# ENERGY SOURCES FOR PHOTOSYNTHETIC CARBON DIOXIDE FIXATION[1]

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The high-energy intermediates, or high-energy states, involved in photophosphorylation (PSP), may be used directly to drive $CO_2$ fixation, without the intercession of ATP. This paper reports an investigation on the amounts of $CO_2$ fixed in isolated spinach chloroplasts, when ATP synthesis was inhibited, but when electron flow and high-energy intermediate synthesis was unaffected. As a corollary to this work, the role of pyrophosphate (PPi) as a source of energy was studied.

Izawa, Winget and Good (1966) reported that phloridzin inhibited ATP formation in PSP, in a manner similar to oligomycin inhibition of mitochondrial ATP synthesis. Nobel (1967) noted that phloridzin, while strongly inhibiting PSP had only a marginal effect on the uptake of $Ca^{++}$ by chloroplasts. It was hoped that the use of this inhibitor would point to a role for high energy states other than for ATP formation.

The second inhibitor used in this investigation was quinacrine. This compound was shown by Izawa (1965) and Dilley and Vernon (1966) to inhibit the synthesis of ATP, while allowing high energy intermediates to perform mechanical work in the chloroplasts.

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Methods:

Fresh spinach leaves were washed, cooled and ground in 100-ml buffer solution (0.01M N-2-hydroxy ethyl piperazine-N'-2 ethane sulphonic acid pH 7.8; 0.33M sorbitol; 1.0mM $MgCl_2$; 1.0mM $MnCl_2$; 1.0mM EDTA) in a chilled mortar. The homogenate was filtered through cheesecloth, and the filtrate spun at 2000g for 50 seconds. The pellet was suspended in 2 ml of the same buffer, but without EDTA, pH 7.5. Reactions were performed in Warburg flasks held in a waterbath (20°C), and illuminated by incandescent lamps (30,000 lux). Reactions were argon flushed, and were terminated by the addition of 0.2 ml 20% trichloro-acetic acid.

Isotope counting was done in a scintillation counter. Dark controls were run in all experiments and the count obtained was subtracted from the experimental values to give a measure of the light-induced isotope uptake.

Examination by phase-contrast microscopy indicated that a high proportion of the chloroplasts used in these experiments were intact. Chlorophyll concentrations were assayed by the method of Arnon (1949).

Results and Discussion:

In order to determine the effects of phloridzin and quinacrine on ATP synthesis, both the light-dependent and light-triggered reactions were studied (Bennun and Avron, 1964; Petrack and Lipman, 1965). Some typical results are shown in Table 1. From this data it was concluded that both mechanisms of ATP synthesis were 60-70% inhibited by a 5-minute pre-incubation with either of the inhibitors. It was now possible to test whether $CO_2$ fixation would proceed even when synthesis of ATP was inhibited.

Table 1. Effects of phloridzin and quinacrine on PSP in spinach chloroplasts.
(expressed as μmoles ATP/mg chlorophyll/hour)

| A. Phloridzin (mM) | | 0 | 0.25 |
|---|---|---|---|
| light-dependent reaction | chloroplast preparation # 1 | 24.7 | 7.4 |
| | 2 | 25.9 | 10.0 |
| light-triggered reaction | 3 | 66.1 | 19.9 |
| | 4 | 61.7 | 20.7 |
| | 5 | 95.1 | 24.2 |
| B. Quinacrine (μM) | | 0 | 10 |
| light-dependent reaction | 6 | 6.4 | 2.1 |
| | 7 | 1.7 | 0.5 |
| light-triggered reaction | 8 | 140.1 | 53.4 |
| | 9 | 160.2 | 58.5 |
| | 10 | 183.6 | 121.8 |

In the light-dependent reaction, 0.2 ml of the chloroplast suspension were added to reaction buffer (see Methods) and incubated in the dark, with or without inhibitor, for 5 min. The reaction buffer also contained 2.0 μmoles ADP, 2.0 μmoles $KH_2PO_4$ and 0.2 μmoles phenazine methosulphate. The total volume was 2 ml. The light reaction lasted for 5 min. ATP was assayed by the luciferase method (Strehler, 1963).
The same buffer was used for the light triggered reaction, except it was made 2.0mM in glutathione, and 10 μmoles $KH_2PO_4$ were added. After the 5 min pre-incubation, $2.0 \times 10^5$ cpm $^{32}Pi$ were added; the light was turned on for 20 sec., and then 20 μmoles ADP were added in the dark, and allowed to react for 1 min. $AT^{32}P$ was assayed by the method of Avron (1960).

