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APPEARANCE OF PHOTOCHEMICAL FUNCTION IN PROTHYLAKOIDS DURING PLASTID DEVELOPMENT

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Summary

1. A method to separate the vesicles of prothylakoids from prolamellar body preparations obtained from etiolated and rapidly greening *Avena* laminae (0.25—4 h illumination) is described. The prothylakoid preparations were found to be free from contaminating prolamellar bodies but enriched prolamellar body preparations (enriched prolamellar body preparations) still contained some adhering prothylakoid material.

2. Only existing β-carotene appears to be transferred from the prolamellar bodies to the prothylakoids during early development and this ceases when freshly synthesized β-carotene becomes available.

3. Prolamellar body structures proper show no positive association of existing or developing photochemical activities; these are only to be found in the developing prothylakoids.

4. Using methylviologen-linked electron transport-dependent oxygen consumption, Photosystem I activities may be detected with added diaminodurene within 15 min of illumination and within 30 min and 1 h with added tetramethylphenylenediamine and dichlorophenolindophenol, respectively.

5. During the 2nd. and 3rd. h of greening, proton-pumping capability and

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Abbreviations: CF_1, chloroplast coupling factor I; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DAD, diaminodurene; DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DMMIB, 2,3-dimethyl-5,6-methyleneioxy-*p*-benzoquinone; DPC, 1,5-diphenylcarbazide; P^+-700, electron-accepting site of PS I; PNDA, *p*-nitrosodimethylaniline; PS, photosystem; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

later ATP formation increased in prothylakoids in the presence of diaminodurene.

6. The first indications of Photosystem II activity using diphenylcarbazide as electron donor are shown at a similar time (2 h) with prothylakoids. The last photochemical activity to appear is the capacity to split water (3 h) and consequently the diphenylcarbazide activity diminishes to zero before 8 h of illumination have passed.

7. The lack of effect of uncouplers such as NH_4^+ prior to 2 h suggests that in spite of some proton-pumping ability there is the possibility of proton-leaky areas existing within prothylakoids. This. lack of a persistent proton gradient before 2 h of illumination may explain the different starting times of phenazine methosulfate- and diaminodurene-dependent photophosphorylation (0.25 and 2 h, respectively).

Introduction

The light-induced formation of photosynthetic membranes in higher plants has been extensively investigated using the greening of etiolated tissue as a model for the plastid development that occurs naturally in light-grown tissues. The use of such material has the advantage that sufficient tissue is available for biochemical analysis at an arrested stage of development which can be transformed within a distinct time regime into a fully photosynthetic system. In all cases, chlorophyll accumulates, protein is synthesized and an ordered thylakoid system appears. There have been many studies on aspects of this transformation such as appearance of oxygen evolution [1—3], the different electron transport reactions [1,4—12], osmotically responsive internal spaces [13,14], photophosphorylation [15—17], and carbon dioxide fixation [18—20]. The problem to date is that such studies have concentrated on one particular aspect of the greening system using differing species with various growth conditions and illumination regimes including those employing flashing lights. Integrated studies such as these where the overall sequence of events are determined are rarely reported. Nevertheless attempts have been made to draw these studies together and the most successful and recent reviews on the subject are those of Boardman and coworkers [21,22].

Criticisms may be made of the validity of the greening model largely on the basis of ultrastructural differences between plastids formed in the leaves of higher plants growing in a normal light-dark cycle and in the dark. In the former, small poorly organized prolamellar bodies may be observed at a certain stage in the development of proplastids to chloroplasts [23—25] but they never achieve the crystalline predominance found in Angiosperm etioplasts. An additinal complication is posed by senescence. Prolamellar structures become larger with increasing age of tissue and the greening process becomes slower. Care must be taken to distinguish developmental events from those involved in ageing [26,27].

In an earlier report on the development of chloroplast coupling factor I (CF_1) particles during greening [28] the possibility was raised of detaching the prothylakoid membranes as vesicles from isolated prolamellar bodies so as to be

able to study assembly of the photosynthetic apparatus without the complication of the prolamellar body presence, obviating some of the criticisms of the use of the greening system. This separation has now been achieved and is reported here using electron microscopy and the trypsin-activated Ca^{2+}-dependent ATPase activity of CF_1 which is associated only with prothylakoids [28] to monitor the purity of such preparations. In addition, an integrated study of the appearance of electron transport functions, proton-pumping activities and photophosphorylation in prothylakoids, by comparison to those in the more developed thylakoid systems, has been carried out. The chlorophyll and carotenoid compositions of prothylakoids and prolamellar bodies have also been determined during the developmental process to allow an appreciation of the pigment requirements of the newly formed photosynthetic apparatus.

Materials and Methods

Etioplasts and (0.25—24 h) etiochloroplasts were prepared using simple differential centrifugation methods [29] from laminae of either dark-grown or dark-grown and partially illuminated (0.25—24 h) 8-day-old *Avena sativa* L. (var. Arnold) seedlings grown at 25°C. The resuspended plastids were lysed for 15 min at 0°C in 5 ml of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.3) and centrifuged (3000 × *g*, 10 min) to sediment the internal membrane fractions. In the case of membranes from etioplasts or 0—4 h etiochloroplasts the pellets are known to contain prolamellar bodies with adhering prothylakoids [28,30]. These resuspended preparations (20 ml) were treated with ultrasound (twice 5 s, 20 kHz) to release a substantial proportion of the prothylakoids from the prolamellar bodies. A centrifugation (3000 × *g*, 10 min) sedimented an enriched prolamellar body fraction (enriched prolamellar body preparations) and a final centrifugation of the supernatant (20 000 × *g*, 10 min) pelletted the prothylakoids.

The electron microscope procedure to monitor these various fractions, including the pre-stain procedure of Oleszko and Moudrianakis [31] to enhance the visualization of the CF_1 particles, has been described earlier [30]. Trypsin-activated Ca^{2+}-dependent ATPase activity to monitor the presence of CF_1 particles was estimated using a modified method [28] of the procedure described by Ryrie and Jagendorf [32] as a criterion of the degree of contamination of the enriched prolamellar body preparation fraction by prothylakoid material.

Carotenoids and chlorophylls were extracted and separated by procedures previously described [33] and estimated spectrophotometrically [34,35]. Protein [36] was used as the main basis of expression of all specific activities in the absence of significant amounts of chlorophyll in preparations from dark-grown laminae illuminated for short periods of time.

