Demonstration of Photophosphorylation by Maize Chloroplasts [1,2]

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Photophosphorylation by isolated chloroplasts has been reported and studied in several species (21), but no published evidence of the reaction by maize chloroplasts could be found. In a study of this reaction by maize chloroplasts initial experiments using existing isolation techniques (2, 12) failed to give active chloroplasts. One previous report of a smiliar inability to demonstrate photophosphorylation has been made by Heber (11) who was unable to obtain chloroplasts capable of photophosphorylating from *Vicia faba*. In this case a substance which inhibited the reaction by spinach chloroplasts was found in the bean cell sap.

The aims of this study were to determine if maize leaf tissue contained a component that inhibited chloroplast activity and to devise methods of extraction that would permit the isolation of chloroplasts from maize leaf tissue which would be capable of carrying out the Hill reaction and cyclic and non-cyclic photophosphorylation.

Materials & Methods

Maize (*Zea mays* L. WF9 × M14) tissue was obtained from plants grown in soil in the greenhouse. All the leaf tissue from plants, 2 to 4 weeks old, was used. Spinach (*Spinacia oleoracea* L.) was purchased from the supermarket. Spinach leaves (5 g) were ground in 30 ml of 0.01 M NaCl, 0.05 M tris[4] pH 7.8, and 0.4 M sucrose (basic grinding medium) in an Omnimixer for 30 seconds at low speed (40 v on the variable transformer) and for 30 seconds at high speed (90 v). The homogenate was then filtered through eight layers of cheesecloth and centrifuged at 2,000 × *g* for 7 minutes. The pellet was resuspended in 10 ml of grinding medium and recentrifuged at the same speed. The chloroplast pellet was finally taken up in 1.5 ml of grinding medium. Although this procedure was satisfactory for spinach tissue, maize chloroplasts isolated by the identical technique were not active. In fact the supernatant fluid (maize leaf extract) from the first centrifugation was used as a source of inhibitor.

The reaction mix used for cyclic photophosphorylation contained the following (in μmoles): tris buffer pH 7.8, 26; sodium chloride, 23; magnesium chloride, 4; potassium phosphate pH 7.8, 4; glucose, 33; ATP, 1; PMS, 0.3; excess hexokinase, and chloroplasts containing 8 to 15 μg chlorophyll, all in a final volume of 1 ml.

The reaction was run in 25 ml test tubes placed in a water bath with Plexiglas sides and illuminated from both sides by six 100-w incandescent bulbs placed 4 inches from the tubes. The temperature was maintained at about 16 C by running tap water. The reaction was continued for 5 minutes and stopped by turning off the light and adding 2 ml of 10 % TCA. The precipitate was spun down and the supernatant fluid assayed for inorganic phosphate esterified. Initially, the Fiske and Subbarow (8) method was used. Later experiments were done using P^{32} in the reaction medium (at a concentration of ca. 100,000 cpm per ml of medium) and labelled organic phosphate counted at the termination of the experiment using Avron's (3) modification of the Nielsen and Lehninger technique (16). Chlorophyll concentration was measured by the method of Arnon (1).

All manipulations during isolation were done at as near 0 C as possible using ice buckets and refrigerated centrifuges.

Results

Inhibition Studies: Mixture experiments were carried out. The addition of either spinach leaf extracts or aqueous extracts of spinach chloroplasts to maize chloroplasts did not stimulate phosphorylation. However, addition of maize leaf extract to spinach chloroplasts inhibited (66 %) their activity (table I). Incubation of spinach chloroplasts in maize leaf extract in the cold for short periods reduced their ability to phosphorylate (table I).

Results pertaining to properties and characteristics of the inhibitory component(s) of maize leaf extract are presented in table II. The heat stability of the inhibitor is demonstrated in experiment a. The leaf extract was boiled for 5 minutes, filtered through glass wool and added to the reaction mix. Experiment b shows that the inhibitor could be removed by adsorption onto activated charcoal. Charcoal (1 g)

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[4] The following abbreviations have been used: tris: tris(hydroxymethyl) aminomethane; ATP: adenosine triphosphate; ADP: adenosine diphosphate; EDTA: ethylenediaminetetraacetic acid; PMS: phenazine methosulfate; TCA: trichloroacetic acid.

Table I

Inhibition of Cyclic Photophosphorylation in Spinach Chloroplasts by Maize Leaf Extract

Treatment	Rate*
Added to complete reaction mix	
Maize leaf extract**	120
Extracting medium** (control)	360
Preincubated with spinach chloroplasts	
Maize leaf extract***	211
Extracting medium*** (control)	310

* μmoles of phosphate esterified per mg chlorophyll per hour.
** 0.2 ml added to 1 ml of reaction mix containing spinach chloroplasts.
*** 3 ml of maize leaf extract were added to 3 ml of spinach chloroplasts and incubated 10 minutes at 2 C. The chloroplasts were then spun down and incubated in the reaction medium.

