THE pH DEPENDENCE OF THE PHOTOSYNTHETIC ELECTRON TRANSPORT IN GLUTARALDEHYDE-TREATED THYLAKOIDS

George C. PAPAGEORGIOU and Joan ISAAKIDOU†

Nuclear Research Center Demokritos, Department of Biology, Aghia Paraskevi, Athens, Greece

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1. Introduction

Conditions which make thylakoid membranes permeable to ions also shift the pH optimum of oxygenic Hill reactions from the alkaline region to about neutrality. According to [1,2], the breakdown of the permeability barrier exposes the H_2O splitting complex, which is accessible only from the interior, directly to the external ionic conditions. Thus, the pH dependence of the Hill reaction rate reflects the pH dependence of the H_2O splitting activity.

A chemical treatment which changes profoundly the physicochemical properties of thylakoids, yet allows substantial fractions of Hill activity to survive, is fixation with glutaraldehyde (review [3,4]). Among other effects, glutaraldehyde lowers the permeability barrier to cations, allowing a faster passive H^+ efflux from thylakoids [5], as well as the penetration of the organic cation PMS to the chl a sites [6]. The possibility that the pH dependence of oxygenic Hill reactions is also affected by glutaraldehyde is examined here.

Abbreviations: chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; FeCN, $K_3[Fe(CN)_6]$; MES, 2-(*N*-morpholino)ethane-sulfonic acid; PMS, *N*-methyl-phenazonium methosulfate; TAPS, tris-(hydroxymethyl)-methyl-amino propane sulfonate

Subscripts: e, i, and ei denote properties of the external (e) and internal (i) phases of a thylakoid suspension, and ei their difference

Superscripts: • and ° denote conditions of continuous darkness and continuous illumination

† Deceased, 16 November 1980

2. Materials and methods

Type C chloroplasts (stacked thylakoids) were prepared as in [7]. The final pellet was resuspended in 100 mM NaCl, 10 mM NaCl, 10 mM $MgCl_2$, and 50 mM tricine–NaOH (pH 7.2) at 1 mg chl/ml. Aqueous purified glutaraldehyde (Sigma grade I, 25% (v/v), A_{235}:A_{280} = 0.25) was used. The reagent concentration, determined according to [8] was found to be 3.250 M. The reagent was diluted and was mixed with chloroplasts at a ratio of 65 μmol glutaraldehyde/mg chl (molar ratio, glutaraldehyde/chl = 58). The fixation reaction was carried out at 0°C. After 5 min, the suspension was diluted 5-fold with buffer, the chloroplasts were pelleted by centrifugation and were washed once, and then they were resuspended in buffer at 1 mg chl/ml. The thylakoid volume of this preparation was completely insensitive to changes in the osmotic pressure of the suspension medium.

Oxygen evolution was measured with a concentration electrode as in [7]. The suspension media contained 200 mM sucrose and 20 mM $MgCl_2$ and were buffered with 100 mM of the following compounds: MES–NaOH, for pH 5.5–7.5; tricine–NaOH for pH 7.5–8.5; and TAPS–NaOH for pH 8.5–9.5. The reaction mixtures contained 20–30 μg chl/ml and either 2 mM FeCN (photosystem II and photosystem I acceptor), or 2 mM FeCN plus 0.6 mM *p*-phenylene diamine (photosystem II acceptor; [8]). Photosystem I activity alone was measured in terms of O_2 uptake in 3 ml samples that contained 60–90 μg chl, 10 μM DCMU, 30 μM DCIP, 2 mM Na-ascorbate and 100 μM methylviologen.

