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Cyclic Photophosphorylation in Vivo and its Relation to Photosynthetic CO_2-Fixation

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Abstract. **Salicylaldoxime (2×10^{-3} M and less) inhibits cyclic photophosphorylation in intact *Chlorella* cells severely whereas photosynthetic O_2-evolution and $^{14}CO_2$-fixation is hardly affected. Cyclic photophosphorylation *in vivo* was measured by following anaerobic light dependent glucose uptake. A similar difference in susceptibility has been observed with carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. Various controls exclude the possibility that the difference in inhibition was caused by differing experimental conditions or, in the case of glucose assimilation, by an inhibition of a reaction other than photophosphorylation.**

There also exists a great difference in light saturation of cyclic photophosphorylation and photosynthesis. Evidence is reported that at light saturation of glucose uptake light driven cyclic phosphorylation is indeed the limiting reaction.

The results are considered as evidence that cyclic photophosphorylation does not contribute ATP stoichiometrically to photosynthetic CO_2-fixation.

According to the Calvin cycle 3 moles of ATP are necessary for each mole of CO_2 fixed photosynthetically. It is quite generally assumed that cyclic photophosphorylation supplies 1 mole of ATP and non-cyclic photophosphorylation 2 moles (2). A number of observations, however, disagree with such an assumption (13, 14, 17). One of these observations has been the different susceptibility of cyclic photophosphorylation and photosynthetic CO_2-fixation towards various poisons. A more detailed study using the 2 inhibitors salicylaldoxime and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone has now been carried out.

Cyclic photophosphorylation *in vivo* was measured by following anaerobic light dependent glucose uptake by *Chlorella vulgaris*. Since it has been shown that 85 % of the glucose taken up under these conditions is present in the cells as oligo- and polysaccharides and only 1 % as free glucose (26), we use the term photoassimilation of glucose synonymously with light dependent glucose uptake. It was found previously that for this process only photosystem I is necessary (26). This has been supported more recently by experiments using Bishop's *Scenedesmus* mutants (27), as well as by the observation that the quantum requirement per glucose assimilated decreased from 6 at 658 mμ to 4 at 712 mμ (29).

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[2] This strain of *Chlorella* had been called *C. pyrenoidosa* before. It has been kindly identified recently as *C. vulgaris* by Dr. Soeder.

Materials and Methods

The same strain of *Chlorella vulgaris*[2] has been used as previously (12, 25) and it has been cultured under the conditions described before (25). *Ankistrodesmus braunii* has been grown at 3000 lux in the medium of Kessler (16). The *Scenedesmus* mutant 11 was grown essentially in the medium of Kessler (16) with the addition of 2 g glucose and 0.25 g yeast extract per liter.

Salicylaldoxime was obtained from E. Merck A.G., Darmstadt. A sample of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (CCP) was kindly supplied by Dr. P. Heytler, Du Pont de Nemours.

Measurement of Glucose Uptake. Before every glucose uptake experiment the cells (200 μg chlorophyll/ml) were preincubated aerobically in the presence of glucose (8 mg/ml) in 0.03 M phosphate buffer pH 6.5, since only after this adaptation period the uptake was linear with time (28). After 60 min the cells were transferred directly to 15 ml rectangular Warburg vessels and the disappearance of glucose from then on, either anaerobically in the light (1800 lux) or aerobically in the dark, was determined as described previously (25).

Manometric Measurements. Photosynthetic O_2-production was measured in 0.1 M carbonate buffer ($NaHCO_3$:Na_2CO_3 = 9:1) under conditions otherwise identical with those of glucose uptake experiments. Photoreduction was measured in an atmosphere of 96 % H_2 and 4 % CO_2 at a light intensity of 700 lux. The *Ankistrodesmus* cells were adapted for 18 hr.

