Biochimica et Biophysica Acta, 440 (1976) 522–530

BBA 47147

PHOSPHORYLATION OF NUCLEOTIDES BOUND TO CHLOROPLAST MEMBRANES AND THEIR ROLE IN PHOTOPHOSPHORYLATION

CLAUDE AFLAFO and NOUN SHAVIT

Department of Biology, Ben Gurion University of the Negev, Beer Sheva (Israel)

(Received February 18th, 1976)

SUMMARY

1. The initial rapid phosphorylation of membrane-bound ADP yields [γ-^{32}P]-ATP. Long term illumination of chloroplasts resulted in the introduction of label in the β position of ADP and ATP.

2. It is concluded that the initial acceptor in photophosphorylation is ADP and not AMP. The appearance of $^{32}P_i$ in the β position of the nucleotide fractions is probably the result of side reactions not directly involved in the photophosphorylation mechanism.

3. The inhibitor phlorizin affects similarly the phosphorylation of bound ADP and the net photophosphorylation reaction.

4. The uncoupler nigericin has different effects on the phosphorylation of membrane-bound ADP and the net photophosphorylation reaction. Dissipation of energy by low concentrations of the uncoupler affects primarily the step(s) of ATP release to the medium, while steps leading to the interconversion of P_i and ADP to form bound ATP are less sensitive and are inhibited by relatively higher uncoupler concentrations.

INTRODUCTION

Recent proposals on the mechanism of energy coupling in mitochondrial and chloroplast membranes introduced the concept that energy is required in order to effect the release of a preformed ATP molecule firmly held at the catalytic site [1, 2]. This change in binding affinities of reactants and products may occur via a conformational change in the coupling ATPase. Indeed, tightly bound nucleotides were detected in several energy-transducing membrane systems [3–5]. Using washed chloroplast membranes, Harris and Slater [5] reported values of 2.5 nmol ATP and 1.3 nmol ADP bound per mg chlorophyll. With energized membranes, complete exchange of bound nucleotides with free nucleotides occurred. An exchange between the bound nucleotides and P_i, in the presence of Mg^{2+}, resulted in the incorporation of labelled phosphate at both the β and γ positions of the nucleotides. Boyer et al. [6] investigated the rapid (up to 50 ms) labelling pattern in ADP and ATP by energized chloroplast membranes in the presence of $^{32}P_i$, using a rapid mixing technique. In double labelling experiments with $^{32}P_i$ and tritiated nucleotides it was demonstrated [7] that the initial ATP formed in an acid-base transition reaction must occur via the phosphorylation of bound ADP.

In this communication, it is shown that the initial incorporation of $^{32}P_i$ into bound nucleotides in chloroplasts depends upon electron transport and yields [γ-^{32}P]-ATP. The appearance of [β-^{32}P]ATP and ADP is probably the consequence of secondary reactions not related to photophosphorylation. The energy requirements of photophosphorylation involving bound nucleotides were analyzed using inhibitors and uncouplers. It is concluded that the newly formed ATP is bound to the membrane and that energy input is required both to aid its formation and subsequent release from the coupling enzyme.

MATERIALS AND METHODS

Chloroplasts were isolated from fresh market lettuce leaves by conventional procedures, washed three times by resuspension in 0.4 M sucrose and 1 mM tricine, pH 8.0, and centrifugation, and finally resuspended in this medium. Chlorophyll content was estimated according to Arnon [8].

Reaction mixtures for $^{32}P_i$ incorporation into organic phosphates contained in a final volume of 1 ml the following components in μmol: tricine, 20; KCl, 20; $MgCl_2$, 5; $^{32}P_i$, 0.5 (containing about $4 \cdot 10^7$ cpm); phenazine methosulfate, 0.005 and thrice washed chloroplasts containing about 100 μg chlorophyll. Samples were illuminated with 100 000 lux white light and the reaction was terminated after turning the light off by trichloroacetic acid addition to a final concentration of 3 %. Total label incorporated into the organic phosphate fraction was determined as described [9].

