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FURTHER STUDIES OF THE RELATIONSHIP BETWEEN CATION-INDUCED CHLOROPHYLL FLUORESCENCE AND THYLAKOID MEMBRANE STACKING CHANGES

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Summary

Salt-induced changes in thylakoid stacking and chlorophyll fluorescence do not occur with granal membranes obtained by treatment of stacked thylakoids with digitonin. In contrast to normal untreated thylakoids, digitonin prepared granal membranes remain stacked under all ionic conditions and exhibit a constant high level of chlorophyll fluorescence. However, unstacking of these granal membranes is possible if they are pretreated with either acetic anhydride or linolenic acid.

Trypsin treatment of the thylakoids inhibits the salt induced chlorophyll fluorescence and stacking changes but stacking of these treated membranes does occur when the pH is lowered, with the optimum being at about pH 4.5. This type of stacking is due to charge neutralization and does not require the presence of the 2000 dalton fragment of the polypeptide associated with the chlorophyll *a*/chlorophyll *b* light harvesting complex and known to be lost during treatment with trypsin (Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100—117).

Using the method of 9-aminoacridine fluorescence quenching it is argued that the surface charge density, on a chlorophyll basis, of unstacked thylakoid membranes is intermediate between digitonin derived granal and stromal membranes, with granal having the lowest value.

The results are discussed in terms of the importance of surface negative charges in controlling salt induced chlorophyll fluorescence and thylakoid

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Abbreviations: Chl, Chlorophyll; DCMU, 3(3′,4′-dichlorophenyl)-1,1-dimethyl urea; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; PS I, Photosystem I; PS II, Photosystem II.

stacking changes. In particular, emphasis is placed on a model involving lateral diffusion of different types of chlorophyll protein complex within the thylakoid lipid matrix.

Introduction

In attempting to give an explanation for the relationship between salt induced chlorophyll fluorescence and thylakoid stacking changes [1—5], Barber and colleagues [6—8] have put forward the idea that these effects reflect lateral movement of different types of chlorophyll-protein complexes in the lipid matrix of the membrane. It has been argued that under conditions when electrostatic screening of surface charges is poor and coulombic repulsion is at a maximum [9—11], the various chlorophyll-protein complexes (and possibly other protein complexes) are more homogeneously distributed in the membrane. Under these conditions energy transfer between different types of chlorophyll-protein complexes is high, such that chlorophyll fluorescence is at a minimum (assuming the existence of chlorophyll-protein complexes having high and low fluorescence yields) and no stacking occurs [8]. On the other hand, it is postulated that the addition of salts, which effectively screen the surface negative charges, gives rise to lateral diffusion of the various protein complexes in such a way as to create domains on the membrane surface having either low or high net surface charge densities. Membrane stacking leading to grana formation is thought to occur at the less charged or electroneutral regions due to the significant reduction in coulombic repulsion, whilst the more charged domains would resist stacking and give rise to the stromal lamellae [6—8].

Freeze fracture studies [1,12—14] do give some support to these ideas, since addition of salts has been shown to induce reorganisation of intramembrane particles by lateral diffusion. Also recently a series of experiments involving changing surface charge characteristics and lipid fluidity have given more direct evidence for the importance of lateral pigment-protein diffusion in controlling the salt-induced fluorescence and thylakoid stacking changes [8].

The concept that membrane appression and grana formation involves the lateral displacement of protein complexes which have exposed surfaces carrying different levels of net negative charge contrasts to some extent to the models of Mullet and Arntzen [15] and Gerola et al. [16]. Mullet and Arntzen advocate that stacking requires specific short range interactions between adjacent membrane surfaces involving a polypeptide exposed on the surface of the light-harvesting chlorophyll *a*/*b* protein complex. Their specific interaction type mehanism does not necessarily require lateral charge displacement and apparently occurs in such a way as to allow controlled interaction between the various pigment protein complexes to account for the changes in fluorescence yield associated with changes in spillover of energy between Photosystem II (PS II) and Photosystem I (PS I).

