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Restoration of Photoreductase Activities in Acetone-extracted Chloroplasts by Plastoquinones and Tocopheryl-quinones

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Identification of a new member of the plastoquinone group in spinach chloroplasts brings the total number of quinones in these chloroplasts to eight. These include plastoquinones A, B, C, and D; vitamin K_1; and α-, β-, and γ-tocopheryl-quinones. Evidence is presented that each of these quinones function with a different pattern of specificity for restoration of photoreductase or for stimulation of photooxidase activities in acetone-extracted spinach chloroplasts.

As a result of studies of the lipids of spinach chloroplasts we have been able to identify eight quinones. These include plastoquinones A, B, C, and D; vitamin K_1; and α-, β-, and γ-tocopheryl-quinone. The presence of several related quinones in spinach chlorplasts raises a question as to their significance in electron transport function. It has previously been shown by Bishop (1959) and Krogmann and Olivero (1962) that plastoquinone A will restore ferricyanide reduction and photosynthetic phosphorylation in heptane-extracted chloroplasts. In this paper we should like to present evidence that the several plastoquinones and tocopheryl-quinones function at different sites in the electron transport system in chloroplasts. The plastoquinones have the same absorption maximum at 255 mμ, which indicates the same quinone chromophore. These can be separated by chromatographic procedures and they show different activities in restoration of electron transport functions in extracted chloroplasts. The isolation of plastoquinones A, B, and C (PQA, B, and C)[1] has been described previously (Kegel *et al.*, 1962). Plastoquinone D (PQD) is found in close association with plastoquinone C. The mixture of plastoquinones C and D is stable for several weeks in ethanol, but when the two quinones are separated they both become quite unstable under all conditions studied. Plastoquinone D is especially difficult to preserve for any length of time, and on standing either as an oil or in ethanol solution it is converted to another compound with a broad absorption maximum at 253 mμ. This degradation product of PQD still shows a decrease in absorbancy upon reduction with potassium borohydride, but there is no isosbestic point at shorter wavelengths which is characteristic of typical quinone spectra during reduction.

α-Tocopheryl-quinone (αTQ) is present in amounts comparable to PQC and PQD in spinach chloroplasts, whereas β- and γ-tocopheryl-quinones (β- and γTQ) are present in almost trace amounts (Henninger *et al.*, 1963). Again, each of these quinones shows different effects in electron transport function.

Vitamin K_1 has been recognized as a component of chloroplasts since the work of Dam (1942). We have recently isolated a purified sample of vitamin K_1 from spinach chloroplasts (Kegel and Crane, 1963). The only effects of vitamin K_1 on electron transport which we have observed are inhibitions.

The properties of the various plastoquinones and tocopheryl-quinones from chloroplasts are shown in Table I. The samples of PQC and PQD are the best

[1] Abbreviations used in this paper: PQ (combined with A, B, C, as PQA), plastoquinone; αTQ, α-tocopheryl-quinone; DCIP, 2,6-dichloroindophenol; PPNR, photosynthetic pyridine nucleotide reductase.

TABLE I
PROPERTIES OF CHLOROPLAST QUINONES

Quinone	R_F Thin Layer 1[a]	R_f Thin Layer 2	Absorbance Maximum (mμ)	$E^{1\%}_{1\ cm}$ at max mμ	Isosbestic Points[b] (mμ)	Mp
PQA	0.81	0.74	255	246	276,233	44
PQB	0.88	0.78	255	202	276,233	35
PQC	0.0	0.49	255,262	96	283,232	Oil
PQD	0.0	0.40	255,262	55	290,226	Oil
αTQ	0.21	0.37	261	414	282,232	Oil
βTQ	0.19	0.33	261	430	280,229	Oil
γTQ	0.16	0.25	258	430	279,230	Oil

[a] Thin layer 1, 15% trichloroethylacetate in benzene; 2, 1% ether in chloroform. All absorbance values in ethanol.
[b] Isosbestic points determined by reduction with potassium borohydride.

we have been able to obtain by the thin-layer method.