The nucleotides present in the trichloroacetic acid extracts were adsorbed on activated charcoal which was washed with cold 0.1 M HCl followed by cold water to remove inorganic phosphates. The adsorbed nucleotides were then eluted with an absolute ethanol 1 M NH_4OH solution (1 : 1, v/v). Chromatographic separation of nucleotides was performed on polyethylenimine-cellulose sheets by a modification of the method of Randerath and Randerath [10]. Labelled nucleotides samples were diluted with 5 nmol of carrier AMP, ADP and ATP and separated by ascending chromatography with 1 M formic acid/1.25 M LiCl/1 mM EDTA. Radioactivity content was determined with a Packard strip counter or in a Nuclear Chicago gas flow counter. [^{32}P]ATP content was estimated from the difference before and after chemical hydrolysis with 1 M HCl at 100 °C for 10 min or as non-hydrolyzable ^{32}P-labelled glucose-6-*P* formed after incubation with 0.6 mg/ml hexokinase, 10 mM glucose and 5 mM $MgCl_2$. Enzymes and reagents were purchased from Sigma. Polyethylenimine-cellulose sheets were obtained from Macherey-Nagel and Co.

RESULTS

Illumination of chloroplasts in the presence of $^{32}P_i$, $MgCl_2$ and phenazine methosulfate, without added ADP, results in the incorporation of ^{32}P into an organic phosphate fraction. The rapid initial incorporation of P_i (Fig. 1; up to 3 s) was

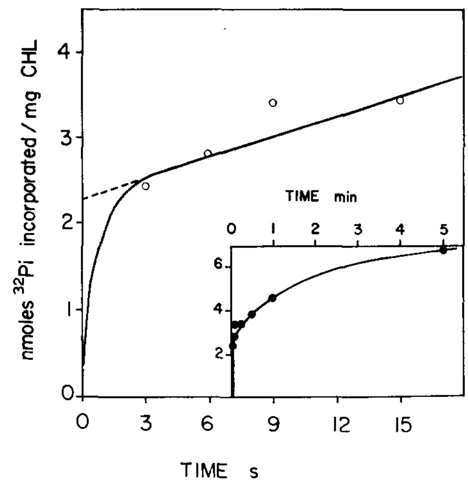

Fig. 1. Incorporation of $^{32}P_i$ into bound nucleotides. Reaction mixtures were as described in Materials and Methods and contained chloroplasts equivalent to 110 μg chlorophyll.

followed by a much slower phase as shown in the inset of Fig. 1. The initial rapid incorporation of $^{32}P_i$ was dependent upon the presence of phenazine methosulfate and Mg^{2+} and both the rapid and slow phase depended upon illumination (Table I). The reaction products were identified chromatographically and the results are given in Table II. The label incorporated during the initial rapid phase of the reaction was almost entirely in the γ-phosphate of ATP. Under these conditions, the distribution of label with added ADP (not shown) is identical to that reported for net photophosphorylation [11]. During longer illumination periods, additional label appeared in the nucleotide fraction as well as in a non-nucleotide fraction. The nucleotide fraction was primarily [γ-^{32}P]ATP with some label incorporated as [β-^{32}P]ATP and [β-^{32}P]ADP. The radioactivity incorporated in [β-^{32}P]ATP and [β-^{32}P]ADP was variable. In several experiments, these values were as high as 12 % of the total label

TABLE I

DEPENDENCE OF $^{32}P_i$ INCORPORATION UPON Mg^{2+}, PHENAZINE METHOSULFATE AND ILLUMINATION

Reaction mixtures and assay conditions as described in Materials and Methods. Thrice washed chloroplasts containing 90 μg chlorophyll were added. Samples were illuminated or incubated in the dark for a total period of 12 s. Aliquots were removed each 3 s and reaction stopped by addition of trichloroacetic acid to a final concentration of 3 %. Values for zero time were extrapolated as shown in Fig. 1.

Incubation conditions	nmol $^{32}P_i$ incorporated/mg chlorophyll	
	0 s	12 s
Complete ; illuminated	1.0	1.52
$-Mg^{2+}$; illuminated	0.22	0.89
−phenazine methosulfate; illuminated	0.17	0.76
Complete ; dark	0.0	0.05

TABLE II

LABEL DISTRIBUTION IN BOUND NUCLEOTIDES

Reaction mixtures and assay conditions as described in Materials and Methods and Figs. 2 and 3. Total $^{32}P_i$ incorporation was determined as label appearing in the water phase after isobutanol/benzene extraction of the phosphomolybdate complex [9]. Nucleotides were absorbed on active charcoal and analyzed as described.