Photosynthetic $^{14}CO_2$-fixation. $^{14}CO_2$-fixation was measured in a small lollipop. The cells (200 μg chlorophyll/ml) were incubated in 0.03 M phosphate buffer pH 6.5 and an air/$^{14}CO_2$ mixture (0.44 % CO_2) with a specific radioactivity of 0.036 μc/μmole was rapidly bubbled through the suspension. The light intensity (~2000 lux) was adjusted to allow the same photosynthetic rate as was observed in the manometric measurement when O_2-evolution was followed. The measurement of the radioactivity incorporated was carried out as described before (25).

Results

Inhibition of Glucose Uptake and of Photosynthetic CO_2-Fixation by Salicylaldoxime. Salicylaldoxime at a concentration of 5×10^{-3} M inhibits in chloroplasts the photosynthetic electron transport between the 2 light reactions (30), most likely close to photosystem II (15, 21). Light-dependent ^{32}P incorporation is inhibited in *Ankistrodesmus braunii* to more than 50 % at 1×10^{-3} M (32).

In *Chlorella vulgaris* concentrations of 5×10^{-3} M salicylaldoxime result in a pronounced inhibition of $^{14}CO_2$-fixation and O_2-evolution (Fig. 1). The anaerobic photoassimilation of glucose, however, is almost completely inhibited at a concentration of 1×10^{-3} M. Fig. 1 also shows that the oxidative assimilation of glucose in the dark is inhibited to the same extent as the anaerobic photoassimilation. The slight difference in inhibition of photosynthesis when O_2-evolution and CO_2-fixation was measured can be explained, since salicylaldoxime acts as an uncoupler of endogenous respiration (table I). When O_2-evolution was measured the apparent photosynthesis has been corrected for the increased O_2-uptake, whereas no correction could be made for the increased CO_2-output leading to a higher dilution of the $^{14}CO_2$.

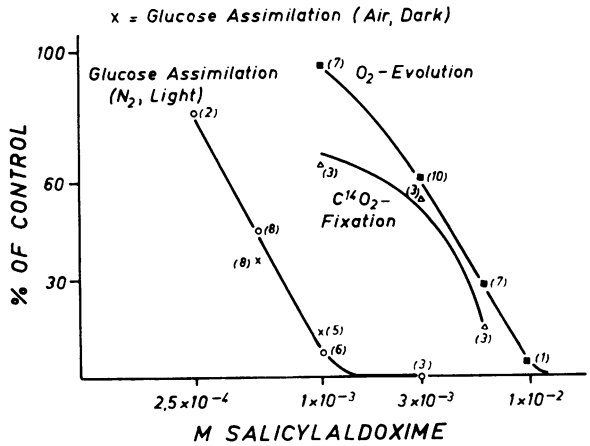

FIG. 1. Salicylaldoxime inhibition of anaerobic photoassimilation and aerobic dark assimilation of glucose, as well as of pthotosynthetic $^{14}CO_2$-fixation and O_2-evolution. Average of (n) experiments. For details see Materials and Methods.

Table I. *Effect of Salicylaldoxime on Endogenous Respiration and Glucose Respiration*

Salicylaldoxime Concn.	Respiratory rate of Endogenous respiration	Glucose respiration
M	% of control	
5×10^{-4}	164	95
1×10^{-3}	160	74
3×10^{-3}	118	44
1×10^{-2}	44	26

Inhibition of Glucose Uptake and of Photosynthetic CO_2-Fixation Under Identical Conditions by Salicylaldoxime. The photoassimilation of glucose has been measured under strictly anaerobic conditions whereas aerobic conditions existed when photosynthetic CO_2-fixation was followed. It seemed possible, therefore, that the different degree of inhibition of these 2 processes was due to the different experimental conditions. Since oxidative dark assimilation of glucose is also completely prevented at the same low salicylaldoxime concentrations, it was possible to measure the inhibition of photoassimilation under aerobic as well as under anaerobic conditions. Photoassimilation of glucose is inhibited to the same extent under both conditions; 3×10^{-3} M salicylaldoxime for example inhibit 100 % which shows that cyclic photophosphorylation also cannot proceed in air in the presence of 2 to 3×10^{-3} M of the poison (table II).

Table II. *Inhibition of Glucose Uptake by Salicylaldoxime Under Aerobic and Anaerobic Conditions*

Experimental conditions are given in Materials and Methods.