In this paper we continue to explore experimentally the relationship between salt-induced chlorophyll fluorescence and thylakoid stacking changes in an attempt to give more credibility to the lateral charge displacement model.

Materials and Methods

Chloroplasts were isolated from pea leaves using a grinding medium which contained 0.3 M sorbitol, 2 mM sodium iso-ascorbate, 0.05 (w/v) bovine serum albumin and 30 mM Hepes taken to pH 7.8 with KOH. Chloroplasts were pelleted and then subjected to a 10 s osmotic shock in distilled water followed by the addition of an equal volume of double-strength medium to give 0.1 M sorbitol, 1 mM HEPES, 1 mM KOH (pH 7.5, HCl). Chloroplasts were collected by centrifugation and resuspended in a medium of the same composition as above, hereafter called the 'basic' medium. When the effect of increasing monovalent cation concentration in the presence of a fixed background concentration of divalent cations was to be studied, the resuspension medium contained 0.5 mM $MgCl_2$. The chloroplast stock was kept on ice at a chlorophyll concentration of 3—5 mg/ml, and aliquots were diluted just before use.

Chlorophyll fluorescence measurements were made using suspensions consisting of the basic medium supplemented by 10 μM DCMU. The excitation light, transmitted by a Schott BG18 (2 mm) and BG38 (2 mm) was about 10 W/m^2 at the cuvette surface. Fluorescence emission was detected through a combination of a Balzer B40 693 nm interference filter and a Schott RG695 cut-off filter.

The relative degree of chloroplast thylakoid stacking or of the cohesion within granal fragments after various treatments was monitored by the effectiveness of digitonin to disrupt the membrane systems [17,18]. Samples were suspended in the basic medium supplemented with salts as indicated, the chlorophyll concentration being 0.1 mg/ml. Upon incubation at room temperature for 8—10 min, they were treated with digitonin for 60 s (4 mg digitonin/ml), subsequently diluted 20-fold with ice-cold basic medium and centrifuged at 10 000 $\times g$ and 0°C for 30 min. The percentage of chlorophyll appearing in the 10 000 $\times g$ pellet was determined and gives a relative measure of stacking. Chlorophyll concentrations were determined according to Arnon [19].

When subchloroplast fragments were to be used in further experiments, they were prepared with the same digitonin concentration and procedure as above, but the chlorophyll concentration was increased to 0.3 mg/ml. Chloroplasts were either unstacked in 10 mM KCl or stacked in 5 mM $MgCl_2$ (or 130 mM KCl) prior to digitonin treatment. In the latter case centrifugation at 10 000 $\times g$ for 30 min yielded granal fragments. In both the stacked and unstacked cases, recentrifugation of the '10 K supernatant' at 100 000 $\times g$ for 1 h yielded light fragments here referred to as the stromal fragments and fragments from unstacked chloroplasts respectively.

Fluorescence from 9-aminoacridine (20 μM) was excited using light transmitted by a Schott UG1 filter and detected at right angles to the excitation beam by a photomultiplier shielded by a Balzer B40 (498 nm) filter. The fluorescence from a suspension (containing 10 μM DCMU) was first brought to a minimum by adding about 30 μM EDTA, and then salts were added as small aliquots from stock solutions to release quenching of fluorescence [20].

Acetic anhydride treatment of chloroplasts or granal fragments was performed by a method similar to that of Prochaska and Dilley [21]. Samples were suspended in a medium at room temperature which contained 100 mM

sorbitol, 5 mM $MgCl_2$ and 30 mM Tricine brought to pH 8.5 with KOH. Acetic anhydride was added as a 1 : 1 mixture in ethanol so that the final concentration was 4 mM in the case of chloroplasts, and 2 mM in the case of granal fragments. After 30 s incubation, each mixture was diluted with ice-cold basic medium, and centrifuged. The effect of various levels of linolenic acid on granal fragments was monitored by the digitonin method outlined above after incubating the preparation with the fatty acid for 10 min.

