# EFFECTS OF EXTERNAL FACTORS ON PHOTOPHOSPHORYLATION AND EXCHANGE OF $CF_1$-BOUND ADENINE NUCLEOTIDES

Susanne BICKEL-SANDKÖTTER and Heinrich STROTMANN
*Botanisches Institut der Tierärztlichen Hochschule, Abteilung für Biochemie der Pflanzen,*
*3000 Hannover, Bünteweg 17, West Germany*

Received 12 March 1976

## 1. Introduction

Chloroplast coupling factor $CF_1$ contains firmly bound adenine nucleotides which were shown to be slowly exchanged in de-energized chloroplasts and in the isolated enzyme [1–9]. On energization of the thylakoids either by light-dependent electron transport [1–3,9] or by an acid–base transition [9], bound adenine nucleotides are rapidly replaced by free ADP or ATP. From $^3H$ incorporation studies [10,11], fluorescence studies of covalently bound fluorescamine [12], and other indirect evidence [13] it was concluded that energization induces a conformational change of $CF_1$. Between the energy-dependent change of $CF_1$ conformation and the alteration of adenylate binding an intrinsic relationship may be suggested.

The functional role of bound adenine nucleotides in the mechanism of photophosphorylation has recently been interpreted [3,7] by analogy with the conformational hypothesis of oxidative phosphorylation [14,15]. In this concept the actual energy-requiring step of the phosphorylation cycle was thought to be the transition of $CF_1$ from an inactive to an adenylate-exchangeable form. By this reaction pre-formed bound ATP was assumed to be released and replaced by free ADP. The formation of tightly bound ATP itself was believed to be energy-independent. Assuming that the dissociation equilibria of $CF_1$–ADP and $CF_1$–$P_i$ complexes were high in comparison to $CF_1$–ATP complex in the inactive state, the equilibrium of the ATPase reaction was predicted to be shifted towards the formation of firmly bound ATP [3].

In the present paper light-induced exchange of bound adenine nucleotides was studied as a function of several external parameters. The results indicate a corresponding behavior of adenylate exchange and photophosphorylation in dependency of light intensity, pH, uncoupling, and electron transport inhibition. On the other hand, arsenate and phlorizin which are known to inhibit photophosphorylation, do not affect the light-dependent exchange of bound adenine nucleotides. $Mg^{2+}$ ions stimulate both, photophosphorylation and adenylate exchange, however in a different way.

## 2. Materials and methods

Chloroplast isolation from spinach leaves and the preparation of [$^{14}C$]ADP pre-labelled membranes has been described elsewhere [9].

Release of bound adenine nucleotides from pre-labelled chloroplasts was measured in small semi-transparent centrifugation tubes with a final volume of 0.3 ml. The standard incubation medium contained 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM methylviologen, 1 mM orthophosphate, and 0.1 mM ADP. After addition of the labelled chloroplasts (final chlorophyll concentration 0.2 to 0.3 mg/ml) and mixing, the tubes were inserted into a centrifuge ('Microfuge 152', Beckman) with illumination equipment. After 30 sec pre-incubation either light was switched on (white light, $1.8 \times 10^5$ ergs/$cm^2$sec inside the tubes) or the samples were kept in the dark. After the indicated times light was extinguished and the chloroplasts were immediately spun down. $^{14}C$ contents of the clear supernatants were measured by liquid scintillation counting in 'Unisolve 1' scintillator cocktail (Koch-Light Lab. Ltd.).

Photophosphorylation was measured under identical experimental conditions, except that orthophosphate was labelled by $^{32}P$. For these experiments unlabelled thrice-washed chloroplasts were used. The samples were illuminated for 15 sec. The reaction was stopped by addition of 20 µl 70% $HClO_4$. Incorporation of $^{32}P_i$ into the organic phosphate fraction was determined as described elsewhere [16].

## 3. Results

In a previous paper [9] we reported that washed broken chloroplasts incorporated $^{14}C$-labelled ADP in a light-dependent reaction. A certain amount of label could not be removed by subsequent washes. This share was specifically bound by coupling factor $CF_1$. Pre-labelled membranes are a suitable material for the study of the exchange of $CF_1$-bound adenine nucleotides.

