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DISTRIBUTION OF PLASTOQUINONES IN FRACTIONATED CHLOROPLASTS

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SUMMARY

The distribution of plastoquinones A and C has been studied in photochemically active fragments derived from chloroplasts. Fractionation procedures include digitonin and Triton X-100 treatment and sonication. After detergent treatment low levels of plastoquinones A and C are present in fragments which show high indophenol photoreductase activity (reaction 2) whereas high levels of the quinones are found in fragments which show high activity in $NADP^+$ reduction dependent on ascorbate (reaction 1). On the other hand, after sonication a fraction is obtained which has high reaction 2 activity but low levels of plastoquinones A and C. It appears that maximum photoreductase activity can be obtained in fractions which have one-tenth the original level of plastoquinone A and C.

INTRODUCTION

Localization of any one of the nine chloroplast quinones in chloroplast fractions that contain different light reactions would help determine the site of function of the quinones. We have studied the distribution of plastoquinones, tocopherolquinone and vitamin K in chloroplast fractions which have been prepared by several procedures.

In previous studies BECKER, GROSS AND SHEFNER[1] have shown high plastoquinone A and chlorophyll content in the most active fractions as determined by photoreduction of ferricyanide. In other words, plastoquinone A content was correlated with activity. The use of digitonin to fragment chloroplasts into fragments which show activities characteristic of two different light reactions has been developed by BOARDMAN AND ANDERSON[2]. A fractionation of this type was used to obtain a different view of quinone distribution. Still another approach was provided by the fractionation with Triton X-100 developed by VERNON AND SHAW[3,4].

We have recently shown a requirement for both plastoquinone A and C for the restoration of indophenol photoreductase activity in heptane-extracted chloroplasts[5]. Therefore we have especially studied the distribution of these two quinones along with the activity in the fractions. Other quinones were estimated also. Photoreduction of indophenol was used to measure the second light reaction, photoreduction of $NADP^+$

Abbreviations: CMU, 1-(*p*-chlorophenyl)-3,3'-dimethylurea; DCIP, 2,6-dichlorophenylindophenol; PQ, plastoquinone.

was used to measure the overall activity of the combined light reactions and photoreduction of $NADP^+$ with ascorbate as substrate along with indophenol as catalytic carrier dye and CMU to inhibit the combined reactions was used to measure the first light reaction.

METHODS

Isolation of chloroplasts

Spinach chloroplasts were prepared in 0.50 M sucrose containing phosphate buffer (pH 7.5) according to the method described previously[6].

Fractionation procedures

Digitonin-fragmented chloroplasts were prepared by incubation of 200 ml chloroplasts (containing 200 mg chlorophyll) at 0° in 0.05 M phosphate buffer (pH 7.2), containing 0.01 M KCl and 0.5 % digitonin. Triton X-100-fragmented chloroplasts were prepared under the same experimental conditions as employed with digitonin with 0.08 % Triton X-100 substituted for digitonin. Sonically fragmented chloroplasts were prepared by vibrating 25-ml suspensions of chloroplasts (1 mg chlorophyll per ml) in 0.15 M NaCl in a Branson sonicator set at 4 A for 3-min periods.

Suspensions of the above fragmented chloroplasts were fractionated by differential centrifugation. Fractions were collected at the following centrifugations: 1000 $\times$ *g* for 10 min, 10000 $\times$ *g* for 30 min, 50000 $\times$ *g* for 30 min and 144000 $\times$ *g* for 60 min. The pellet from each spin was resuspended in 0.015 M NaCl (pH 6.8) for photoreductase assays.

Photoreduction assays were measured spectrophotometrically in 3.0 ml of reaction mixture. $NADP^+$ photoreduction with water as the source of electrons was carried out by the method of KEISTER, SAN PIETRO AND STOLZENBACH[7]. Photoreduction of $NADP^+$ with ascorbate–DCIP as the reductant was assayed by the method of VERNON AND ZAUGG[8]. Indophenol reduction was carried out in a reaction mixture of 1 ml 0.02 M phosphate (pH 7.4); 2.0 ml DCIP (10^{-5} g/ml); 0.01 ml chloroplasts (1.5 mg chlorophyl per ml).

