# *p*-CHLOROMERCURIPHENYL SULPHONIC ACID AS A SPECIFIC INHIBITOR OF THE PHOSPHATE TRANSPORTER IN ISOLATED CHLOROPLASTS

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Received 22 March 1977

## 1. Introduction

It has been established that there are at least three specific transporters on the chloroplast envelope membranes [1]. These transporters almost certainly play an important role in the regulation of photosynthesis and of cytoplasmic metabolism. The phosphate transporter facilitates counter-exchange of inorganic phosphate, 3-phosphoglycerate, dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate across the envelope membranes [1,2]. The dicarboxylate transporter is specific for dicarboxylic acids and is thought to be involved in transfer of reducing equivalents between chloroplast and cytoplasm [1,2]. The adenine nucleotide transporter exchanges ATP and ADP across the envelope membranes although the rates of transfer are low compared to the other two transporters [1,3].

The use of specific inhibitors of these transporters would provide further information about the transporters themselves and about their role in photosynthesis. Werdan and Heldt [4] described the effect of *p*-chloromercuriphenyl sulphonic acid (CMS), a reagent for SH-groups, on two of the transporters. CMS strongly inhibited the transport of phosphate and of 3-phosphoglycerate but had little effect on transport of dicarboxylates. This suggests that CMS specifically inhibits the phosphate transporter although its effect on the adenine nucleotide transporter and on $CO_2$ fixation have not been tested.

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*Abbreviations:* CMS, *p*-chloromercuriphenyl sulphonic acid

Using pea chloroplasts, in which the rate of adenine nucleotide transport is higher than for spinach [5,6], we have confirmed that CMS is a specific inhibitor of the phosphate transporter and shown that CMS does not inhibit the adenine nucleotide transporter. In addition, evidence is provided that the inhibition of $CO_2$ fixation by phosphate [7] requires entry of phosphate via the transporter.

## 2. Methods

Chloroplasts were isolated from young pea shoots as described previously [5,6]. Chlorophyll was determined by the method of Arnon [8]. Oxygen evolution was measured with a Rank oxygen electrode. The vessel was maintained at 20°C and was illuminated by a slide projector giving a light intensity of $2 \times 10^5$ erg. $cm^{-2}$. $s^{-1}$. Back exchange of adenine nucleotides was measured as described previously [5,6]. Adenylylimidodiphosphate was obtained from Boehringer and Soehne (Mannheim, Germany) and all other biochemicals from the Sigma Chemical Company (Saint Louis, USA).

## 3. Results and discussion

$CO_2$-Dependent oxygen evolution by isolated chloroplasts is stimulated by low concentrations of phosphate (0.2–0.5 mM) but is inhibited by higher concentrations (2–5 mM) [5,7]. If CMS inhibits the phosphate transporter then CMS should prevent, or at least decrease, the inhibition of $CO_2$ fixation by higher concentrations of phosphate. As no

phosphate was included in the reaction medium for fig.1, entry of small amounts of phosphate would stimulate oxygen evolution whereas entry of gross amounts would inhibit. $CO_2$-Dependent oxygen evolution was inhibited by 30–40% if CMS was included in the reaction medium from the outset (fig.1). In the presence of CMS, low concentrations of phosphate (0.2 mM) did not stimulate oxygen evolution suggesting that CMS inhibited the phosphate transporter and therefore prevented the entry of phosphate. In the absence of CMS, addition of high concentrations of phosphate (3 mM) resulted in a marked inhibition of oxygen evolution (fig.1B) whereas chloroplasts treated with CMS showed a stimulation with the same concentration of phosphate (fig.1A). The lack of inhibition of oxygen evolution in CMS-treated chloroplasts, by concentrations of phosphate which almost completely inhibited oxygen evolution in untreated chloroplasts, suggests that the phosphate transporter was strongly inhibited by CMS, thus preventing the entry of gross amounts of phosphate. The stimulation of oxygen evolution

