Effects of Purine Nucleotides on Photosynthetic Electron Transport in Isolated Chloroplasts

Takao Yagi and Yasuo Mukohata

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Japan 560

Abstract

The effects of guanylates and inosinates (and adenylates) on phosphorylation, ferricyanide reduction, and light-induced H^+ uptake in spinach chloroplasts were studied. GDP, GTP, IDP, and ITP (but not GMP and IMP) stimulated the light-induced H^+ uptake and partially inhibited ferricyanide reduction. Phosphate, arsenate, and phlorizin increased the extent of inhibition by these nucleotides and decreased the values of their apparent dissociation constants for the inhibition process. In the presence of phosphate (or arsenate), restoration of ferricyanide reduction from the level inhibited by guanylates and inosinates was observed as phosphorylation (or arsenylation) proceeded. These results suggest that phosphorylation of GDP and IDP as well as ADP takes place after two steps of nucleotide binding to the chloroplast coupling factor 1. The apparent dissociation constants of GDP and IDP for these two binding steps were estimated to be about 34 and 38 µM for the first and 110 and 160 µM for the second step, respectively (at pH 8.3, 15°C). Above pH 9, the ratio ($P/\Delta e$) of the extent of phosphorylation to the increment of electron transport from the basal level measured in the presence of [ATP + Pi] or [ADP + Pi + phlorizin], became increasingly large. When the electron transport level inhibited by dicyclohexyl-carbodiimide was taken to be the basal activity, the $P/\Delta e$ ratio remained almost constant (~ 1) from pH 7.0 up to 10.

Introduction

We have reported that the regulation of electron transport and phosphorylation by adenylates in isolated spinach chloroplasts could be

interpreted by the sequential binding to two kinds of sites on CF_1* [1]. As ADP (or ATP) binds to the site of higher binding affinity on CF_1 (the inhibition site), a conformation change of CF_1 [2] is induced, which reduces leakage of H^+ through CF_1. This results in stimulation of the light-induced H^+ uptake [2] and inhibition of electron transport to the basal level [1]. The electron transport is then restored when phosphorylation or arsenylation turns over after another ADP binds to the other site (the coupling site) in the presence of Pi or As [1, 3–5].

GDP and IDP as well as ADP can be a substrate of photophosphorylation [6–8]. However, inhibition of Fecy reduction [1, 9] and stimulation of light-induced H^+ uptake [2] were not observed with the addition of GDP [9], GTP [2], IDP [1], or ITP [2]. If the two-step binding of nucleotides to CF_1 is found in the phosphorylation mechanism of every nucleotide diphosphate, these reports are contradictory. We conducted a detailed survey comparing the regulation of electron transport and phosphorylation by guanylates, inosinates, and adenylates.

Materials and Methods

Chloroplasts were prepared from market spinach leaves in a choline medium by a method described previously [10, 11]. The reaction mixture for Fecy reduction was composed of 0.1 M sucrose, 5 mM $MgCl_2$, 10 mM tricine (pH 8.3), 600 µM Fecy, and chloroplasts equivalent to 20 µg/ml in the chlorophyll concentration determined by the method of Arnon [12]. Nucleotides, Pi, As, phlorizin, and DCCD were added to the reaction mixture as required.

Fecy-reducing activity was calculated from the absorbance difference at 420 nm before and after actinic illumination (5×10^4 lux, white light) for a few minutes. The amount of ^{32}Pi esterified during Fecy reduction was determined by the method of Asada et al. [13] with slight modification.

In the pH-dependence experiments, a mixed buffer composed of citrate, piperazine-N-N'-bis(2-ethane sulfonic acid), tricine and sarcosine (10 mM each) was used instead of a single tricine buffer.

In the experiments for the light-induced H^+ uptake, an arrangement of a pH meter (2 pH full scale) and a recorder (0.3 pH full scale on a recorder chart) was used with actinic light (> 500 nm) from a 750-W projector lamp. The reaction mixture was composed of 0.1 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 20 µM PMS, and chloroplasts of 50 µg/ml chlorophyll con-

---

* Abbreviations: CF_1: chloroplast coupling factor 1; As: arsenate; Fecy: ferricyanide; tricine: tris(hydroxymethyl)methylglycine; DCCD: dicyclohexylcarbodiimide; PMS: phenazine methosulfate.

centration. Nucleotides and Pi were added as required. The initial pH of the reaction mixture in the dark was adjusted to 7.3 ± 0.03 with NaOH.