The different membrane fractions plus unresolved internal membranes from 5—24-h etiochloroplasts, which contain few if any prolamellar bodies [28], were resuspended in Hepes (20 mM, pH 8) and the activity of Photosystem I (PS I) was determined in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 10^{-5} M) by the measurement of the rate of oxygen uptake after the addition of ascorbate ($5 \cdot 10^{-3}$ M), methylviologen ($4 \cdot 10^{-4}$), NaN_3 ($5 \cdot$

10^{-5} M), together with either *N,N,N',N'*-tetramethylphenylenediamine (TMPD, 10^{-4} M), 2,6-dichlorophenolindophenol (DCIP, $2 \cdot 10^{-4}$ M), or diaminodurene (DAD, 10^{-3} M). As a measure of PS II activity, oxygen consumption in the light was recorded in the presence of the plastoquinone antagonist dibromothymoquinone (DBMIB, 10^{-6} M) and of 2,3-dimethyl-5,6-methyleneioxy-*p*-benzoquinone (DMMIB, 10^{-5} M) channelling electrons to oxygen after PS II [38,39] with H_2O or 1,5-diphenylcarbazide (DPC, 10^{-3} M) [40] as electron donors for PS II. In addition, photoreduction of DCIP (10^{-4} M) in the same system, but omitting DPC and DMMIB, was determined by the rate of oxygen evolution. Cooperation between the two photosystems was determined by the rates of oxygen consumption in the presence of methylviologen or of oxygen evolution associated with the photobleaching of *p*-nitrosodimethylaniline (PNDA, 10^{-5} M) with H_2O or DPC as the electron donors [41]. Ferredoxin-NADP oxidoreductase was measured by the rates of oxygen consumption after the addition of a NADPH-generating system and anthraquinone-2-sulfonate according to the method of Elstner and Heupel [42]. All changes in the amount of oxygen in the incubation medium (total volume 2 ml) associated with electron transport reactions were measured at 20°C using a water-jacketed oxygen electrode [43]. The illumination was from a forced air-cooled, 250 W slide projector, resulting in a radiant light flux of about 180 klux within the assay solution.

Proton-pumping activities were determined with the oxygen electrode equipment described above, but modified by the insertion of a pH electrode (Philips Model PW 9414) and calibrated with NaOH [44] in a weakly buffered suspension (0.2 mM Hepes, pH 6.9).

The estimation of ATP formation in the presence of different electron donor and acceptor systems (see Results) was carried out using the sensitive luciferase assay [45] as modified by Chow and Hope [46].

All values given in this paper are averages of at least three different experiments.

Results

As a consequence of previous work [28,30] on isolated prolamellar body fractions, an isolation procedure to detach prothylakoids from prolamellar body preparations has been developed. In the electron microscope, the prothylakoid fraction (Fig. 1) from either etioplasts or 0.25—4-h etiochloroplasts appeared to consist exclusively of small vesicles of 0.18 μM average diameter heavily studded on their outside faces with particles microscopically identical to CF_1 [31]. No trace of prolamellar body material was observed in these preparations nor evidence for inverted prolamellar vesicles (i.e. CF_1 particles towards the inside). However, when the enriched prolamellar body fraction was examined the prolamellar bodies still retained some attached prothylakoids (Fig. 2) although considerably fewer than in a conventional prolamellar body preparation (cf. Ref. 30) obtained without using ultrasound.

The fact that CF_1 particles only occur on the prothylakoid membranes and not on prolamellar body structures proper [28] enables the comparison of CF_1 (i.e. trypsin-activated Ca^{2+}-dependent ATPase activities) in both enriched

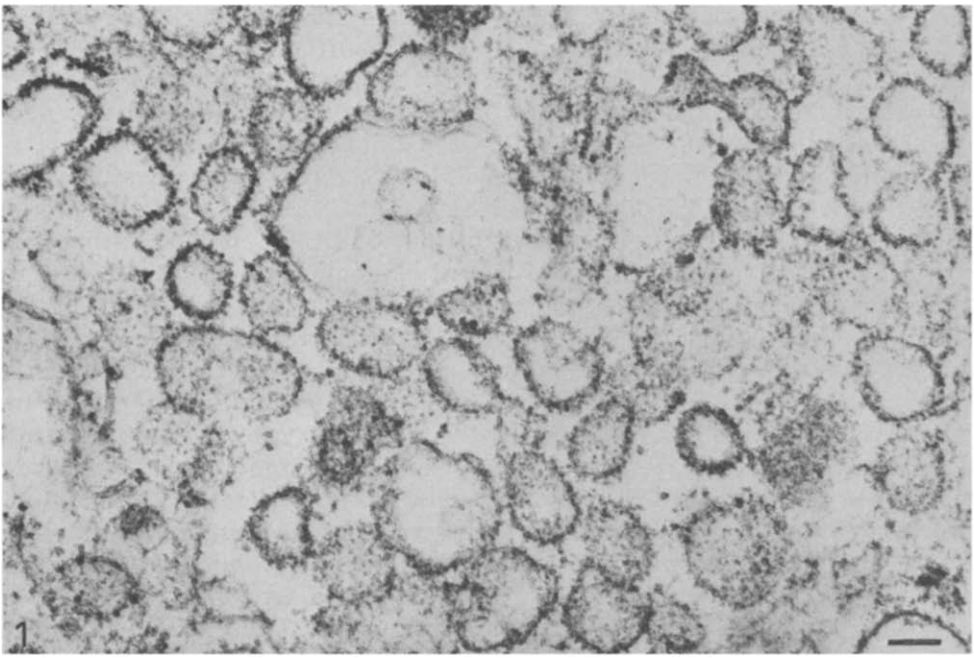

Fig. 1. Electron micrograph of a prothylakoid vesicle (PTV) preparation obtained from etioplasts isolated from etiolated *Avena* laminae as described in Materials and Methods. The method of preparation for electron microscopy was one which enhances the visualization of the CF_1 particles (Ref. 31) and the full procedure is described elsewhere (Ref. 30). The solid bar represents a distance of 0.1 μm and the reproduced figure is at a magnification of ×70 500 diameters.