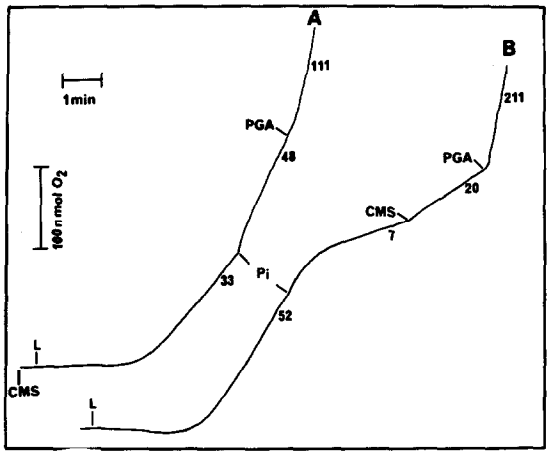


Fig.1. Effect of CMS on inorganic phosphate inhibition of $CO_2$-dependent oxygen evolution. Oxygen evolution was assayed in a medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes (pH 7.6), 4 mM $NaHCO_3$ and chloroplasts equivalent to 100 μg chlorophyll in total vol. 2.1 ml. CMS (50 μM) was included in the reaction medium in trace A. Additions were: inorganic phosphate ($P_i$) 3 mM; 3-phosphoglycerate (PGA) 2 mM; and CMS 50 μM, as indicated. The numbers along the traces are rates of oxygen evolution expressed as μmol. mg. chlorophyll$^{-1}$. h$^{-1}$.

in CMS-treated chloroplasts, by high phosphate concentrations, suggests that a slow uptake of phosphate occurred, resulting in rates of phosphate entry similar to those normally seen with much lower phosphate concentrations. The stimulation of oxygen evolution in CMS-treated chloroplasts by 3-phosphoglycerate (fig.1A) also suggests that the phosphate transporter was not totally inhibited by CMS and that a slow rate of entry of phosphate and 3-phosphoglycerate was maintained. We have found that chloroplasts must be preincubated with CMS for 2–3 min to achieve maximum inhibition of the phosphate transporter. This is in agreement with the results of Werdan and Heldt [4]. Thus in fig.1B the addition of CMS subsequent to phosphate inhibition had little effect and the phosphate inhibition was still reversed by 3-phosphoglycerate. Flugge and Heldt [9] showed inhibition of phosphate transport in spinach chloroplasts by *p*-(diazonium)-benzene sulphonic acid. However, this inhibitor required lengthy preincubations and did not produce a strong inhibition of the transporter [9]. In plant mitochondria, the phosphate transporter is inhibited by mersalyl [10] and by *N*-ethylmaleimide [11]. In experiments with pea chloroplasts, we have found mersalyl to be almost totally ineffective in preventing phosphate inhibition. Addition of *N*-ethylmaleimide (50 μM) totally inhibited $CO_2$ fixation. Possibly, *N*-ethylmaleimide entered the chloroplasts and inhibited phosphorylation as it is known to do in broken chloroplasts [12].

$CO_2$ fixation by chloroplasts isolated from young pea shoots is inhibited by addition of ATP analogs [6]. The analogs enter the chloroplasts, via the adenine nucleotide transporter, in exchange for endogenous adenine nucleotides thereby depleting the chloroplasts of ATP and inhibiting $CO_2$ fixation. If CMS acted as an inhibitor of adenine nucleotide transport, it should prevent inhibition of $CO_2$ fixation by ATP analogs. Figure 2 shows the effect of one analog, adenylyl-imidodiphosphate, on $CO_2$ fixation. Preincubation with CMS (fig.2B) did not prevent inhibition of oxygen evolution by the ATP analog. Direct measurement of adenine nucleotide transport was made to confirm the resistance of adenine nucleotide transport to CMS. Uptake of ADP was measured as the kinetics are slower than for ATP and would enable any inhibition by CMS to be detected. Chloroplasts which had been preincubated with CMS

