Biochimica et Biophysica Acta, 430 (1976) 135–144

BBA 47078

STUDIES ON PHOTOPHOSPHORYLATION UTILIZING METHYLENE DIPHOSPHONATE ANALOGS OF ADP AND ATP

ARNOST HORAK and SAUL ZALIK

Department of Plant Science, University of Alberta, Edmonton, Alberta (Canada)

(Received August 1st, 1975)

SUMMARY

Spinach chloroplasts were able to photophosphorylate the ADP analog α,β-methylene adenosine 5′-diphosphate (AOPCP). Phosphorylation of AOPCP was catalyzed by chloroplasts that were washed or dialyzed to remove free endogenous nucleotides. In the presence of glucose, hexokinase, AOPCP and $^{32}P_i$, the ^{32}P label was incorporated into α,β-methylene adenosine 5′-triphosphate (AOPCPOP).

In contrast to photophosphorylation of AOPCP, the ATP analog AOPCPOP was a poor substrate for the ATP-P_i exchange reaction and its hydrolysis was neither stimulated by light and dithiothreitol nor inhibited by Dio-9.

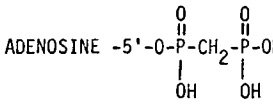
Photophosphorylation of AOPCP was inhibited by the α,β- and β,γ-substituted methylene analogs of ATP, while phosphorylation of ADP was unaffected by them. The ATP-P_i exchange was also unaffected by both ATP analogs, while the weak AOPCPOP-P_i exchange was inhibited by the β,γ-methylene analog of ATP.

Direct interaction of methylene analogs with the chloroplast coupling factor ATPase was indicated by the enzymatic hydrolysis of AOPCPOP on polyacrylamide gels.

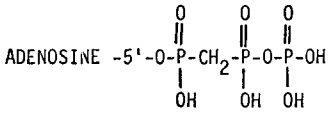
INTRODUCTION

Previously we used the ADP analog α,β-methylene adenosine 5′-diphosphate (AOPCP, Fig. 1) in an attempt to determine whether ADP undergoes a transphosphorylation to AMP and ATP during photophosphorylation as proposed by Roy and Moudrianakis [1, 2]. It was found that AOPCP can serve as a substrate for photophosphorylation [3] and that the reaction resembles the phosphorylation of ADP in its requirement for $MgCl_2$ and phenazine methosulfate to obtain full activity [4]. The two reactions also had a similar pH optimum and a similar sensitivity to the energy transfer inhibitor Dio-9 and the uncoupler NH_4Cl [4]. While these results indicated that photophosphorylation of AOPCP occurred in the same manner as that

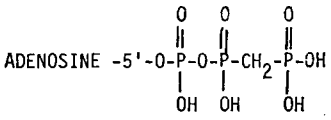
Abbreviations: AOPCP, α,β-methylene adenosine 5′-diphosphate; AOPCPOP, α,β-methylene adenosine 5′-triphosphate; AOPOPCP, β,γ-methylene adenosine 5′-triphosphate; TES, tris (hydroxymethyl) methylaminoethane sulfonic acid.



α,β-methylene adenosine 5'-diphosphate



α,β-methylene adenosine 5'-triphosphate



β,γ-methylene adenosine 5'-triphosphate

Fig. 1. The methylene diphosphonate analogs of ADP and ATP used.

of ADP, they did not exclude the possibility that the phosphorylation of the analog was mediated by endogenous nucleotides. Thus the major objective of the present study was to determine whether endogenous nucleotides were required for the reaction to proceed.

The results presented show that AOPCP is photophosphorylated directly by the photosynthetic membranes of chloroplasts without the involvement of free endogenous nucleotides. In contrast to photophosphorylation of AOPCP, the ATP analog AOPCPOP was found to be a poor substrate for the ATP-P_i exchange and ATPase activities which are considered to be partial reactions of photophosphorylation.

MATERIALS AND METHODS

Chloroplast preparation and photophosphorylation. Spinach chloroplasts were prepared in 0.4 M sucrose containing 40 mM TES/NaOH and 0.2 % fatty acid poor bovine serum albumin at pH 8 as described previously [3]. For most experiments the chloroplasts used were supplemented with 25 % glycerol and stored in liquid nitrogen [3] for periods of up to 18 months without loss of activity. For some experiments freshly prepared plastids were washed with 10 mM NaCl, or frozen plastids were thawed and dialyzed against 10 mM NaCl and acid-washed Norit charcoal (1 g/l) to remove endogenous nucleotides. Photophosphorylation of ADP or AOPCP was measured as described previously [4] using 2 mM ADP or AOPCP as substrate.