	Glucose taken up		
	Light/N_2	Dark/air	Light/air
	mg	*mg*	*mg*
Control	4.43	5.25	4.96
Salicylaldoxime 1×10^{-3} M	1.64	1.32	1.20
Salicylaldoxime 3×10^{-3} M	0.00	0.00	0.00

In a further experiment $^{14}CO_2$-fixation and glucose uptake was measured in one and the same vessel (table III). Again it was observed that photoassimilation of glucose can be inhibited without severely affecting photosynthetic $^{14}CO_2$-fixation. Even an increase in radioactivity could be observed in the presence of salicylaldoxime. This is due to the fact that salicylaldoxime inhibits the high respiratory rate in the presence of glucose in contrast to its effect on the lower endogenous respiration, which is significantly increased (table I). Therefore, the in-

Table III. *Inhibition of $^{14}CO_2$-Fixation and of Glucose-Uptake by Salicylaldoxime*

A total volume of 3 ml phosphate buffer (0.02 M, pH 6.5) contained glucose adapted algae equivalent to 0.6 mg chlorophyll and 8 mg glucose. The side arm of a rectangular Warburg vessel contained 30 μmoles of $^{14}CO_2$-bicarbonate (2.8 μc) which was tipped into the main compartment after temperature equilibration. After 2 and one-fourth hr at 28° and 1500 lux the amount of $^{14}CO_2$ fixed was determined in 0.1 ml aliquots. The residual glucose was determined as described in Materials and Methods. The measurements were made simultaneously.

	$^{14}CO_2$ fixation	Glucose taken up
	cpm	*mg*
Control	72,441	7.20
Salicylaldoxime		
2×10^{-3} M	85,169	0.78

hibitor simulates a too strong inhibition of $^{14}CO_2$-fixation in the latter case, since the $^{14}CO_2$ supplied is diluted by the increased amount of nonradioactive respiratory CO_2 (see also Fig. 1). When $^{14}CO_2$-fixation is measured in the presence of glucose, on the other hand, the reduced output of respiratory CO_2 leads to the opposite effect. The exact inhibition of photosynthetic CO_2-fixation in this experiment, therefore, remains unknown. However, it has been shown by the data of Fig. 1 (O_2-evolution) that the inhibition is very low indeed.

The fixation products also are not different in the presence of salicylaldoxime: 70 % of the total radioactivity fixed after 2 and one-fourth hr was present in sucrose and starch as this was also the case in the control (14).

The Specificity of the Salicylaldoxime Inhibition. About 80 % of the glucose taken up by *Chlorella* under anaerobic conditions in the light is incorporated into sucrose and starch (26). The high-energy phosphate necessary for the assimilation is supplied by cyclic photophosphorylation. In case high-energy phosphate is also necessary for the actual uptake, it would also have to be supplied by photophosphorylation. It is possible, therefore, that salicylaldoxime interferes in 3 different ways with glucose assimilation: it could inhibit A) the actual uptake, B) one or more of the enzymes necessary for the assimilation, C) the generation of energy required for (B) and if necessary also for (A). It is obvious that we are measuring an inhibition on photophosphorylation only if case (C) is true.

It was of interest, therefore, to measure the inhibition of a process which is independent of the factors mentioned above under A) and B), but does require cyclic photophosphorylation. Photoreduction was chosen as such a process; it requires photosystem I and most likely cyclic photophosphorylation only (5, 6). In table IV the mean values of 3 parallel samples of an experiment with *Ankistrodesmus braunii* are given. Photoreduction is inhibited by

Table IV. *Salicylaldoxime Inhibition of Photosynthesis and Photoreduction in* Ankistrodesmus braunii

For details see Materials and Methods.

Salicylaldoxime Concn.	Inhibition	
	O_2-evolution	Photoreduction
M	%	%
3×10^{-3}	18	58
5×10^{-3}	31	72

salicylaldoxime and it also is much more sensitive than photosynthetic O_2-evolution. The difference in inhibition between the 2 processes is not quite as drastic as in the case of glucose assimilation and photosynthesis in *Chlorella vulgaris*. In comparison to *Chlorella*, however, *Ankistrodesmus* is altogether less affected by salicylaldoxime.

