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# 5-AMINOLEVULINATE DEHYDRATASE ACTIVITY IN THYLAKOID-RELATED STRUCTURES OF ETIOCHLOROPLASTS FROM RADISH COTYLEDONS

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**Key Word Index**—*Rhaphanus sativus*, Cruciferae, morphogenesis, phytochrome; chlorophylls, etiochloroplasts, thylakoids, 5-aminolevulinate dehydratase.

**Abstract**—5-Aminolevulinate dehydratase activity was studied in etiochloroplasts purified from radish cotyledons grown for 120 hr under continuous far-red light. The activity was mainly recovered from the stroma of the organelles, as previously observed, but also from the membrane fraction. The bound activity was not altered after incubation in the presence of NaCl or detergents indicating that the enzyme was neither associated with the pelletable fraction by electrostatic forces nor entrapped in lipid vesicles After separation of prothylakoids and prolamellar bodies on a sucrose gradient, 5-aminolevulinate dehydratase activity and two-thirds of the total protein were observed in prothylakoids. Carotenoids were mainly present in the prolamellar bodies fraction. A few chlorophylls were observed in prothylakoids and prolamellar bodies, indicating that impure fractions were obtained, but that 5-aminolevulinate dehydratase activity was actually bound to thylakoid-related structures

## INTRODUCTION

The etioplasts of higher plants grown in darkness contain membranous prothylakoids (Pts) and crystalline-like prolamellar bodies (PLBs). These two structures are considered to be precursors or storage sites for the functional chloroplast membranes appearing after exposure to light.

Recent work on the biochemical composition of both Pts and PLBs has highlighted the differences in their composition and probable functions [1] Most of the proteins and pigments involved in the photosynthetic apparatus are found in Pts [1–4] whereas the PLBs contain only products considered to accumulate as a result of the operation of abnormal metabolic pathways in the dark [5].

This assumption was in part confirmed by studies on the photochemical functions appearing during rapid greening of *Avena* laminae [6]. This study also showed that carotene is stored in PLBs before transfer to Pts, indicating a possible relationship between both structures in the light. Ikeuchi and Murakami [7], along with Ryberg and Sundqvist [8] found that the protochlorophyll–NADPH reductase complex in *Avena* and *Triticum* respectively is concentrated in PLBs, while the latter authors implicate phototransformable protochlorophyllide as a constituent of both PLBs and PTs [2] Thus, conflicting results have been obtained on the composition and therefore function of Pts and PLBs in etioplasts, possibly as a result of the different experimental protocol used for their preparation However, despite these contradictory data on the actual role of Pts and PLBs, and their relationships, it is considered that they are involved in the preliminary processes of the establishment of the photosynthetic apparatus [9].

The tetrapyrrole compounds of chlorophyll haem, and probably the last enzymes involved in the metabolic pathway are bound to internal membranes of etioplasts [10, 11] and some of them are present in Pts [8]. The 5-aminolevulinic acid-synthetizing system is located in the stroma of green chloroplast [12, 13], but the enzymes catalyzing the intermediary stages in the pathway have not been unambiguously located inside the organelles Furthermore, all the data available on enzyme localization in organelles were obtained mainly with etioplasts from dark grown plants or chloroplasts formed after rapid greening (mainly induced using flashes of white light)

Light not only acts as a photochemical effector on the transformation of protochlorophylls to chlorophylls, but also modulates, via phytochrome, protein synthesis [14] By using continuous standard far-red (FR) light [15], photomorphogenetic responses are observed [16], as this irradiation establishes a low but active amount of $P_{FR}$ in the cells Only traces of chlorophylls are found, estimated at 5% of the total amount of pigment detected under white light [17]. If, under such irradiation, PLBs become smaller, and a large amount of Pts are observed [18], no grana stacking is observed [19], despite the appearance of some preliminary well-characterized photosynthetically related reactions [17]. Thus, these etiochloroplasts represent the ultimate differentiation state which can be reached by the organelles without actinic light They are,

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therefore, a material of interest for the study of metabolic processes taking place immediately before greening