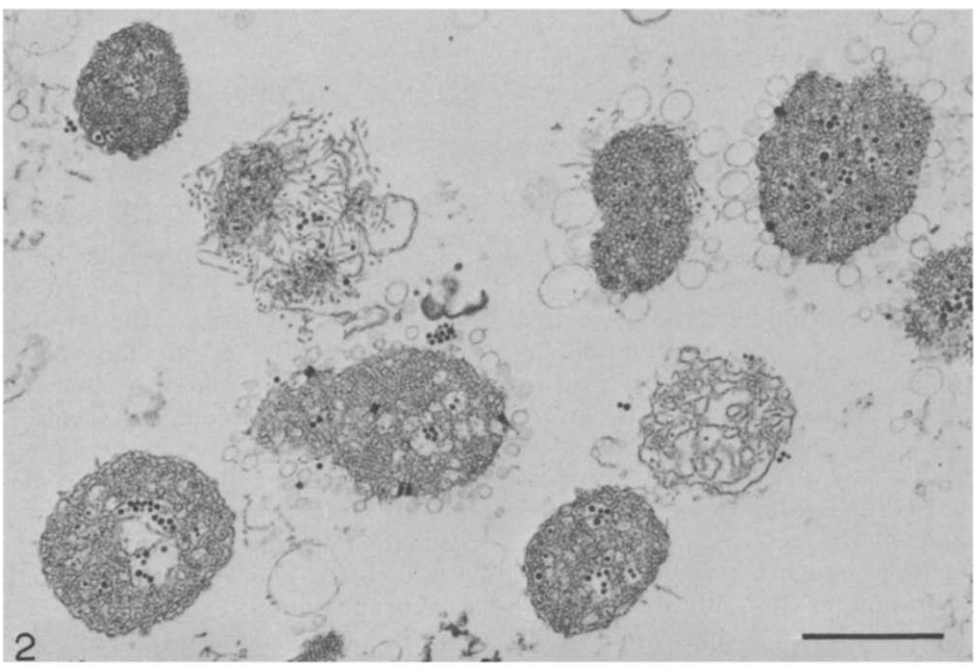

Fig. 2. Electron micrograph of enriched prolamellar body (EPLB) preparation obtained from etioplasts isolated from etiolated *Avena* laminae as described in Materials and Methods section. The method of preparation for electron microscopy was the same as that of Fig. 1. The solid bar represents a distance of 1 μM and the reproduced figure is at a magnification of ×18 000 diameters.

TABLE I

LEVELS OF CF_1 ATPase ACTIVITY IN PROTHYLAKOID AND ENRICHED PROLAMELLAR BODY PREPARATIONS

Estimation of trypsin-activated Ca^{2+}-dependent ATPase activities in preparations of unresolved inner membrane fractions, prothylakoid vesicles (PTVs) and enriched prolamellar bodies (EPLBs) prepared from etioplasts and 0.5, 2 and 4-h etiochloroplasts obtained from *Avena*. The modified method for the estimation of ATPase activity is fully described elsewhere [28] and is based upon an earlier method employed by Ryrie and Jagendorf [32]. The rates of activity are expressed in terms of μmol inorganic phosphate released/mg protein per h and the protein concentration was about 2 mg/ml for each preparation. All determination were made in triplicate are the values displayed below and the mean values obtained from three different preparations of each experimental treatment and fractionation. PLBs, prolamellar bodies; PTVs, prothykaloid vesicles.

Period of illumination (h)	μmol phosphate · mg^{-1} protein · h^{-1}			Contamination of EPLBs by PTVs (%)
	Unresolved internal membrane preparations (PLBs)	PTVs	EPLBs	
0	1.64	5.62	0.77	14
0.5	2.01	5.97	0.93	16
2	2.46	7.22	1.17	16
4	3.48	10.79	2.23	21

prolamellar body preparations and prolamellar vesicle fractions to be used to determine the degree of contamination of the enriched prolamellar body preparations by prothykaloids. Table I shows the respective specific. ATPase activities in preparations of both enriched prolamellar body preparations and prothylakoids and the prolamellar body fractions from which they were derived. The results indicate a 14% (enriched prolamellar body preparations from etioplasts) to 20% (enriched prolamellar body preparations derived from 4-h etiochloroplasts) contamination of enriched prolamellar body preparations by prothylakoids. This increase is undoubtedly due to the gradual disappearance of prolamellar bodies during development coupled to a progressive loss of crystallinity which makes separation of the two components more difficult. Few if any prolamellar bodies persist in dark-grown *Avena* seedlings after 4 h illumination and consequently further separation, by these means, of later internal membranes is unnecessary. Nevertheless two further domains, those of the grana and stroma thylakoids, progressively appear together from this point onwards. Ultrastructural characterization of these unresolved preparations has already appeared [28].

Small amounts of prothylakoid material from the very early stages of development (0—1 h etiochloroplasts) considerably hampers pigment extraction and analysis. Detectable chlorophyll *a* appears extremely early in the prothylakoid fractions (Fig. 3) and rises steadily throughout the first 24 h of greening. Chlorophyll *b* appears more slowly and consequently the chlorophyll *a/b* ratio falls from infinity to a steady value around 3 within the same period. The changes in the amounts of the individual carotenoids in prothylakoids and later (5—24 h) unresolved inner membranes are more complicated. Fig. 4 shows that the first major carotenoid to rise in amount is β-carotene followed 3 h later by the predominant xanthophyll, lutein. The ratio of β-carotene to total

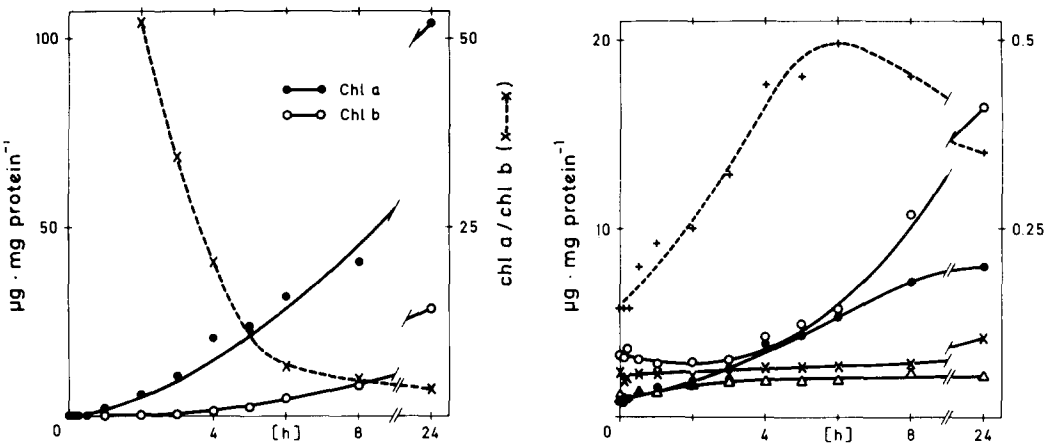

Fig. 3. Levels of chlorophyll *a* (●———●) and chlorophyll *b* (○———○) in preparations of prothylakoid vesicles (PTVs) prepared from etioplasts and 0.25—4-h etiochloroplasts and in unresolved internal membranes isolated from 5—24-h etiochloroplasts are shown with respect to protein concentration. The ratio of chlorophyll *a* to chlorophyll *b* (X- - - - -X) is also shown. The methods of chlorophyll extraction and estimation are given in Materials and Methods section and bulked preparations of each developmental stage containing a total of 100 mg of protein were analysed.