All experiments were carried out at $15 \pm 0.1\,°C$.

Results and Discussion

Figure 1 shows the effects of guanylates on Fecy reduction in isolated chloroplasts. With an increase in GDP or GTP concentration above 10 μM, Fecy-reducing activity was gradually inhibited and reached the lowest level at around 300 μM. The inhibition profiles with IDP and ITP were similar to those with GDP and GTP (data not shown). The percentage inhibition was about 16% for GDP and GTP and 27% for IDP and ITP. These values are considerably smaller than those for ADP and ATP (about 50% [1]). The values of the apparent dissociation constant (K_D^{app}) for the inhibition process of GDP (and GTP) and IDP (and ITP) were about 120 and 170 μM, respectively. GMP (>about 20 μM) and IMP (>about 20 μM; not shown) depressed Fecy-reducing activity to a smaller extent than GDP and GTP (or IDP and ITP).

Light-induced H^+ uptake by chloroplasts is stimulated by ADP and ATP [2, 14], but not by GDP, GTP, IDP, or ITP [2]. However, as shown in Fig. 2, when the concentration of one of these nucleotides increased, the light-induced H^+ uptake was stimulated. Therefore, these guanylates and inosinates as well as adenylates can be considered to bind to CF_1 and change the conformation of CF_1 [2] to depress H^+ leakage through CF_1 [14]. The K_D^{app} values of these nucleotides were about 50 μM for GDP and
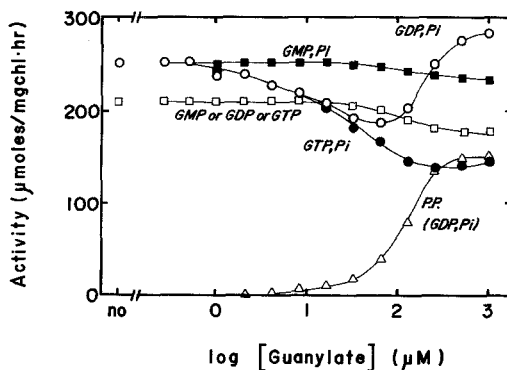

Figure 1. Effects of guanylates on Fecy-reducing activity in isolated chloroplasts (pH 8.3, 15°C). The phosphorylation activity with GDP (△, P.P.) is also shown. Pi concentration was 1 mM.
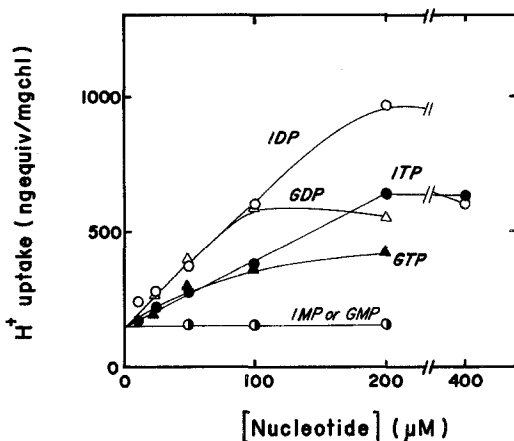
Figure 2. Effects of nucleotides on light-induced H$^+$ uptake (15°C). For details, see Materials and Methods.

GTP, 70 µM for IDP, and 90 µM for ITP. These results differed from the negative results obtained by McCarty et al. [2], who were unable to detect such stimulation, probably because they used these nucleotides at 10 µM in their experiments.

The difference in the K_D^{app} values estimated from the inhibition of Fecy reduction (at pH 8.3: Fig. 1 and not shown) and from the stimulation of H$^+$ uptake (at pH 7.3: Fig. 2) can be roughly interpreted as the decrease of K_D^{app} with pH decrease (see Fig. 4).