Illumination time (s)	$^{32}P_i$ incorporated into fraction (nmol/mg chlorophyll)		$\frac{[^{32}P]AXP \times 100}{\text{Total nucleotide } [^{32}P]}$			
	Total	Nucleotide	ATP	ADP	AMP	$[\gamma\text{-}^{32}P]ATP$
3	2.4	2.4	99	0.8	0	99
900	7.7	4.4	94	3.5	2.5	93

incorporated into the nucleotide fraction. The pattern of labelling in the non-nucleotide fraction, which was not absorbed on activated charcoal, was not elucidated. This non-nucleotide fraction is apparently not pyrophosphate, since it was not hydrolyzed by pyrophosphatase, nor did the presence of pyrophosphatase during illumination prevent its formation.

The initial rapid incorporation of $^{32}P_i$ results from phosphorylation of ADP present endogenously in the chloroplast preparation. The endogenous ADP content, estimated by extrapolation (Fig. 1), varied between 1.0 and 2.5 nmol/mg chlorophyll. The variability observed may be due to seasonal effects and leaf storage conditions (summer chloroplasts gave lower values). The effectiveness of ADP removal by the washing procedure may also contribute to this variability. Further, it was shown that the initial rapid $^{32}P_i$ incorporation into ATP is not the result of exchange between ATP and $^{32}P_i$. Preillumination in the presence of unlabelled P_i followed by a subsequent illumination with added $^{32}P_i$, markedly reduced the amount of $[^{32}P]ATP$

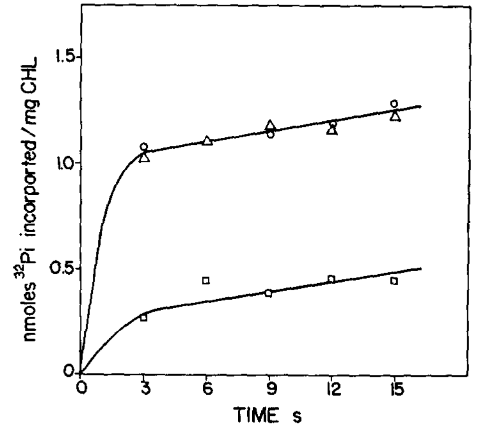

Fig. 2. Effect of preillumination on the rapid phase of $^{32}P_i$ incorporation. Chloroplasts containing 177 μg chlorophyll were illuminated as described in Materials and Methods. ○, not preilluminated; △, preilluminated for 15 s in the absence of P_i; □, preilluminated for 15 s in the presence of $^{31}P_i$ and $^{32}P_i$ added afterwards. In all cases total P_i concentration was identical.

TABLE III

FORMATION OF GLUCOSE 6-PHOSPHATE FROM PREFORMED ATP

Reaction mixtures and assay conditions as described in Materials and Methods, except that thrice washed chloroplasts containing 142 μg chlorophyll were added and illuminated for 10 s. The reaction was stopped after turning the light off at the time intervals indicated, by addition of trichloroacetic acid or by incubating the aliquots in the dark with hexokinase-glucose and $MgCl_2$ for 10 s and then terminating the reaction by addition of trichloroacetic acid.

Time elapsed after illumination (s)	Without hexokinase-glucose (nmol/mg chlorophyll) $[^{32}P]ATP$	With hexokinase-glucose (nmol/mg chlorophyll)	
		$[^{32}P]ATP$	Glc-6-^{32}P
0	1.9	0.05	1.9
10	1.8	0.00	1.7
20	1.4	0.06	1.4
30	1.2	—	—

TABLE IV

INCORPORATION OF $^{32}P_i$ INTO ATP WITH AND WITHOUT HEXOKINASE-GLUCOSE

Reaction mixtures and assay conditions were as described in Materials and Methods except that thrice washed chloroplasts containing 178 μg chlorophyll were added and illuminated for the time indicated. $[^{32}P]ATP$ and glucose 6-phosphate were calculated from the values obtained before and after hydrolysis with 1 M HCl for 10 min at 100 °C.

Additions	Illumination time (s)	$^{32}P_i$ incorporated (nmol/mg chlorophyll)	
		ATP	Glc-6-*P*
None	5	1.8	0.0
None	10	1.9	0.0
+hexokinase-glucose	5	0.7	3.4
+hexokinase-glucose	10	0.6	6.6

TABLE V

EFFECT OF PHLORIZIN ON $^{32}P_i$ INCORPORATION INTO BOUND NUCLEOTIDES AND ON NET PHOTOPHOSPHORYLATION

Reaction mixtures and assay conditions as described in Materials and Methods, except that thrice washed chloroplasts containing 90 μg chlorophyll and 0.5 mg/ml hexokinase/10 mM glucose or 0.5 mM ADP were added as indicated. Illumination time was 15 s. Phlorizin was added as indicated.