Fig. 4. Levels of β-carotene (●———●), lutein (○———○), violaxanthin (X———X), and neoxanthin (△———△) in preparations of prothylakoid vesicles (PTVs) isolated from etioplasts and 0.25—4-h etiochloroplasts and in unresolved internal membranes prepared from 5—24 h etiochloroplasts are shown with respect to protein concentration. The ratio of β-carotene to the xanthophylls (+- - - - -+) is also shown. The methods of carotenoid extraction and estimation are given in Materials and Methods and bulked preparations of each developmental stage each containing a total of 100 mg of protein were analysed.

xanthophyll as a consequence rises to a maximum around 6 h of greening and later declines. Similar results have been reported previously [47,48]. The specific amounts of violaxanthin and neoxanthin by contrast scarcely change throughout the greening process. The interpretation of the pigment changes in the enriched prolamellar body preparation fractions is complicated by the known contamination of prothylakoids. Measurements of chlorophylls in these fractions can be completely accounted for by the 14—20% contamination indicated by the ATPase determinations. However β-carotene, and later xanthophyll values decline in the enriched prolamellar body preparation fractions (Fig. 5) possibly indicating real displacement of stored carotenoid from the prolamellar body structures for the purpose of prothylakoid development before freshly biosynthesized material is available. The later rise of the specific amount of the β-carotene and the ratio of β-carotene to total xanthophylls is probably wholely due to prothylakoid contamination.

Preliminary studies of the electron transport functions using oxygen electrodes revealed that the light requirement was extremely high and 180 klux radiant flux was required for saturation of all preparations tested (0—4 h). The effect of red instead of white light was investigated but the light requirement characteristics remained approximately the same. Relatively high rates of unspecific photooxidation (i.e. oxygen uptake of the order of 0.75 and 1.5 µmol $O_2 \cdot h^{-1} \cdot mg^{-1}$ protein for prothylakoids and enriched prolamellar body preparations, respectively) are experienced especially with preparations from etioplasts

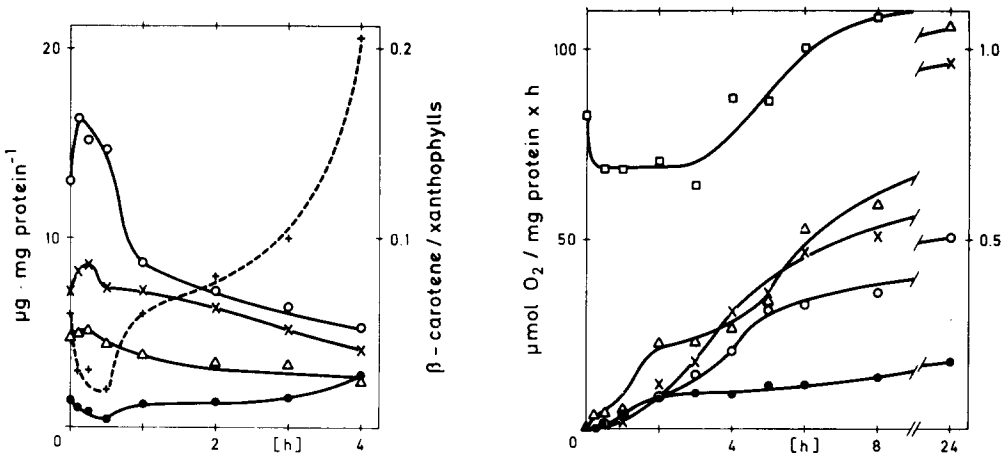

Fig. 5. Levels of β-carotene (●———●), lutein (○———○), violaxanthin (X———X), and neoxanthin (△———△) in preparations of enriched prolamellar bodies (EPLBs) isolated from etioplasts and 0.25—4-h etiochloroplasts are shown with respect to protein concentration. The ratio of β-carotene to the xanthophylls (+- - - - -+) is also shown. The methods of carotenoid extraction and estimation are given in Materials and Methods and bulked preparations of each developmental stage each containing a total of 100 mg of protein were analysed.

Fig. 6. Photochemical activities of Photosystem I and rates of ferredoxin-NADP oxidoreductase in unresolved internal membranes of etioplasts (0 h) and etiochloroplasts (0.25—24 h). Rates of electron transport were measured by the amounts of O_2 consumption as described in Materials and Methods. In addition to 0.2 mM DCIP in the absence (●———●) or presence (○———○) of 20 mM NH_4Cl; 1 mM DAD (△———△) or 0.1 mM TMPD (X———X), both without added NH_4Cl are shown here. The level of activity of the oxidoreductase (□———□) was measured as the rate of oxygen consumption in the presence of a NADPH-generating system (20 µg glucose-6-phosphate dehydrogenase, 5 µmol glucose 6-phosphate, 0.1 µmol NADP in a total volume of 1.5 ml) and of anthraquinone sulfonic acid, 0.1 mM in the dark (right-hand scale, all the rest left-hand scale). Protein concentration was about 2.0 mg/ml.

and 0.25—1 h etiochloroplasts. Appropriate light and dark controls were always required to account for them. Normally prothylakoids from etioplasts exhibit 50% less photooxidation than enriched prolamellar body preparations. Presumably these higher rates may be attributed to the unusually high lipid content of the prolamellar body structure proper [50].

The development of electron transport reactions associated with PS I, as measured by oxygen consumption using methylviologen as an autooxidizable acceptor in the presence of reduced DAD, TMPD or DCIP, in unresolved preparations of inner membranes is shown in Fig. 6. With DAD an appreciable activity was detected within 0.25 h whereas TMPD and DCIP were somewhat slower requiring a lag of 0.5—1 h after the start of illumination before preparations were active. Thereafter all systems showed an increase in electron transport-mediated oxygen consumption on a protein basis up to 8 h of illumination. Only NADP-dependent oxidoreductase activity could be measured in the etiolated preparations which, after a lag phase of 3 h, also showed an increase with plastid development.