METHODS

Plastoquinones A, B, and C are isolated as previously described by chromatography of a propanol-heptane extract from chloroplasts on a silicic acid–super cell column. PQD is obtained by further fractionation of the original PQC fraction. The PQC- and PQD-containing fraction is passed through a small column of pure silicic acid using chloroform for elution. The mixture of quinones obtained is then chromatographed as a strip on thin-layer silica gel G using chloroform to develop the chromatogram. PQC and PQD are obtained in two closely associated bands. A small amount of an additional compound with an absorption maximum at 263 mμ is also obtained which shows a higher R_F than the PQC and PQD. Treatment of an ethanolic solution of this compound with borohydride causes disappearance of the 263 mμ absorbance but does not produce an increase of absorbance around 290 mμ as would be expected of a typical quinone. For this reason we will refer to this additional component of the PQC-PQD complex as R263. We do not see any evidence for this compound in the spectrum of the original PQC-PQD mixture so we must consider the possibility that R263 arises by conversion of one of the quinones during the latter stages of purification. The purified PQC and PQD must be used immediately for assay. PQC obtained by the procedure described will maintain the typical plastoquinone spectrum for 4 or 5 days if stored at −15°. PQD will decompose in 2 or 3 days. R263 is quite stable.

Assay of activity of the quinones were carried out using chloroplasts from which quinones had been removed by means of acetone extraction. Chloroplasts were isolated from spinach leaves in 0.5 M sucrose as previously described. The chloroplasts were then resuspended in distilled water using repeated centrifugation to remove the sucrose. After suspension in water the chloroplasts were lyophylized. The dry chloroplasts were then extracted with dry acetone, using 10 ml of acetone per gram of dried chloroplasts and shaking the acetone suspension on a reciprocal shaker for 90 minutes at room temperature (25°). After extraction the chloroplasts were removed from suspension by centrifugation and the residual acetone was removed under vacuum. The pale green extracted chloroplasts were then suspended in 0.1 M Tris-HCl, *p*H 7.0.

The following assay systems were used to determine the effect of the quinones on electron transport function in the extracted chloroplasts.

Photoreduction of ferricyanide was measured by observing the decrease in absorbancy at 420 mμ when the cuvet was placed in light for 1 minute. The assay system contained 0.75 μmole potassium ferricyanide, 0.20 μmole potassium phosphate buffer, *p*H 7.5, and chloroplasts containing 0.0092 mg chlorophyll in a total volume of 3.0 ml. In some experiments the reliability of this assay was increased by centrifugation of the cuvet after exposure to light to eliminate possible changes of turbidity caused by differential settling of chloroplasts.

Reduction of 2,6-dichloroindophenol (DCIP) (Eastman) was determined in a system the same as used for ferricyanide except that the ferricyanide was replaced with 1 μmole of indophenol and chloroplasts containing 0.037 mg of chlorophyll were used. Cytochrome c reduction was determined in a system similar to that of Keister *et al.* (1962) using chloroplasts containing 0.08 mg chlorophyll for each assay. Reduction of NADP in the presence of ascorbic acid and a trace of DCIP was determined according to the method of Vernon and Zaugg (1960). In this reaction a small amount of chlorophenyldimethylurea was added to inhibit the oxygen-producing light reaction. Chloroplasts used contained 0.064 mg chlorophyll. Light was supplied by a 100-w tungsten bulb separated from the reaction cuvet by a 100-ml beaker of water. This system provides about 4000 ft-c. Activity cannot be restored in extracted chloroplasts which have been heated at 100° for 2 minutes in aqueous suspension.

Chlorophyll was determined by the method of Arnon (1949). The plastoquinones were determined by the borohydride reduction procedure (Crane, 1959) on the fractions obtained by thin-layer chromatography, and were dissolved in ethanol. For estimation of each plastoquinone we have used the micromolar extinction coefficient of 15 assuming the same chromophore as plastoquinone A.

RESULTS AND DISCUSSION

In a typical acetone extraction of chloroplasts most of the quinones and chlorophylls are removed. There is a tendency for a small amount of PQC and PQD and significant amounts of αTQ to remain in the extracted chloroplasts. About 10% of the original chlorophyll remains in the extracted chloroplasts. As would be expected, there is a change in the binding of chlorophyll to the extracted chloroplasts as evidenced by the fact that the adsorption maximum of the chlorophyll, measured as a suspension of chloroplasts in water, shifts from 678 mμ (untreated chloroplasts) to 668 mμ (after acetone extraction). An example of the effects of acetone extraction on the chlorophyll and quinone content of spinach chloroplasts is shown in Table II. This sample of extracted chloroplasts was used for assays of photoreduction and photooxidation shown in Table III.

