$3 \cdot 10^{-5}$	−2.95	3.40	2790
Light	$6 \cdot 10^{-5}$	−5.90	0.45	350
Light	10^{-4}	−6.00	0.35	280

Effect of light and CMU on ATP level in leaves

As previously reported[9], illumination of spinach leaves causes an increase of ATP in the tissues in the presence of CMU. Table II shows the time-course of this effect; it can be seen that the ATP increase is almost complete after 2 min illumination, whereafter the ATP concentration remains constant at a level considerably higher than in the dark controls. On the other hand, CMU has no effect on the ATP concentration in the leaves kept in the dark[9]. In the experiment of Table II, the leaf discs were used immediately after their preparation. It has been previously shown[9] that the ATP level falls to very low values if the leaves are kept in darkness for several hours. This effect can be obtained more rapidly and reproducibly on incubation of the leaf discs in the dark in an atmosphere of nitrogen. After 1 h in these conditions, air was drawn through the flasks containing the leaf discs in the presence of CMU, and the time-course of ATP changes was followed, with and without illumination (Table III). As can be seen from the results of the two experiments reported, ATP concentration increases very rapidly in the light and slowly in darkness. The presence of $8 \cdot 10^{-5}$ M CMU rules out the possibility that light acts by increasing rapidly the concentration

of O_2 in the cells, oxygen evolution being completely inhibited under these conditions. The ATP increase is almost maximal after 2 min illumination (Expt. I), while at this time no ATP increase can be observed in the dark. In Expt. II (Table III), the ATP increase in darkness (*i.e.* ATP formation by oxidative phosphorylation) is considerably slower; probably as a consequence of the damage suffered by the leaves during the dark anaerobic period, and still photophosphorylation induces a rapid rise of ATP level in 2 min or less. In all cases observed, the steady state concentration of ATP attained in darkness after a more or less long time is considerably lower than that reached in the light in a few minutes.

TABLE II

EFFECT OF LIGHT AND CMU ON ATP LEVEL IN SPINACH LEAF DISCS

Each flask contained 20 discs of 22 mm diameter (4.5 g fresh weight). Incubation at 20°, in $3 \cdot 10^{-5}$ M CMU. Illumination: 20000 lux at flask level.

Time of incubation (min)	*ATP (mμmoles/g fresh weight)*	
	Dark	*Light*
0	59	—
2	—	75
10	—	76
30	58	82

TABLE III

TIME-COURSE OF THE ATP-LEVEL INCREASE IN SPINACH LEAVES IN LIGHT AND DARK

Spinach leaf discs (see Table II) preincubated 60 min at 20°, under N_2, in CMU ($7.5 \cdot 10^{-5}$ M). At time 0, air was bubbled into the flasks at a rate of 50 l/h, and light was supplied (30 000 lux) where indicated.

Expt.	*Incubation time (min)*	*mμmoles of ATP/g of leaves (fresh weight)*	
		Dark	*Light*
I	0	9	—
	2	4	48
	10	45	52
	30	43	60
II	0	0.0	—
	2	0.0	36
	10	0.0	37
	30	47.0	63

The independence of light-induced ATP synthesis on O_2 has been demonstrated in experiments in which leaves were incubated under nitrogen, in the presence of CMU, in light or dark. Table IV shows that when Saxifraga leaf discs containing very little ATP are incubated under N_2 in darkness, the ATP concentration remains extremely low. In the light, however, the ATP level increases dramatically, and only under prolonged anaerobiosis it tends to decrease. Here again, the presence of CMU com-

pletely inhibits any oxygen evolution, so that ATP generation by oxidative phosphorylation can be excluded.

TABLE IV

PHOTOPHOSPHORYLATION UNDER NITROGEN IN SAXIFRAGA LEAVES

Saxifraga leaf discs (20 discs, 0 g fresh weight) were preincubated a few minutes in CMU ($7.5 \cdot 10^{-5}$ M), in air. At 0 time, nitrogen was bubbled in to the flasks at a rate of 3 l/min at 30°.

Incubation time in N_2 (min)	*mμmoles ATP/g fresh weight*	
	Dark	*Light*
0	5	—
15	0	60
60	0.5	29

DISCUSSION

The results reported here demonstrate that the light-dependent synthesis of ATP in leaves occurs, in air as well as in nitrogen, in the presence of CMU at a concentration such as to inhibit completely photosynthetic O_2 evolution and CO_2 fixation (Table I), and therefore is similar to the cyclic process observed with chloroplast preparations in the presence of the redox dye PMS[1,6]. It appears thus reasonable to assume that the CMU-insensitive, oxygen-independent photophosphorylation demonstrated in leaves must be coupled to a cyclic electron transport from the reductant to the oxidant photochemically generated. As an alternative mechanism, the possibility could be suggested that some reductant present in the cells could substitute for water as an electron donor to the photo-oxidant, thus making the electron transport system insensitive to CMU. This possibility, however, is in contrast with the observation that CMU inhibits to the same extent both O_2 evolution and $^{14}CO_2$ fixation by leaves (Table I); indeed, if a by-pass of the CMU-sensitive O_2 evolution from water existed, a CMU-insensitive fixation of $^{14}CO_2$ should be observed, independent of oxygen evolution. This is clearly not the case. Furthermore, CMU not only does not prevent light-induced ATP-increase in leaves, but actually enhances it; and when healthy, freshly prepared leaf discs are used, CMU is required for the light-dependent increase of ATP level[9]. This finding was tentatively interpreted as indicating that ATP generated in cyclic photophosphorylation can accumulate to a much larger extent if its utilization for CO_2 assimilation is prevented, as is the case in the presence of CMU. Accordingly, one would expect the ATP increase in the light to be larger, in the absence of CMU, in a CO_2-free atmosphere, while the presence of CMU should render immaterial the simultaneous presence or absence of CO_2. This was indeed found to be the case in a number of experiments[13], but the results are scarcely reproducible and their interpretation is complicated by variable and often very large effect of CO_2 on the ATP level in darkness. These effects, probably related to various metabolic reactions, are unexplained at this moment.

The data reported in Table III permit a comparison of the efficiencies of oxidative phosphorylation and of cyclic photophosphorylation in leaves. It can be seen that cyclic photophosphorylation can operate much more promptly and probably at a higher

rate, than oxidative phosphorylation. This might be due to the fact that the photophosphorylation rate depends only on the ADP and phosphate supply at one particular site in the chloroplast, light being continuously provided in very large excess, while the oxidative phosphorylation rates are controlled by a very large number of metabolic reactions, all of them contributing to the over-all process. The high capacity for converting light energy into phosphate transfer potential with which green cells are endowed enables them, on the other hand, to perform metabolic work other than CO_2 assimilation at the expense of this energy. It has been reported elsewhere[14] that active transport as well as polysaccharide synthesis are stimulated by light in the presence of CMU; these findings are best explained in terms of availability of ATP, although more complex effects of light cannot be excluded.

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