Photophosphorylation in presence of hexokinase. The design of the experiment was similar to that described by Bennun and Avron [5]. Freshly prepared chloroplasts

were washed twice with 10 mM NaCl and aliquots containing 100 μg chlorophyll were incubated in a volume of 3 ml at pH 8 with: 300 μmol TES/NaOH, 15 μmol $MgCl_2$, 9 μmol sodium [^{32}P]phosphate, 3 mg fatty acid poor bovine serum albumin, 90 nmol phenazine methosulfate, 50 μmol glucose, 6 μmol of ADP or AOPCP and 100 units of dialyzed yeast hexokinase where indicated. After 5 min of illumination 0.2 ml of 60 % trichloroacetic acid was added and the precipitate was removed by centrifugation. A 0.5-ml aliquot of the supernatant was used for determination of total esterified phosphate (organic ^{32}P) by isobutanol benzene extraction [6]. Another 2-ml aliquot was mixed with 0.1 g of acid-washed Norit charcoal and the charcoal centrifuged down. 1 ml of this supernatant was used for determination of esterified phosphate (glucose 6-[^{32}P]phosphate). The charcoal was washed three times with water, suspended in 2 ml of 1 M HCl and heated for 7 min on a boiling water bath. After centrifuging down the charcoal, an aliquot of the supernatant (1 ml) was counted to obtain a measure of phosphate esterified into nucleotide triphosphate.

ATP-P_i exchange. The ATP-$^{32}P_i$ exchange was measured under conditions similar to those described by Shavit [7] and McCarty and Racker [8]. The incubation mixtures contained in 0.8 ml at pH 8: 100 μmol TES/NaOH, 5 μmol $MgCl_2$, 30 nmol phenazine methosulfate, 10 μmol dithiothreitol and chloroplasts (58 μg of chlorophyll). After 5 min at 20 °C and 40 000 lux of incandescent light, the light was switched off and 0.2 ml of a solution containing 2 μmol sodium [^{32}P]phosphate and 2 μmol of ATP were added. After a 15 min dark incubation, 0.1 ml of 30 % trichloroacetic acid was added. The denatured plastids were removed by centrifugation, dithiothreitol was oxidized by H_2O_2 and the esterified ^{32}P determined according to Nielsen and Lehninger [6].

ATPase activity. The light- and dithiothreitol-activated ATPase activity of spinach chloroplasts was measured as described previously [9] using 5 mM ATP or AOPCPOP as substrate.

Chlorophyll determination. Chlorophyll concentration was measured in 80 % acetone extracts of chloroplasts using the equation of MacKinney [10].

Chemicals. The nucleotide analogs AOPCP, AOPCPOP and AOPOPCP were purchased from Miles Laboratories, ADP, ATP and yeast hexokinase (Type C-301, suspension in $(NH_4)_2SO_4$) from Sigma and fatty acid poor bovine serum albumin was from Calbiochem. Dio-9 was a generous gift from Dr. R. D. Hill, Plant Science Department, University of Manitoba.

RESULTS

Photophosphorylation

In order to show that photophosphorylation of AOPCP is not due to contamination of this compound by ADP, small amounts of AOPCP were chromatographed on cellulose as well as on polyethyleneimine-cellulose thin-layer sheets. Chromatography on cellulose sheets with the solvent system isobutyric acid/concentrated ammonia/water (66 : 1 : 33, v/v) [11] separated AOPCP from ADP, but the two nucleotides ran close to each other (R_F = 0.56 and 0.46, respectively). On polyethyleneimine-celullose sheets [12] the separation of AOPCP and ADP was very good (R_F = 0.71 and 0.44, respectively) when 1.0 M LiCl was used for development. In either case there was no detectable contamination of AOPCP by ADP. These methods

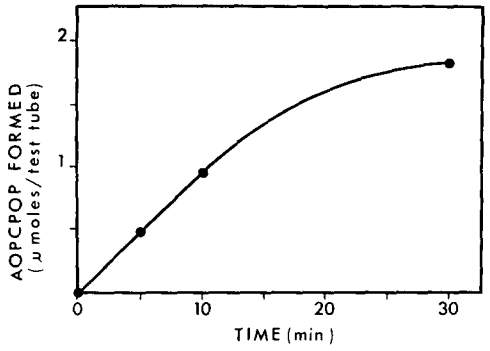


Fig. 2. Time course of photophosphorylation of AOPCP. Each incubation mixture contained 92 μg chlorophyll and 6 μmol AOPCP in a volume of 3 ml.

could reveal 5 % or more ADP contamination in AOPCP. Furthermore, experiments of Horak and Barton [13] on the effects of AOPCP on blood platelet aggregation indicated that AOPCP (Miles Laboratories) does not contain any ADP. Their assay is very sensitive to ADP and would reveal less than 1 % of ADP contamination in AOPCP.