The corresponding activities exhibited by prothylakoid preparations over a shorter and earlier period are shown in Fig. 7. It may be observed that prothylakoid material exhibits higher specific activities than any other preparation. Pronounced increases in activities are shown during development with all artifi-

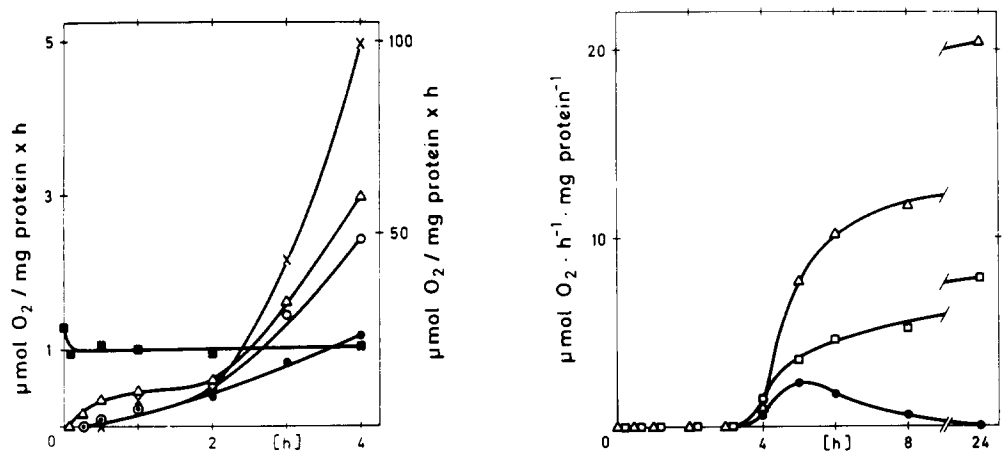

Fig. 7. Photochemical activities of Photosystem I (right-hand scale) and rates of ferredoxin-NADP oxidoreductase (left-hand scale) in prothylakoid vesicles of etioplasts (0 h) and etiochloroplasts (0.25—4 h). Experimental conditions were the same as in Fig. 6. DCIP → O_2, in the absence (●———●) or presence (○———○) of 20 mM NH_4Cl; DAD → O_2 (△———△); TMPD → O_2 (X———X); oxidoreductase (■———■). Protein concentration was about 2.0 mg/ml.

Fig. 8. Development of Photosystem II activities in unresolved internal membranes of etioplasts (0 h) and etiochloroplasts (0.25—24 h). As a measure of Photosystem II activity, oxygen consumption in the light was recorded in 20 mM Hepes (pH 8) in the presence of 10 µM DBMIB, 10 µM DMMIB and 50 µM NaN_3 with H_2O (□———□) or DPC (0.1 mM, ●———●) as electron donors for Photosystem II. Photoreduction of 0.1 mM DCIP (△———△) was determined by the rates of oxygen evolution in the same system, but omitting DPC. Protein concentration was about 2 mg/ml.

cial electron donors; DAD being the first to start within the first 5 min, followed by DCIP (0.25 h) and then TMPD (0.5 h). The specific activities with the enriched prolamellar body preparation fractions (values not shown) are well below 20% of those found in prothylakoids and most likely due to the contamination of the enriched prolamellar body preparation fraction by prothylakoids described earlier. These findings provide strong evidence that there are probably no photochemical activities within the prolamellar body structure proper at any stage of development. By contrast the specific activities of the NADP-dependent oxidoreductase are similar in both enriched prolamellar body preparations and prothylakoids and could mean that this particular protein may form part of the prolamellar bodies proper and may be transferred to the extending prothylakoid membranes during development.

By contrast to PS I, significant activities associated with PS II using the oxygen electrode are not revealed until 4 h and later. Fig. 8 shows the results obtained with unresolved inner membranes and Table II contains details of small PS II and PS II + PS I-associated activities that were detected earlier than 4 h in prothylakoid and enriched prolamellar body preparations. Besides a low amount of DPC-induced oxygen consumption in prothylakoids after 2 h, water splitting and concomitant electron transport only starts after 3 h showing appreciable rates after 4 h. Once again prothylakoid contamination of the enriched prolamellar body preparations would account for any detected photochemical activity in these preparations. The ability to feed in electrons from either water to DCIP or from water or DPC to oxygen via DMMIB differs

TABLE II

TIME OF APPEARANCE OF PS II AND PS II + PS I ACTIVITIES

Development of Photosystem II activities and of the cooperation between both photosystems in preparation of prothylakoid vesicles (PTVs) and enriched prolamellar bodies (EPLBs). Activities are expressed as electron transport-mediated rates (μmol · mg^{-1} protein · h^{-1}) of oxygen consumption or evolution. Experimental conditions for the measurement of Photosystem II activities (H_2O → DCIP; H_2O → DMMIB; DPC → DMMIB) were the same as in Fig. 8; those for a cooperation between Photosystem I and Photosystem II (H_2O → methylviologen (MV); DPC → MV; H_2O → PNDA) as in Fig. 9. $K_3Fe(CN)_6$ concentration was 1.25 mM; protein concentration was about 2 mg/ml.

Function	PTVs			EPLBs		
Period of illumination (h):	2	3	4	2	3	4
H_2O → DCIP	0	0	2.62	0	0	0.50
H_2O → PS II → DMMIB (with DBMIB inhibition)	0	0.03	3.35	0	0	0.64
DPC → PS II → DMMIB (with DBMIB inhibition)	0.01	0.04	1.50	0	0	0.29
H_2O → PS II → ferricyanide	0	0.02	1.17	0	0	0.11
H_2O → PS II → PS I → MV	0	0	1.44	0	0	0.28
DPC → PS II → PS I → MV	0	0	0.96	0	0	0.18
H_2O → PS II → PS I → PNDA	0	0	2.08	0	0	0.40

during development. With water as electron donor there is a steady increase in oxygen consumption and DCIP reduction whilst the rates involving DPC show an optimum between 4 h and 6 h of illumination and thereafter decrease to zero.