Results

Figs. 1 and 2 show the characteristic changes in thylakoid stacking and chlorophyll fluorescence measured with stacked thylakoid membranes which were subsequently diluted into media of various levels of monovalent cations. These changes are similar to those previously reported [22—24]. The figures also show that the characteristic changes in stacking and chlorophyll fluorescence are not observed for salt-induced grana fragments obtained by the digitonin technique outlined in the Methods. Apparently the removal of stromal membranes inhibits the unstacking and associated chlorophyll fluorescence decrease seen with normal lamellae suspended in media containing low levels of monovalent cations. The reason for this could be the inability for net charge to be introduced into the appressed regions by lateral protein diffusion from stromal lamellae as suggested in our previous publications [6—8]. It is argued that this mechanism of charge injection would give rise to coulombic repulsion between the membrane surfaces and thus bring about unstacking. Indeed, as Fig. 3 shows, treatment of these granal fragments with the negatively charged fatty acid, linolenic acid, brought about unstacking as monitored by a decrease

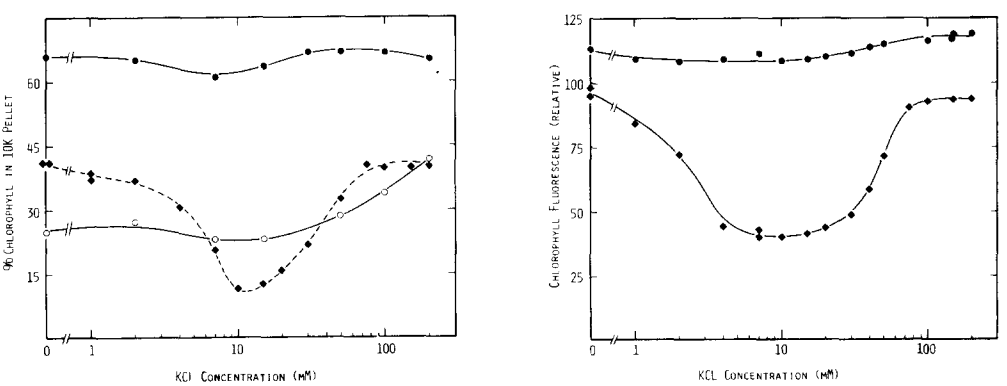

Fig. 1. Digitonin treatment of chloroplasts (♦) and granal fragments (●, ○) in mixed electrolyte media. Sample stocks in 0.5 to 1 mM $MgCl_2$ were diluted into the basic medium containing various concentrations of KCl such that the background $MgCl_2$ concentration was about 0.03 mM in all cases. Upon incubation at room temperature for 8—10 min, digitonin treatment was applied. Granal fragments were obtained and were either treated (○) or not treated (●) with acetic anhydride prior to the experiment. 10 k pellet: 10 000 × *g* pellet.

Fig. 2. Chlorophyll fluorescence of chloroplasts (♦) and granal fragments (●) in mixed electrolyte media. As in Fig. 1, the background $MgCl_2$ was about 0.03 mM and incubation time was 8—10 min at room temperature. Chlorophyll concentration was 10 μg/ml.

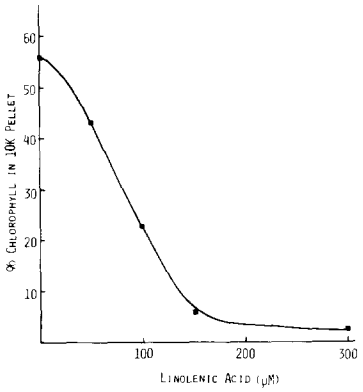

Fig. 3. Digitonin treatment of granal fragments in the presence of linolenic acid. Granal fragments were obtained, allowed to incubate for 10 min at room temperature in the basic medium supplemented with 10 mM KCl, and then treated with digitonin (see Methods).

in the 10 000 × *g* pellet obtained after digitonin treatment. It has been shown previously that treatment with linolenic acid inhibited stacking of normal thylakoids and also increased the net negative charge density of the membrane surfaces [8,25].