We have studied 5-aminolevulinate dehydratase (ALA-D, E C 4 2.1.24) activity in etiochloroplasts of radish seedling cotyledons grown under continuous FR light The enzyme, after light promoted synthesis in the cytoplasm [20, 21] is transported into the etiochloroplasts under continuous FR light [22] and is probably involved in chlorophyll biosynthesis there [23], condensing two 5-ALA into porphobilinogen However, ALA-D localization inside the organelles is not precisely known as pointed out by a few conflicting results [24, 25] On the other hand, in a previous study, the activity was considered as entirely stromal [22] However, after slightly changing the experimental procedure and using another radish cultivar we observed that some ALAD activity is recovered from membranes after plastid disruption Furthermore, any enzymatic activities, recognized as stromal, can be associated with thylakoids after various light treatments [26]. Here we present evidence that a small amount of enzymatic ALA-D activity can be observed bound to thylakoids As we have previously shown that the total activity increases in etiochloroplasts with time from sowing, up to 120 hr, we have chosen this time of seedling differentiation for the present study

## RESULTS

The activity of ALA-D and an estimation of total carotenoids measured during the major purification steps of thylakoids are shown Table 1 When etiochloroplasts were pelleted after grinding tissues (P), only 9% of total ALA-D activity was recovered When the organelles were further purified after two washes in isotonic buffer (Pe), a significant loss of ALA-D activity was observed· from 25 2 to 5 3 pmol/s/ml This represents only 21% of the activity detected in (P), and 1 9% of the total. This decrease was not due to the rupture of etiochloroplasts followed by leakage of the stromal contents, as carotenoids showed a similar trend Some ghosts of organelles, possibly entrapping released material during grinding, and considered in (P) as true organelles, may have lysed during the washes in isotonic medium

After disruption of the purified organelles in hypotonic buffer and centrifugation, ALA-D activity was measured in the supernatant, the stromal fraction (St = 3 1 pmol/s/ml) and the pellet (thylakoids, $T_1$ = 2 pmol/s/ml), but carotenoids were only observed in the latter fraction, along with 9 1% of the ALA-D activity detected in (P) Thylakoids were then washed with hypotonic buffer and centrifuged again An ALA-D activity of 1 95 pmol/s/ml was observed after the first wash ($T_2$ = 6 5% from P) and 1 4 pmol/s/ml after a second wash ($T_3$ = 5 5% from P) Along with these results, a parallel decrease in carotenoids was also observed, though no significant measurement could be performed on the corresponding supernatant (data not shown)

The enzymatic activity observed in thylakoids was very low in comparison to those detected in the crude extract (0 7% of total activity in $T_1$ and 0 5% in $T_2$), and artifacts may easily have been generated by osmotic shock and extensive washing Membranes are negatively charged in buffered solutions [27], and electrostatic interactions are favoured by the low ionic strength of the hypotonic buffers Pellets ($T_3$) were incubated in the presence of NaCl solutions, giving final concentrations ranging from 0 25 to 1 M [28] Only a slight release of ALA-D activity was observed, along with a decrease in the carotenoid content of the pellets after washing and recentrifugation (data not shown) Another source of artifacts might be aggregation or swelling of membrane fragments after their disruption in aqueous medium [8]

Table 1 ALAD activity and distribution of carotenoids in etiochloroplasts and thylakoids after washings in hypotonic buffer

| Preparation | | ALAD activity (pmol/sec/ml) | (%) | | Carotenoids (OD 480 nm/ml) |
|---|---|---|---|---|---|
| Crude extract | S | 256 | From pellet P | From total activity | 13 5 |
| | P | 25 2 | 100% | 9% | 4 3 |
| (Tris–HCl, 0 1 M, 2-mercaptoethanol, 5 mM, $MgCl_2$, 10 mM, and D-mannitol, 0 6 M) | | 281 2 | | 100% | 17 8 |
| Purified etiochloroplast | Pe | 5 3 | 21% | 1 9% | 0 52 |
| Broken etiochloroplast (Tris–HCl, 10 mM, $MgCl_2$, 10 mM, and 2-mercaptoethanol, 5 mM) | ST | 3 1 | | | Not-detectable |
| Washed thylakoids (Tris–HCl, 10 mM, $MgCl_2$, 10 mM, and 2-mercaptoethanol, 5 mM) | $T_1$ | 2 | 9 1% | 0 7% | 0 51 |
| | $T_2$ | 1.95 | 6 5% | 0 7% | 0 41 |
| | $T_3$ | 1 4 | 5 5% | 0.5% | 0 32 |