Quinone determination was made by extracting chloroplasts containing 3–5 mg of chlorophyll. The chloroplasts were extracted with 25–50 ml of ethanol until the residue was colorless. The total extract was evaporated to dryness, taken up in 3 ml of heptane–diethylether (2:1, v/v) and streaked on a silica gel GHR plate. For development chloroform solvent is allowed to ascend to a height of 15 cm. Quinone bands on the plates were located by spraying a single spot of extract at one side of the plate with leuco methylene blue[9]. A strip of adsorbent silica gel corresponding to the developed quinone spots is scraped off the plate in 3 ml of ethanol and centrifuged for 3 min at 1000 $\times$ *g* to remove silica gel. Amounts of quinone were determined spectrophotometrically. Chlorophyll was determined by the method of ARNON[10]. The ratio of chlorophyll *a*: chlorophyll *b* was calculated according to ARNON's procedure[10].

RESULTS

Component and activity distribution

The membrane fractions prepared with digitonin according to the BOARDMAN AND ANDERSON procedure[2] show the distribution of chlorophyll and separation of

activity which they have described. The ratio of chlorophyll *a*: chlorophyll *b* in our preparation shifts from 2.4 in the heaviest fraction to 5.4 in the lightest fraction. The intermediate fraction still possesses a high level of $NADP^+$ reduction activity which indicates both reactions 1 and 2 are functioning together. The quinone distribution shows the majority of quinones of all types in the lighter fraction whereas only one-tenth remains in the heavy fraction associated with high indophenol reduction activity. The distribution of activity and plastoquinones A and C are shown in Table I. Vitamin K_1, α-tocopherol and α-tocopherylquinone were also concentrated in the 50000 × *g* fraction in a pattern similar to plastoquinone A, but quantitative values for the former are more difficult to obtain on the material available.

The fractionation procedure does not appear to produce the distribution of activity by selective inhibition of different reactions in the separated fractions because the overall recovery of each partial activity (reaction 1 and 2) in the sum of fractions is good. A slight overall increase in indophenol photoreductase activity may be related

TABLE I

DISTRIBUTION OF CHLOROPHYLL, PLASTOQUINONES AND PHOTOREDUCTASE ACTIVITY IN CHLOROPLAST FRAGMENTS OBTAINED BY DIGITONIN TREATMENT

Fraction	*Chlorophyll a/chlorophyll b*	*Total chlorophyll (mg)*	*Reductase activities* (μmoles reduced/min per mg chlorophyll)*			*Quinone content (μmoles/mg chlorophyll)*	
			DCIP	*$H_2O \rightarrow NADP^+$*	*DCIP⁻ ascorbate→ $NADP^+$*	*PQ A*	*PQ C*
Original	2.73	200.0	1.65	1.86	0.85	0.13	0.057
1 000 × *g*	2.39	33.4	1.48	0.09	0.60	0.01	0.009
10 000 × *g*	2.44	130.8	2.68	1.10	1.08	0.01	0.006
50 000 × *g*	4.70	23.1	0.002	0.50	1.64	0.41	0.069
144 000 × *g*	5.41	6.3	0.00	0.00	1.27	0.05	0.015
Supernatant	—	1.2	0.0	0.00	0.18	0.03?	0.01?

* DCIP indicates reduction of indophenol in light, $H_2O \rightarrow NADP^+$ indicates the overall photoreduction of $NADP^+$ by chloroplasts without an artificial electron donor, DCIP–ascorbate $\rightarrow NADP^+$ indicates photoreduction of $NADP^+$ with ascorbate as electron donor and DCIP as catalyst.

TABLE II

DISTRIBUTION OF CHLOROPHYLL, PLASTOQUINONES AND PHOTOREDUCTASE ACTIVITY IN FRAGMENTS OBTAINED BY TREATMENT OF CHLOROPLASTS WITH TRITON X-100

Fraction	*Chlorophyll a/chlorophyll b*	*Total chlorophyll (mg)*	*Reductase activities (μmoles reduced/min per mg chlorophyll)*			*Plastoquinones (μmoles/mg chlorophyll)*	
			DCIP	*$H_2O \rightarrow NADP^+$*	*DCIP⁻ ascorbate→ $NADP^+$*	*PQ A*	*PQ C*
Original	3.04	450	2.06	5.81	0.6	0.1	0.05
1 000 × *g*	2.29	367	5.47	3.14	0.06	0.07	0.02
10 000 × *g*	3.45	64	1.47	2.84	0.3	0.11	0.085
50 000 × *g*	4.25	1	0.41	0.90	0.004	0.08	0.02

to increased availability of dye in the fragmented particles. Likewise in several runs there has been good recovery of total quinones which would preclude any breakdown or conversion of quinones.