Additions	$^{32}P_i$ incorporated (μmol/mg chlorophyll per h)	Inhibition (%) +phlorizin (mM)	
		0.6	6.0
None	1.0*	75	98
+hexokinase-glucose	1.2	62	99
+ADP	193.4	80	99

* Extrapolated value in nmol/mg chlorophyll.

TABLE VI

EFFECT OF NIGERICIN ON $^{32}P_i$ INCORPORATION INTO BOUND NUCLEOTIDES AND ON NET PHOTOPHOSPHORYLATION

Reaction mixtures and assay conditions as described under Table V except that thrice washed chloroplasts containing 162 μg chlorophyll were illuminated for 12 s.

Additions	$^{32}P_i$ incorporated (μmol/mg chlorophyll per h)	Inhibition (%) +nigericin (μM)	
		0.1	0.3
None	1.0*	14	56
+hexokinase-glucose	2.2	30	68
+ADP	359.0	58	86

* Extrapolated value in nmol/mg chlorophyll.

formed (Fig. 2). The label incorporation in the slower phase of the reaction may be due to exchange between unlabelled ATP attached to the membranes and ^{32}Pi.

Effect of addition of hexokinase-glucose. Treatment with hexokinase, glucose and $MgCl_2$ included in the washing procedure did not increase the amount of ADP available as phosphate acceptor. Addition of hexokinase-glucose immediately after illumination resulted in the conversion of almost all the [^{32}P]ATP into glucose 6-phosphate, as shown in Table III. More than 95 % of the [γ-^{32}P]ATP appears to have been released to the medium and was available to interact with hexokinase in the dark period following illumination. It is also shown that this newly formed [γ-^{32}P]ATP can apparently be partially hydrolyzed by the chloroplast system (in the absence of hexokinase) during the post-illumination dark incubation. In another

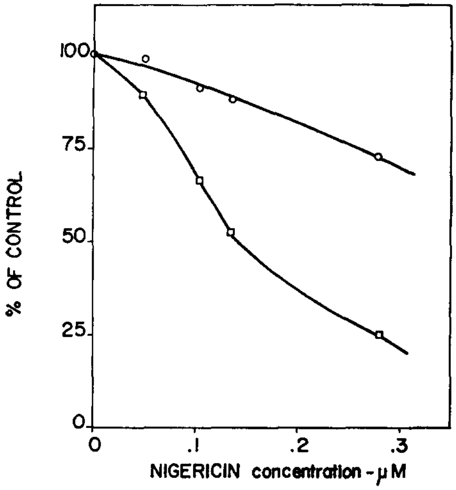

Fig. 3. Effect of nigericin on the incorporation of $^{32}P_i$. For assay conditions see Materials and Methods. Chloroplasts containing 117 μg chlorophyll were illuminated for 15 s without added ADP (○), and with added ADP, 0.5 mM (□). Control phosphorylation rates: without added ADP, 1.1 nmol/mg chlorophyll and with ADP, 150 μmol/mg chlorophyll per h.

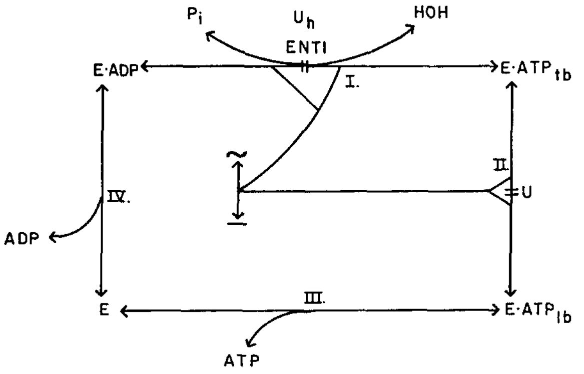

Fig. 4. A reaction cycle for catalytic participation of CF_1-bound ADP and ATP in photophosphorylation. E, coupling factor one; ENTI, energy transfer inhibitor; U and U_h, uncoupler at low and high concentrations, respectively; tb and lb, tightly and loosely bound nucleotides, respectively; ~, energized state.

experiment, we tested the ability of hexokinase to convert small amounts of medium ATP to glucose 6-phosphate by incubating up to 0.4 nmol [γ-^{32}P]ATP/unit hexokinase (without chloroplasts). More than 98 % of the ATP was converted to glucose 6-phosphate in 3 s. When hexokinase plus glucose were present during illumination, we observed an enhancement in label incorporation due to the recycling of the ADP acceptor (Table IV), and glucose 6-phosphate accumulation. However, a considerable amount of [^{32}P]ATP remained unavailable to the hexokinase, appearing as [γ-^{32}P]ATP even after several seconds of illumination. The value of 0.6 nmol/mg chlorophyll of ATP found represents about 30 % of the bound ATP detected in the absence of hexokinase.