Light-induced quenching of 9-aminoacridine fluorescence was measured with a Perkin-Elmer MPF-3L spectrofluorimeter, which was fitted with a fiber

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optics actinic light attachment. The latter provided 300 W/m² of red light ($\lambda > 650$ nm) at right angles to the directions of fluorescence excitation ($\lambda = 400$ nm, $\Delta\lambda = 2$ nm, ~0.8 ω/m^2) and fluorescence detection ($\lambda = 528$ nm, $\Delta\lambda = 18$ nm). The excitation beam which emerged from the monochromator passed through a color filter (Corning CS 5.58), while another color filter (CS 4-96) guarded the entrance of the analyzing monochromator. We ascertained that the actinic light did not leak to the photomultiplier tube. At the employed [chl], the actinic light was saturating, while the weak excitation beam did not energize the chloroplasts. This was ascertained by the constancy of 9-aminoacridine fluorescence when 5 mM NH_4Cl were introduced to the suspension. The samples contained 4–32 μg chl/ml, 30 mM KCl, 5 mM $MgCl_2$, 10 μM 9-aminoacridine, 1 mM FeCN, and were buffered either at pH 6.5 with 15 mM MES–KOH, or at pH 8.5 with 15 mM tricine–KOH. The fluorescence quenching signal was corrected for membrane binding of 9-aminoacridine as in [9] by extrapolating linear concentration plots of the signal to zero chlorophyll. The equations developed in [10] were used to convert quenching to proton concentrations. The osmotic volume/sample was estimated from the molarity of the chlorophyll and the osmolarity of the suspension medium [10]. Glutaraldehyde-treated samples were assumed to have the same internal volume/sample as the respective untreated controls.

All preparations were made at 0–4°C, and all assays were performed at room temperature (23 ± 1°C); [chl] was determined as in [11].

3. Results

Fig.1 shows pH curves of the O_2 evolution rate in the presence of PD_{ox} for control (A) and glutaraldehyde-treated chloroplasts (B). Addition of 5 mM NH_4Cl narrows the pH profile of the control prepara-