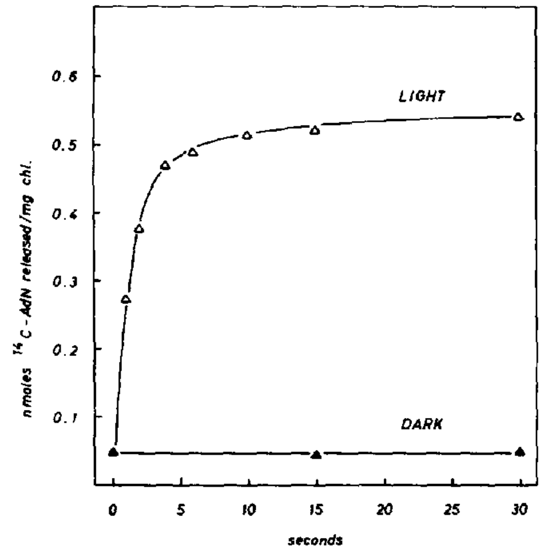

Fig. 1. Kinetics of dark and light release of $CF_1$-bound [$^{14}C$] adenine nucleotides by chloroplasts in the presence of 0.1 mM ADP and 1 mM $P_i$. Pre-labelling of the chloroplasts was performed with 22.5 µM [8-$^{14}C$]ADP [9]. Release incubation was carried out as described in Materials and methods.

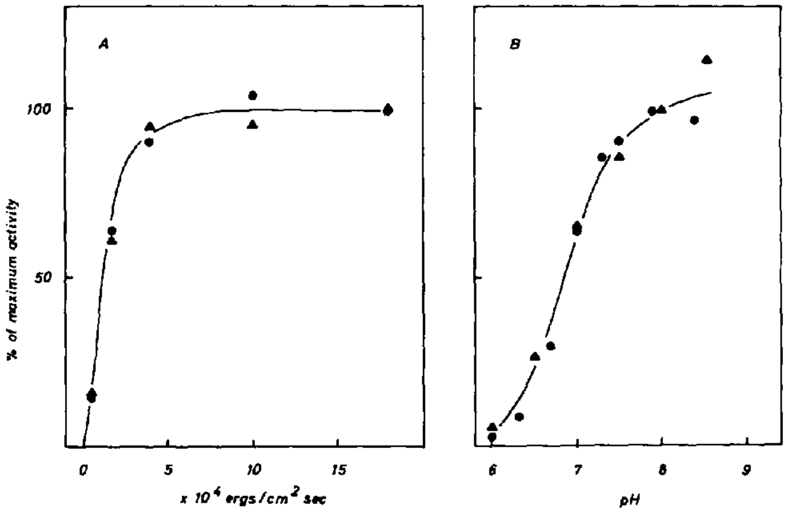

Fig.2. Photophosphorylation and adenine nucleotide exchange as affected by light intensity (A) and pH (B). Adenine nucleotide exchange activity was determined by the initial rate of [$^{14}C$]adenylate release by pre-labelled chloroplasts in the presence of 1 mM $P_i$ and 0.1 mM ADP. The initial rate was computed from $^{14}C$ appearance in the medium after 2 sec illumination. Corresponding dark backgrounds were subtracted. For photophosphorylation measurements see Materials and methods. In (B) the following buffers were used: pH 6.0–6.5 MES, pH 6.5–7.5 HEPES, pH 7.5–8.5 Tricine. The rates of overlapping pH values were averaged. Control rates (A), photophosphorylation: (●): 0.246 µmol $^{32}P_i$ incorporated/mg chlorophyll × 15 sec; adenine nucleotide exchange (▲): 0.213 nmol [$^{14}C$]AdN released/mg chlorophyll × 2 sec. (B) photophosphorylation (●): 0.234 µmol/mg chl × 15 sec; adenine nucleotide exchange (▲): 0.142 nmol/mg chl × 2 sec.

In these experiments labelled membranes were incubated in a medium which contained unlabelled ADP and phosphate as well as an electron acceptor of a non-cyclic system (methylviologen). Adenylate exchange was followed by the release of bound $^{14}$C-adenine nucleotides into the medium. In fig.1 the kinetics of $^{14}$C release in the dark and light are shown. In the dark for 30 sec no exchange was observed. In the light a rapid release of bound labelled adenine nucleotides occurred. The half time was about 1 sec and the reaction was nearly complete after 10 sec. The initial rate of light-induced release of $^{14}$C can be taken as a measure of adenine nucleotide exchange activity.