Etiochloroplasts were purified by differential centrifugation and the pellets were washed two times with extraction buffer Stromal fraction (St) and crude thylakoids ($T_1$) were separated by centrifugation after disruption of the organelles in hypotonic buffer Thylakoids were washed twice in the same buffer

Soluble material can then be pelleted inside these vesicles and considered as bound molecules. Pellets ($T_3$) were incubated in the presence of increasing concentrations of three kinds of detergents [29] After a one hr incubation in their presence and centrifugation, the results shown in Fig. 1 were obtained. Clearly both SDS and Triton X100 alter the location of carotenoid pigments (Fig. 1 a,b) Thus they were solubilized from the pellet and released after centrifugation into the supernatant. This behaviour was not observed after incubation with CTB (Fig. 1c). The effect of SDS treatment on ALA-D activity was an immediate decrease both in the supernatant and in the pellet This effect had been observed during preliminary experiments on purified, soluble, ALA-D and certainly represents denaturation [30] Triton X100 or CTB have no effect on ALA-D activity in such control experiments, as confirmed in Fig. 1 b,c when the measurement was performed on the supernatant collected after the incubation. However, an increase of activity was observed in the pellet ($T_3$) incubated in the presence of Triton X100 or CTB and then recentrifuged. Therefore, because neither NaCl nor detergent treatment can release this ALA-D activity from pelletable material, it was considered that some ALA-D molecules are tightly bound to pelletable material obtained after disruption of purified etiochloroplasts.

In etiolated material, after isopycnic centrifugation, thylakoid membranes can be separated into prothylakoids (Pts) and prolamellar bodies (PLBs [31]). When pellet ($T_3$) was mechanically broken and applied to the top of a continuous sucrose gradient, the carotenoids banded mainly at a sucrose concentration of 1.25 g/ml, but ALA-D activity was mainly recovered at 1.13 g/ml (Fig. 2). These results, by comparison to those reported by other authors [1, 8], indicated that the carotenoids were localized mainly in PLBs, whereas the enzymatic activity was more closely associated with Pts. The two fractions were pooled and protein concentration estimated in both of them One-third of the proteins was detected in PLBs, and the remaining protein was located principally in Pts, as previously noted [2, 7] One half of the Pts and PLBs obtained after isopycnic centrifugation was pelleted again, after dilution in hypotonic buffer to decrease the sucrose concentration. The pellets were washed and extracted with 80% acetone [32], in order to analyse pigments by spectrophotometry (Fig. 3a) Scanning from 700 to 350 nm revealed that both fractions exhibit similar profiles with peaks at 420, 440, and 472 nm due to carotenoids However, the absorption at 480 nm, which is used for carotenoid estimation [32], was about one-half as intense in Pts as in the PLB preparation (Pts, O D. 0.08, PLBs, O.D. 0 18), confirming our former observation (Fig 2) Pts exhibited a small peak at 485 nm which might be due to carotene, and which was absent from PLBs preparation. However, this latter maximum was not detected in all suspensions which were scanned, despite the standardization of the procedure used. In the red region of the spectrum, both preparations absorb at 662 nm, due to chlorophylls. As for carotenoids, the pigment was mainly observed in PLBs No absorption due to protochlorophylls was observed at 635 nm, but a broad peak at 620–630 nm, of unknown origin was present Figure 3b shows the concentration of chlorophylls extracted by grinding 25 radish seedling cotyledons in 80% acetone at different times from sowing As can be seen, the amount of chloro-