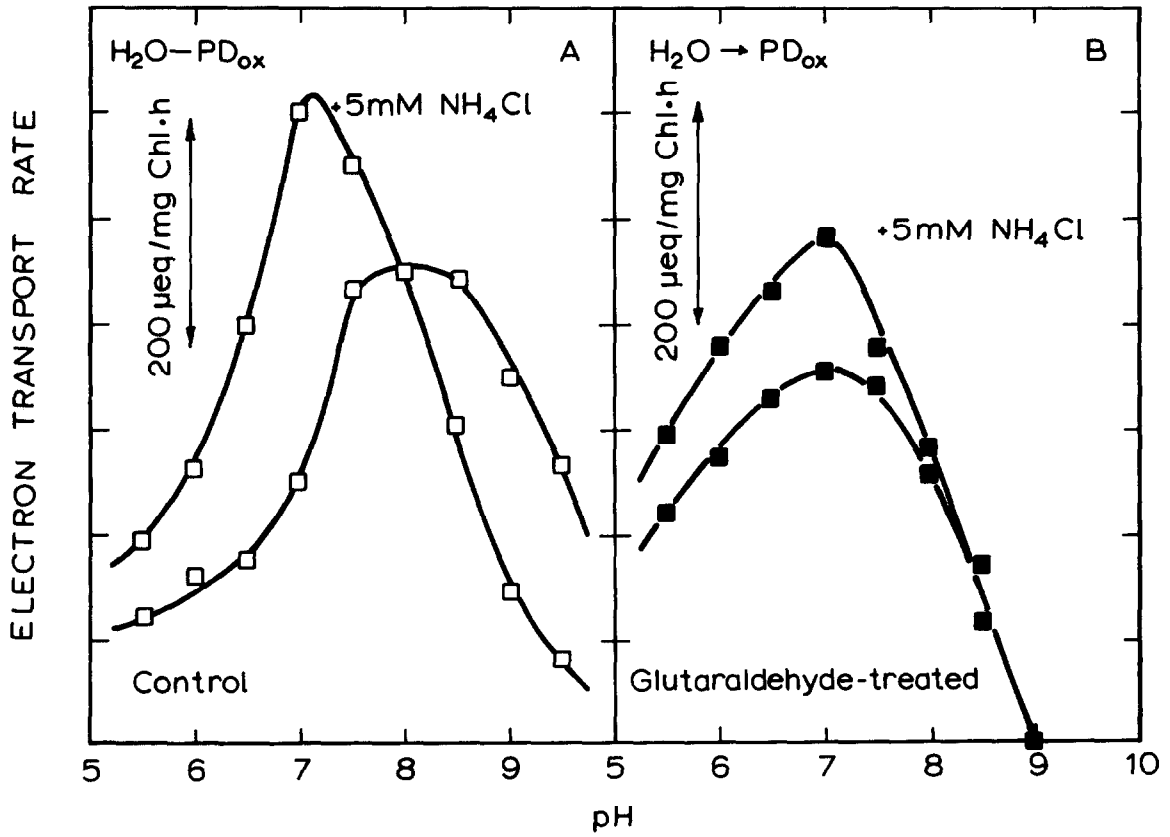

Fig.1. The pH dependence of the rate of PD_{ox}-supported photosynthetic O_2 evolution by isolated chloroplasts, in the absence and in the presence of 5 mM NH_4Cl: (A) control; (B) glutaraldehyde-treated chloroplasts.