In the following experiments exchange activity was compared with steady state photophosphorylation under the same conditions. Fig.2 shows that the two processes exhibit an identical dependency on light intensity and pH of the medium. In fig.3 the

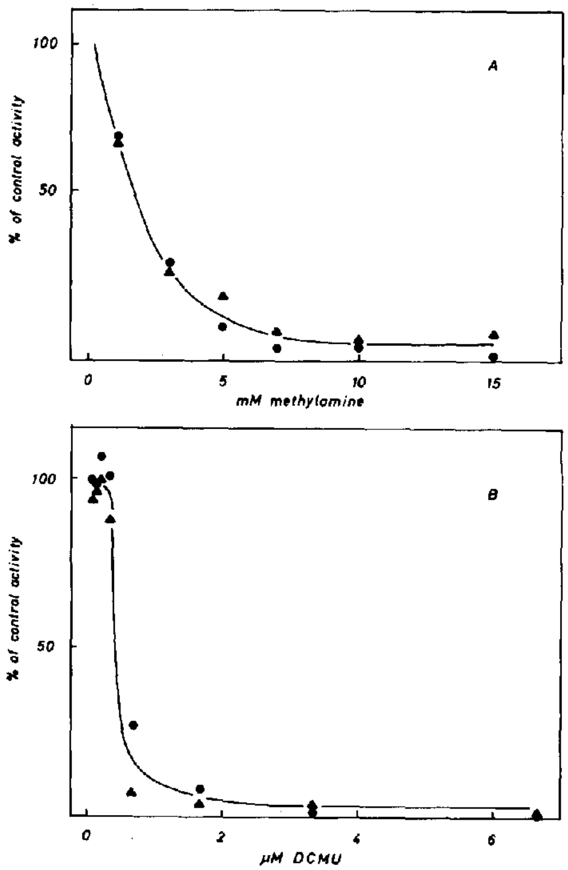

Fig.3. Photophosphorylation and adenine nucleotide exchange as a function of methylamine (A) and DCMU (B) concentration. The experimental conditions were the same as in fig.2. Control rates (A), photophosphorylation (●): 0.268 µmol/mg chl × 15 sec; adenine nucleotide exchange (▲): 0.170 nmol/mg chl × 2 sec. (B), photophosphorylation (●): 0.356 µmol/mg chl × 15 sec; adenine nucleotide exchange (▲): 0.227 nmol/mg chl × 2 sec.

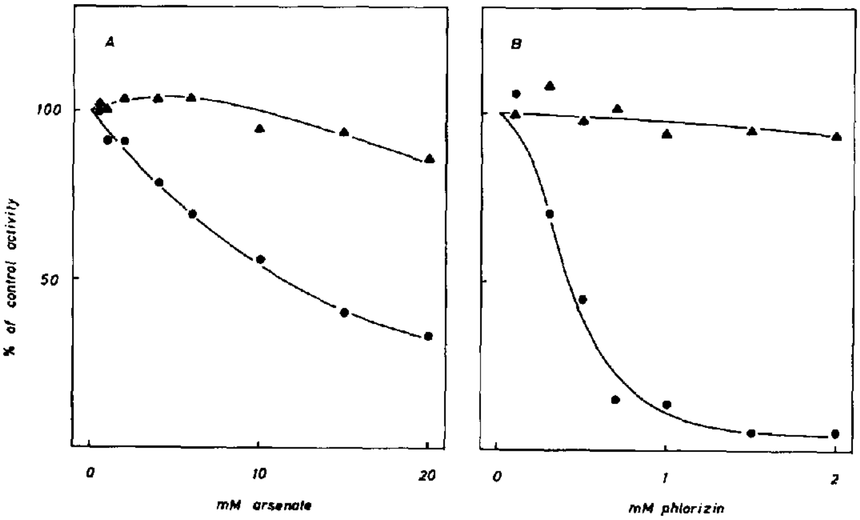

Fig.4. Photophosphorylation and adenine nucleotide exchange as a function of arsenate (A) and phlorizin (B) concentration. Experimental conditions as in fig.2. Control rates (A), photophosphorylation (●): 0.299 µmol/mg chl × 15 sec; adenine nucleotide exchange (▲): 0.228 nmol/mg chl × 2 sec. (B), photophosphorylation (●): 0.363 µmol/mg chl × 15 sec; adenine nucleotide exchange (▲): 0.255 nmol/mg chl × 2 sec.