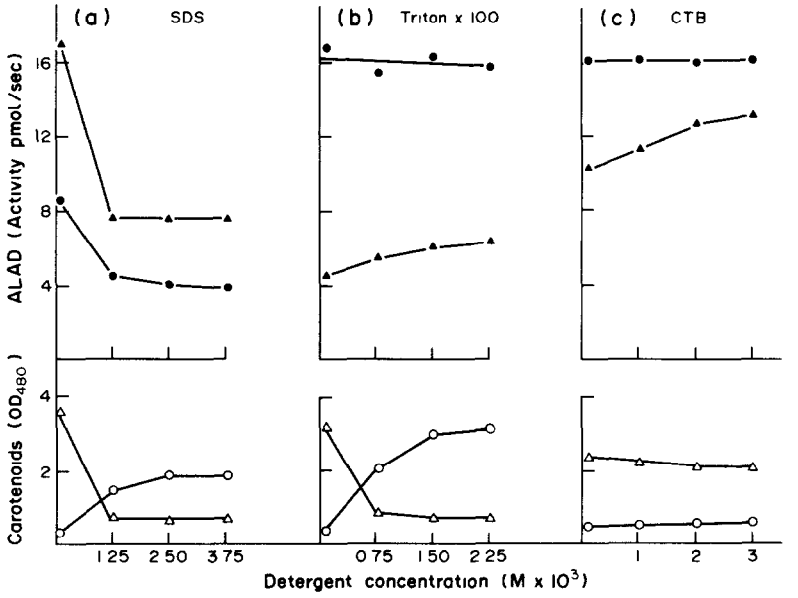


Fig 1 ALAD activity after incubation of thylakoids for 1 hr in the presence of increasing concentrations of detergent and after centrifugation at 32000 *g* for 20 min. (a) SDS treatment, (b) Triton X100 treatment, (c) CTB treatment, ●—● , ALAD activity recovered in supernatant, ▲—▲, ALAD activity remaining in pellet, ○—○, carotenoids in supernatant, ——, carotenoids in pellet

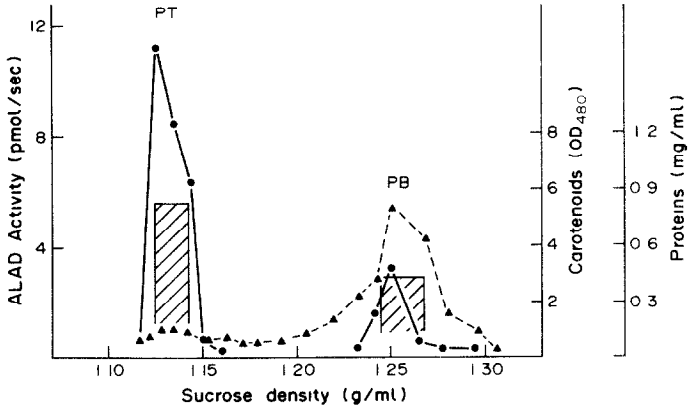


Fig 2 Elution profile of sucrose density gradient (15–60% w/v) after isopycnic centrifugation (87 000 *g* for 30 min) of mechanically sheared thylakoids ▲---▲, carotenoids, ●——●, ALAD activity, □□□, proteins Sucrose density was assayed by refractometry