Approx. 30 % of the AOPCP added was phosphorylated in 30 min of incubation with chloroplasts containing 92 μg of chlorophyll (Fig. 2). Over 50 % of added AOPCP was phosphorylated in the same period by plastids containing 184 μg chlorophyll. Since such a large amount of nucleotide was phosphorylated and there was no evidence of ADP contamination of AOPCP it is evident that the nucleotide phosphorylated was AOPCP itself.

The previously reported experiments on photophosphorylation of AOPCP were all performed with plastids prepared in sucrose/TES/bovine serum albumin medium and stored in liquid nitrogen [3, 4]. Such chloroplasts may contain endoge-

TABLE I

PHOTOPHOSPHORYLATION OF ADP AND AOPCP BY VARIOUS CHLOROPLAST PREPARATIONS

The following chloroplast preparations were used: I, plastids prepared in 0.4 M sucrose containing 40 mM TES/NaOH and 0.2 % fatty acid poor bovine serum albumin at pH 8 and stored in liquid nitrogen [3]; II, plastids freshly prepared as in I; III, plastids freshly prepared as in I and washed once with 10 mM NaCl; IV, plastids prepared and stored as in I, but after thawing dialyzed for 20 h against two changes of 10 mM NaCl and acid-washed Norit charcoal (1 g/l).

Chloroplast preparation	P_i esterified (μmol/mg chlorophyll per h)		$\frac{\text{AOPCP}}{\text{ADP}}$ ratio
	ADP	AOPCP	
I	670	68	0.101
II	650	75	0.115
III	830	84	0.101
IV	199	24	0.121

TABLE II

EFFECT OF ADP ON PHOTOPHOSPHORYLATION OF AOPCP BY DIALYZED PLASTIDS

Chloroplast preparation IV (Table I) was used. Chloroplasts contained 55 μg chlorophyll. The volume was 3 ml and the incubation time 5 min.

Nucleotide added	P_i esterified (nmol/test tube)
1. 2 mM ADP	910
2. 2 mM AOPCP	109
3. 10 μM ADP	28
4. 2 mM AOPCP + 10 μM ADP	136
4 minus 3	108

nous nucleotides which could be phosphorylated first. In a subsequent reaction, these phosphorylated nucleotides could phosphorylate AOPCP. The latter reaction might even be catalyzed by an enzyme not associated with photosynthetic lamellae.

To determine if endogenous nucleotides were involved in the phosphorylation of AOPCP we used a variety of spinach chloroplast preparations and followed the photophosphorylation of ADP and AOPCP by them (Table I). Washing of plastids with 10 mM NaCl should have removed free endogenous nucleotides and enzymes not associated with chloroplast lamellae. However, there was no decrease in phosphorylation of AOPCP (preparation III). Furthermore, even 20 h of dialysis against 10 mM NaCl and charcoal (preparation IV) did not eliminate the ability of the plastids to phosphorylate AOPCP. The AOPCP to ADP ratio remained fairly constant in all these types of chloroplast preparations suggesting that AOPCP is phosphorylated directly by the photosynthetic membranes and the reaction is not mediated by endogenous nucleotides. This finding was substantiated by the observation that the addition of low concentrations of ADP (10 μM) did not stimulate photophosphorylation of AOPCP (Table II).

TABLE III

EFFECT OF HEXOKINASE ON PHOTOPHOSPHORYLATION OF ADP AND AOPCP

Details of the experiment are given in Materials and Methods.