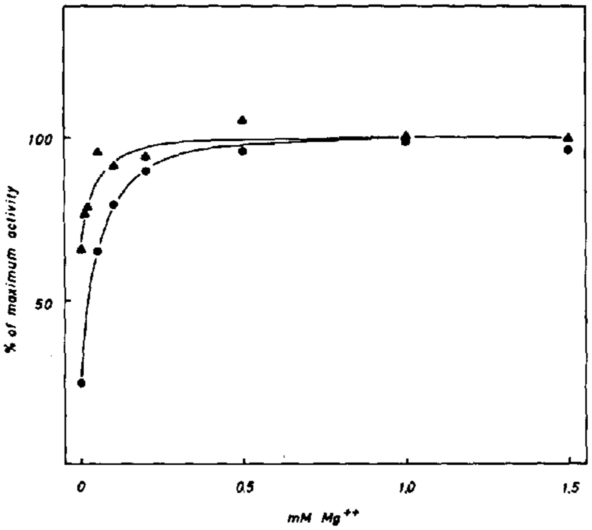

Fig.5. Photophosphorylation and adenine nucleotide exchange as a function of $Mg^{2+}$ concentration. Experimental conditions as in fig.2. Control rates, photophosphorylation (●): 0.255 μmol/mg chl × 15 sec; adenine nucleotide exchange (▲): 0.196 nmol/mg chl × 2 sec.

rates of the two reactions are drawn as a function of methylamine and DCMU concentrations. The results demonstrate that uncoupling and electron transport inhibition likewise affect both processes in the same way. With the uncoupler FCCP (not shown) corresponding concentration curves were obtained, too.

Unlike methylamine and FCCP the uncoupler arsenate does not affect adenine nucleotide exchange, although it inhibits photophosphorylation (fig.4A). In a similar manner the energy transfer inhibitor phlorizin inhibits photophosphorylation but does not injure adenylate exchange (fig.4B).

$Mg^{2+}$ is well known to be an essential cofactor in photophosphorylation [17]. In fig.5 photophosphorylation and adenine nucleotide exchange are shown as a function of $Mg^{2+}$ concentration. $Mg^{2+}$ shortage decreases both, photophosphorylation and adenine nucleotide exchange; however the effect on photophosphorylation is much more pronounced.

## 4. Discussion

Following the conformational hypothesis as developed by Harris and Slater [3], the photophosphorylation cycle may be subdivided into two partial processes, the formation of $CF_1$-bound ATP and the exchange of bound ATP for free ADP. In the experiments reported in the present paper, adenine nucleotide exchange was directly studied by measurement of the release of bound [$^{14}$C]adenine nucleotides in the presence of unlabelled ADP. This reaction was shown to be strictly energy-dependent. Release of bound ATP is believed to be caused by an energy-dependent conformational change of $CF_1$ [3]. If this is true, adenylate exchange activity is a measure for the conformational change.

Photophosphorylation is controlled by several external factors. With regard to $CF_1$ related reactions these factors. With regard to $CF_1$ related reactions these factors either affect adenine nucleotide exchange or the synthesis of ATP on the enzyme. In our experiments a discrimination between the two sites is possible. Adenylate exchange is controlled by the pH value of the medium and by light intensity; moreover electron transport inhibition and uncoupling by methylamine and FCCP impair adenylate exchange and photophosphorylation in a corresponding way. The results indicate that a high energy state established by photosynthetic electron transport, is required for adenine nucleotide exchange on $CF_1$. The close relationship between this reaction and overall photophosphorylation indicates the essential role of adenine nucleotide exchange in the mechanism of energy transformation. In a previous paper [9] we demonstrated that an artificial pH gradient across the thylakoid membrane likewise is able to induce adenylate exchange in the absence of an electron transport. Thus, a ΔpH seems to link the primary and the final energetic events, as proposed by the chemiosmotic theory [18]. This is in accordance with earlier reports by Ryrie and Jagendorf [11] who demonstrated that $^3$H incorporation (as a measure of conformational change of $CF_1$) can be brought about by an acid–base transition, too.

