

Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts

The effect of hydrogen peroxide and of Paraquat

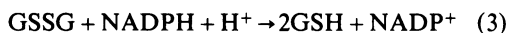
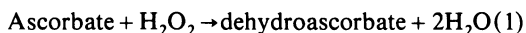
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The stroma of spinach chloroplasts contains ascorbic acid and glutathione at millimolar concentrations. [Reduced glutathione]/[oxidized glutathione] and [ascorbate]/[dehydroascorbate] ratios are high under both light and dark conditions and no evidence for a role of oxidized glutathione or dehydroascorbate in the dark-deactivation of fructose biphosphatase could be obtained. Addition of H₂O₂ to chloroplasts in the dark decreases the above ratios, an effect that is reversed on illumination. Addition of Paraquat to illuminated chloroplasts caused a rapid oxidation of reduced glutathione and ascorbate, and apparent loss of dehydroascorbate. Paraquat rapidly inactivated fructose biphosphatase activity, as assayed under physiological conditions.

Ascorbic acid and glutathione are present in the stroma of spinach (*Spinacia oleracea*) chloroplasts (for review see Halliwell, 1981). Since chloroplasts produce H₂O₂ upon illumination (Nakano & Asada, 1981) and yet contain little, if any, catalase or 'non-specific' peroxidase activity, Foyer & Halliwell (1976) suggested that the H₂O₂ was disposed of by an 'ascorbate-glutathione cycle' as follows:



An ascorbate peroxidase enzyme catalysing reaction (1) (Grodén & Beck, 1979) and a glutathione reductase (reaction 3; Halliwell & Foyer, 1978) enzyme are present in spinach chloroplasts. Reaction (2) appears to be non-enzymic in spinach chloroplasts (Foyer & Halliwell, 1976, 1977), although a dehydroascorbate reductase may be present in pea chloroplasts (Jablonski & Anderson, 1981). However, no direct evidence for a role of GSH or As in removing H₂O₂ in intact chloroplasts has yet been reported.

Upon illumination of chloroplasts the activity of the Calvin-cycle enzyme fructose biphosphatase, as assayed at physiological concentrations of substrate

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; As, ascorbate; DAs, dehydroascorbate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); PQ, Paraquat.

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and Mg²⁺, increases (Leegood & Walker, 1980a; Laing *et al.*, 1981), probably owing to reductive conversion of the enzyme into a form with greater affinity for its substrate (Charles & Halliwell, 1981a). In the dark, enzyme activity decreases again. Wolosiuk & Buchanan (1977) found that fructose biphosphatase could be deactivated *in vitro* by GSSG or DAs and suggested that they were formed for this purpose in darkened chloroplasts. However, Halliwell & Foyer (1978) discovered that the [GSH]/[GSSG] ratio in spinach chloroplasts was high under both light and dark conditions. Since they made the latter measurement after 10 min of darkness, this does not rule out the possibility that a 'burst' of GSSG or DAs formation immediately after switching off the light could deactivate fructose biphosphatase (Buchanan, 1980).

In the present paper we report the concentrations of As, DAs, GSH and GSSG in type A spinach chloroplasts in both light and dark, and the effect of H₂O₂ and of the herbicide PQ upon the amounts present. The effect of H₂O₂ on the activity of fructose biphosphatase in isolated spinach chloroplasts has been reported previously (Charles & Halliwell, 1981a). The present paper also reports the effect of PQ on this enzyme. PQ is reduced by Photosystem I and generates H₂O₂ and the superoxide radical, O₂⁻, within the chloroplast (Dodge, 1975; Halliwell, 1981).

Materials and methods

Reagents

Fresh spinach leaves were obtained from New Covent Garden Market, London, S.W.8, U.K.

Glutathione reductase was from Boehringer, 2-vinylpyridine from Aldrich and all other reagents from Sigma Chemical Corp., including PQ (Methyl Viologen).

Chloroplast isolation

Type A chloroplasts (Hall, 1972) were isolated from washed mature spinach leaves as described by Charles & Halliwell (1981a), except that Mg^{2+} , Mn^{2+} and EDTA were omitted from the preparation and resuspension medium. Their percentage intactness was determined by the ferricyanide method (Lilley *et al.*, 1975). Chlorophyll was measured by the method of Arnon (1949). Spinach chloroplast fructose biphosphatase was purified, reduced and assayed as described by Charles & Halliwell (1980a). The enzyme assay contained $100\ \mu M$ substrate and $5\ \text{mM-MgCl}_2$.