Nucleotide	Hexokinase	P_i esterified (μmol/mg chlorophyll per h)		
		Organic ^{32}P	^{32}P-labeled nucleotide triphosphate	glucose 6-[^{32}P]phosphate
–	–	0	2	0
–	+	1	0	0
ADP	–	696	692	9
ADP	+	889	7	868
AOPCP	–	73	69	0
AOPCP	+	74	65	2

TABLE IV

EFFECT OF METHYLENE ANALOGS OF ATP ON PHOTOPHOSPHORYLATION OF ADP AND AOPCP

Chloroplasts (38 μg chlorophyll) were incubated in a volume of 1 ml. Concentration of all nucleotides was 2 mM. Figures in parentheses indicate percent activity of ADP phosphorylation.

Nucleotide	P_i esterified (μmol/mg chlorophyll per h)	Activity (%)
ADP	551	100
ADP+AOPCPOP	537	97
ADP+AOPOPCP	590	107
AOPCP	61	100 (11)
AOPCP+AOPCPOP	38	62 (7)
AOPCP+AOPOPCP	39	64 (7)

The conclusion that photophosphorylation of AOPCP is direct, rather than mediated by free endogenous nucleotides was further supported by the experiments in which photophosphorylation of ADP and AOPCP was carried out in the presence of excess hexokinase and glucose (Table III). When ADP was used as substrate in the presence of hexokinase and glucose the esterified ^{32}P was found in glucose 6-phosphate. In contrast when AOPCP was used as substrate, the ^{32}P label remained in the nucleotide fraction. This indicated that photophosphorylation of AOPCP was not mediated by free endogenous ATP since in such a case the hexokinase would have trapped the ^{32}P label in glucose 6-phosphate. Unlike ATP, AOPCPOP could not serve as a substrate in the hexokinase reaction (we observed no NADP reduction with hexokinase and glucose 6-phosphate dehydrogenase in presence of AOPCPOP) and the ^{32}P label therefore stayed in AOPCPOP.

From these results it appeared that phosphorylation of AOPCP may occur in the same manner and at the same site as that of ADP. As a further test of this the sensitivity of photophosphorylation of ADP and AOPCP to inhibition by α, β- and β, γ-methylene analogs of ATP was studied. It can be seen from Table IV that photophosphorylation of AOPCP was inhibited equally by both methylene analogs of ATP, resulting in about 30–40 % inhibition when their concentration was 2 mM. At the same concentration, there was no inhibition of ADP phosphorylation. This could be due to the higher affinity of ADP to the photophosphorylation system [4] or due to a difference in the sites of interaction of the methylene analogs and of ADP with the chloroplasts.

ATP-P_i exchange

It is assumed that the light- and dithiothreitol-activated ATP-P_i exchange in chloroplasts represents a partial reaction of photophosphorylation [8]. Since AOPCP serves as a substrate in the photophosphorylation reaction, one could expect the product of this phosphorylation, AOPCPOP, to be able to take part in an AOPCPOP-P_i exchange reaction. The β, γ-analog AOPOPCP, on the other hand, should not take part in the exchange reaction due to the stability of the P-C-P bond. It could, however, act as an inhibitor of the AOPCPOP-P_i and ATP-P_i exchange. Results in

TABLE V

EFFECT OF METHYLENE ANALOGS OF ATP ON THE ATP-$^{32}P_i$ EXCHANGE REACTION

The concentration of all nucleotides was 2 mM. Figures in parentheses indicate percent activity of ATP-P_i exchange.

Nucleotide	P_i exchanged (μmol/mg chlorophyll per h)	Activity (%)
ATP	13.6	100
ATP+AOPCPOP	14.1	104
ATP+AOPOPCP	13.9	102
AOPCPOP	0.16	100 (1.2)
AOPCPOP+AOPOPCP	0.05	31 (0.4)
AOPOPCP	0	

Table V showed that the exchange activity with AOPCPOP was only about 1 % of the activity with ATP. This weak exchange was inhibited by AOPOPCP which itself did not react in the exchange reaction at all. Neither of the methylene analogs had any effect on the ATP-P_i exchange. As in the case of photophosphorylation, this could be due to lower affinity of the analogs to the sites involved in the exchange or due to an interaction with a site different from the site for ATP.

ATPase activity

Another chloroplast activity which seems to be closely related to the photophosphorylation process is the light- and dithiothreitol-activated ATPase [8]. We, therefore, tested whether the ATP analog AOPCPOP could serve as a substrate in this reaction. The results are shown in Table VI. The hydrolysis of ATP was five times faster after activation by light and dithiothreitol than in the dark and it was inhibited strongly by the energy transfer inhibitor Dio-9. In contrast, the hydrolysis of AOPCPOP was neither stimulated by light and dithiothreitol nor inhibited by Dio-9.