Following acetone extraction there is a decrease in the rates of reduction of ferricyanide, cytochrome c

TABLE II
EXTRACTION OF PLASTOQUINONES BY ACETONE[a]

	Chlorophyll (mg)	PQA (μmoles)	PQB (μmole)	PQC + D (μmole)	αTQ (μmole)	β + γTQ (μmole)
Original chloroplasts	15.7	2.03	0.36	0.37	0.21	0.005
Extracted chloroplasts	2.8	0	0	0.17	0.16	0
Acetone extract	16.2	2.50	0.39	0.24	0.06	0.002

[a] 10 g dry wt of spinach chloroplasts extracted with 100 ml acetone for 1.5 hours at room temperature.

TABLE III
RESTORATION OF PHOTOREDUCTASE ACTIVITIES BY QUINONES

Additions	μmoles Added[a]	Acceptor NADP	Cyt C	DCIP	Ferricyanide[c]
αTQ	0.0047	−1.76	4.02	1.7	27
βTQ	0.0089	−1.61	3.20	0.9	70
γTQ	0.0012	2.92	3.96[b]	−0.1	54
PQA	0.026	−2.52	−0.16	1.5	50
PQB	0.026	2.44	0.47	1.5	44
PQC	0.013	4.54	1.01	1.2	42
PQD	0.013	−2.95	−0.46	−0.9	48
PQC + PQD	0.013 + 0.013	−0.88	2.43	3.5	20
Extracted chloroplasts	None	1.28	0.77	2.1	24
Dried chloroplasts	None	3.59	7.86	1.1	53

[a] The amounts added refer only to NADP and cytochrome c assays. Amounts added for indophenol and ferricyanide restoration are 0.026 for all quinones except γTQ, 0.052 μmole. [b] Activity with 0.0006 μmole γTQ added. [c] Rates of reduction expressed in the following units: NADP, μmoles/10 min/mg chlorophyll; cyt c, μmoles/2 min/mg chlorophyll; DCIP, μmoles/5 min/mg chlorophyll; and ferricyanide, μmoles/5 min/mg chlorophyll. Negative values indicate photooxidation of the reduced acceptor.

TABLE IV
EFFECT OF PPNR ON RESTORED REDUCTASE ACTIVITY

Addition	mg Added	NADP −PPNR	NADP PPNR[a]	Cytochrome c −PPNR	Cytochrome c PPNR[a]
		μmoles/10 min/mg Chlor.		μmoles/2 min/mg Chlor.	
Dried chloroplasts	None	0.57	3.59	0.77	7.86
Extracted chloroplasts	None	0.89	1.28	0.25	0.77
αTQ	0.0047			0.60	4.02
PQA	0.026	−0.40	−2.52		
PQB	0.026	2.50	2.44		
PQC + D	0.013 + 0.013			0.80	1.62

[a] Photosynthetic pyridine nucleotide reductase (PPNR) equal to one unit as described by San Pietro and Lang (1956).

and NADP, but on a chlorophyll basis there is an increase in the photoreduction of indophenol. On a dry weight basis there is a decrease in all reductase activities.

Two general statements can be made about the restoration of photoreductase activities by plastoquinones and tocopheryl-quinones as shown in Table III. First, none of the quinones stimulates all activities. Secondly, the tocopheryl-quinones tend to be effective in restoration of certain activities at much lower levels than the amounts of plastoquinones required for restoration of activity. Ferricyanide reduction is the least selective process and is stimulated to some extent by all quinones except α-tocopheryl-quinone. Indophenol reduction is stimulated only by the mixture of PQC and PQD. Recombined samples of PQC and PQD after separation of R263 are also active, which indicates that the R263 component is not responsible for the activity. Cytochrome c reduction is the only activity restored by α-tocopheryl-quinone. β- and γ-tocopheryl-quinones are also active in the restoration of cytochrome c reduction as is the mixture of PQC and PQD. Purified PQC is also slightly active in this system. The ascorbate-dependent NADP reduction is stimulated by γ-tocopheryl-quinone, PQB, and PQC.