Determination of As and DAs

A modification of the method of Okamura (1980) was used. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by As in acidic solution. The Fe^{2+} then forms complexes with bipyridyl, giving a pink colour that absorbs at 525 nm. Total ascorbate (As + DAs) is determined through a reduction of DAs to As by dithiothreitol. For the concentrations quoted, half a sample of chloroplast preparation was assayed for total ascorbate content, and the other half was assayed for As only. DAs concentrations were then deduced from the difference.

A sample ($400\ \mu l$; $250\text{--}350\ \mu g$ of chlorophyll) of the chloroplast preparation was added to $200\ \mu l$ of 10% (w/v) trichloroacetic acid. After vortex-mixing it was allowed to stand in ice for 5 min. NaOH ($10\ \mu l$, $5\ M$) was added, mixed and the mixture centrifuged for 2 min in a Microfuge. To a $200\ \mu l$ sample of the supernatant was added $200\ \mu l$ of $150\ \text{mM-NaH}_2\text{PO}_4$ buffer, pH 7.4, and $200\ \mu l$ of water. To another $200\ \mu l$ of supernatant sample was added $200\ \mu l$ of buffer, $100\ \mu l$ of $10\ \text{mM-dithiothreitol}$ and, after thorough mixing and being left at room temperature for 15 min, $100\ \mu l$ of 0.5% (w/v) *N*-ethylmaleimide. Both samples were vortex-mixed and incubated at room temperature for $>30\ \text{s}$. To each was then added $400\ \mu l$ of 10% (w/v) trichloroacetic acid, $400\ \mu l$ of 44% (v/v) H_3PO_4 , $400\ \mu l$ of 4% (w/v) bipyridyl in 70% (v/v) ethanol and $200\ \mu l$ of 3% (w/v) $FeCl_3$. After vortex-mixing, samples were incubated at $37^\circ C$ for 60 min and the A_{525} was recorded. A standard curve in the range 0–40 nmol of As or DAs was used for calibration.

Determination of GSH and GSSG

The method used employed the specificity of glutathione reductase and was a modification of that of Griffith (1980). A portion ($50\ \mu l$; about $25\ \mu g$ of chlorophyll) of chloroplast suspension was quickly

mixed with $250\ \mu l$ of resuspension medium ($330\ \text{mM-betaine}/50\ \text{mM-Hepes}/KOH$, pH 7.6) and $150\ \mu l$ of 10% (w/v) sulphosalicylic acid. After centrifugation, $150\ \mu l$ of the supernatant was added to $700\ \mu l$ of $0.3\ \text{mM-NADPH}$, $100\ \mu l$ of $6\ \text{mM-DTNB}$, $50\ \mu l$ of glutathione reductase ($10\ \text{units/ml}$), all reagents being prepared in $125\ \text{mM-NaH}_2\text{PO}_4$ buffer, containing $6.3\ \text{mM-EDTA}$, at pH 7.5. Reaction was followed as ΔA_{412} and the total glutathione content was calculated from a standard curve.

To measure GSSG, $150\ \mu l$ (about $75\ \mu g$ of chlorophyll) of chloroplast suspension was added to $75\ \mu l$ of sulphosalicylic acid. After mixing and centrifugation, to $180\ \mu l$ of the supernatant was added $12\ \mu l$ of 2-vinylpyridine followed by $20\ \mu l$ of 50% (v/v) triethanolamine, the latter being placed on the side of the tube above the level of the liquid. The solution was vortex-mixed for 30 s and left at $25^\circ C$ for 25 min. A portion ($150\ \mu l$) of the resultant solution was assayed as above. Calibration curves were carried out using GSSG samples treated exactly as above, since triethanolamine slightly alters the rate of colour development.

Results

Type A chloroplasts (Hall, 1972) were prepared from different batches of leaves and analysed for their contents of ascorbate and glutathione by sensitive assay methods. Table 1 summarizes the results obtained. GSH, GSSG, As or DAs added to the chloroplasts in known amounts could be quantitatively detected in subsequent assay. The $[GSH]/[GSSG]$ and $[As]/[DAs]$ ratios were always high and did not change under light or dark conditions. There was no evidence for a burst of GSSG or DAs formation on switching off the light (Fig. 1 shows a typical example). The intactness of the chloroplast preparations used was 70% or more, as determined by the ferricyanide method.

Table 1. Ascorbate and glutathione concentrations in illuminated spinach chloroplasts

Values are given as means \pm s.d. for *n* chloroplast preparations from different batches of leaves. Concentrations are calculated by assuming an internal chloroplast volume of $23\ \mu l$ per mg of chlorophyll. Several chloroplast preparations contained no detectable DAs. The chloroplast intactness was 70% or more as measured by the ferricyanide method.