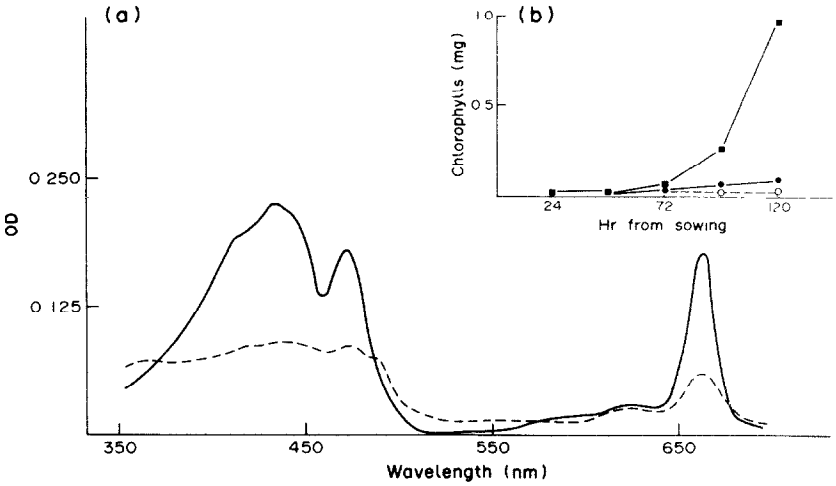


Fig 3 Chlorophyll estimation in radish cotyledons grown under continuous standard FR light (a) Absorbance spectra of PLBs (——) and Pts (----) in 80% acetone, (b) Chlorophyll (mg) detected in 25 radish seedling cotyledons grown up to 120 hr from sowing (■——■, under continuous white light, ●——●, under continuous standard FR light, ○——○, in complete darkness)

phylls appearing under continuous FR light is consistent with the 5% amount of those observed under white light and also reported previously by Weistrop and Stern [17]

## DISCUSSION

The enzymes involved in chlorophyll biosynthesis are localized in chloroplasts or etioplasts [33]. The regulation of ALA formation occurs in the stroma of these organelles [12, 13] and the last steps of haem modification occur in the thylakoids [10]. However, the few reports on ALA-D localization in organelles are quite confusing. In tissue cultures of *Kalanchoe*, ALA-D was reported to be intimately associated with internal membranes of chloroplasts [34], but in *Cucumber* etioplastids, only soluble enzyme was found [24]. In a previous report, we considered the enzyme as entirely stromal However, after a change in radish cultivar, we re-examined the enzymatic activity found in pellets after disruption of organelles A low activity was observed in etiochloroplast membranes Starting from a large amount of material, this activity is however easily detectable As the numerous manipulations may generate artifacts, we have taken great care to prevent their formation, starting with the hypothesis that our observation would come from artificially entrapped enzyme in biological vesicles arising after drastic changes of osmotic strength However, neither NaCl treatments, used to release ionic linkages, nor washes with detergents caused the enzyme to be released into the supernatant Measurements of carotenoids in the same experiments using detergents show that these pigments can be solubilized from the membrane fraction, while ALA-D activity remains constant in the pelletable material, if not increased after Triton X100 One explanation would be that ALA-D and carotenoids have a different localization within membrane structures

Treatment using increasing concentrations of CTB induces an increase in ALA-D activity, but is without effect on carotenoids This might confirm the above assumption. If CTB acts only near the enzyme in removing some unknown molecules, it could give greater access to substrate and therefore, lead to an increase in overall activity. Triton X100 would have a similar effect but in removing the structures containing carotenoids.

This hypothesis had to be confirmed, as different experimental procedures have been used to prepare and purify the main parts of internal etiochloroplast membranes [2, 7, 31]. After mechanical shearing of etiochloroplast membranes, gradient centrifugation gave two distinct fractions, at 1 13 and 1 25 g/ml sucrose. In agreement with published data, these fractions were identified as Pts and PLBs respectively [2, 31] The ALA-D activity was mainly localized in the lightest fraction corresponding to Pts. The presence of two-thirds of the total amount of proteins recovered there agrees also with the presence of the enzyme mainly in Pts. Other membrane-associated activities where also found principally in Pts such as NADPH–chlorophyll oxidoreductase (chlorophyllase, [4]) and ATPase [35]. However, Ikeuchi and Murakami [7] find chlorophyllase in PLBs instead of Pts.

The determination of accessory pigments in photosynthesis has also given rise to controversial results. Carotenoids have been reported to occur in both fractions [7, 31] or, only, in Pts [5] Wellburn and Hampp [6] indicated possible transfer of carotene from PLBs to Pts upon irradiation during greening, but Lütz did not consider any structural relationship between the two structures [3].