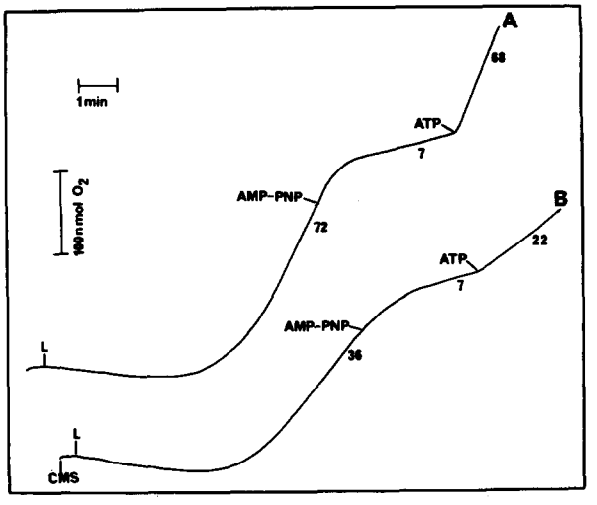


Fig.2. Effect of CMS on inhibition of $CO_2$-dependent oxygen evolution by an ATP analog. Oxygen evolution was measured as described in fig.1 except that 0.2 mM inorganic phosphate was included in the reaction medium. CMS (50 μM) was included in the reaction medium in trace B. Adenylyl-imidodiphosphate (AMP-PNP), 0.7 mM and ATP, 0.8 mM were added as indicated.

showed ADP uptake kinetics which were not significantly different from control chloroplasts (fig.3).

Thus Werdan and Heldt [4] showed that CMS does not inhibit the dicarboxylate transporter and we have now shown that the adenine nucleotide transporter is not inhibited by CMS (figs 2,3). However, if chloroplasts are preincubated with CMS the phosphate transporter is strongly inhibited, as judged by (a) the inhibition of $P_i$ and PGA uptake [4] and (b) the prevention of inhibition of $CO_2$ fixation by high phosphate concentrations (fig.1).

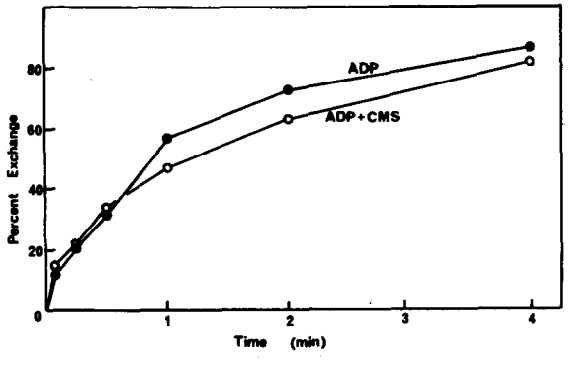


Fig.3. Effect of CMS on adenine nucleotide back exchange. The back exchange was measured as described in ref. [6]. Chloroplasts were preincubated with CMS (50 μM) for 3 min prior to ADP addition.

It has been suggested that phosphate inhibition of $CO_2$ fixation is the result of uptake of the added phosphate in exchange for internal sugar phosphates [13]. The efflux of sugar phosphates would deplete the pools of photosynthetic intermediates and so inhibit $CO_2$ fixation. Implicit in this model is the uptake of the added phosphate via the phosphate transporter. The lack of phosphate inhibition of CMS-treated chloroplasts (fig.1) together with the known inhibitory properties of CMS on the phosphate transporter [4] thus support the above model.

The use of CMS, which is a potent and specific inhibitor of the phosphate transporter in chloroplasts should provide a useful tool for the investigation of metabolite transport between chloroplast and cytoplasm and its role in regulating photosynthesis.

## Acknowledgements

Financial support was provided by the Australian Research Grants Committee and by a Commonwealth Postgraduate Research Award (S.P.R.).

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