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# The Effect of Diamide and Buthionine Sulfoximine on Glutathione Pools and Transmembrane Electron Transport by Cultured Carrot Cells

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## Introduction

The role of glutathione, a tripeptide ($\gamma$-L-glutamyl-L-cysteinylglycine) is well established in the sulfur cycle as a storage and transport form for reduced sulfur (10). It also functions in detoxification of pesticides by plants (10), but its role as a possible internal electron donor for transplasma membrane electron transport has not been studied previously. This study was undertaken with the purpose of establishing whether glutathione had an effect on plasma membrane redox reactions of cultured carrot cells. A link between electron transport and glutathione content of cells was established by decreasing the internal glutathione concentration. This was accomplished by growing cells in presence of diamide (7), a chemical known to oxidize the reduced form of glutathione (GSH) to its oxidized from (GSSG) and buthionine sulfoximine, a glutathione biosynthesis inhibitor (6).

## Materials and Methods

Carrot cells (*Daucus carota* L.) were grown for 8 days in liquid suspension culture on Murashige and Skoog's medium without agar as previously described (2). Control cells received no diamide or buthionine sulfoximine but 3 replications each received 0.2, 0.4, 0.6 and 0.8 mM diamide or 100, 200 or 300 $\mu$M buthionine sulfoximine immediately after the initial transfer of cells on day 1. After inoculation, the flasks were placed on a gyrating shaker at 24 °C under room lights. On day 8, cells were harvested by centrifugation in a table model non-refrigerated International centrifuge at setting 4 (200 $\times$ g) for 2 min. For assaying plasma membrane redox reactions or H + excretion, cells were washed twice with sucrose-salts solution (0.1 M sucrose with 10mM each NaCl, KCl and $CaCl_2$) and suspended in 50 ml of the same. For extracting glutathione from cells, they were washed twice with distilled water and stored frozen until used.

For dry weight determination, 1 ml aliquots of cells in triplicate were washed with distilled water and placed in a 100 °C oven overnight.

Plasma membrane electron transport was measured as ferricyanide reduction in an Aminco DW-2A double beam spectrophotometer as the difference between 420 nm (measuring wavelength) and 500 nm (reference wavelength), following the reduction of ferricyanide. The reaction mixture contained 25mM Tris-Mes, pH 7, sucrose-salts and 0.1 ml cells (about 5$\mu$g dry wt.). 100$\mu$M ferricyanide was added to start the reaction. Ferricyanide reduction rates were calculated, using a millimolar extinction coefficient of 1.

Proton excretion by carrot cells was measured with a pH meter equipped with a Corning combination pH electrode in a water-jacketed chamber at 24 °C. Air was bubbled through the chamber's sidearm continuously to dissipate $CO_2$. The reaction mixture contained 4.9 ml sucrose-salts, 25$\mu$M phosphate buffer, pH 7, and 0.1 ml cells (about 5$\mu$g dry wt.). After measuring the basal rate for 5 min., 100$\mu$M ferricyanide was added to measure the excretion of redox-driven protons. All rates were recorded with a Linear recorder. Proton excretion rates were calculated from adding known amounts of HCl at the end of each assay.

Glutathione (GSH in combination with GSSG) was extracted by the method of Aker-

boom and Sies (1), except the NEM step was omitted. Frozen cells (2.5-3g wet wt.) were ground in 3 ml of 10% perchloric acid with a mortar and pestle. The extract was filtered through Miracloth and the residue reground in 7 ml perchloric acid solution. The combined acid extracts were centrifuged at 5000 × g for 5 min to remove the precipitated proteins. The supernatant, containing the extracted mixture of reduced and oxidized forms of glutathione and mixed acid-soluble disulfides, was neutralized with a few drops of 2 M NaOH dissolved in 0.3 M Tris-Mes buffer. The spectrophotometric assay for reduced and oxidized glutathione was performed immediately after neutralization of the extract. The reaction mixture contained 1 ml buffer (0.1 M potassium phosphate, pH 7, with 0.001 M EDTA, 0.1 ml neutralized extract, 50$\mu$l NADPH of 4mg/ml stock, dissolved in the above buffer, and 20$\mu$l glutathione reductase (6 units per ml), dissolved in buffer. The reaction was started by the addition of 20$\mu$l DTNB (1.5 mg dissolved in 0.5% $NaHCO_3$). The reaction rates, seen as an increase of absorbance of 412 nm, were recorded with a Linear recorder for 3 min. At the end of the assay, the absorbance at 412 was taken manually as a single absorbance reading. The reaction rates for the recorded assay were calibrated from known amounts of glutathione added to the reaction mixture in place of the neutralized carrot cell extract. The glutathione concentration from a single absorbance reading at 412 nm after a 3 min incubation was obtained by dividing the O.D. per min at 412 nm by the extinction coefficient for the DTNB product $A_{412nm}$ = 13.6mM) and by the dry weight of cells used per assay.