Pi (1 mM) stimulated Fecy-reducing activity about 16% in the absence of nucleotide (Fig. 1), as reported by Avron et al. [15]. On the contrary, Pi (or As, 1 mM) increased the extent of inhibition of Fecy reduction (ΔFecy$_{inhibited}$) not only by ATP [1, 16], but also by GTP and ITP, while Pi did not enhance the depression of Fecy reduction by GMP or IMP (Table I). The percentage inhibition in the presence of Pi became over 40% for GTP and ITP, the order of effectiveness of Pi (and As) usually being GTP>ITP>ATP. As shown later in Fig. 4, Pi (and As) decreased the K_D^{app} values for the inhibition by these nucleotides and increased the values of the apparent Hill constant. Pi also enhanced the percentage stimulation of the light-induced H$^+$ uptake by GTP and ITP (200 µM each) about 1.3 times. In the presence of GMP or IMP, Pi could not stimulate the light-induced H$^+$ uptake (data not shown). These results may suggest that Pi (and As) is a kind of allosteric effector (when the other effector [16], nucleotide di- or tri-phosphate is present), for the conformation change of CF$_1$ in the inhibition process, although many factors in thylakoids are

influenced by Pi and induce the same changes in K_D^{app} and the Hill constant.

As shown in Table I, phlorizin as well as Pi increased $\Delta Fecy_{inhibited}$ with GDP, GTP, IDP, or ITP. Phlorizin did not affect Fecy-reducing activity depressed by GMP, IMP, and AMP (data not shown). Since phlorizin and Pi compete in phosphorylation, although the inhibition by phlorizin is not perfectly competitive [17], they may have the same role in the enhancement of $\Delta Fecy_{inhibited}$. However, the K_D^{app} value for ITP, IDP, or GDP in the presence of phlorizin differed from that with Pi. In the presence of phlorizin, the K_D^{app} value for GDP differed from that for GTP. The latter difference disappeared when 1 mM Pi was added. These phenomena have not been understood completely but can be explained partially by the anomalous nature of phlorizin [18].

Avron et al. [15] and McCarty et al. [2] reported that the apparent inhibition by ADP may actually be caused by ATP formed through phosphorylation of added ADP. The same argument can be given in the case of GDP or IDP. However, the inhibition profile by ADP, GDP, or IDP with both phlorizin (1 mM) and Pi (or As, 1 mM) was coincident with that obtained with the corresponding triphosphate with Pi (1 mM) (data not shown). Even under the arsenylation condition where the product is hydrolyzed as soon as it is formed [1, 19], GDP and IDP as well as ADP inhibited Fecy reduction at a concentration as low as 30 μM. Furthermore, $1,N^6$-etheno ADP, which cannot be a substrate of adenylate kinase [20], also inhibited Fecy reduction [16]. Therefore, we conclude that electron

TABLE I. Comparison of the effects of phlorizin and Pi[a] on the inhibition processes by nucleotides at pH 8.3 (15°C)

	Maximum inhibition (%)			K_D^{app} (μM)		Apparent Hill constant	
	No addition	Phlorizin	Pi	Phlorizin	Pi	Phlorizin[b]	Pi
GMP	10	11	10	—	—	—	—
GDP	16	35	46[c]	95	34[c]	0.8	1.1[c]
GTP	16	33	46	35	34	0.8	1.1
IMP	10	9	10	—	—	—	—
IDP	27	40	42[c]	60	38[c]	0.6	1.1[c]
ITP	27	40	42	60	38	0.6	1.2
ATP	48	42	65	1.0	1.6	0.8	1.3

[a] Concentrations of phlorizin and Pi were 1 mM.
[b] Values of apparent Hill constants were much smaller with phlorizin than with Pi.
[c] In the presence of both phlorizin and Pi.

transport is inhibited not only by these nucleotide triphosphates but also by the nucleotide diphosphates.

Nucleotide monophosphates, GMP and IMP (and AMP), did not stimulate the light-induced H$^+$ uptake (Fig. 2) and did not affect the inhibition process by nucleotide di- or tri-phosphates. These monophosphates may not bind to CF$_1$. The mechanism of their apparent depression of Fecy reduction (Fig. 1) would be different from that by the di- and tri-phosphates. Another difference was the absence of Pi- (and phlorizin-) enhancement of electron transport depression by monophosphates (Table I). We had reported [1] that IDP could not regulate electron transport since the observed inhibition profile of IDP was identical to the depression profile of AMP. We correct ourselves here since stimulation of the light-induced H$^+$ uptake and phlorizin-enhanced electron transport inhibition by IDP have now been observed.