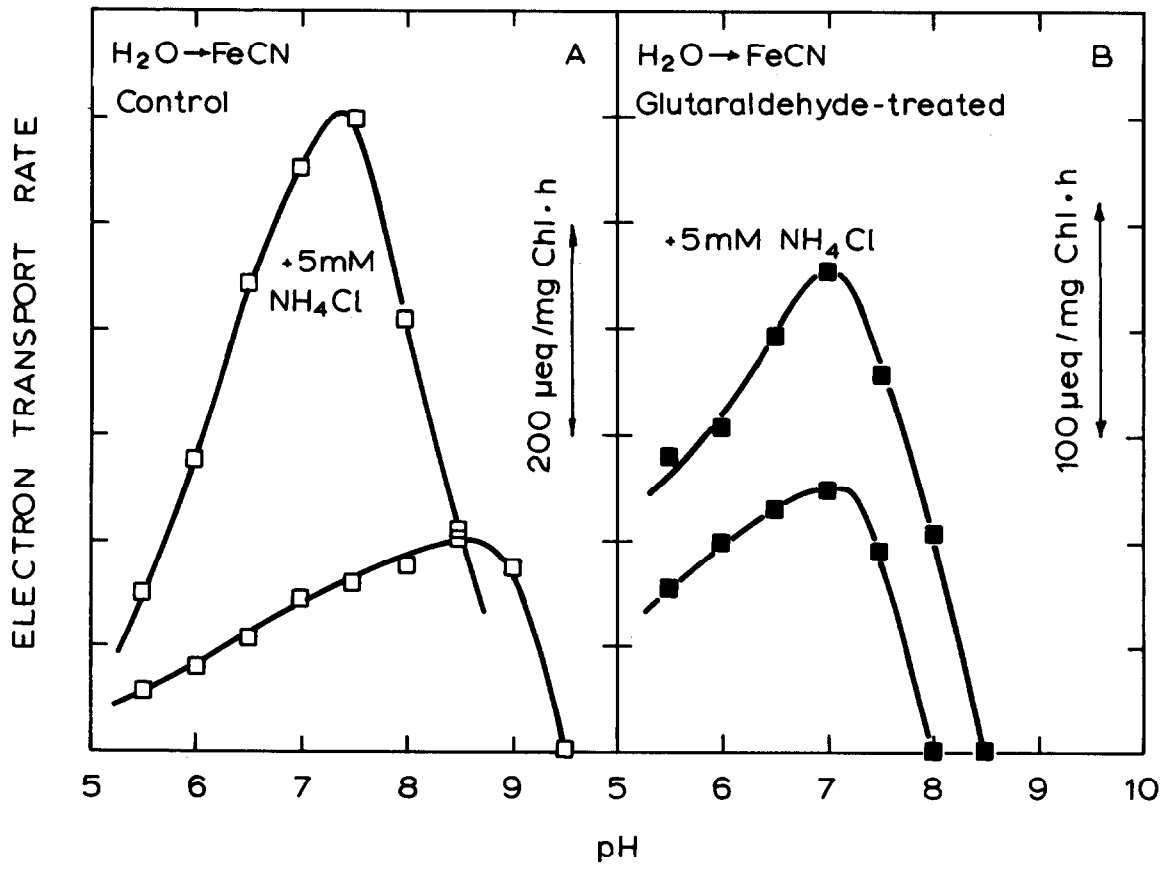

Fig.2. The pH dependence of the rate of FeCN-supported photosynthetic O_2 evolution by isolated chloroplasts, in the absence and in the presence of 5 mM NH_4Cl: (A) control; (B) glutaraldehyde-treated chloroplasts.

tion rate and shifts its optimum from pH ≃ 8 to pH ≃ 7. Maximal rate stimulation by the uncoupler (×2.4) occurs at pH 6.5–7.0. Above pH ≃ 9, the uncoupled preparation is inhibited completely. In the glutaraldehyde-treated preparation, on the other hand, the PD_{ox} Hill reaction is optimal at pH 7, both in the presence and in the absence of NH_4Cl. Maximal rate stimulation (×1.3) by the uncoupler is also at pH 7. Above pH 9, no O_2 is photoevolved.

The pH profiles of the H_2O to FeCN Hill reaction are shown in fig.2. The photoreduction of FeCN, a photosystem I acceptor primarily [2,8], is more drastically inhibited by glutaraldehyde than the photoreduction of PD_{ox} [11]. Addition of 5 mM NH_4Cl narrows the pH profile of the control preparation and shifts its optimum from pH 8.5 to pH 7.3 (A). In glutaraldehyde-treated thylakoids, however, the FeCN Hill reaction is optimal at pH 7, irrespectively of the presence of the uncoupler, while above pH ≃ 8.5 the reaction ceases altogether. Maximal rate stimulation by the uncoupler occurs at pH 7, both for the control and the glutaraldehyde-treated preparations.

In contrast to the above oxygenic Hill reactions, the methyl viologen-mediated O_2 uptake by suspensions of illuminated chloroplasts, in the presence of the O_2 evolution inhibitor DCMU, and of photosystem I electron donating couple DCIP/ascorbate, obeys an entirely different pH profile (fig.3). In the absence of NH_4Cl, optimal rates for the control (A) and the glutaraldehyde-treated chloroplasts (B) are observed at pH 8.5. A slight acid shift of the pH optimum (~0.5 units) after the addition of NH_4Cl is observed only in the glutaraldehyde-treated chloroplasts. Maximal, uncoupler-induced rate stimulation occurs at pH 8–8.5.

The observed rate stimulation in the presence of NH_4Cl suggests that glutaraldehyde-treated thylakoids