The localization of protochlorophylls is also subject to controversy They have been assigned to Pts [4, 8], to both Pts and PLBs [2] with one-third in Pts and two-thirds in PLBs after calculations based on the presence of saponins [1]. These last results are ambiguous because saponins are markers of questionable validity. Initially they were employed to identify PLBs [36], but were subsequently considered mere contaminants [2]. Our results show that carotenoids are mainly associated with PLBs but are also observed in Pts. This could be consistent with the observation of a transfer during greening [6], as etiochloroplasts are temporary structures which, at 120 hr from sowing under continuous FR light, do not undergo further transformations [18]

We did not observe any protochlorophylls absorbing at 638 nm, but chlorophylls which exhibit a clear peak at 662 nm, as observed also previously in bean leaves with a similar light source [19] This important observation indicates unambiguously that the fractions prepared come from the inner membranes of organelles. We can assume also, as chlorophylls should be found only in thylakoids, that, although Pts are relatively pure, PLBs are heavily contaminated by Pts, as previously observed [6]. The presence of chlorophylls under continuous FR light (Fig. 3a) was reported earlier [17, 19]. This is probably due to the long tail of the absorbance of this pigment up to 690 nm, which can then absorb light from standard FR filters [37]. The presence of this pigment may also explain why we did not observe any protochlorophylls. As reported before, protochlorophylls which accumulate in the dark, are transformed to chlorophylls, then allowing new molecules to be formed from ALA [14]. However, under FR light, after 120 hr from sowing, not enough material would be available for synthesis of new haems, as reserves of any kind are probably low in cotyledons [38].

The co-incident presence of ALA-D activity, of a small quantity of carotenoids and of chlorophylls bound to the fraction recovered at 1.13 g/ml sucrose, lead us to the conclusion that we have prepared prothylakoids, albeit impure, with enzymatic activity attached to them. The question arising now is the exact roles of stromal and bound ALA-D activities In studying the evolution of ALA-D activity during etiochloroplast synthesis [22], we tried to extrapolate from the measurements of carotenoids detected in low-speed supernatants to the amount of enzyme *in situ* in organelles. If the same calculation is performed on pellet ($T_3$), roughly 25% of total ALA-D activity would be associated with the membranes. Thus, at 120 hr from sowing under continuous FR light, 50–60% of ALA-D activity is localized in organelles [22], and one-half of this activity should be membrane associated. This enzymatic activity will be lost if any purification step is performed before the measurement of activity This could possibly explain the different values we obtained either by measuring the activity in crude extracts [20], or after purification through Sephadex G25 gel sieving [21]. After purification, the activity of the enzyme detected after culture of radish cotyledons under FR light is the same as that observed in complete darkness. If crude extract is noted a clear difference is observed between the enzymatic activity observed in the light or in darkness [21]. In this latter observation, the higher activity observed should be due to the bound enzyme, which is not detected after purification

The role and origin of this bound activity is of crucial importance Perhaps membranous ALA-D comes from the stromal species, which then represents a transitory state. Both forms may then participate in the formation of chloroplastic haems. Alternatively, the enzymes are part of separate pools working in separate haem biosynthetic pathways such as for chlorophylls and cytochromes. These possibilities are now under investigation

## EXPERIMENTAL

*Seedling growth and light conditions.* Radish seeds (*Raphanus sativus* cv. national, Vilmorin, Le Menitré, 49250 Beaufort en Vallée, France) were grown 120 hr under continuous FR light as described elsewhere [20, 22, 39]. Harvesting and organelles purification were performed under green safelight, and all operations took place in crushed ice or at 4°