TABLE VI

ATPase ACTIVITY WITH ATP AND AOPCPOP

Chloroplasts containing 58 and 115 μg chlorophyll were used in experiments with ATP and AOPCPOP, respectively. Figures in parentheses indicate percent activity of the light- and dithiothreitol-activated hydrolysis of ATP.

Substrate	Conditions	P_i released (μmol/ml chlorophyll per h)	Activity (%)
ATP	Light, dithiothreitol	35	100
ATP	Light, dithiothreitol, Dio-9 (2.9 μg/ml)	3.5	10
ATP	Dark, no dithiothreitol	6.5	19
AOPCPOP	Light, dithiothreitol	4.6	100 (13)
AOPCPOP	Light, dithiothreitol, Dio-9 (5.8 μg/ml)	4.8	104 (14)
AOPCPOP	Dark, no dithiothreitol	4.6	100 (13)

In view of these differences it was unclear whether the hydrolysis of AOPCPOP was catalyzed by the coupling factor/ATPase which is known to be responsible for the light- and dithiothreitol-activated ATPase of chloroplasts [8]. Therefore an experiment to find out whether the coupling factor/ATPase can catalyze hydrolysis of AOPCPOP was conducted.

It was shown previously [14] that the coupling factor/ATPase present in EDTA extracts of chloroplasts can be localized on polyacrylamide disc electrophoresis gels by staining specifically for ATPase activity. Because crude EDTA extracts of plastids might contain besides the coupling factor other enzymes capable of AOPCPOP hydrolysis, demonstration of AOPCPOP hydrolysis by this staining technique was used rather than measuring its hydrolysis directly in crude EDTA extracts. It was found that the enzyme was capable of hydrolyzing AOPCPOP

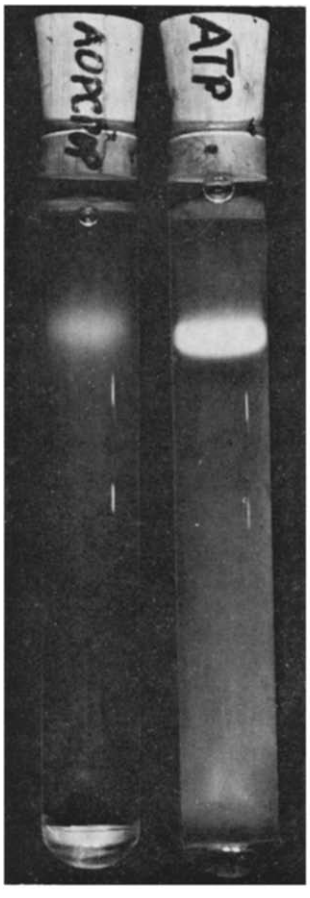


Fig. 3. ATPase activity in EDTA extracts of chloroplasts electrophoresed on polyacrylamide gels. Chloroplasts were washed twice with 10 mM NaCl to remove soluble proteins and the ATPase was extracted by 0.75 mM EDTA [8]. The EDTA extract was concentrated, electrophoresed and stained for ATPase activity as described previously [14] using 5 mM ATP or AOPCPOP as substrate. The gels were photographed after 6 days in the staining solution.

(Fig. 3). However, this reaction was much slower than that with ATP. The ATPase band was discernible after about 1 h in the staining solution containing ATP. In contrast with AOPCPOP the band became visible only the second day after the staining was started Nevertheless, the results indicated that AOPCPOP can interact directly with the coupling factor/ATPase.

DISCUSSION

It has been shown that chloroplasts are able to photophosphorylate a whole range of nucleotide diphosphates including ADP, GDP, IDP, UDP and CDP [5], as well as the fluorescent ADP analog ethenoadenosine diphosphate [15]. All of these nucleotides are modified in the heterocycle moiety when compared to ADP. Our observation that chloroplasts can also phosphorylate AOPCP extends the specificity range of the photophosphorylation reaction to a nucleotide modified in the diphosphate side chain.

It is still not clear whether the phosphorylation of ADP analogs proceeds in the same manner as that of ADP. To resolve this the following three alternatives need to be examined. Firstly, the analogs are phosphorylated directly by chloroplasts without involvement of endogenous adenine nucleotides. Secondly, the analogs are phosphorylated via adenine nucleotides which are firmly bound to the photosynthetic membranes and act as coenzymes in phosphorylation. Thirdly, the analogs are phosphorylated via free endogenous adenine nucleotides in a reaction catalyzed by nucleotide diphosphokinase.