We also found that pretreatment of digitonin prepared granal fragments with acetic anhydride resulted in their unstacking (Fig. 1). In this case the ability of the reagent to acetylate certain amino groups on membrane surfaces induces an increase in the net negative surface charge density of the exposed proteins (see Table I). Like linolenic acid, acetic anhydride also inhibits stacking and the characteristic fluorescence changes observed on adding salts to normal thylakoids (Table I). That acetic anhydride increases the surface charge density of the membrane was tested by measuring the ability of treated and untreated thylakoids to quench 9-aminoacridine fluorescence under low salt conditions. This dye accumulates near a negatively charged surface, resulting in quenching of its fluorescence [26,27]. The degree of fluorescence quenching is a measure of the surface charge density and, as Table I shows, the quenching of 9-amino-

TABLE I

EFFECTS OF ACETIC ANHYDRIDE PRE-TREATMENT

	Control	Treated
(i) $MgCl_2$ (5 mM) induced chlorophyll fluorescence increase	175%	4%
(ii) Percentage Chl in 10 K pellet after digitonin treatment		
10 mM KCl *	7.4	1
5 mM $MgCl_2$	51.7	3.2
(iii) 9-aminoacridine fluorescence F_{min}/F_{max} **		
10 μg Chl/ml	0.634	0.540
20 μg Chl/ml	0.510	0.375

* Chloroplasts were incubated in the basic medium supplemented with either 10 mM KCl or 5 mM $MgCl_2$ prior to digitonin treatment.

** Fluorescence of 9-aminoacridine was measured in the basic medium with 30 μM EDTA (F_{min}) or 10 mM $MgCl_2$ (F_{max}). DCMU was also present (10 μM).

acridine emission was increased by pretreating the membranes with acetic anydride. The data in Table I indicate that, for the particular extent of acetic anydride treatment used, the surface charge density was increased by a factor of about 2, judging from the fact that the degree of quenching by treated membranes at 10 μg Chl/ml was nearly the same as untreated membranes at 20 μg Chl/ml.

It has been consistently found that extensive grana formation occurs only when light-harvesting complex is present [1,2]. Also, mild treatment of the exposed portion of this complex with the proteolytic enzyme trypsin inhibits salt induced grana formation and the associated increase in chlorophyll fluorescence yield [5,15,28]. It has been found that this is due to the removal of a 2000 molecular weight segment of the major polypeptide of the light harvesting complex, and has led Artnzen and colleagues to suggest that this polypeptide plays a specific role in grana formation [15]. Such a mechanism may operate but we would like to emphasize that membrane-membrane appression can occur if charge displacement or neutralization occurs, without the need to advocate a specific interaction mechanism. It could be that the loss of the 2000 molecular weight fragment from the light-harvesting complex is sufficient to change the surface charge characteristics of this complex [29] so that specific protein clustering or domain formation is not possible. Indeed, Nakatani and Barber [29] have recently shown, using the technique of particle electro-