Arsenate and phlorizin likewise inhibit photophosphorylation but do not impair adenylate exchange. As an obvious explanation we may conclude that these substances interfere with reactions in close relationship to the formation of bound ATP on $CF_1$. This is quite comprehensible in the case of arsenate which can be regarded as a kind of competitive inhibitor in the formation of pyrophosphate bonds. However, with phlorizin deviating and unexpected results were obtained by Pflugshaupt and Bachofen [7]. They reported that $^{32}P_i$ incorporation into membrane-bound ATP was not affected by this energy transfer inhibitor in sonicated chloroplasts. As yet this discrepancy can not be explained.

$Mg^{2+}$ seems to exhibit a function in adenylate exchange as well as in ATP formation. However, photophosphorylation is more severely inhibited by $Mg^{2+}$ deficiency than nucleotide exchange. Even in the presence of 1 mM EDTA (with excess $Na^+$ ions for preservation of $CF_1$-membrane binding) more than 50% of the exchange activity was retained, whereas under the same conditions photophosphorylation was completely suppressed. $Mg^{2+}$ requirement for light-dependent incorporation of $^{32}P_i$ into membrane bound ATP has been reported by Harris and Slater [3].

Our results definitely demonstrate the energy dependency of adenine nucleotide exchange on $CF_1$ in accordance with the conformational hypothesis. However, energy independency of the formation of bound ATP has not yet been established. In this respect quite contrary experimental results were obtained by two different groups [3,7].

## Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

## References

[1] Roy, H. and Moudrianakis, E. N. (1971) Proc. Natl. Acad. Sci. US 68, 464.

[2] Roy, H. and Moudrianakis, E. N. (1971) Proc. Natl. Acad. Sci. US 68, 2720.

[3] Harris, D. A. and Slater, E. C. (1975) Biochim. Biophys. Acta 387, 335.

[4] Girault, G., Galmiche, J. M., Michel-Villaz, M. and Thiery, J. (1973) Eur. J. Biochem. 38, 473.

[5] Vambutas, V. and Bertsch, W. (1975) Biochim. Biophys. Acta 376, 169.

[6] Cantley, Jr., L. C. and Hammes, G. G. (1975) Biochemistry 14, 2968.

[7] Pflugshaupt, C. and Bachofen, R. (1975) Bioenergetics 7, 49.

[8] Vandermeulen, D. L. and Govindjee, (1975) FEBS Lett. 57, 272.

[9] Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194.

[10] Ryrie, I. J. and Jagendorf, A. T. (1971) J. Biol. Chem. 246, 3771.

[11] Ryrie, I. J. and Jagendorf, A. T. (1972) J. Biol. Chem. 247, 4453.

[12] Kraayenhof, R. and Slater, E. C. (1975) in: Proc. of the Third Intern. Congr. on Photosynthesis, (M. Avron, ed.) Vol. II, p. 985, Elsevier, Amsterdam.

[13] McCarty, R. E. and Fagan, J. (1973) Biochemistry 12, 1503.

[14] Boyer, P. D. (1974) in: Dynamics of Energy-Transducing Membranes, (L. Ernster, R. W. Estabrook and E. C. Slater, eds.) BBA Library 13, p. 289, Elsevier, Amsterdam.

[15] Slater, E. C., Rosing, J., Harris, D. A., van de Stadt, R. A. and Kemp, Jr., A. (1974) in: Membrane Proteins in Transport and Phosphorylation, (G. F. Azzone, M. E. Klingenberg, E. Quagliariello and N. Siliprandi, eds.) p. 137, North-Holland, Amsterdam.

[16] Strotmann, H. (1970) Ber. Dtsch. Bot. Ges. 83, 443.

[17] Avron, M., Krogmann, D. W. and Jagendorf, A. T. (1958) Biochim. Biophys. Acta 30, 144.

[18] Mitchell, P. (1966) Biol. Rev. 41, 445.