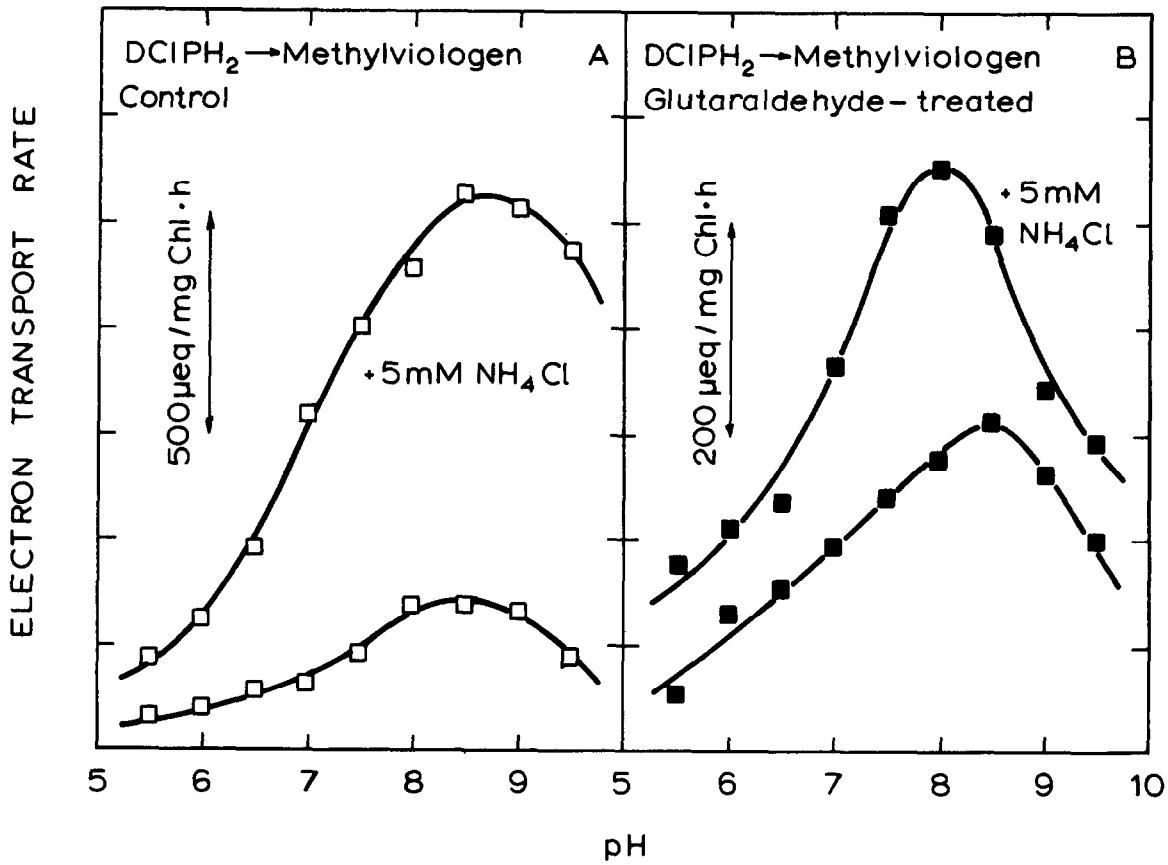

Fig.3. The pH dependence of the rate of methylviologen-mediated O_2 uptake by isolated chloroplasts, in the absence and in the presence of 5 mM NH_4Cl: (A) control; (B) glutaraldehyde-treated chloroplasts.

Table 1
Light-induced quenching[a] of 9-aminoacridine fluorescence and calculated pH changes in control and glutaraldehyde-treated chloroplasts[b] measured in the presence of 1 mM $K_3FeC(N)_6$ in media buffered at pH 6.5 and pH 8.5[c]

Chloroplast stocks	pH_e = 6.50			pH_e = 8.50		
	ϕ	ΔpH°_{ei}	pH_i	ϕ	ΔpH°_{ei}	pH_i
Control: Buffered						
at pH 6.5	0.10	2.40	4.10	–	–	–
at pH 7.2	0.10	2.39	4.11	3.55	3.95	4.55
at pH 8.5	–	–	–	3.03	3.88	4.62
Glutaraldehyde-treated:						
at pH 6.5	0.09	2.36	4.14	–	–	–
at pH 7.2	0.04	2.02	4.48	0.26	2.82	5.68
at pH 8.5	–	–	–	0.11	2.43	6.07