A selective distribution of chlorophylls is also shown in fractions prepared by addition of Triton X-100 to chloroplasts. Again the heaviest fraction has the highest ratio of chlorophyll *b*:chlorophyll *a*. Unfortunately we have only been able to recover good indophenol reductase activity in these fractions. The activity is concentrated in the heavy fraction as shown in Table II. The ascorbate-dependent $NADP^+$ reduction is decreased considerably from the original. The quinone distribution pattern again follows the distribution observed with the digitonin fractionation. Only low levels of plastoquinone A and C are found in the most active indophenol photoreductase fraction whereas the rest of the two quinones are found in the fraction with a higher chlorophyll *a*:chlorophyll *b* ratio: a fraction which shows only limited indophenol reduction activity.

Sonication of the chloroplasts also produces particles of different density[11]. The distribution of chlorophylls in these fractions shows less change in ratio of chlorophyll *a*:chlorophyll *b*, but there is some accumulation of chlorophyll *a* in lighter fractions. The heaviest fraction 1000 × *g* obtained by sonication shows a doubling of plastoquinone A over the original but no increase in plastoquinone C (*cf.* Table III). The various activities in this fraction are not greatly different from the original so the excess PQ A in this fraction is not associated with any of the activities under study. The 10000 × *g* fraction shows an increase in both indophenol reduction and $NADP^+$ reduction activities and a decline in ascorbate–indophenol-based $NADP^+$ reduction. PQ A is at one-half the original level whereas PQ C is twice the original level in this fraction. The 50000 × *g* and 144000 × *g* fractions show reduced indophenol reduction and $NADP^+$ reduction but enhanced ascorbate–indophenol $NADP^+$ reduction. Both of these fractions have low levels of both PQ A and PQ C. Addition of plastoquinones A and C separately or in combination did not stimulate activity in any of the fractions which show a low quinone content. The same levels or lower levels of plastoquinone A and C (0.1 μmole and 0.01 μmole per mg chlorophyll) will restore indophenol reduction activity in chloroplasts which have been extracted with heptane.

TABLE III

DISTRIBUTION OF CHLOROPHYLLS, PLASTOQUINONES AND PHOTOREDUCTASE ACTIVITIES IN FRAGMENTS OBTAINED BY SONICATION OF CHLOROPLASTS

Fraction	*Chlorophyll a/chlorophyll b*	*Total chlorophyll (mg)*	*Reductase activities (μmoles reduced/min per mg chlorophyll)*			*Plastoquinones (μmoles/mg chlorophyll)*	
			DCIP	*$H_2O \rightarrow NADP^+$*	*DCIP-ascorbate→ NADP*	*PQ A*	*PQ C*
Original	3.01	124.6	1.87	2.72	0.97	0.08	0.018
1 000 × *g*	2.52	79.8	2.02	2.42	1.51	0.18	0.019
10 000 × *g*	2.66	32.4	3.58	4.48	0.82	0.04	0.046
50 000 × *g*	5.20	1.7	1.26	1.27	1.58	0.013	0.011
144 000 × *g*	4.64	0.8	1.16	1.40	2.72	0.04	0.012
Supernatant	—	0.7	1.86	0.39	0.0	trace	0.002