## Results and Discussion

The role of glutathione in plant cells has been reveiwed by Rennenberg (10) and in animal cells by Kosower and Kosower (7) and by Meister (8). In green plants glutathione serves as the main long-distance transport form of sulfur from the leaves to the roots and as a storage form of sulfur. Glutathione also participates in the detoxification of hydrogen peroxide and fungicides. According to Reese and Wager (9), buthionine sulfoximine, an inhibitor of glutathione biosynthesis, causes a decrease in the levels of a cadmium-binding peptide, which leads to reduced growth of cultured tobacco cells. Recently it has been shown by Earshaw and Johnson (5) that the addition of 0.1 mM glutathione to wild carrot suspension cultures caused these cells to proliferate whereas the addition of 0.3 mM buthionine sulfoximine, which decreased cellular glutathione levels by inhibiting the action of $\gamma$-glutamylcysteine sythetase (6), promoted the generation of somatic embryos. Since Crane et al. (4) had shown previously that plasma membrane electron transport reactions were associated with growth of cultured cells, it was decided to find out what effect glutathione levels in carrot cells had on transmembrane electron transport.

As shown in Table I, adding diamide to growing carrot cells decreased the internal glutathione content to a maximum 23% by oxidation of blutathione (8). Buthionine sulfox-

Table 1. The Effect of Diamide and Buthionine Sulfoximine on Total Gluthathione Levels of Cultured Carrot Cells.

| Treatment | Gluthatione (GSH + GSSG) ($\mu$moles g dry wt.$^{-1}$) | Decrease (%) |
|---|---|---|
| None | 2.21 | — |
| 0.2mM diamide | 2.20 | 0 |
| 0.4mM diamide | 1.70 | 23 |
| 0.6mM diamide | 1.85 | 16 |
| 0.8mM diamide | 1.80 | 18 |
| None | 2.54 | — |
| 100$\mu$M buthionine sulfoximine | 1.41 | 44 |
| 200$\mu$M buthionine sulfoximine | 0.51 | 80 |
| 300$\mu$M buthionine sulfoximine | 0.18 | 93 |

imine, an inhibitor of γ-glutamylcysteine synthetase (10) proved to be more effective than diamide in reducing the internal glutathione levels in carrot cells with a decrease to 93% of control cell level (Table I). Oxidation of reduced glutathione by diamide and a block of its biosynthesis by buthionine sulfoximine affected plasma membrane redox in a similar manner by causing 25 to 37% inhibition of ferricyanide reduction rates (Fig. 1, A and