Effects of phlorizin and nigericin on bound ATP. The effect of phlorizin, an energy transfer inhibitor, on the initial rapid phase of $^{32}P_i$ incorporation, with and without hexokinase-glucose, was similar to that on the net photophosphorylation (with added ADP) under these assay conditions (Table V). However, with nigericin, a differential inhibition of photophosphorylation was observed (Table VI). Although the relative degree of inhibition of reactions whose rates are different is being compared, the uncoupling of net photophosphorylation by nigericin does not inhibit the phosphorylation of bound ADP (Fig. 3). In the presence of hexokinase-glucose, a sequence of reactions requiring the release of membrane-bound [^{32}P]ATP, stronger inhibition was observed (Table VI).

DISCUSSION

These results demonstrate that the initial acceptor of P_i in chloroplasts is most probably membrane-bound ADP, rather than AMP. No formation of β-^{32}P-labelled nucleotides was detected during the initial phase of phosphorylation of membrane-bound nucleotides resulting in ATP formation. Some incorporation into the β position does occur upon more prolonged illumination, under conditions similar to those reported by Harris and Slater [5]. The role of AMP as the initial acceptor of P_i in photophosphorylation was suggested by Roy and Moudrianakis [12, 13] who reported the participation of nucleotides tightly bound to the coupling ATPase, in the process of ATP formation. However, washed chloroplast membranes, found to be virtually devoid of bound AMP, could nevertheless catalyze the incorporation of $^{32}P_i$ into ADP and ATP, independent of added AMP [5]. While the incorporation of $^{32}P_i$ into membrane-bound nucleotides via exchange reactions resulted in the incorporation of label into the β and γ positions, the pattern of labelling differs from that observed in net photophosphorylation with added ADP (refs. 5 and 11; Aflalo, C. and Shavit, N., unpublished). On the basis of the data presented and the considerations mentioned above, we conclude that the incorporation of label into the β position of the membrane-bound nucleotides is most probably the result of exchange reactions not on the main path of the photophosphorylation process, as previously suggested [5, 7].

The rapid initial incorporation of $^{32}P_i$ into the γ position of ATP permits an estimation of the ADP content in these washed lettuce chloroplasts membranes. Although some variability was observed, the estimated value is in close agreement with the amounts determined in spinach chloroplast membranes [5].

The phosphorylation of membrane-bound ADP requires a proton electrochemical gradient, since it depends upon electron transport (Table I). It can also be achieved by an acid-base transition of chloroplasts [6] (Moalem, S. and Shavit, N., unpublished). In the presence of an hexokinase-glucose system, about one-third of the ATP (compared to the amount of ATP formed in its absence) appears to be bound and unavailable for the hexokinase catalyzed glucose 6-phosphate formation (Table IV). The detection of amounts of ATP significantly above the expected steady-state level for a system involving both phosphorylating chloroplast membranes and a hexokinase-glucose "trap", lend support to the assumption that the rate-limiting step in the overall reaction sequence is the release of ATP from the coupling enzyme. From the effects of an uncoupler and an energy transfer inhibitor on the phosphorylation of membrane-bound ADP, we conclude that both the condensation of ADP and P_i and the release of the newly formed ATP from the enzyme may require energy (Fig. 3; Tables V and VI). Harris and Slater [5] suggested a single "priming" turnover of the coupling system as an energy-requiring step before phosphorylation could occur. This activation step was inhibited by uncoupling agents but not by an energy transfer inhibitor, such as Dio-9. However, the relationship between the exchange reactions they observed and the capacity for phosphorylation of membrane-bound ADP or net photophosphorylation with free ADP, is not clear. Our results show that the initial phosphorylation of bound ADP is inhibited by phlorizin, an energy transfer inhibitor, and by relatively high concentrations of the uncoupler nigericin. Boyer et al. [14, 15] have suggested that the uncoupler-insensitive $P_i \rightleftharpoons$ HOH exchange in submitochondrial particles represents the binding of P_i at the catalytic site with the formation of a tightly bound ATP and its reversal, which may occur in the presence of an uncoupler, with little or no energy input. More recent experiments [7] on the effects of an uncoupler on the two components of the $P_i \rightleftharpoons$ HOH exchange reaction, allowed localization of the uncoupler-insensitive exchange to the reactions of P_i, ADP and ATP while bound to the catalytic site. The entry of P_i from the medium to the catalytic site is prevented by energy dissipation in the presence of an uncoupler. No data is available on a similar uncoupler-sensitive $P_i \rightleftharpoons$ HOH exchange in chloroplasts membranes involving bound P_i, ADP and ATP. From the observed effects of uncouplers (see also Harris and Slater [5]), it is clear that the incorporation of medium $^{32}P_i$ into bound ATP does require energy input. The presence of bound P_i in these washed chloroplast membranes was not considered. Yamamoto et al. [16] reported the presence of membrane-bound P_i in chromatophores of *Rhodospirillum rubrum*. However, the incorporation of $^{32}P_i$ into bound nucleotides in chromatophores was not affected by oligomycin while photophosphorylation was strongly inhibited.