Figure 3 shows the inhibition profiles by ATP in the presence of Pi (1 mM) at various pH values. Figure 4 shows the pH dependence of the apparent Hill constants and the K_D^{app} values for the inhibition processes by ATP, ITP, and GTP with or without Pi (1 mM) (from Fig. 3 and data not shown) according to the procedure described previously [1]. The pH dependence of K_D^{app} of ADP is similar to that of ATP (data not shown). At pH 8.3, the K_D^{app} values of GDP (and GTP, about 34 μM) and IDP (and ITP, about 38 μM) were about 20 times greater than that of ADP (and ATP, about 1.8 μM) in the presence of Pi (or As). As shown in Fig. 4, the profiles of K_D^{app} with and without Pi parallel each other, suggesting that the dissociation of H$^+$ from Pi is only slightly responsible for the pH dependence of K_D^{app}.
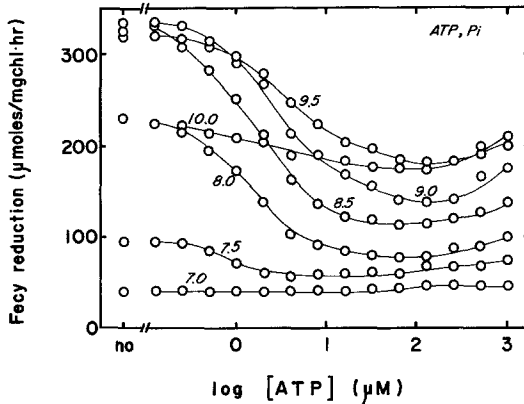

Figure 3. Effects of ATP on Fecy-reducing activity at various pH values in the presence of Pi (1 mM, 15°C). The numerals show the pH values of the reaction mixture.
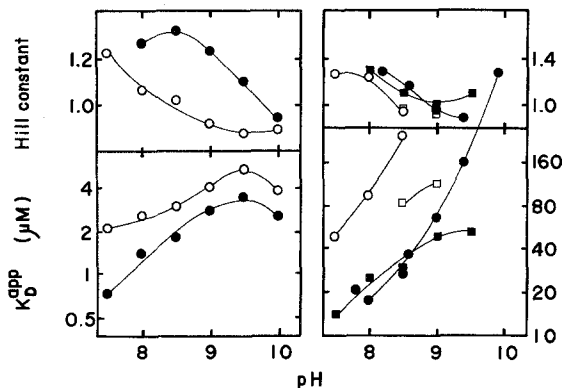
Figure 4. pH Dependence of K_D^{app} values and apparent Hill constants for the inhibition processes by nucleotides with or without Pi (1 mM, 15°C). (A) In the presence of O: ATP only, ●: both ATP and Pi. (B) In the presence of O: ITP only, ●: both ITP and Pi, □: GTP only, ■: both GTP and Pi.

When the GDP (or IDP) concentration increased in the presence of Pi (1 mM), Fecy reduction was inhibited at about 1 μM then restored at concentrations higher than 40 μM and became maximum at around 1 mM as shown in Fig. 1. The restoration profile nearly paralleled the profile of phosphorylation activity. As described previously [1], the true initial rates of Fecy reduction under phosphorylation condition are higher than the activities calculated from the absorbance difference before and after actinic illumination (due to ADP shortage during illumination). The rates under arsenylation condition where the product was hydrolyzed immediately to regenerate ADP could be substituted for the true initial rates for phosphorylation conditions [1, 19]. The apparent dissociation constant of GDP and IDP for the restoration process (K_m^{app}) was thus determined under arsenylation condition (1 mM As) with and without 1 mM phlorizin, which was used to determine the activity of basal electron transport [17]. At pH 8.3, the K_m^{app} values for GDP (about 110 μM) and IDP (about 160 μM) were about ten times greater than those for ADP (about 14 μM). These results, together with the difference in K_D^{app} shown above, imply that the binding affinity of nucleotides to both inhibition and coupling sites [1] in CF$_1$ greatly decreases when the amino group in the 6 position of the purine ring of ADP and ATP is substituted by a hydroxyl group. A similar trend was found with 1,N^6-etheno adenylates [16]. However, the K_m^{app} values obtained with Swiss chard chloroplasts by Bennun and Avron [7] were 60 and 70 μM for ADP and GDP, respectively. Such disagreement may be ascribed to plant species.
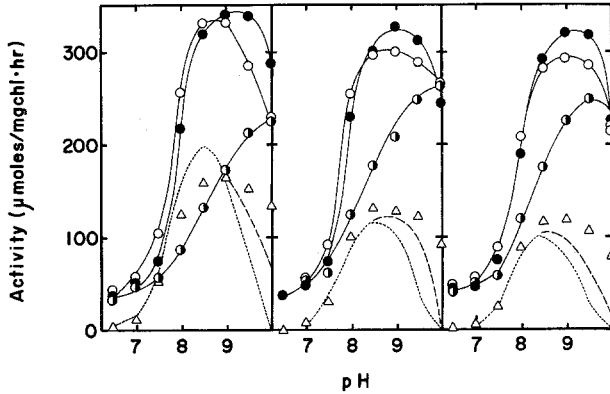
Figure 5. pH Dependence of Fecy-reducing and phosphorylation activities in isolated chloroplasts (15°C). (A) ●: the level in the presence of Pi (1 mM) only, $Fecy_{Pi}$; ◐: the maximal inhibition level in the presence of both ATP (300 μM) and Pi (1 mM), $Fecy_{ATP,Pi}$; ○: the level in the presence of ADP (1 mM) and Pi (1 mM), $Fecy_{ADP,Pi}$; ———: $\Delta Fecy_{inhibited} = Fecy_{Pi} - Fecy_{ATP,Pi}$; -----: $\Delta Fecy_{coupled} = Fecy_{ADP,Pi} - Fecy_{ATP,Pi}$; △: phosphorylation activity in the presence of ADP (1 mM) and Pi (1 mM).
(B), (C) These activities were measured as in Fig. 5A, except adenylates were replaced by inosinates (B) or guanylates (C).