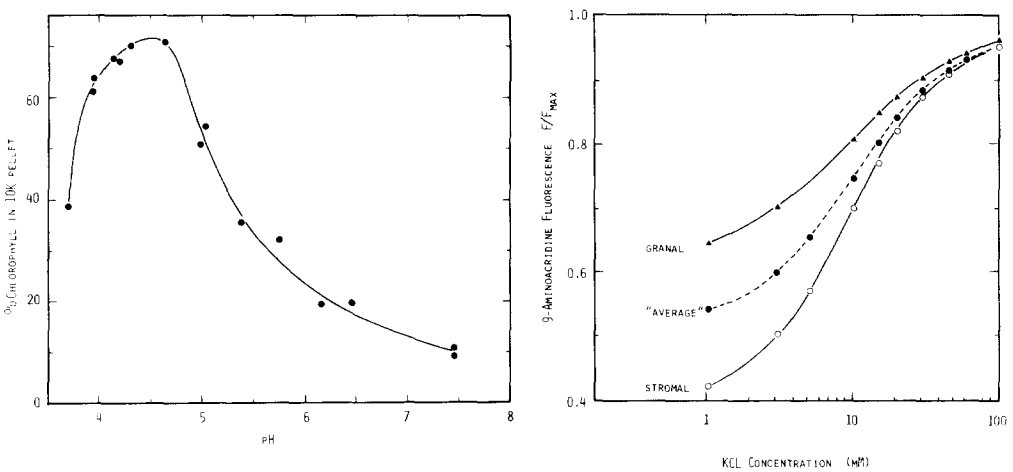

Fig. 4. Digitonin action on trypsin-treated chloroplasts. Chloroplasts (0.2 mg chl/ml) were pre-treated with trypsin (Sigma, 1 μg/ml) for 3 min at room temperature, then mixed with a 10-fold excess of trypsin inhibitor, and washed by dilution and centrifugation. Treated chloroplasts showed a $MgCl_2$-induced chlorophyll fluorescence increase of 18%. These chloroplasts were allowed to stand at room temperature for 8—10 min in the basic medium supplemented by 5 mM $MgCl_2$, 1 mM Mes and 0.5 mM succinate at the pH values indicated, and then treated with digitonin.

Fig. 5. The release of 9-aminoacridine fluorescence quenching by KCl in suspensions of digitonin-derived subchloroplast fragments. F is the 9-aminoacridine fluorescence for a particular KCl concentration added to the basic medium containing 20 μM 9-aminoacridine and F_{max} is the maximum fluorescence in the presence of 20 mM $MgCl_2$. Granal fragments (▲, Chl a/b = 2.40) were present at 7.8 μg Chl/ml; fragments from 'average' unstacked chloroplasts (●, Chl a/b = 2.99) at 7.4 μg chl/ml, and stromal fragments (○, Chl a/b = 4.61) at 7.0 μg chl/ml.

phoresis, that trypsin or pronase treatment sufficient to inhibit the salt induced chlorophyll fluorescence changes increases the amount of net negative charge on the thylakoid membrane surface. If the additional negative charge has been exposed on the light-harvesting complex surface, which seems very likely, then both salt-induced grana and domain formation would be inhibited due to increased coulombic repulsion. Under these circumstances stacking can occur only if charge neutralization is brought about. To emphasize this point, we have monitored pH induced stacking of trypsin-treated thylakoids (Fig. 4). These treated membranes showed little salt induced stacking or chlorophyll fluorescence changes but were able to stack when the pH was lowered, with maximum appression occurring at about pH 4.5.

If the salt induced stacking and associated chlorophyll fluorescence changes involves lateral charge displacement then, as previously pointed out [7], the surface charge density of unstacked ('average') thylakoids should be intermediate to the surface charge density of the stromal and granal membranes. To show this is not simple, since the granal membrane surface (i.e. the surface in the partition gaps) cannot be readily exposed as indicated in Fig. 2. Morever, the surface charge densities of the stromal and average fragments are difficult to determine by particle electrophoresis because of their very small dimensions. We have attempted to make relative measurements of the surface charge densities of the three types of membrane using the technique of 9-aminoacridine fluorescence quenching [20]. As Fig. 5 shows, based on equal chlorophyll levels, the trend in the F/F_{max} ratio in low salt conditions was indicative of the following order of surface charge densities: stromal > average > granal.

Discussion

In this paper we have tried to emphasise three important points in support of the lateral charge displacement model outlined in the introduction and discussed in depth in previous papers [6—8].

(1) Unstacking of digitonin prepared granal particles does not occur in a low salt medium unless they are treated in such a way as to introduce electrical charge onto the surfaces of the appressed regions.

(2) Although trypsin treatment inhibits salt induced grana formation, thylakoid stacking can be induced after this treatment by lowering the pH of the suspension medium to the isoelectric point of the membrane.