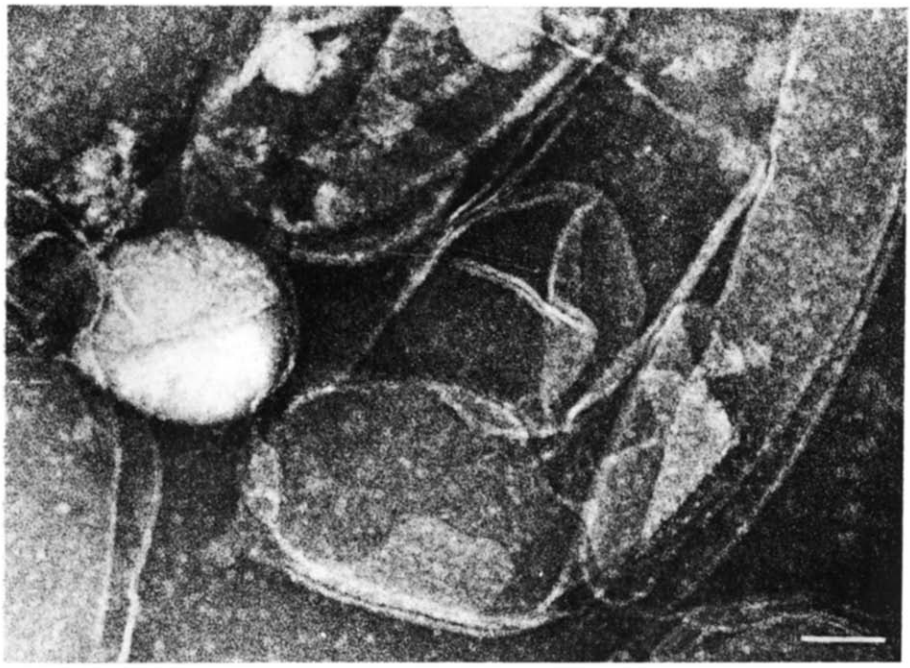

Fig. 1. Untreated chloroplast fraction stained with 0.2% phosphotungstate (pH 6.8). The sheets of membrane are relatively large and show a rough globular surface. 134900 ×. The marker indicates 0.1 μ.

Structure of the fragments

The fragments prepared by digitonin fractionation and those produced by sonication have been examined in the electron microscope. Negatively stained fragments of untreated chloroplasts are shown in Fig. 1. The heavy fragments from the digitonin treatment show sheets of membrane smaller than the original grana which under phosphotungstic acid treatment appear very thin and with a tendency to roll up at the edges. The light fraction on the other hand shows small disk-like sheets or particles; again very thin and closely adherent to the substrate (*cf.* Fig. 2). The thin sheets of membrane could develop by a longitudinal splitting of grana membranes under the influence of digitonin penetrating between the membrane layers. Separation of particles from a membrane has been suggested by ANDERSON AND BOARDMAN[15] as the basis for the separation obtained by digitonin fractionation. The structures which we observe would be consistent with that view.

Sonication shows membrane fragments of a different type as has been described by BECKER, SHEFNER AND GROSS[12]. The membrane appears to be broken up into smaller sheets under phosphotungstic acid staining. The size of the membrane sheets decreases in proportion to the centrifugal force required to sediment each fraction.

DISCUSSION

In view of previous studies which have indicated that plastoquinone A is involved between the first and second light reactions presumably at a site required for