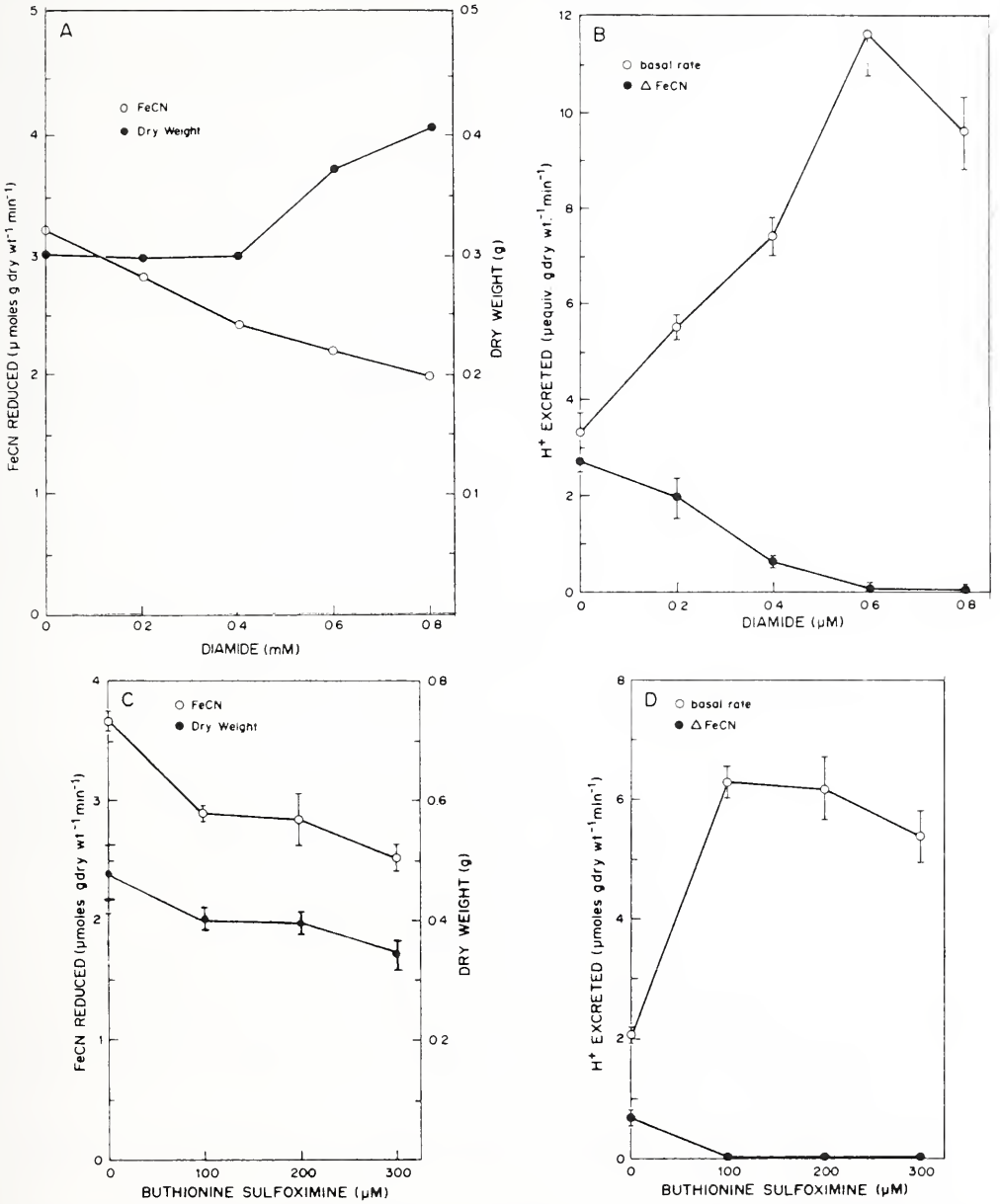


FIGURE 1. The effect of growing carrot cells with inhibitors. A). Transmembrane ferricyanide reduction rate and dry weight of carrot cells grown for 8 days with various concentrations of diamide. B). Proton excretion by carrot cells grown for 8 days with various concentrations of diamide. C). Transmembrane ferricyanide reduction and dry weight of carrot cells grown for 8 days with various concentrations of buthionine sulfoximine. D). Proton excretion by carrot cells grown with various concentrations of buthionine sulfoximine. Reaction conditions for each assay as described in the Materials and Methods section. The H + excretion rate with ferricyanide is the net rate after subtraction of the basal rate.

1, C). On the other hand, these agents produced different effects on cell proliferation. Diamide (0.8mM) stimulated carrot cell growth, while buthionine sulfoximine inhibited growth 30%. The proton excretion patterns (Fig. 1, B and 1, D) were similar after treatment with both compounds. The basal rate of proton excretion, attributed mainly to the action of the plasma membrane H+ -ATPase, was stimulated 3-fold by both compounds. Proton excretion due to plasma membrane electron transport in presence of potassium ferricyanide was inhibited 100% in both cases.

Since green tobacco cells cultured under photoheterotrophic conditions are known to release 30× more glutathione (up to 3.5mM) into the culture medium than cells devoid of chlorophyll (10), it can be argued that transmembrane ferricyanide reduction in such cells may be due to the release of glutathione. In our studies with carrot cells a rapid rate of glutathione release is not likely because chloroplasts are absent from the yellow callus cells and the small amount of glutathione released is too slow to account for the ferricyanide reduction rates observed (about 4 $\mu$moles g dry wt.$^{-1}$ min $^{-1}$). We have shown that release of reducing agents from carrot cells is less than 10% of the ferricyanide reduction rate (3).

In summary, the present data indicates that growing carrot cells for 8 days with diamide, an oxidizing agent, and buthionine sulfoximine, an inhibitor of glutathione biosynthesis, result in decreased levels of glutathione in the cells, which leads to a partial inhibition (up to 37%) of plasma membrane redox reactions and 100% inhibition of oxidant associated proton excretion, while the proton excretion by the plasma membrane H+ -ATPase is stimulated 3-fold with both treatments. The difference in growth responses may be attributed to the difference in action of the reagents. Diamide causes oxidation of reduced glutathione, whereas buthionine sulfoximine causes loss of both oxidized and reduced forms.

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