Our present knowledge can be accommodated in a minimal scheme based on the sequence of reactions suggested by Boyer et al. [6] and Harris and Slater [5]. The dissipation of energy by an uncoupler will affect the conformational change responsible for the change in the affinities for P_i, ADP and ATP. Most of the energy appears to be required at the step of ATP release to the medium (affected by low concentrations of uncoupler) while steps involving the binding of P_i from the medium are less sensitive to uncoupler and may be affected directly or even indirectly by the presence of higher uncoupler concentrations.

ACKNOWLEDGEMENTS

We wish to thank Dr. A. San Pietro and Dr. S. Lien for helpful discussions during the preparation of this manuscript and Miss Z. Maimon for excellent technical assistance. We would like to thank Dr. P. D. Boyer for making the manuscript of his paper [7] available to us prior to publication. This work was supported in part by grant 21-A of the Israel National Science Foundation.

REFERENCES

1 Boyer, P. D. (1974) in Dynamics of Energy-Transducing Membranes (Ernster, L., Estabrook, R. W., Slater, E. C., eds.), pp. 289–301, Elsevier, Amsterdam

2 Slater, E. C. (1974) in Dynamics of Energy-Transducing Membranes (Ernster, L., Estabrook, R. W., Slater, E. C., eds.), pp. 1–20, Elsevier, Amsterdam

3 Slater, E. C., Rosing, J., Harris, D. A., Van de Stadt, R. J. and Kemp, Jr., A. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G. F., Klingenberg, M. E., Quagliariello, E. and Siliprandi, N., eds.), pp. 137–147, North-Holland Publ. Co., Amsterdam

4 Abrams, A., Nolan, E. A., Jensen, C. and Smith, J. B. (1973) Biochem. Biophys. Res. Commun. 55, 22–29

5 Harris, D. A. and Slater, E. C. (1975) Biochim. Biophys. Acta 387, 335–348

6 Boyer, P. D., Stokes, B.O ., Wolcott, R. G. and Degani, C. (1975) in Symposium on Biological Energy Transductions, Fed. Proc. 34, 1711–1717

7 Boyer, P. D., Smith, D. J., Rosing, J. and Kyalar, C. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. and Siliprandi, N., eds.), pp. 361–372, North-Holland Publ. Co., Amsterdam

8 Arnon, D. I. (1949) Plant Physiol. 24, 1–15

9 Avron, M. (1961) Anal. Biochem. 2, 535–543

10 Randerath, K. and Randerath, E. (1967) in Methods in Enzymology (Collowick, S. P. and Kaplan, N. O., eds.), Vol. 12, pp. 323–347, Academic Press, New York

11 Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272

12 Roy, H. and Moudrianakis, E. N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 464–468

13 Roy, H. and Moudrianakis, E. N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2720–2724

14 Boyer, P. D., Cross, R. L. and Momsen, W. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2837–2839

15 Cross, R. L. and Boyer, P. D. (1975) Biochemistry 14, 392–398

16 Yamamoto, N., Yoshimura, S., Higuti, T., Nishikama, K. and Horio, T. (1972) J. Biochem. Tokyo 72, 1397–1406