In addition it has been observed that the inhibition of photoassimilation of glucose by salicylaldoxime can be reversed to a large extent by increasing the light intensity (table V). This phenomenon has been previously observed with antimycin A (25) and also occurs with CCP, as will be shown below. This observation can be best explained if the poisons inhibit the light-dependent reactions, *i.e.* photophosphorylation. Urbach and Simonis (32) observed that salicylaldoxime at relatively low concentrations inhibits the light-dependent ^{32}P-incorporation. All these various observations make it seem very unlikely that the salicylaldoxime inhibition of photoassimilation of glucose is due to an inhibition of the actual glucose uptake or of one of the assimilatory enzymes.

Table V. *The Effect of Light Intensity on Salicylaldoxime Inhibition of Photoassimilation of Glucose*

The data are average values of 3 experiments. The concentration of inhibitor was 1×10^{-3} M.

Light intensity	Inhibition
lux	%
1,200	75
7,500	32
20,000	19

The Inhibition of Glucose Uptake and of O_2-Evolution by CCP. Uncoupling agents belonging to the class of carbonylcyanidephenylhydrazones have been introduced by Heytler and Prichard (8). It has been shown that in chloroplasts cyclic and noncyclic photophosphorylation is inhibited by CCP (3). Wiessner observed an inhibition of photoassimilation of acetate by *Chlamydobotris* (34).

In Fig. 2 the influence of CCP on anaerobic photoassimilation and on oxidative assimilation of glucose as well as on photosynthetic CO_2-evolution can be seen. Photoassimilation of glucose by *Chlorella* is strongly inhibited by CCP; 3×10^{-5} M prevents glucose assimilation to 90 %. The oxidative assimilation is somewhat less sensitive, a concentration of

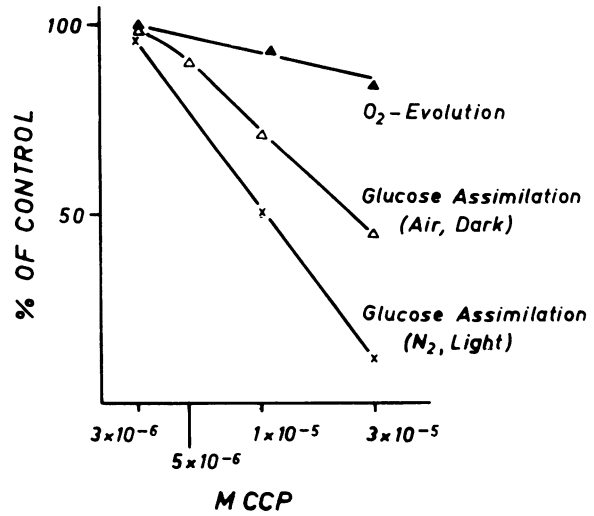

FIG. 2. CCP inhibition of anaerobic photoassimilation and aerobic dark assimilation of glucose and of photosynthetic O_2-evolution. Each point represents average values of 2 or 3 experiments.

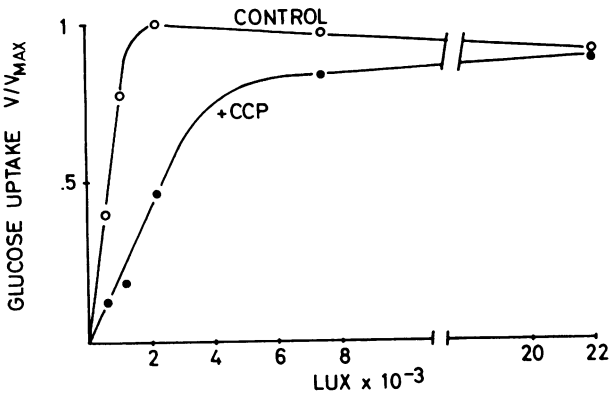

FIG. 3. Effect of light intensity on CCP inhibition of anaerobic photoassimilation of glucose. Each point represents average values of 2 or 3 experiments. V = rate of uptake, V_{max} = maximal rate of uptake.