After acetone extraction there is appearance of a new type of activity namely a photooxidation of reduced cytochrome c or NADP in the presence of certain added quinones. This type of activity is illustrated by negative values shown in Table III. In the experiments illustrated in the table where negative values are shown, addition of quinone in the dark leads to a partial reduction of cytochrome c or NADP. When the system is exposed to light, the reduced acceptor is oxidized. This type of activity is best seen when PQA or PQD is added to the acetone-extracted chloroplasts in the NADP system. Similar effects can be shown when reduced NADP is added at the start with subsequent oxidation occurring in the light. These dark-reduction and photooxidation processes are readily reversible. After photooxidation in the light the NADP can be shown to return partially to the reduced form when the system is returned to the dark. It would appear that the addition of certain quinones makes a pool of reducing power available for reduction of NADP in the dark,

and that the electron flow is reversed in light to regenerate the pool of reducing power with consequent oxidation of the NADPH.

The photoreduction of both NADP and cytochrome c has been shown by Keister *et al.* (1962) to require the presence of an enzyme, photosynthetic pyridine nucleotide reductase. The restoration of cytochrome c reduction by α-tocopherylquinone and by the PQC plus PQD mixture also requires addition of photosynthetic pyridine nucleotide reductase. The reduction of NADP restored by PQB, however, is different from the activity of the original chloroplasts since no requirement for photosynthetic pyridine nucleotide reductase can be shown (see Table IV). It is, of course, possible that a small amount of photosynthetic pyridine nucleotide reductase is present in the extracted chloroplasts and that this endogenous PPNR works with high efficiency when PQB is added. For the present, we must say that the restoration of NADP reduction by PQB does not resemble the original system in this lack of a requirement for photosynthetic pyridine nucleotide reductase. On the other hand, the photooxidation of reduced NADP which occurs when PQA is added to extracted chloroplasts is stimulated by addition of photosynthetic pyridine nucleotide reductase.

These results lead us to the proposal that there are several sites for quinone function in chloroplasts which show considerable specificity with regard to quinone type. Further experimentation will be necessary to determine the site of function of each of these quinones.

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In Vivo Studies with Radioactive Steroid Conjugates. I. The Fate of Randomly Tritiated Androsterone Glucuronoside in Humans*

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Randomly tritiated androsterone glucuronoside was administered intravenously in trace amounts to three normal individuals. Urine was collected for 2–3 days and from these specimens pure androsterone glucuronoside was reisolated by chromatographic techniques. The distribution of the tritium between androsterone and glucuronic acid portions of the conjugate isolated from the urine was the same as that of the injected tracer. In one instance all the radioactivity present in the urine was found to be contained in androsterone glucuronoside. Furthermore in no instance was tritium found in the urinary androsterone sulfate. These results indicate that the androsterone glucuronoside present in urine is a metabolic end-product which, once formed, is neither cleaved *in vivo* nor further metabolized.

Metabolites of the steroid hormones as well as those of many other classes of compounds are found in the urine conjugated with either glucuronic acid or sulfuric acid. In the past, these "detoxified" products have been regarded merely as a means by which the excretion of waste products is facilitated. More recently the suggestion has been made (Fishman, 1961) that these conjugates may, in addition, serve as vehicles for the transport of biologically active compounds through the blood or perhaps through the walls of the target cells. The recent findings that dehydroisoandrosterone sulfate (Roberts *et al.*, 1961) and estrone sulfate (Twombley and Levitz, 1960) are freely interconvertible *in vivo* with their respective steroidal aglycones has caused a renewal of interest in this subject since it is evident that conjugated as well as free steroids must be taken into account before the metabolic pathways and the mode of action of these hormones can be fully understood. Further emphasis has been given to this facet of steroid metabolism by the recent demonstration that dehydroisoandrosterone sulfate is a normal secretory product of the adrenal gland in humans (VandeWiele *et al.*, 1963). That the adrenal gland can *in vivo* sulfate dehydroisoandrosterone (Wallace and Lieberman, 1963; Cohn *et al.*, 1962) as well as estrone (Sneddon and Marrian, 1963) has also been unequivocally demonstrated. Finally, the demonstration that pregnenolone sulfate may serve as a direct *in vivo* precursor of dehydroisoandrosterone sulfate without the cleavage of the sulfate ester group reveals a still undefined metabolic role for sulfate conjugates (Calvin *et al.*, 1963).

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