Table II

Cyclic Photophosphorylation Rates of Spinach Chloroplasts As Affected by Treated Maize Leaf Extract

Treatment*	Experiment			
	a	b	c	d
Control	284**	328	210	200
Unboiled extract	125	...	...	...
Boiled extract	125	180	150	70
Charcoal adsorption	...	325	...	...
Collagen adsorption	...	...	...	110
Cation exchange resin***	...	...	151	...
Anion exchange resin†	...	...	154	...
Chloroform ether extraction††	...	...	148	...

* The leaf extract was treated in various ways and then 0.2 ml added to the reaction medium containing spinach chloroplasts. For all controls the relevant amounts of extracting medium were subjected to the same treatment and then 0.2 ml added to the reaction medium.
** Figures in the body of the table are rates (μmoles of phosphate esterified per mg chlorophyll per hour) of phosphorylation by spinach chloroplasts.
*** Dowex 50. pH of extract 2.8.
† Amberlite IRA 200. pH of extract 8.5.
†† Double extraction of leaf extract with equal volumes of chloroform ether (1:1 v/v).

was added to 15 ml of extract and shaken for 10 minutes; the charcoal was then filtered off and 0.2 ml of the extract added to the reaction medium. Further investigation showed that the substance was not removed by anion (Amberlite IRA 200) or cation (Dowex 50) exchange resins or by extraction with chloroform ether (1:1, v/v) (experiment c). Thus it is unlikely that the inhibitor possesses strongly charged groups or that it is a simple phenol or similar hydrophobic compound readily soluble in chloroform ether.

Clendenning et al. (5) have previously shown that tannins are natural inhibitors of the Hill reaction. Tannins may be adsorbed onto collagen (6, 17). Collagen was treated with chrome alum (17) and 1.5 g of collagen (containing 70 % moisture) were added to 5 ml of leaf extract and shaken for 10 minutes [table II (experiment d)]. The removal of tannins alleviated part, but not all of the inhibition.

Development of an Isolation Procedure for Maize Leaf Tissue: Since maize leaf extracts contained chloroplast inhibitors attempts were made to isolate active (photophosphorylating) chloroplasts using protective agents. McClendon (15) has shown the protective nature of Carbowax 4000[5] for chloroplasts and Clendenning et al. (6) further demonstrated its usefulness in isolating chloroplasts for the Hill reaction, especially in plants having a high tannin content. The use of McClendon's medium in an attempt to produce active chloroplasts was not successful. Neither the addition of EDTA, ascorbate, glutathione, nor cysteine to this medium brought any results. At this stage the use of high concentrations of Carbowax was abandoned.

Modifications of the basic grinding medium used for isolating chloroplasts from spinach were made by adding protective agents. Diethyldithiocarbamate and cysteine, when added to this basic medium, led to the isolation of active chloroplasts from corn. Their separate effects were not additive, cysteine alone being more effective (table III). Reduced glutathione was also capable of protecting the chloroplasts during isolation. The results of differing concentrations of glutathione and cysteine are shown in table IV. It can be seen that the optimal concentrations for both substances was 0.005 M, neither substance being effective at high concentrations. The slight advantage for glutathione was borne out in subsequent experiments.

The results shown in table II suggest that at least part of the inhibition is due to tannins. Because Carbowax has been cited as blocking tannin absorption onto chloroplasts (6), theoretically it should be beneficial. Carbowax was added to the glutathione

Table III

Enhancement of Cyclic Phosphorylation in Maize Chloroplasts by Adding Cysteine & Diethyldithiocarbamate to Isolation Medium

Addition to basic isolation medium**	Rate*
None	3
+ 0.01 M diethyldithiocarbamate	53
+ 0.01 M cysteine	142
+ 0.01 M cysteine + 0.01 M diethyldithiocarbamate	75

* μmoles of phosphate esterified per mg of chlorophyll per hour.
** Basic isolation medium was 0.1 M NaCl, 0.05 M tris, pH 7.8, and 0.4 M sucrose.

[5] Trade name for polyethylene glycol. Manufactured by the Union Carbide Chemicals Co.