(3) Granal, stromal and unstacked lamellae carry different amounts of surface electrical charge.

These findings coupled with earlier work [8], seem to be in line with the general idea that salt induced thylakoid stacking and chlorophyll fluorescence changes involve the lateral movement of pigment protein complexes. It seems to be a combination of differences in the level of net electrical charge on exposed surfaces of different types of pigment protein complex and the existence of a fluid lipid matrix which allows the lateral protein movement and domain formation to occur in response to an increase in electrostatic screening of the surface charges [8]. Accepting this view it would be reasonable to identify the light-harvesting complex with a surface carrying a low net electrical charge so that it allows membrane appression to occur where it forms do-

mains. Whether short range specific interactions of the type postulated by Mullet and Arntzen [15] exist under these conditions is not clear and may not be necessary as long as the surface charge density is low in this region and attraction by van der Waals forces dominates in the way discussed by Sculley et al. [30]. The displacement of protein complexes carrying net electrical charges to the stromal membranes could involve some of the PS I complexes [7]. This would account for the corresponding increase in chlorophyll fluorescence resulting from a decrease in spillover from PS II to PS I [6,31].

Apparently the ability of screening cations to induce different types of protein domains is sensitive to modification of the surface electrical properties of the exposed segments of the two types of chlorophyll-containing protein complexes. It is proposed that either exposing net charge on the surface of the light-harvesting complex or neutralizing charge on the PS I complex inhibits the salt induced domain formation and the associated chlorophyll fluorescence and spillover changes [8]. However these treatments do not inhibit pH-induced membrane stacking, except that, unlike the salt-induced stacking, the resulting 'grana' are less enriched in chlorophyll *b* [8].

This idea of lateral diffusion of proteins in the lipid matrix of the thylakoid membrane induced by changing forces of interaction between them may not only be relevant to pigment proteins but also to pigment-free proteins having functional activity (electron transport proteins, coupling factor, etc.). Whether such lateral diffusion and reshuffling of different types of protein complexes within the plane of the membrane is of physiological significance, in terms of regulation, is yet to be established. Nevertheless, since the salt induced spillover changes are closely related to the State 1-State 2 changes in intact organisms [31—34] it seems likely that the in vivo interaction between PS II and PS I could involve lateral movements of the appropriate pigment-protein complexes. The control of these interactions could either be via changes in the ionic levels close to the membrane surface [31] or by changing the charge density on some of the exposed protein surfaces. With regard to the latter, the light-induced phosphorylation of the light-harvesting complex is worth noting [35]. In relating the lateral charge displacement model with State 1-State 2 changes it should be noted that only a few nanometres change in spatial separation would lead to significant changes in energy transfer between adjacent chlorophyll-protein complexes. Such changes could occur in vivo at the periphery of the grana stacks and some changes in the degree of stacking would therefore need to be anticipated for the State 1-State 2 transition. Indeed, a decrease of about 20% in the degree of stacking has been observed for in vivo *Chlamydomonas* thylakoids on going from State 1 to State 2 [36].

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References

1 Arntzen, C.J. (1978) in Current Topics in Bioenergetics (Sanadi, R. and Vernon, L., eds.), Vol. 8, pp. 111—160, Academic Press, New York