Substrate measured	Concentration (mM)
As (<i>n</i> = 40)	12.9 ± 2.8
DAs (<i>n</i> = 38)	1.12 ± 1.2
Total ascorbate (<i>n</i> = 39)	13.5 ± 1.9
GSH (<i>n</i> = 42)	4.2 ± 1.7
GSSG (<i>n</i> = 41)	0.3 ± 0.17
Total glutathione (<i>n</i> = 40)	4.5 ± 1.6

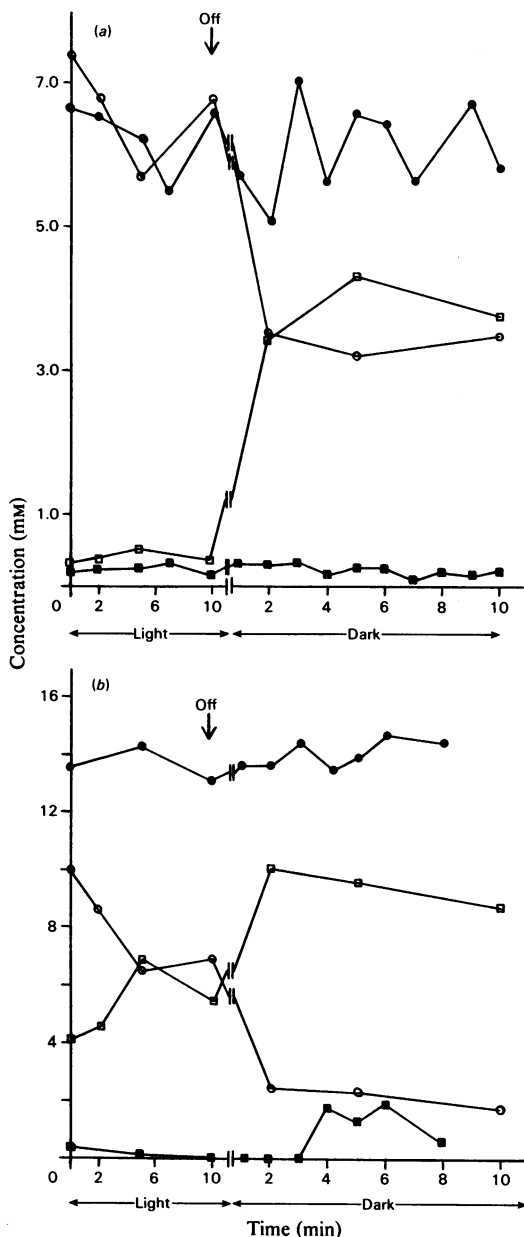


Fig. 1. Effect of 100 μM-H₂O₂ on the concentrations of (a) GSH and GSSG and (b) As and DAs in illuminated and darkened type A chloroplasts

(a) The light was switched on at time zero and off as indicated by the arrow. Where indicated, H₂O₂ at a final concentration of 100 μM was added at time zero. Portions were removed at the times indicated and assayed for GSH and GSSG, as described in the Materials and methods section. Symbols: ●, GSH; ■, GSSG; ○, GSH, +H₂O₂; □, GSSG, +H₂O₂. (b) Conditions were as described for (a). Portions were removed at the times indicated and assayed for As and DAs as described in the Materials and methods section. Symbols: ●, As; ■, DAs; ○, As, +H₂O₂; □, DAs, +H₂O₂. Similar results were

H₂O₂ is a fairly non-polar molecule and crosses the chloroplast envelope readily (Egneus *et al.*, 1975). The effect of addition of H₂O₂ at a final concentration of 100 μM to illuminated type A chloroplast preparations is shown in Figs. 1(a) and 1(b). There was a slow oxidation of part of the As present to DAs but little change in the GSH and GSSG levels. On turning off the light, however, the [As]/[DAs] ratio decreased markedly within 2 min and GSH was equally quickly converted into GSSG (Figs. 1a and 1b). Under both light and dark conditions, a fall in the concentration of total ascorbate on addition of H₂O₂ was recorded. A high [GSH]/[GSSG] ratio rapidly re-appeared upon illumination of H₂O₂-treated dark-adapted chloroplasts (Table 2).

Addition of PQ to illuminated type A chloroplasts caused a rapid loss of GSH and transient formation of GSSG (Fig. 2a). However, despite a massive loss of As on paraquat addition, there was no significant increase in DAs detected (Fig. 2b). The loss of total ascorbate may be an indication of immediate degradation of DAs formed from oxidation of As. GSSG is also quickly lost. Addition of PQ to darkened chloroplasts produced much slower, although significant, changes (Table 3). PQ also caused a rapid inactivation of fructose biphosphatase activity, as assayed at physiological concentrations of substrate and Mg²⁺ (Fig. 3).