The development of cooperation between the two photosystems in

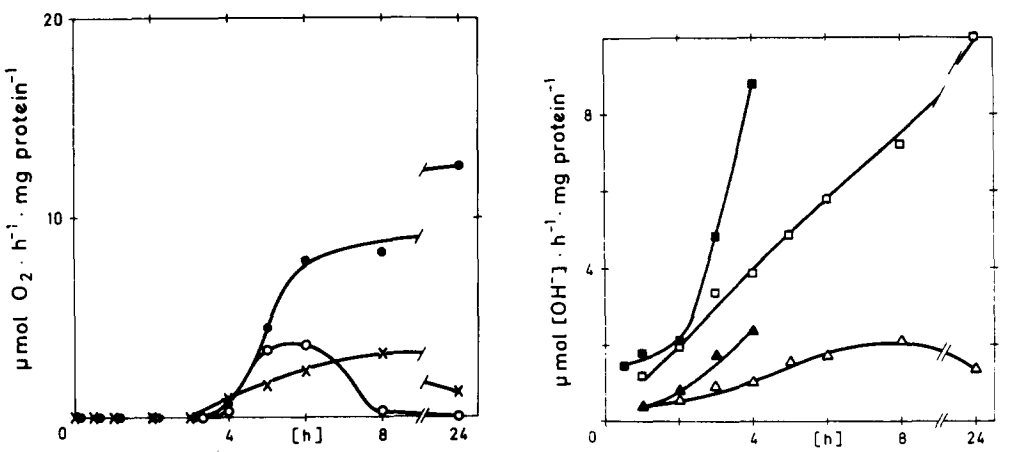

Fig. 9. Development of cooperation between Photosystem I and Photosystem II in unresolved internal membranes of etioplasts (0 h) and etiochloroplasts (0.25—24 h). Cooperation was measured as electron transport-associated consumption (●———●, H_2O, or ○———○, 0.1 mM DPC → 0.4 mM methylviologen → O_2) or evolution (H_2O → 10 μM PNDA (X———X)) of oxygen in 20 mM Hepes (pH 8). Protein concentration was about 2 mg/ml.

Fig. 10. Development of proton-pumping activities in unresolved internal membranes (□, △) and prothylakoid vesicles (■, ▲) of etioplasts (0 h) and etiochloroplasts (0.25—24 h). Light-driven rates of changes in $[OH^-]$ in the incubation medium (0.2 mM Hepes, pH 6.9; ascorbate, 0.5 mM; methylviologen, 0.4 mM; NaN_3, 50 μM) were monitored in the presence of 1 mM DAD (□, ■) or 0.1 mM DCIP (△, ▲) by a pH electrode. Protein concentration was about 2 mg/ml.

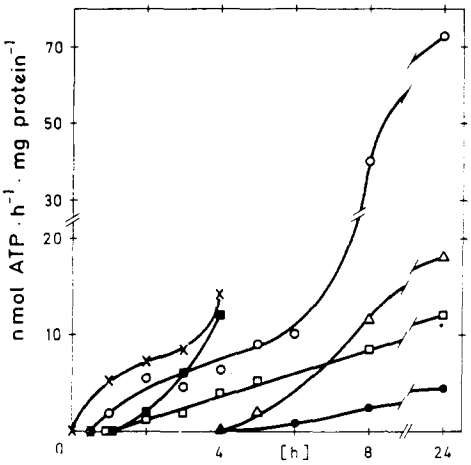

Fig. 11. Development of photophosphorylation in unresolved internal membranes (i.m.) and prothylakoid vesicles (PTVs) of etioplasts (0 h) and etiochloroplasts (0.25—24 h). Light-dependent formation of ATP was measured with the luciferin-luciferase assay after incubation of the membranes in a medium containing 20 mM Hepes (pH 8), 3 mM ADP, 6 mM KH_2PO_4, and 15 μg/ml phenazine methosulfate (○———○, i.m.; X———X, PTVs), 1 mM DAD (□———□, i.m.; ■———■, PTVs), 0.1 mM DCIP (●———●, i.m.) or 1.25 mM $K_3Fe(CN)_6$ (△———△, i.m.). In the case of DAD- and DCIP-mediated electron transport 5 mM ascorbate, 0.4 mM methylviologen and 50 μM NaN_3 were added. Protein concentration was about 3 mg/ml.

unresolved inner membrane preparations is shown in Fig. 9. Electron transport-associated consumption (H_2O or DPC → methylviologen → O_2) or evolution of oxygen (H_2O → PNDA) shows a similar time-dependent sequence as observed for PS II alone. Table 2 indicates that with prothylakoids there is a delay of approximately 1 h between the first appearance of most PS II activities (3 h) and PS II + PS I reactions (4 h).

Data derived from unresolved internal membranes (0—24 h) and prothylakoids (0—4 h) shown in Fig. 10 demonstrates the progressive development of a light-dependent proton gradient (measured as a change in the OH^- concentration in the incubation medium by the various preparations). As before (cf. Fig. 10 with Figs. 6 and 7) the couple ascorbate/DAD was found to be much more effective than that of ascorbate/DCIP).

Light-driven ATP formation (Fig. 11) by the same couples follows exactly the same form, with a delay of 1—2 h between the two functions in terms of development time (cf. Fig. 11 with Fig. 10). By contrast phenazine methosulfate induces appreciable rates of photophosphorylation with prothylakoid preparations after 0.25 h and only slightly later with unresolved internal membranes.

Because reduced phenazine methosulfate will donate electrons directly to the electron-accepting site of PS I (P^+-700) [51] whereas DAD and DCIP require plastocyanin to feed electrons to P^+-700 [38], the sequential comparison of not only the appearance of electron transport, proton gradient creation and the ability to form ATP, but also the effect of uncoupling by NH_4^+, was evaluated (Table III). Prior to 2 h of illumination the addition of NH_4Cl does not enhance electron flow-dependent oxygen consumption in prothylakoids nor does it reduce the ratio of proton uptake to oxygen consumption

TABLE III

COMPARISON OF ELECTRON TRANSPORT, PROTON PUMPING AND ATP FORMATION

Effect of NH_4^+ (20 mM) on electron transport-dependent oxygen consumption on the ratio of (OH^- formation)/(O_2 consumption) with 0—4-h prothylakoid vesicles. The values are compared to the rates of photophosphorylation with 15 μg/ml phenazine methosulfate (PMS) and 1 mM DAD as cofactors. Experimental conditions for DAD-dependent O_2 consumption, OH^- and ATP formation in the light were the same as in Figs. 6, 10 and 11, respectively. Protein concentration was about 2 mg/ml.