*Isolation of etiochloroplasts* Seedlings (50 g fr. wt) were ground with 150 ml 0 1 M Tris–HCl buffer pH 9, containing $5 \times 10^{-3}$ M 2-mercaptoethanol, $10^{-2}$M $MgCl_2$ and 0 6 M D-mannitol, in a Waring Blendor fitted with a special razor blade bucket [40] The brei was squeezed through 5 layers of cheesecloth to remove large uncut material The filtrate was centrifuged (750 g × 10 min, Beckman J 21, rotor JA 20) to settle intact cells and the remaining debris The pellet was discarded and the supernatant was centrifuged again at 2500 *g* × 15 min Aliquots were pipetted off from the supernatant (S) and the pellet (P) to serve as controls Pellet was washed (× 2) with 5 ml extraction buffer from which it was separated by centrifugation (2500 *g* × 15 min) The remaining etiochloroplast were layered onto a density cushion containing 1 3 M sucrose and were centrifuged at 2500 *g* × 30 min Organelles were pelleted and resuspended in 10 ml extraction buffer The mixture was then layered onto a

discontinuous sucrose gradient (1 3–2 M) After a 20 min centrifugation at 4000 *g*, etiochloroplasts were pipetted off from the interface (Pe) and diluted in hypotonic buffer ($10^{-3}$ M Tris–HCl, pH 8 5, containing $5 \times 10^{-3}$ M 2-mercaptoethanol and $10^{-2}$ M $MgCl_2$)

*Preparation of washed membranes and separation of prothylakoids and prolamellar bodies* The broken etiochloroplasts were centrifuged 32 000 *g* × 20 min The yellow pellet contained thylakoidal membranes (T1) The supernatant represented the stromal part of etiochloroplatids (St) Membranes were washed (× 2) in hypotonic buffer and aliquots were taken from pellets (T2, T3)

Thylakoidal membranes in (T3) were disrupted by the use of a glass homogenizer(Potter, 5 strokes) and the extract was layered onto a continuous sucrose gradient (30 ml, 15-60% w/v) and centrifuged 87 000 *g* × 2 hr (Beckman L5-50, rotor 60 Ti), as described previously [31] One ml fractions were collected from the top (density gradient fractionator, Beckman) and assayed for pigment, protein concentration and ALAD activity Sucrose density was measured by refractometry (Atago refractometor, 78671)

*Treatment of washed thylakoidal membranes by* NaCl *and detergents* (i) NaCl Purified membranes T3 were incubated for 2 hr in the presence of different NaCl solns giving final concns ranging from 0 25 to 1 M buffered with 0 01 M Tris–HCl, pH 8 5, containing $5 \times 10^{-3}$ M 2-mercaptoethanol and $10^{-2}$ M $MgCl_2$ (ii) Detergents Three different detergents types were used cationic· Cetyl trimethylammonium bromide (CTB 1 to 3 $\times 10^{-3}$ M), anionic SDS (1 25 to 3 75 $\times 10^{-3}$ M), non-ionic Triton X100 (0 75 to 2 25 $\times 10^{-3}$ M ) [29]

Membranes T3 were incubated for 1 hr in the above buffer in the presence of enough detergent soln to give the indicated final concentrations

After incubation with detergents or salt, the mixture was centrifuged at 80 000 *g* for 30 min (Beckman L5-75-rotor 75 Ti) and measurements were performed on either supernatant or pellet [27]

*Measurements and expression of results* ALAD activity was measured as previously described [20] Aliquots were taken during incubation and the results were plotted vs time to check the linearity of the reaction Porphobilinogen (PBG) concentration was estimated according to [41] and ALAD activity was expressed as pmol PBG/ml formed from 5-aminolevulinic acid/s

Pigments were measured after extraction in 80% $Me_2CO$ Carotenoids were estimated according to [32] after correction of the optical density (O.D ) measured at 480 nm by the absorbancies at 645 and 663 nm Chlorophylls was extracted from 25 radish seedling cotyledons grown either in white light, standard FR light, or complete darkness and ground in 80% $Me_2CO$ After centrifugation, chlorophyll was estimated according to [32]

Fractions containing either Pts or PLBs were pooled separately and diluted with hypotonic buffer in order to decrease the sucrose concentration (1 to 5). Membrane structures were pelleted again (Beckman L5-50, rotor 60 Ti, 32 000 *g* for 20 min ) and material was resuspended with 80% $Me_2CO$ according to [32] Spectra were recorded using a Shimatzu spectrophotometer Protein concentration was estimated according to the procedure described by Bradford [42]

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