3×10^{-5} M results in an inhibition of 55 %. The same concentrations of CCP inhibit photosynthetic O_2-evolution only slightly (Fig. 2). The uncoupler behaves similar to salicylaldoxime and to antimycin A (25).

Effect of Light-Intensity on CCP-Inhibition. The degree of inhibition with CCP is dependent on light-intensity; it decreases with increasing intensity a phenomenon also observed in *in vitro* experiments (3). The effect is illustrated in Fig. 3. The decrease in inhibition starts at light intensities where photoassimilation is saturated already in the control.

The possibility that at high light intensities the inhibitor is destroyed could be excluded by the data of table VI. In these experiments the degree of inhibition was determined at strong light intensity followed by a period of low light intensity. There was a clear inhibition at the low light intensity in spite of the strong light treatment which had preceeded. In the first interval (= strong light) the usual lack of inhibition was observed.

In addition the possibility has been excluded that at stronger light intensities a noncyclic or pseudocyclic photophosphorylation comes into play. This is shown with experiments using *Scenedesmus* mutant number 11 of Bishop which is incapable of carrying out a noncyclic electron flow. Also with this orga-

Table VII. *Effect of Light Intensity on CCP Inhibition of Glucose Uptake by* Scenedesmus *Mutant Nr. 11*
CCP concentration was 2×10^{-5} M.

Expt.	Conditions	Glucose taken up	Inhibition
		mg	%
1	Control 10,000 lux	1.98	
	CCP "	1.26	36
	Control 2,000 lux	1.44	
	CCP "	0.33	77
II	Control 10,000 lux	2.00	
	CCP "	1.73	13
	Control 2,000 lux	1.62	
	CCP "	0.92	43

Table VI. *Effect of a Preceding Strong Light Period on the Inhibition of Glucose Uptake by CCP in Subsequent Weak Light*
CCP concentration was 1.5×10^{-5} M. For details see Materials and Methods.

Expt			Glucose taken up	Inhibition
			µg	%
1	Control	10.000 lux	1350	
	CCP		1365	0
	Control	(10,000 +) 2.000 lux	1800[1]	
	CCP	(10,000 +) 2,000 lux	0960	47
II	Control	10,000 lux	1960	
	CCP	10.000 lux	1880	4
	Control	(10,000 +) 2.000 lux	2240[1]	
	CCP	(10,000 +) 2,000 lux	1320	41

[1] Glucose taken up during the 2000 lux period only.

nism the inhibition of CCP decreased at higher light intensities (table VII).

Discussion

According to the Calvin cycle the third ATP for photosynthetic CO_2-fixation would have to be supplied by cyclic photophosphorylation in strictly stoichiometric amounts. An inhibition of this phosphorylation should, therefore, rapidly lead to a strong inhibition of photosynthesis. The experiments reported here as well as previous ones (14, 25) show, however, that this is not the case. Even in one and the same experimental sample of *Chlorella* (table III) cyclic photophosphorylation can be almost completely blocked without affecting photosynthetic $^{14}CO_2$-fixation for 2 and one-fourth hr severely. This speaks against a stoichiometric participation of cyclic photophosphorylation in photosynthesis.

Similar results have been obtained by other investigators. Simonis and Urbach (23) observed that X-ray irradiation is much more harmful to light dependent ^{32}P incorporation than to $^{14}CO_2$-fixation. Trebst and Burba (31) as well as Gimmler *et al.* (7) observed that cyclic photophosphorylation *in vivo* can be severely inhibited at concentrations of disalicylidenepropanediamine which do not impair photosynthetic O_2-evolution to any extent.