The degree of inhibition varied from one chloroplast preparation to another (the age of the tissue was found to be of importance in determining the degree of inhibition). Nevertheless, $CO_2$ fixation was only slightly affected by the inhibitors. This may signify the presence of a mechanism capable of operating in the absence of an ATP synthesizing system.

Table 2. Effects of phloridzin and quinacrine on $CO_2$ fixation. (expressed as μmoles $^{14}CO_2$ fixed/mg chlorophyll/hour)

| A. Phloridzin (mM) | | 0 | 0.25 |
|---|---|---|---|
| chloroplast preparation | #1 | 79 | 79 |
| | 2 | 64 | 60 |
| | 3 | 48 | 48 |
| | 4 | 45 | 39 |
| **B. Quinacrine (μM)** | | **0** | **10** |
| | 5 | 89 | 88 |
| | 6 | 77 | 57 |
| | 7 | 56 | 50 |
| | 8 | 36 | 33 |

0.2 ml chloroplasts (0.2 - 0.25 mg chlorophyll) were added to buffer (Methods) pH 7.5. The samples were pre-incubated for 5 min. The 50 μmoles $NaH^{14}CO_3$ ($1.2 \times 10^6$ cpm) were mixed in with the reaction mixture, followed by 5 min light. The experiment was terminated by the addition of TCA, and the unreacted bicarbonate removed by heating to 50-60°C for 5 min.

Jensen and Bassham (1966) showed that PPi (5mM) stimulated the fixation of $CO_2$ by spinach chloroplasts. (Another role for PPi has been shown by Baltscheffsky, 1967, who reported roles for both ATP and PPi in the energy-dependent cytochrome spectral changes of chromatophores and mitochondria of micro-organisms). Studies by Kalberer, Buchanan and Arnon (1967) suggested that the stimulation of $CO_2$ fixation by PPi was a result of the formation, by the action of chloroplast pyrophosphatase, of low levels of inorganic phosphate (Pi) which would stimulate the reaction. Our work indicated that increased levels of inhibition resulted from the addition of a range of Pi from 0.1 μmoles upwards. In Table 3 a 10-20% inhibition of $CO_2$ fixation was observed if 10 μmoles inorganic phosphate were included in the reaction mixture.

Table 3. Effects of Pi and PPi on $CO_2$ fixation. (expressed as cpm $^{14}C$ fixed/mg chlorophyll x $10^3$)

| Inorganic phosphate (μmoles) | Inorganic pyrophosphate (mM) | Chloroplast preparation #1 | #2 |
|---|---|---|---|
| 0 | 0 | 149 | 46 |
| 10 | 0 | 137 | 36 |
| 0 | 5 | 345 | 128 |
| 10 | 5 | 168 | 94 |

Reaction mixtures were the same as in Table 2. There was no pre-incubation period, prior to the 5-min light reaction.

On the other hand, if 10 μmoles Pi were added to a reaction mixture 5mM in PPi, the amount of $CO_2$ fixed was considerably increased, suggesting an additional role for PPi other than as a source of Pi.

The results shown in Table 4 indicated that both ATP and PPi could increase the amount of $CO_2$ fixed by approximately equal amounts.

Table 4. Effects of added ATP or PPi on $CO_2$ fixation. (expressed as cpm $^{14}C$ fixed/mg chlorophyll x $10^3$)

| Addition | Chloroplast preparation #1 | #2 |
|---|---|---|
| ATP (1mM) | 38.7 | 35.0 |
| PPi (1mM) | 35.5 | 39.1 |
| Control | 23.0 | 25.8 |

Reaction conditions were as shown in Table 2; there was no pre-incubation period.

In this light dependent system, the energy from ATP and PPi is utilized for $CO_2$ fixation in chloroplasts. (An energetic role for PPi has also been noted by Siu and Wood, 1962, who reported the enzymatic synthesis of phosphoenol pyruvate from

oxaloacetate and PPi.) It is possible, that ATP is formed following PPi breakdown, or both the ATP and PPi may act by inducing the formation of a high-energy compound or condition, which is itself the energy donor for $CO_2$ fixation.

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