Figures 5A, B, and C show the pH dependence of the maximum values of $\Delta Fecy_{inhibited}$ with ATP, ITP, or GTP in the presence of Pi (1 mM), $\Delta Fecy_{coupled}$ with ADP, IDP, or GDP (+1 mM Pi), and the phosphorylation activity. It was reported previously [1] for adenylates that $\Delta Fecy_{inhibited}$ and $\Delta Fecy_{coupled}$ paralleled the phosphorylation activity up to around pH 9. As shown in Fig. 5, at pH higher than 9, the phosphorylation activity decreased slowly but both $\Delta Fecy_{inhibited}$ and $\Delta Fecy_{coupled}$ decreased very sharply. Avron [21] claimed that the extent of phosphorylation-coupled electron transport could not be the $\Delta Fecy_{coupled}$ $(=\Delta e)$ that Izawa et al. [17] defined with phlorizin (and we with ATP + Pi [1]), since at pH higher than 9, the ratio P/e_2 $(=2P/\Delta e$ in our notation) became absurdly large.

Intuitively, above pH 9 the maximum inhibition level by ATP and Pi seemed not to show the true basal level. Since the pH dependence of the inhibition by [ADP + Pi + phlorizin] was roughly identical with that by [ATP + Pi], we assume the same mechanism of inhibition in both cases. Then, at pH higher than 9, phlorizin would not depress electron transport to the true basal level either. Since phlorizin regulates neither H^+ leakage through CF_1 by itself nor electron transport [1], DCCD, another energy transfer inhibitor that inhibits H^+ leakage through CF_1 [22], was tested. As shown in Fig. 6, phosphorylation in the presence of DCCD was abolished completely, while the pH profile of Fecy reduction in the presence of
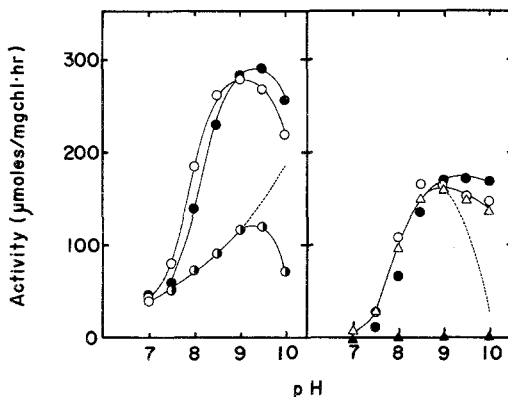
Figure 6. (A) pH Dependence of Fecy-reducing and phosphorylation activities in isolated chloroplasts (15°C). ●: Fecy reduction in the presence of Pi (1 mM) only, $Fecy_{Pi}$. ◑: Fecy reduction in the presence of DCCD (170 μM) with Pi (1 mM) and ADP (1 mM), $Fecy_{DCCD}$. This profile almost coincides with those obtained with DCCD only, DCCD + Pi, DCCD + ADP + Pi, and DCCD + ATP + Pi. ○: Fecy reduction in the presence of both ADP (1 mM) and Pi (1 mM), $Fecy_{ADP,Pi}$. ----: the maximal inhibition level in the presence of ATP and Pi (1 mM), $Fecy_{ATP,Pi}$. (B) pH Dependence of phosphorylation and the incremental activities of Fecy reduction shown in A. ▲ and △: phosphorylation activities in the presence of ADP (1 mM) and Pi (1 mM) with and without DCCD (170 μM), respectively. ●: $\Delta Fecy_{inhibited} = Fecy_{Pi} - Fecy_{DCCD}$; ○: $\Delta Fecy_{coupled} = Fecy_{ADP,Pi} - Fecy_{DCCD}$; ----: $\Delta Fecy_{inhibited} = Fecy_{Pi} - Fecy_{ATP,Pi}$.