There exists in addition a second strong argument which rules out a stoichiometric participation of cyclic photophosphorylation in CO_2-fixation, that is the early light-saturation of cyclic photophosphorylation. Thus the photoassimilation of glucose is saturated at much lower light intensities than photosynthesis (25). Approximately 1 to 2 times the light intensity necessary for compensation is sufficient to saturate glucose assimilation. This saturation could be due of course to limiting enzymic reactions necessary for assimilation or for the actual uptake process. There exist, however, 2 lines of evidence that not the forementioned possibilities but indeed the generation of ATP by cyclic photophosphorylation is limiting at light saturation: A) Several other quite different physiological phenomena, for example light dependent isocitrate-lyase synthesis (24) and the Kok-effect (9), which both are dependent on cyclic photophosphorylation show the same early light saturation as the photoassimilation of glucose. Syrett (24) directly compared the light saturation for the synthesis of isocitrate lyase in *Chlorella pyrenoidosa* with the saturation for photoassimilation of glucose: the light curves were found to be identical.

Anacystis shows the maximal Kok-effect at 1 to 2 times the compensation point (9). Ried (22) measuring O_2-exchange of *Chlorella pyrenoidosa* has shown clearly that the socalled transient T_3 has the same cause as the Kok-effect. Light saturation for T_3 was observed at one-tenth the light intensity necessary to saturate photosynthesis, a result which again agrees with the observations made with *Chlorella vulgaris* concerning the photoassimilation of glucose. Also light dependent ^{32}P incorporation under conditions where only cyclic photophosphorylation takes place is saturated at approximately one-tenth the light intensity necessary to saturate photosynthesis (Urbach, personal communication). A very early light saturation has previously also been reported for the rate of P_i disappearance in *Chlorella* after the light was turned on (13). Since this phenomenon is independent of CO_2 it is most likely also caused by cyclic photophosphorylation. Based on these data it has been concluded already in 1957 (13) that photosynthesis proceedes *in vivo* independent of this photophosphorylation.

B) In case the light saturation of glucose assimilation is indeed due to a limiting cyclic photophosphorylation, it was expected to be possible to advance the light saturation towards higher intensities in the presence of uncoupling agents. This has been observed for CCP (Fig. 3). In the presence of the uncoupler phosphorylation is not limiting any more the electron flow rate in the cycle and thus more light can be used, although less efficient. This also explains the release of CCP inhibition of glucose assimilation at high light intensities.

Since cyclic photophosphorylation does not contribute ATP to CO_2 fixation stoichiometrically the third ATP—if necessary at all—could be either supplied by pseudocyclic photophosphorylation (1, 19) or more than 1 ATP/2 e are generated in the noncyclic electron flow (10, 36). Taking a quantum requirement of not much more than 8 for photosynthetic CO_2 fixation and the photosynthetic scheme based on 2 consecutive light reactions as it is accepted by most workers, it seems impossible, however, that a third ATP is supplied in a pseudocyclic manner.

A further question remains: what physiological function does cyclic photophosphorylation have? Obviously quite a number of ATP requiring reactions can use this ATP: CO_2-assimilation in the presence of H_2 (5, 6), assimilation of organic substances (12, 35), ion uptake (11, 18), protein synthesis (24, 28). The latter has recently been found to be the case in isolated chloroplasts, too (20). ATP generated in cyclic photophosphorylation could, however, also be involved in photosynthetic CO_2-fixation in a non-stoichiometric manner. It could support, for example, oligo- and polysaccharide biosynthesis. Since glucose is photoassimilated almost exclusively to sucrose and starch (26), this ATP obviously can serve this purpose.

It seems likely, therefore, that cyclic photophosphorylation does not serve a specific function. Rather it seems to be an ATP generating system, comparable in capacity with respiration (maximally 30–45 μmoles ATP per mg chlorophyll per hr) and also competing with the respiratory system for ADP (9, 22). Noncyclic photophosphorylation does not seem to inhibit respiration (9), possibly because no

free ATP is generated but an energy rich intermediate is used directly for photosynthetic CO_2 fixation (33).

Finally it shall be pointed out that most of the results reported and discussed are only explainable when 2 different phosphorylating sites for cyclic and noncyclic phosphorylation exist with different susceptibility towards various poisons. Two different phosphorylating sites have been assumed to exist by various authors (4, 14).

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