2 Boardman, N.K., Anderson, J.M. and Goodchild, D.J. (1978) in Current Topics in Bioenergetics (Sanadi, R. and Vernon, L. eds.), Vol. 8, pp. 35—109, Academic Press, New York
3 Staehelin, L.A. and Arntzen, C.J. (1979) in Chlorophyll Organisation and Energy Transfer in Photosynthesis; Ciba Foundation Symp. No. 61 (new series), pp. 147—175, Elsevier, Amsterdam
4 Argyroudi,-Akoyunoglou, J.H. and Akoyunoglou, G. (1977) Arch. Biochem. Biophys. 179, 370—377
5 Jennings, R.C., Forti, G., Gerola, D. and Garlaschi, F.M. (1978) Plant Physiol. 62, 879—884
6 Barber, J. (1980) FEBS Lett. 118, 1—10
7 Barber, J. and Chow, W.S. (1979) FEBS Lett. 105, 5—8
8 Barber, J., Chow, W.S., Scoufflaire, C. and Lannoye, R. (1980) Biochim. Biophys. Acta 591, 92—103
9 Barber, J., Mills, J.D. and Love, A. (1977) FEBS Lett. 74, 174—181
10 Duniec, J.T., Sculley, M.J. and Thorne, S.W. (1979) J. Theor. Biol. 74, 473—484
11 Rubin, B.T. and Barber, J. (1980) Biochim. Biophys. Acta 592, 87—102
12 Ojakian, G.K. and Satir, P. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2052—2056
13 Staehelin, L.A. (1976) J. Cell Biol. 71, 136—158
14 Wang, A.Y.I. and Packer, L. (1973) Biochim. Biophys. Acta 305, 488—492
15 Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100—117
16 Gerola, P.D., Jennings, R.C., Forti, G. and Garlaschi, F.M. (1979) Plant Sci. Lett. 16, 249—254
17 Akoyunoglou, G. and Argyroudi-Akoyunoglou, J. (1974) FEBS Lett. 42, 135—140
18 Chow, W.S., Thorne, S.W., Duniec, J.T., Sculley, M.J. and Boardman, N.K. (1980) Arch. Biochem. Biophys. 201, 347—355
19 Arnon, D.I. (1949) Plant Physiol. 24, 1—15
20 Chow, W.S. and Barber, J. (1980) Biochim. Biophys. Acta 589, 346—352
21 Prochaska, L.J. and Dilley, R.A. (1978) Arch. Biochem. Biophys. 187, 61—71
22 Gross, E.L. and Hess, S.C. (1973) Arch. Biochem. Biophys. 159, 832—836
23 Gross, E.L. and Prasher, S.H. (1974) Arch. Biochem. Biophys. 164, 460—468
24 Vandermeulen, D.L. and Govindjee (1974) Biochim. Biophys. Acta 368, 61—70
25 Shaw, A.B., Anderson, M.M. and McCarty, R.E. (1976) Plant Physiol. 57, 724—729
26 Searle, G.F.W., Barber, J. and Mills, J.D. (1977) Biochim. Biophys. Acta 461, 413—425
27 Searle, G.F.W. and Barber, J. (1978) Biochim. Biophys. Acta 502, 309—320
28 Steinback, K.E., Burke, J.J. and Arntzen, C.J. (1979) Arch. Biochem. Biophys. 195, 546—557
29 Nakatani, H.Y. and Barber, J. (1980) Biochim. Biophys. Acta 591, 82—91
30 Sculley, M.J., Duniec, J.T., Thorne, S.W., Chow, W.S. and Boardman, N.K. (1980) Arch. Biochem. Biophys. 201, 339—346
31 Barber, J. (1976) in The Intact Chloroplast, Vol. 1, Topics in Photosynthesis (Barber, J., ed.), pp. 89—134, Elsevier, Amsterdam
32 Duysens, L.N.M. and Talens, A. (1969) in Progress in Photosynthesis Research (Metzner, H.H., ed.), Vol. 2, pp. 1073—1081, University of Tübingen, Tübingen
33 Bonaventura, C. and Myers, J. (1969) Biochim. Biophys. Acta 189, 366—383
34 Williams, W.P. (1977) in Primary Processes of Photosynthesis, Vol. 2, Topics in Photosynthesis (Barber, J., ed.), pp. 99—147, Elsevier, Amsterdam
35 Bennet, J. (1980) Trends Biochem. Sci. 4, 268—271
36 Bennoun, P. and Jupin, H. (1974) in Proc. 3rd Intl. Congr. Photosynthesis (Avron, M., ed.), Vol. 1, pp. 163—169, Elsevier, Amsterdam