Time of illumination (h)	Electron transport (= O_2 consumption) (μmol · mg^{-1} protein · h^{-1})		(OH^- formation)/(O_2 consumption)		ATP formation (nmol · mg^{-1} protein · h^{-1})	
	DAD	DAD + NH_4Cl	DAD	DAD + NH_4Cl	DAD	PMS
0.5	7.5	7.3	1.00	1.00	0	3.1
1	8.9	8.8	1.08	1.04	0	5.4
2	12.1	18.2	0.86	0.67	2.0	7.2
3	32.0	54.6	0.76	0.52	6.0	8.4
4	59.1	126.0	0.74	0.39	12.2	14.0

using ascorbate/DAD. From 2 h onwards uncoupling starts at the same time as DAD-induced ATP formation takes place but long after phenazine methosulfate-induced ATP formation has existed. This critical period of the appearance of uncoupling coincides with the appearance of PS II activities (cf. Fig. 7 and Table II). Similar results (not shown) to those quoted here were also obtained using the uncoupler, CCCP.

Discussion

The isolation of prothylakoids from prolamellar body preparations was an obvious step from earlier work [28,30] and the main problem was to find the optimum sonication frequency and period to obtain the best results. Insufficient ultrasound treatment produced poor yields of prothylakoids plus high contamination of the enriched prolamellar body preparation fraction with prothylakoids; whilst excessive agitation induced an increase in the rates at which CF_1 particles were dislodged from the membranes and the prolamellar body structures themselves started to disassociate. Consequently the time and frequency of ultrasound treatment employed was an optimum between these factors and explains why the contamination levels of the enriched prolamellar body preparations could not be reduced further.

The methods described here should be equally applicable to other etioplast-chloroplast transformation systems. The advantage offered by oats (*Avena*) and also wheat (*Triticum*) over barley (*Hordeum*) is the fact that their prolamellar bodies have tighter crystalline structures and tend to dissociate less readily. Their plastid envelopes also lyse more readily [30]. In our experience the *Avena* development system is slightly slower than the *Hordeum* system especially during the first hour and this allows closer inspection of the early events of greening. *Zea* is also commonly employed but the dimorphism of the maize plastids is a complication to be avoided at the moment. In general, for the study of greening systems, there are considerable advantages of the mono-

cotyledonous systems over the dicotyledonous due to the availability of sufficient tissue to macerate and enable high yields of preparations, combined with the greater speeds of greening and the simplicity of ontogeny. This is in contrast to studies of green tissues where there is no strong monocotyledonous competitor to spinach or young peas for ease of isolation and study of photosynthetic performance of plastids.

In studies on development the basis of expression of various specific activities is a problem. Chlorophyll appears during greening and so is almost unusable for the very early stages of development, giving rise to falsely high estimates of some activities such as phenazine methosulfate-dependent photophosphorylation [17]. For whole plastids expression of activities based on a plastid number is ideal [28,52] but for preparations involving isolated inner membranes this is difficult to derive. In all the experiments described in this paper we have expressed specific activities on a protein basis. This has proved satisfactory. Very similar results were obtained when a plastid number expression was used. The exceptions to this were the 24-h results which were always lower than when protein was the basis of expression, reflecting the increase in volume and stromal content of plastids over the 8—24 h period.

The prolamellar body is clearly shown to be involved in the storage of β-carotene which may be used for insertion into the prothykaloids before biosynthetic production and direct insertion into the developing membrane is sufficient to meet demands (Figs. 4 and 5) but it is far from clear that a similar situation with respect to photostimulated reduction of protochlorophyllide exists. Previously it has always been presumed that the protochlorophyllide holochrome was associated with the structures of the prolamellar body proper, and the first chlorophyllide to appear then formed part of the newly active membrane, possibly *P*-700. Lütz [50] has recently prepared vesicles very similar to our prothylakoids and has evidence that the protochlorophyllide-chlorophyllide transformation takes place on the prothylakoids and not within the prolamellar body structure proper. This has the advantage that a shorter distance for chlorophyllide transfer to sites of utilization exists. The general availability of prothylakoid preparations should enable studies to be made to confirm or refute these initial indications.

The use of the trypsin-activated Ca^{2+}-dependent ATPase activity of the CF_1 particles as a marker for prothylakoid membranes [28] was extremely useful and has enabled certain deductions to be made concerning the properties of the prolamellar body proper. Perhaps the most important of these is the conclusion that no photochemical activity associated with photosynthetic electron transport exists within the structure making up the prolamellar body proper. To facilitate discussion, Table IV has been drawn up to summarize the acquisition of various photochemical activities throughout the developmental process for prothylakoids (0—4 h) and unresolved inner membranes (5—24 h).

The first electron transport reactions to appear during greening for both unresolved membranes and prolamellar vesicles are those associated with PS I (Figs. 6 and 7, respectively). The only difference between the two types of membrane preparation being much higher specific activities in the latter due to the absence of non-contributing prolamellar body material. All systems showed a more or less steady increase of electron transport-mediated oxygen consump-

TABLE IV

TIME OF APPEARANCE OF VARIOUS COMPONENTS OF PHOTOSYNTHESIS

Summary of results described earlier (Figs. 6—11, Table II and III) in this paper to indicate the approximate time of appearance of most of the basic function associated with photosynthesis in greening *Avena* laminae. With the exception of the use of intact 4-h etiochloroplasts to identify the onset of CO_2 fixation (Ref. 20) and unresolved inner membranes from 8-h etiochloroplasts, all times quoted are the earliest obtained from prothylakoid vesicles (PTVs) isolated from etioplasts and 0.25—4-h etichloroplasts. MV, methylviologen; PMS, phenazine methosulfate.

Time of development (h)	Appearance of photosynthetic function
0	NADP-dependent oxidoreductase activity
0.25	Ascorbate/DAD → PS I → MV electron flow PMS-dependent photophosphorylation
0.5	Ascorbate/TMPD → PS I → MV electron flow
1	Ascorbate/DCPIP → PS I → MV electron flow
2	DPC → PS II → DMMIB electron flow NH_4^+-induced uncoupling of ascorbate/DAD electron flow DAD-dependent proton pumping
3	H_2O → PS II → DMMIB, DCIP or ferryicyanide electron flow DCIP-dependent proton pumping DAD-dependent photophosphorylation
4	H_2O or DPC → PS II → PS I → MV or PNDA electron flow DCIP-dependent photophosphorylation Light-dependent CO_2 fixation (see Ref. 20)
8	Disappearance of DPC → PS II → DMMIB electron flow indicating completion of water-splitting capability

tion on a protein basis up to 8 h of illumination. Similar results have been reported before unresolved membrane fractions from barley with TMPD [7,9,11]. The reason for the different developmental stages at which DAD, TMPD and DCIP start to take part in the photochemical reactions could be due to differing lipophilic properties of these cofactors (DCIP being the least lipophilic) and to changes in the constitution of the prothylakoid membrane during greening. The presence of NADP-dependent oxidoreductase in the etiolated condition is also in accordance with the results of other authors [4] but the enriched prolamellar body preparation results indicate that this function may also exist within the prolamellar body structure proper unlike any of the other electron transport functions examined.