Physiological concentrations of GSSG (0.3 mM; see Table 1) and DAs (1 mM; see Table 1) had no effect on the activity of fructose biphosphatase,

Table 2. Effect of illumination on the concentrations of GSH and GSSG in H₂O₂-dark-adapted type A chloroplasts

A final concentration of 100 μM-H₂O₂ was added to isolated type A chloroplasts in the dark. After 2 min, portions were removed for the assay of GSH and GSSG as described in the Materials and methods section. At the same time, the light was switched on and further portions were removed after 2 min and 5 min of illumination and assayed for GSH and GSSG. Similar results were obtained in three different experiments using chloroplasts from different batches of leaves.

Time (min)	[GSSG] (mM)	[GSH] (mM)
Before addition	0.12	4.21
After addition		
2 (in the dark)	2.82	1.40
2 (illuminated)	0.47	3.52
5 (illuminated)	0.32	3.83

obtained in ten experiments using chloroplasts from different batches of leaves.

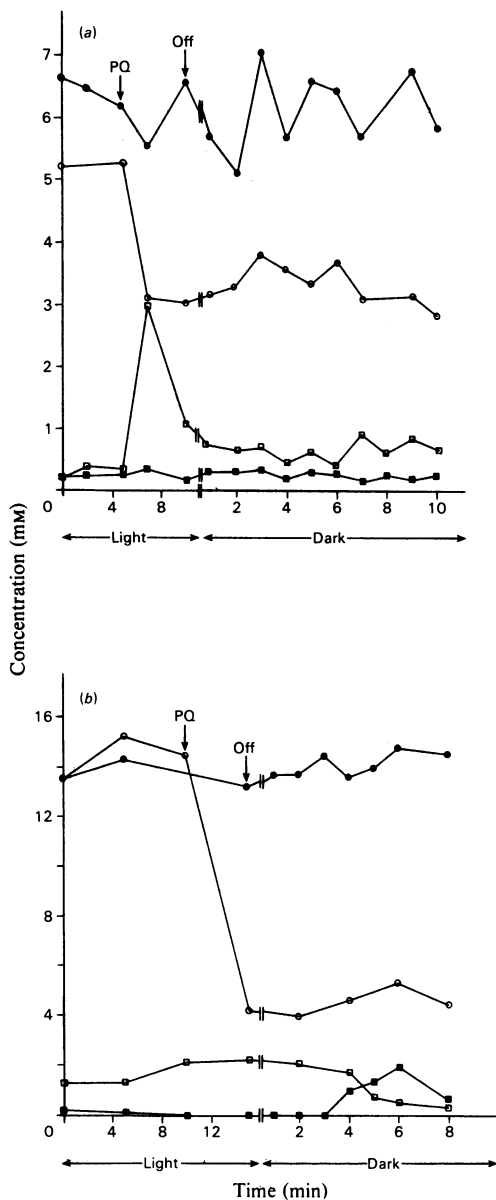


Fig. 2. Effect of 100 μM -Paraquat on the concentrations of (a) GSH and GSSG and (b) As and DAs in illuminated and darkened type A chloroplasts (a) The light was switched on at time zero and off as indicated by the arrow. Where indicated Paraquat at a final concentration of 100 μM was added. Portions were removed at the times indicated and assayed for GSH and GSSG as described in the Materials and methods section. Symbols: ●, GSH; ■, GSSG; ○, GSH, +PQ; □, GSSG, +PQ. (b) Conditions were as described for (a). Portions were removed at the times indicated and assayed for As and DAs as described in the Materials and methods section. Symbols: ●, As; ■, DAs; ○, As, +PQ; □, DAs, +PQ. Similar results were obtained in eight different experiments using chloroplasts from different batches of leaves.

Table 3. Effect of 100 μM -Paraquat on ascorbate and glutathione levels in type A chloroplasts maintained in the dark

A final concentration of 100 μM -Paraquat was added to isolated type A chloroplasts maintained in the dark. Portions were removed at the times indicated and assayed for GSH, GSSG, As and DAs as described in the Materials and methods section. Similar results were obtained in eight different experiments using chloroplasts from different batches of leaves.