[a] Quenching is expressed in terms of $\phi = (F^{\bullet}:F^{\circ})-1$
[b] Glutaraldehyde:chl = 58
[c] The assay media contained KCl 30 mM, $MgCl_2$ 5 mM and were buffered either with 15 mM MES–NaOH (pH 6.5) or 15 mM tricine–KOH (pH 7.2 and pH 8.5)

are capable of creating and of maintaining a pH difference (ΔpH°_{ei}) across their membrane when they are illuminated. This was tested by means of light-induced quenching of 9-aminoacridine fluorescence by chloroplast suspensions in the presence of FeCN. Chloroplasts treated with glutaraldehyde at 3 different pH-values (6.5, 7.2, 8.5) while untreated stocks were used as controls. The quenching reaction was carried out in media buffered at pH_e 6.5 and 8.5. The results are listed in table 1. Fixed thylakoids are indeed capable of ΔpH°_{ei}, which is greater at pH_2 8.5 than at pH_e 6.5. In the case of stocks fixed and maintained at pH 7.2, the observed ΔpH°_{ei} is less by ~0.4 unit at pH_e 6.5, and by ~1.1 unit at pH_e 8.5 from those observed with the respective control chloroplasts. NH_4Cl, 5 mM, and gramicidin, 3.3 μg/ml, completely abolished the quenching in all preparations tested. Less fluorescence quenching, and correspondingly smaller ΔpH°_{ei} are calculated, as the pH of the fixation reaction is increased. Controls, from which the glutaraldehyde had been omitted, are comparatively little affected by an increase of the suspension pH.

We have also examined whether glutaraldehyde engenders similar shifts in the pH optimum of Hill reactions of unstacked thylakoids, since the organic cation PMS partitions to their chl domains more extensively than it does in the case of glutaraldehyde-treated stacked thylakoids [7,12]. Chloroplast unstacking, glutaraldehyde-treatment, and DCIP photoreduction were performed as in [7]. The results listed in table 2 show that glutaraldehyde, NH_4Cl, and gramicidin cause the same shift in the optimum pH of the DCIP photoreduction both in the stacked and in the unstacked thylakoid preparations.

Table 2
Optimal pH of the DCIP-dependent O_2 evolution in control and glutaraldehyde-treated thylakoids in the absence and presence of uncouplers

Thylakoids preparation	Additions	
	None	NH_4Cl[a] or gramicidin[b]
Control		
Stacked	8.5	7.5
Unstacked	8.5	7.5
Glutaraldehyde-treated		
Stacked	6.5	6.5
Unstacked	6.5	6.5

[a] 5 mM; [b] 4 μM

4. Discussion

We have shown here that treatment of thylakoids with glutaraldehyde causes an acid shift of the pH optimum of oxygenic Hill reactions (fig.1, 2), but no shift at all in the pH optimum of a non-oxygenic Hill reaction (fig.3). Since these phenomena parallel those caused by photophosphorylation uncouplers [1,2], they may be attributed to the same cause, namely the exposure of the H_2O splitting complex to the external pH because of easier diffusion of H^+ to the thylakoid interior. These results, therefore, provide independent support to findings for facilitated H^+ [5] and PMS [6,7] movements in glutaraldehyde-modified thylakoid membranes.

Uncouplers stimulate electron transport in glutaraldehyde-treated preparations without attendant pH optimum shifts such as those observed in the case of control preparations (table 2). This stimulation may signify the release of a high H^+ concentration which exerts a control on the rate of photosynthetic electron transport. A possible mechanism for this control can be based on the hypothesis that non-protonated intermediates are more efficient electron donors than their protonated counterparts. Accordingly, the uncoupler-effected alkalinization of the lumen shifts the equilibrium in favor of the unprotonated forms of the intermediates that operate inside the thylakoid, accelerating thus the electron transport rate [13]. However, conflicting data concerning the ability of glutaraldehyde-treated thylakoids for an electrogenic pH difference (ΔpH°_{ei}) have been reported. The sources of discrepancy can be several and they pertain both to the fixation and to the activity assays conditions. An obviously important factor is the ratio glutaraldehyde:chl. At very high molar ratios (6900, [14]), the thylakoids become inactive, at very low ratios (6, [15]) they are nearly as active as the controls, while at intermediate ratios (200–500; [5]), they preserve ~75% of their original capacity for ΔpH°_{ei}.