With the oxygen electrode, activities associated with PS II are only detected after 2—4 h of illumination (Figs. 8 and 9, Table II). This has been described many times [6,7,9,21,22]. Nevertheless when primary photochemical activities are measured spectrophotometrically by photooxidation of *P*-700 and cytochrome *b*-559 and the photoreduction of C550 in intact leaves frozen to −196°C the reaction centres of both PS I and PS II appear within minutes of illumination and with PS II activities accumulating slightly prior to those of PS I [12]. This difference is taken to mean that a block exists in secondary electron transfer reactions perhaps partly on the water-splitting site of PS II. Vernon and Shaw [40] were the first to demonstrate that DPC is only an electron donor for PS II when the water-splitting site of PS II is inactivated (e.g. by

Tris). Accordingly, the transient additional electron transport rates in the presence of DPC compared to those involved wih H_2O splitting suggest a step by step coupling of PS II activities and H_2O splitting. Starting from about 4 h after the onset of illumination PS II-associated electron flow increases rapidly, exhibiting a lag between pigment synthesis and the later formation of the H_2O-splitting system. During this period PS II should be readily accessible for DPC. With increasing time of development the rate of construction of the H_2O-splitting site catches up with that of the pigment system. This process is then obviously finished after about 8 h of illumination as indicated by the inaccessibility of PS II for DPC. DPC as a donor for PS II was also used by Hennigsen and Boardman [11] for developmental studies, but these authors were not able to detect similar effects because of the use of Tris-washed organelles. It is not known if another block exists somewhere after the PS II reaction centre and before plastoquinone. Electron transport-associated consumption (H_2O or DPC → methylviologen → O_2) or evolution (H_2O → PNDA) of oxygen shows the same time-dependent sequence as observed for PS II alone. This finding supports the idea that all endogenous redox carriers between PS II and PS I are present before the start of PS II activities, or that at least they are built in parallel to PS II. What is certain is that (0.25—3 h) prothylakoids allow investigations concerning PS I with measurable activities in the oxygen electrode cell which are independent and operate without the need to add inhibitors such as DCMU or DBMIB to stop electron flow from PS II.

The prothylakoids develop an active proton pump shortly after PS I activities are functioning (Fig. 10). Reduced DAD induces a light-driven ATP formation after about 2 h of illumination with higher specific activities in the prothylakoid fraction whereas there is no reduced DCIP or ferricyanide-dependent photophosphorylation detected until 4—5 h of illumination. By 15 min there exist appreciable rates of photophosphorylation with phenazine methosulfate using prothylakoid preparations although not with preparations derived from completely etiolated tissue (Fig. 11). Plesničar and Bendall [17] also observed cyclic phosphorylation with phenazine methosulfate as a cofactor immediately after the onset of illumination, but according to the properties of this compound, phenazine methosulfate will give artificial rates of photophosphorylation [51]. It has been shown that reduced phenazine methosulfate is able to react even directly with P^+-700 and therefore can support cyclic electron flow at extremely high rates, showing nearly no light saturation [51]. Other cofactors of cyclic photophosphorylation like DAD or DCIP require plastocyanin to close the cycle around PS I (see Ref. 38). Because of this, the use of these latter electron donors should reflect the conditions for photophosphorylation in vivo more closely. Accordingly ATP formation in developing *Avena* plastids is unlikely to start in significant quantity before 2 h of illumination have passed. This also fits the results obtained with uncoupling agents such as NH_4^+ and CCCP (see Table III and Fig. 6). These suggested that until the full functioning of PS II there are proton-permeable areas within the prothylakoid membrane, leading to a discharge of the small proton gradient built up by the electron flow around PS I. Because of this uncontrolled discharge, protons will not effectively pass through the already existing CF_1 particles. With the beginning of PS II activity and the availability or insertion of water-

splitting capacity, these proton leaks disappear giving advent to a progressive rise in proton gradients across the prothylakoid membranes accompanied by the synthesis of ATP (compare Fig. 10 with Fig. 11). Following the same intimation, the early occurrence of phenazine-methosulfate-induced photophosphorylation may also be explained. Due to the very high rates of proton transport across the prothylakoid membrane by this cofactor (especially at high light intensities) the uptake of protons will exceed the uncontrolled discharge across the membrane, thereby channelling some protons through the CF_1 particles, which then leads to ATP formation.

In conclusion, the benefits from this integrated study of the prothylakoid, enriched prolamellar body preparation and unresolved inner membrane preparations have answered many questions, principally that of where the early photochemical activities are located. It has also shown that certain components such as β-carotene and NADP-dependent oxidoreductase are stored in the prolamellar body and may be transferred from the prolamellar body to the prothylakoids during development. Nevertheless, perhaps the most important finding is that, if prolamellar bodies are removed from inner membrane preparations of early development states, the sequence or order of appearance of components in either prothylakoids or unresolved inner membranes is basically the same, and that only the specific activities based on protein in prothylakoids are higher. The reduction in unspecific photooxidation is a bonus. Consequently, with the removal of the complication of the prolamellar bodies from these preparation, a major objection to conclusions drawn from greening studies in the past, we suggest that this modified etioplast to chloroplast transformation mirrors more exactly the direct proplastid-chloroplast transformation of light-grown tissues. As the results deviate so little from most of the existing literature [1--11,13—17] and discounting the existence of prolamellar body structures proper in one, the two membrane transformations may be said to be similar in all respects.

We believe this study also to be one of the first to have considered the integration of electron transport, proton pumping and ATP formation and await with interest the use of other techniques (e.g. spectral changes of the *C*-550 component) to derive information from prothylakoid preparations. We ourselves are particularly interested in the capacity of developing plastids to form ATP. The delay (at least 2 h, Fig. 10) in the generation of an effective proton gradient will handicap the ability of the developing plastid to produce ATP which is then available to assist the assembly of photosynthetic components. As this process is likely to demand large quantities of energy the question must be asked from whence it comes especially in the first 2 h of development. In later papers we will present evidence that the mitochondria are specifically synchronized within the developmental process and mechanisms exist which facilitate this additional energy formation.

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