Table IV

Comparison of Glutathione & Cysteine as Protectants of Maize Chloroplasts During Isolation

Molarity	Rate	
	+ Glutathione*	+ Cysteine
0.0001	32**	16
0.0005	40	20
0.001	65	40
0.005	95	73
0.01	85	55
0.05	4	5

* Glutathione and cysteine were added to 0.01 M NaCl 0.05 M tris pH 7.8, 0.4 M sucrose in the concentrations indicated and the maize chloroplasts extracted in that medium.

** Figures in the body are rates (μmoles of phosphate esterified per mg chlorophyll per hour) of phosphorylation by maize chloroplasts.

enriched medium in various concentrations (fig 1). Addition of low concentrations of Carbowax gave pronounced protection with chloroplast activities (180–200 %) of controls. The actual optimum was in the range of 6 to 10 mg of Carbowax added per ml of medium (insert). This is low compared with previously used concentrations of 400 mg of Carbowax added per ml of medium (15). The shape of the curve is unusual for it rises to a sharp maximum and then gradually decreases with increasing concentration. Table V shows that the protections provided by Carbowax and glutathione are additive.

Recommended Procedure: On the basis of these results the following technique has been used for routine isolation of maize chloroplasts capable of photophosphorylation.

Maize leaves (2 g) are ground, with 30 ml of 0.01 M NaCl, 0.05 M tris pH 7.8, 0.4 M sucrose, 0.005 M glutathione with 6 mg of Carbowax 4000 added per ml, in an Omni-mixer at 40 v for 40 seconds followed by a further 40 seconds at 100 v on the variable transformer. The chloroplasts are then spun down at 2,500 × g for 8 minutes. The pellet is resuspended in 5 ml of 0.01 M NaCl, 0.05 M tris pH 7.8, 0.4 M sucrose, and then recentrifuged. The final pellet is taken up in 1.5 ml of the same medium. With this technique rates of over 400 μmoles of phosphate esterified per mg of chlorophyll per hour have been achieved. This technique has also proved successful for isolating chloroplasts for non-cyclic photophosphorylation, i.e., phosphorylation accompanying the Hill reaction. In this case care must be taken that the final preparation is free from glutathione. With this precaution, results were obtained that gave a molar ratio of phosphate esterified to ferricyanide reduced of 0.5.

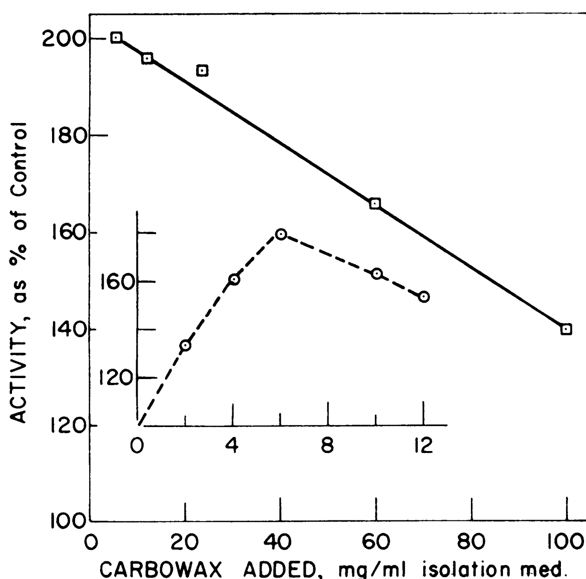

Fig. 1. Enhancement of cyclic photophosphorylation in maize chloroplasts by adding Carbowax to the isolation medium. The basic isolation medium was 0.01 M NaCl, 0.05 M tris pH 7.8, 0.4 M sucrose, 0.005 M glutathione.

Table V

Addition of Carbowax & Glutathione to Isolation Medium for Maize Chloroplasts

Additions to basic isolation medium**	Rate*
None	0
+ Glutathione (0.05 M)	58
+ Carbowax (6 mg/ml)	90
+ Glutathione (0.05 M) & Carbowax (6 mg/ml)	135

* μmoles phosphate esterified per mg chlorophyll per hour.

** Basic isolation medium was 0.01 M NaCl, 0.05 M tris pH 7.8, 0.4 M sucrose, 0.005 M glutathione.

Discussion

This work, together with that of Heber (11), demonstrates that existing isolation techniques based on simple salt or salt sucrose media are not applicable for the extraction of active (photophosphorylating) chloroplasts from all plant species. The failure of these techniques does not indicate that the plants have inactive chloroplasts but that there are inhibitory substances present in leaf extracts. The inhibition can be overcome in young maize plants by adding glutathione (or cysteine) and Carbowax to the isolation medium.