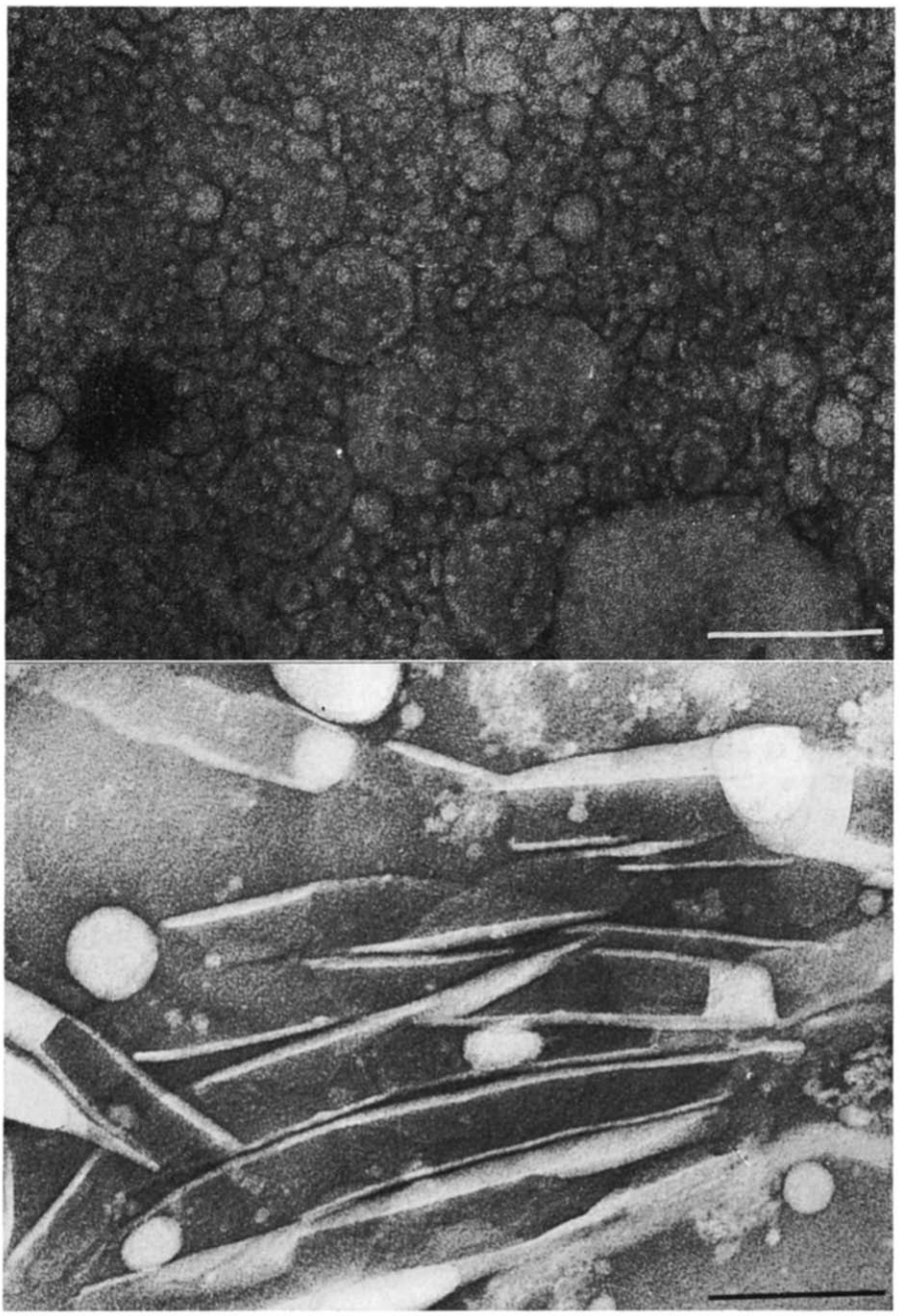

Fig. 2. Top. A portion of the fraction obtained by digitonin treatment and centrifugation at 50000 × *g* stained as for Fig. 1. Very small pieces of membrane and small 50–100-Å globules are present. 269800 ×. Marker indicates 0.1 μ. Bottom. A portion of the fraction obtained by digitonin treatment and centrifugation at 10000 × *g* stained as for Fig. 1. Thin smooth strips of membrane predominate with very few globules. 269800 ×. Marker indicates 0.1 μ.

indophenol reduction the results of the distribution studies come as a surprise. This is especially surprising in view of evidence by WITT *et al.*[13] that a plastoquinone is required as the primary acceptor of electrons for the second light reaction. One might expect that a diminution of the supply of acceptor would decrease the rate of reaction especially since the supply of chlorophyll is in great excess.

On the other hand it has been noted by WITT that the maximum amount of plastoquinone in chloroplasts which shows rapid changes during flashes of light is only one-tenth of the total and that the remainder of the quinone only shows slow redox changes incompatible with a role on the direct electron-transfer chain. Similarly AMESZ[14] has shown only a small portion of the total plastoquinone in blue-green algae undergoing rapid oxidation and reduction.

The low amount of plastoquinones found in the fraction showing full indophenol reduction activity can also be considered reasonable on the basis of restoration studies. After extensive heptane extraction both plastoquinone A and C have been shown to be required to restore full indophenol reduction activity. Maximum activity is restored by about 0.13 μmole plastoquinone A per mg chlorophyll and 0.01 μmole plastoquinone C. Since the usual amount found in chloroplasts is 0.1 μmole and 0.03 μmole per mg chlorophyll of A and C, respectively, it would appear that if the original concentration of quinone is mandatory for full activity the per cent of added quinone returning to the original site must be very high. It is doubtful if all the quinone added would go back to a functional site since some must become attached to inactive areas in the membrane and to the walls of the vessel. It is much more logical to assume that only one-half to one-tenth of the added quinone is returning to an active site. This amount at an active site would be consistent with the amounts of plastoquinones A and C found in the heavy digitonin particles.

The distribution of quinone after sonication indicates that a high level of plastoquinone is not necessarily associated with high activity in the ascorbate–indophenol $NADP^+$ reduction assay. The 144000 $\times$ g fraction shows three times the original activity by this assay and contains only one-half the original PQ A level and two-thirds of the original PQ C level.

By the different procedures we thus obtain chloroplast fragments which contain low levels of plastoquinones A and C with high levels of either indophenol reduction activity or ascorbate–indophenol $NADP^+$ reduction activity. These activities are presumably representative of light reactions 2 and 1 respectively. A low level of plastoquinones A and C may therefore be associated with either reaction. As much as nine-tenths of the plastoquinone A seems to migrate indiscriminately through the various fragments without influencing the activity pattern. The fragments with low plastoquinone content should be useful in spectrophotometric studies and restorations studies because a larger proportion of the plastoquinone should be functional in these fractions if it is important in the portion of the electron-transport system under study.

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