At present the last of these three alternatives has been eliminated. Thus phosphorylation via free endogenous adenine nucleotides can be excluded in the case of AOPCP (Tables I–III), GDP [5] and probably also IDP, UDP and CDP [5]. The resolution of the other two alternatives is more difficult. Should AOPCP be phosphorylated directly at the active site of the coupling factor protein, then the proposed transphosphorylation reaction involving two ADP molecules [1, 2] could be ruled out. On the other hand, should the phosphorylation of AOPCP occur only via firmly bound adenine nucleotides, the involvement of a transphosphorylation mechanism in photophosphorylation will still not be resolved.

Adenine nucleotides have been shown to bind strongly to the mitochondrial and chloroplast coupling factor [1, 16, 17] and evidence has been presented for their involvement in phosphorylation of substrate ADP [18, 19]. Direct interaction of AOPCP with the spinach chloroplast coupling factor was observed by Girault et al. [20] using circular dichroism. In our experiments, direct interaction of AOPCPOP with the coupling factor/ATPase was suggested by its hydrolysis on polyacrylamide gels (Fig. 3).

In contrast to photophosphorylation of the ADP analog, the ATP analog AOPCPOP did not resemble ATP in its interaction with the ATP-P_i exchange and the light- and dithiothreitol-activated ATPase systems of spinach chloroplasts (Tables V and VI). It is interesting to compare these results to those obtained by Sahak et al. [15] with the ethenoadenosine analogs. They observed that ethenoadenosine diphosphate was a good substrate for photophosphorylation while ethenoadenosine triphosphate was a poor substrate for the ATPase and an even worse substrate for the exchange reaction. Results of both studies suggest that the sites involved in photo-

phosphorylation might be different from those involved in the ATP-P_i exchange and in ATPase activity.

ACKNOWLEDGEMENTS

This research was supported by a grant to Saul Zalik from the National Research Council of Canada. We would like to thank Mr. Barry Zytaruk for preparing the figures.

REFERENCES

1 Roy, H. and Moudrianakis, E. N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 464–468
2 Roy, H. and Moudrianakis, E. N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2720–2724
3 Horak, A. and Zalik, S. (1974) Nature 249, 858–860
4 Horak, A. and Zalik, S. (1974) Proc. 3rd Int. Congr. Photosynthesis (Avron, M., ed.), pp. 1067–1072, Elsevier, Amsterdam
5 Bennun, A. and Avron, M. (1965) Biochim. Biophys. Acta 109, 117–127
6 Nielsen, S. O. and Lehninger, A. L. (1955) J. Biol. Chem. 215, 555–570
7 Shavit, N. (1972) in Methods in Enzymology (San Pietro, A., ed.), Vol. XXIV, pp. 318–321, Academic Press, New York
8 McCarty, R. E. and Racker, E. (1968) J. Biol. Chem. 243, 129–137
9 Horak, A. and Hill, R. D. (1971) Can. J. Biochem. 49, 207–209
10 MacKinney, G. (1941) J. Biol. Chem. 140, 315–322
11 Myers, T. C., Nakamura, K. and Flesher, J. W. (1963) J. Am. Chem. Soc. 85, 3292–3295
12 Randerath, K. and Randerath, E. (1967) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. XII, part A, pp. 323–347, Academic Press, New York
13 Horak, H. and Barton, P. G. (1974) Biochim. Biophys. Acta 373, 471–480
14 Horak, A. and Hill, R. D. (1972) Plant Physiol. 49, 365–370
15 Sahak, Y., Chipman, D. M. and Shavit, N. (1973) FEBS Lett. 33, 293–296
16 Hilborn, D. A. and Hammes, G. G. (1973) Biochemistry 12, 983–990
17 Harris, D. A., Rosing, J., Van De Stadt, R. J. and Slater, E. C. (1973) Biochim. Biophys. Acta 314, 149–153
18 Cross, R. L. and Boyer, P. D. (1973) Biochem. Biophys. Res. Commun. 51, 59–66
19 Lutz, H. U., Dahl, J. S. and Bachofen, R. (1974) Biochim. Biophys. Acta 347, 359–370
20 Girault, G., Galmiche, J. M., Michel-Villaz, M. and Thiery, J. (1973) Eur. J. Biochem. 38, 473–478