DCCD ($Fecy_{DCCD}$) did not change even when several combinations of adenylates and/or Pi were added.

Up to around pH 9, $Fecy_{DCCD}$ agreed with $Fecy_{ATP,Pi}$, and at higher pH the former decreased steadily unlike the latter. The profiles of $\Delta Fecy_{inhibited}$ and $\Delta Fecy_{coupled}$ thus obtained with DCCD resemble that of phosphorylation fairly well (Fig. 6B). The $P/\Delta e$ value becomes roughly constant (around 1) within the pH range from 7.0 to 10, when the basal activity of Fecy reduction is represented by the activity in the presence of DCCD instead of [ATP + Pi] or [ADP + Pi + phlorizin].

References

1. Y. Mukohata, T. Yagi, Y. Sugiyama, A. Matsuno, and M. Higashida, *J. Bioenerg.*, 7 (1975) 91.
2. R. E. McCarty, J. S. Fuhrman, and Y. Tsuchiya, *Proc. Natl. Acad. Sci. USA*, 68 (1971) 2522.
3. Y. Mukohata, T. Yagi, and Y. Sugiyama, *J. Bioenerg.*, 7 (1975) 103.
4. Y. Mukohata and T. Yagi, *J. Bioenerg.*, 7 (1975) 111.
5. T. Yagi and Y. Mukohata, *J. Bioenerg.*, 8 (1976) 247.

6. M. Avron, *Biochim. Biophys. Acta,* **40** (1960) 257.
7. A. Bennun and M. Avron, *Biochim. Biophys. Acta,* **109** (1965) 117.
8. A. R. Krall and M. R. Purvis, *Arch. Biochem. Biophys.,* **94** (1961) 14.
9. P. Felker, S. Izawa, N. E. Good, and A. Haug, *Arch. Biochem. Biophys.,* **162** (1974) 345.
10. Y. Mukohata, T. Yagi, A. Matsuno, M. Higashida, and Y. Sugiyama, *Plant and Cell Physiol.,* **15** (1974) 163.
11. Y. Mukohata, T. Yagi, M. Higashida, A. Matsuno, and Y. Sugiyama, *Plant and Cell Physiol.,* **15** (1974) 619.
12. D. I. Arnon, *Plant Physiol.,* **24** (1949) 1.
13. K. Asada, M. Takahashi, and M. Urano, *Anal. Biochem.,* **48** (1972) 311.
14. A. Telfer and M. C. W. Evans, *Biochim. Biophys. Acta,* **256** (1972) 625.
15. M. Avron, D. W. Krogmann, and A. T. Jagendorf, *Biochim. Biophys. Acta,* **30** (1958) 144.
16. Y. Mukohata and Y. Sugiyama, *Plant and Cell Physiol.,* **17** (1976) 733.
17. S. Izawa, T. N. Connolly, G. D. Winget, and N. E. Good, *Brookhaven Symposia in Biol.,* **19** (1966) 169.
18. J. West and D. O. Hall, *Biochem. Soc. Trans.,* **1** (1973) 888.
19. M. Avron and A. T. Jagendorf, *J. Biol. Chem.,* **234** (1959) 967.
20. J. A. Secrist III, J. R. Barrio, N. J. Leonard, and G. Weber, *Biochemistry,* **11** (1972) 3499.
21. M. Avron, *Proceedings 2nd Internatl. Congr. Photosynthesis Res., Stresa,* G. Forti et al., Eds, pp. 861, Dr. W. Junk, N.V. Publishers, The Hague (1971).
22. R. E. McCarty and E. Racker, *J. Biol. Chem.,* **242** (1967) 3435.