We employed here 9-aminoacridine as a fluorescent probe for the measurement of ΔpH°_{ei} in chloroplasts which lost the ability for osmotic volume changes after treatment with glutaraldehyde. (glutaraldehyde: chl = 58). The suitability of this probe was discussed in [16]. Since part of the fluorescence quenching signal can be caused by the binding of the probe to the membrane, we included an appropriate correction for it. According to our results (cf. table 1) osmotically immobilized thylakoids are capable of ΔpH°_{ei}, whose

magnitude depends on the pH of the fixation, and of the activity assays media. Smaller ΔpH°_{ei} is observed as the fixation pH is raised. Bacterial cell membranes [17,18] and chloroplast envelope membranes [19] are also more severely modified by alkaline glutaraldehyde. At the present, we do not know whether to ascribe the smaller ΔpH°_{ei} of alkaline-fixed chloroplasts to a reduced H^+ influx or to an accelerated H^+ efflux, or to both. Table 1 shows also a greater disparity with respect to ΔpH°_{ei} between control and glutaraldehyde fixed chloroplasts at an external pH 8.5, than at pH 6.5.

In conclusion, we may say that although glutaraldehyde has been often employed as a means of stabilizing the morphology and the activity of chloroplasts [3,4], insufficient attention has been generally paid to the optimization of the fixation and the activity assay conditions. The importance of H^+ concentration was demonstrated here.

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References

[1] Harth, E., Reimer, S. and Trebst, A. (1974) FEBS Lett. 42, 165–168.
[2] Trebst, A. (1974) Annu. Rev. Plant Physiol. 25, 423–458.
[3] Papageorgiou, G. C. (1979) in: Photosynthesis in Relation to Model Systems (Barber, J. ed) pp. 211–241, Elsevier Biomedical, Amsterdam.
[4] Papageorgiou, G. C. (1980) Methods Enzymol. 69, 613–625.
[5] West, J. and Packer, L. (1970) Bioenergetics 1, 405–412.
[6] Zilinskas, B. A. and Govindjee (1976) Z. Pflanzenphysiol. 89, 449–452.
[7] Isaakidou, J. and Papageorgiou, G. C. (1979) Arch. Biochem. Biophys. 195, 280–287.
[8] Saha, S., Ouitrakul, R. and Izawa, S. (1971) J. Biol. Chem. 246, 3204–3209.
[9] Slovacek, R. E. and Hind, G. (1981) Biochim. Biophys. Acta 635, 393–404.
[10] Haraux, F. and De Kouchkovsky, Y. (1979) Biochim. Biophys. Acta 546, 455–471.
[11] Hardt, H. and Kok, B. (1976) Biochim. Biophys. Acta 449, 125–135.
[12] Papageorgiou, G. C. and Isaakidou, J. (1978) Z. Pflanzenphysiol. 89, 449–452.
[13] Hauska, G. and Trebst, A. (1977) in: Current Topics in Bioenergetics (Sanadi, D. R. ed) pp. 151–200, Academic Press, New York.
[14] Oku, T., Sugahara, K. and Tomita, G. (1973) Plant Cell Physiol. 14, 385–396.
[15] Boardman, N. K. and Thorne, S. W. (1977) Plant Cell Physiol. spec. iss. Photosynthetic Organelles, pp. 157–163.
[16] Haraux, F. and De Kouchkovsky, Y. (1980) Biochim. Biophys. Acta 592, 153–168.
[17] Munton, J. J. and Russel, A. D. (1973) J. Appl. Bacteriol. 36, 317–
[18] Papageorgiou, G. C. and Tzani, H. (1980) J. Appl. Biochem 2, 230–240.
[19] Papageorgiou, G. C. and Karaoulani, E. (1982) Z. Pflanzenphysiol. in press.