Time after Paraquat addition (min)	[DAs] (mM)	[As] (mM)	[GSSG] (mM)	[GSH] (mM)
0	3.23	7.47	0.10	3.26
2	3.05	7.83	0.12	3.90
5	0.57	7.66	0.32	3.07
10	1.42	6.97	1.24	1.98

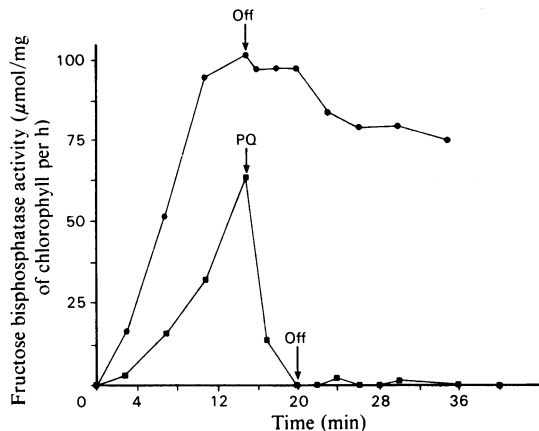


Fig. 3. Effect of 100 μM -Paraquat on the physiological activity of fructose biphosphatase in isolated type A chloroplasts

Illumination was commenced at time zero to generate the physiological (reduced) form of fructose biphosphatase. Where indicated, Paraquat at a final concentration of 100 μM was added and the light turned off as indicated by the arrows. Portions were removed at the times indicated and assayed for enzyme activity as described in the Materials and methods section. Similar results were obtained in five different experiments using chloroplasts from different batches of leaves.

either purified or in the reduced form (results not shown).

Discussion

The [GSH]/[GSSG] and [As]/[DAs] ratios are maintained high in chloroplasts under both light and dark conditions – there is no sign of a burst of GSSG or DAs formation when the light is turned off

(Figs. 1 and 2). Deactivation of enzymes such as fructose biphosphatase must therefore be achieved by other mechanisms, as already suggested (Charles & Halliwell, 1980a,b, 1981a,b; Leegood & Walker, 1980a,b; Soulie *et al.*, 1981), especially as physiological concentrations of GSSG and DAs have no effect on the purified reduced form of fructose biphosphatase.

The oxidation of As on addition of H₂O₂ to chloroplasts in the light or dark is consistent with the operation of an ascorbate peroxidase enzyme (Grodén & Beck, 1979), which must therefore be catalytically active under both conditions. Perhaps the net amount of As oxidized in the light is limited by the recycling of DAs at the expense of GSH, which is in turn recycled by glutathione reductase. Consistent with this, addition of H₂O₂ in the dark produced a greater oxidation of As accompanied by a fall in the [GSH]/[GSSG] ratio (Fig. 1); presumably the rate of the glutathione reductase reaction is limited by the supply of NADPH in the dark. Further evidence for this suggestion comes from the observation that illumination of H₂O₂-treated dark-adapted chloroplasts caused a marked rise in the [GSH]/[GSSG] ratio (Table 2).

Addition of PQ to illuminated chloroplasts causes a rapid uptake of O₂ and inhibition of CO₂ fixation (Dodge, 1975), accompanied by the formation of H₂O₂ and O₂^{-•}. Removal of electrons from the Photosystem I acceptor by PQ also prevents generation of NADPH and so glutathione reductase cannot operate. There is a rapid oxidation of both GSH and As (Fig. 2) and a loss of the physiologically-active form of fructose biphosphatase (Fig. 3), presumably due to the action of H₂O₂ (Charles & Halliwell, 1980c, 1981a) and to the fact that electrons will no longer be available to reduce thioredoxin and activate the enzyme (Leegood & Walker, 1980a,b). Once formed, the DAs disappear rapidly. In the absence of GSH to recycle it, DAs might be degraded into oxalic acid and L-threonic acids (Habermann *et al.*, 1968). GSSG is also lost. In illuminated chloroplasts the addition of PQ obviously imposes a great 'oxidative stress'. PQ had a much slower action on As and GSH in the dark, consistent with the light-dependence of its toxic effect on plants (Dodge, 1975). Nevertheless it can probably be reduced slowly in the dark by a 'back flow' of electrons from NADPH to the electron transport chain (e.g. Leegood & Walker, 1981) or even by glutathione reductase itself (Richmond & Halliwell, 1982) and thus give rise to some formation of H₂O₂ and O₂^{-•}.

In summary all these results are consistent with the operation of an ascorbate/glutathione cycle (Foyer & Halliwell, 1976) in the intact chloroplast and support the conclusion that GSSG and DAs are not involved *in vivo* in dark deactivation of fructose biphosphatase.

Note added in proof (Received 6 January 1983)

Foyer *et al.* (1983) have independently performed experiments that support the conclusions reached in the present paper.

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