The inhibitory substances present in maize leaf extract have not been finally identified, but certain predictions are possible on the basis of the results in table II. It is likely that at least part of the inhibition is due to tannins. Besides the evidence presented here it has been known since 1890 that tannins are present in maize tissue (19). Further, they have been found to inhibit that part of the chloroplast system responsible for the Hill reaction (4, 5). It

is also probable that more than one inhibitory substance is present in the leaf extract. This would explain the additive effects of glutathione and Carbowax.

It is presumed that the beneficial effect of reduced glutathione is due to the protection of sulfhydryl groups. The presence of such groups is implied by the inhibition of photophosphorylation by *p*-chloromercuribenzoate (2) and similar sulfhydryl poisons (13). Glutathione has previously been found to stimulate photophosphorylation by chloroplast fragments when added to the reaction medium (14). The need for glutathione or cysteine is also in accord with work on the isolation of various enzymes from maize leaf tissue showing that 0.01 M cysteine or glutathione was required for active preparations (9).

Creswell (7) has shown that adding cysteine to the isolation medium is mandatory for extracting nitrate reductase from maize, tobacco, and tomato leaves. No activity could be observed in the absence of cysteine. Thus it appears that certain plant species, including maize, contain substances that will inhibit enzymes or enzyme systems that contain sulfhydryl groups. Therefore, negative results with respect to isolation of enzymes and enzyme systems from such plants may only reflect the lack of protective agents during the extraction rather than the absence of the enzyme from the tissue.

Carbowax has the following known effects: A, it increases the osmotic pressure (15); B, it protects against tannins (6), and C, it precipitates proteins (20). For the previously reported osmotic pressure effects a high concentration of Carbowax was used. It is unlikely that the small amounts added to a relatively high concentration of sucrose would so alter the osmotic pressure as to give the observed stimulation. Carbowax added at a concentration of 400 mg/ml removed the majority of the protein from the supernate of the first centrifugation. This confirms the work of Stocking (20). Even at the low concentrations used, some protein may be precipitated onto the chloroplasts. Whether or not any deposition of protein on the chloroplasts would be beneficial is open to question. Hanson (10) has found that incubating mitochondria with protein (zein or bovine serum albumin) decreases their activity and that incubation with proteolytic enzymes causes a stimulation. From this, it was concluded that adsorbed cytoplasmic protein interferes with oxidative phosphorylation either by limiting diffusion rates or by steric hinderance. It is suggested that a similar phenomenon may occur when higher levels of Carbowax cause the precipitation of proteins onto the chloroplast. Such protein might impede the flow of reactants (ADP & ATP) between the reaction medium and the active sites in the chloroplast. High Carbowax concentrations stimulate rather than inhibit the Hill reaction (6) while inhibiting phosphorylation, and thus it is presumed that they have an uncoupling action. Here again protein adsorption may be implicated, for experiments with plant mitochondria (18) have demonstrated the uncoupling nature of basic proteins. Thus by a process of elimination and on the basis of the data in table II the conclusion may be reached that the sole beneficial effect of Carbowax may be ascribed to its action in blocking the adsorption of tannins onto the chloroplasts.

The Carbowax concentration curve (fig 1) consists of two parts. The second part, the gradual decline in activity, may be explained by Carbowax's property of protein precipitation, as discussed above. It is thought that the curve could, therefore, be the result of the summation of the stimulatory effect of tannin removal and the gradual inhibition due to protein adsorption onto the chloroplast.

The final isolation technique described should prove useful as a basis for isolating chloroplasts, in an active state, from other species known to contain tannins or other natural inhibitors (4). The optimum concentrations of Carbowax may well have to be adjusted for species and age of tissue. Preliminary work with older field grown maize tissue has indicated that the optimum concentration of Carbowax is higher (up to 20.0 mg/ml) than that for the material used in these experiments.

Summary

Extraction of chloroplasts from maize (*Zea mays* L.) leaf tissue by existing techniques failed to yield chloroplasts that were capable of cyclic and non-cyclic photophosphorylation. Further, extracts from maize leaves have been found to inhibit photophosphorylation by spinach (*Spinacia oleoracea* L.) chloroplasts. Some facets of this inhibition were studied.

A procedure has been developed that will permit the isolation of chloroplasts, from young maize leaves, that will carry out the Hill reaction and cyclic and non-cyclic photophosphorylation. Leaves are ground in an isolation medium of 0.01 M NaCl, 0.05 M tris pH 7.8; 0.4 M sucrose; 0.005 M glutathione with 6 mg of Carbowax 4000 added per ml. The remainder of the procedure is similar to previously used methods. The stimulation by glutathione and Carbowax is maximal at the concentrations indicated, notably higher concentrations of either, inhibit activity. Their effects are separate and additive. The possible mechanisms of stimulation are discussed.

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