INHIBITOR STUDIES ON THE PHOTOPHOSPHORYLATION IN VIVO BY UNICELLULAR ALGAE (ANKISTRODESMUS) WITH ANTIMYCIN A, HOQNO[1], SALICYLALDOXIME AND DCMU.

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Little work has been done on the elucidation of the mechanism of photophosphorylation in vivo (Krall and Bass 1962, Urbach and Simonis 1962, Forti and Parisi 1963). On the other hand, the photosynthetic phosphorylation in isolated chloroplasts has been investigated in many experiments by which it is known that ATP-formation in light is possible in a cyclic as well as in a non-cyclic process (Arnon 1960). By means of inhibitor studies using inhibitors that have been applied with success in photosynthetic phosphorylation systems with isolated chloroplasts it was intended to continue our former studies (Simonis and Urbach 1960, 1963; Urbach and Simonis 1962) and to find out more clearly whether the photosynthetic phosphorylation systems working in vitro are also active in vivo. The results of our new studies with inhibitors additionally suggest a regulation between oxidative phosphorylation and photosynthesis in green algal cells. Although now it is known that in intact cells the

[1] Abbreviations: HOQNO, 2n heptyl-4-hydroxyquinoline-N-oxide; DCMU, dichlorophenyldimethylurea; DNP, 2,4-dinitrophenol; TCA, trichloro acetic acid; Po, TCA-soluble organic phosphate compounds.

penetration of labelled inorganic phosphate into chloroplasts is much slower than into the cytoplasm (Santarius et al. 1964, Heber et al. 1964) the following findings show that the short-time incorporation of ^{32}P into the TCA-soluble organic phosphate compounds (Po) in the light can be used as an indirect test reaction of the photosynthetic phosphorylation in vivo. The same conclusion was drawn from the recently published results of the action spectrum of incorporation of ^{32}P into Po in Ankistrodesmus (Simonis and Mechler 1963).

Materials and Methods: Ankistrodesmus braunii (Naegeli) was grown synchronously in liquid culture medium of Pirson and Ruppel (1962). Before the incorporation of ^{32}P the algae were kept in a phosphate-free medium for 4 hours in the light and for one hour in the dark. The incorporation of ^{32}P was carried out in Warburg vessels with two side arms at 25°C. The reaction mixture was composed as indicated in table 1. One side arm contained 0,5 ml $^{32}PO_4$ (10-20 µC), the second side arm contained 0,5 ml TCA (65%). For the incorporation in the light (6500 lux) and in the dark ^{32}P was added from the side arm. After 5 min of incorporation the reaction was stopped by addition of TCA from the second side arm. After 30 min extraction with TCA the labelled Po was obtained from the supernatant liquid by centrifugation and by the fractionation method according to Avron (1960). Experiments in the presence of N_2 were performed under a stream of highly purified nitrogen freed of traces of oxygen by pyrogallol. The gas passed through the algal suspension for more than 10 min before and during the actual experiment. DCMU, antimycin A and salicylaldoxime were dissolved in methanol. Fresh solutions of HOQNO were prepared daily in 10^{-3}M KOH.

Results and Discussion: In experiments of Arnon (1960) the cyclic photophosphorylation in isolated chloroplasts with vitamin K is not inhibited by DNP. On the basis of our results

with DNP in Ankistrodesmus in which the incorporation of ^{32}P into Po is slightly inhibited in the light in contrast to a strong inhibition in the dark (Simonis and Urbach 1960) we assumed at that time that also in vivo a cyclic photophosphorylation occurs besides a non-cyclic photophosphorylation. However it was shown by Wessels (1960) that DNP in light will be reduced and employed as a catalyst of photophosphorylation. Therefore it seemed necessary to prove the existence of a cyclic photophosphorylation in vivo by means of other inhibitors. Addition of DCMU, a potent inhibitor of O_2-production in photosynthesis, in concentrations of 2×10^{-5}M, results in a total inhibition of O_2-evolution, an inhibition of only about 50% of ^{32}P incorporation in the light and no inhibition of ^{32}P incorporation in the dark (Urbach and Simonis 1962). In an N_2-atmosphere the phosphorylation in the light is more strongly inhibited by DCMU than under aerobic conditions (table 1). Since in an N_2-atmosphere, together with a total inhibition of the O_2-production in photosynthesis by DCMU, the respiration and the oxidative phosphorylation will be suppressed by lack of O_2, the finding of a remaining ^{32}P incorporation in light suggest a cyclic photophosphorylation in vivo. This result was confirmed in the last time by Forti and Parisi (1963) with Elodea.

Antimycin A completely inhibited the respiration of isolated mitochrondria at 10^{-7} - 10^{-8}M (Ahmad et al. 1950). With the same concentration the bacterial photophosphorylation was also inhibited (Baltscheffsky 1960). In contrast to these results with bacteria, antimycin A in the same concentration had no effect on the photophosphorylation in isolated chloroplasts. In our experiments antimycin A at 10^{-4}M nearly comple-

tely inhibited the incorporation of ^{32}P into Po in the dark but no inhibition was found in the light (table 1). On the other

Table 1:
Action of inhibitors on ^{32}P incorporation into Po in light and dark in air and in N_2-atmosphere (counts/µg chlorophyll)

		^{32}P incorporation into Po			
		air		N_2	
		cts/µg chl	%	cts/µg chl	%
light					
control		31 500	100	31 800	100
+ antimycin A	(200µg)	31 850	101	28 300	89
+ HOQNO	(200µg)	25 800	82	30 600	96
+ salicylaldoxime	($2x10^{-2}$M)	-	-	640	2
+ "	($1x10^{-2}$M)	320	1	-	-
+ "	($1x10^{-3}$M)	15 750	50	12 400	39
+ DCMU	($2x10^{-5}$M)	18 400	58	8 300	26
+ DCMU + antimycin A		3 800	12	2 550	8
+ " + HOQNO		3 460	11	1 910	6
+ " + salic.ald.	($2x10^{-2}$M)	-	-	320	1
+ " + " "	(10^{-3}M)	1 730	5,5	540	1,7
dark					
control		15 500	100	1 550	100
+ antimycin A	(200µg)	990	6	-	-
+ HOQNO	(200µg)	1 085	7	-	-
+ salicylaldoxime	($2x10^{-2}$M)	695	4,5	-	-
+ "	($1x10^{-3}$M)	1 400	9	-	-
+ DCMU	($2x10^{-5}$M)	14 850	96	1 535	99

The reaction mixture in a final volume of 4,5 ml contained in µmoles; tris buffer (pH 8,0), 65; KNO_3, 24; $NaNO_3$, 24; $MgSO_4 \cdot 7H_2O$, 3; $Ca(NO_3)_2 \cdot 4H_2O$, 0,3; $ZnSO_4 \cdot 7H_2O$, 0,01; microelements according to Pirson and Ruppel (1962); algae containing 70 µg chlorophyll; inhibitors as indicated. The values of the table are representative examples of a number of experiments. The values of different experiments are always related to the dark control.

hand, when antimycin A was added together with DCMU, we observed a strong inhibition of ^{32}P incorporation in the light in air as well as in an N_2-atmosphere. This inhibition was considerably greater than with DCMU alone. These results show that the remaining photophosphorylation after inhibition with DCMU, which we have considered as a cyclic photophosphorylation, is blocked by antimycin. In accordance with results obtained by Tagawa et al. (1963), who found an inhibition of the endogenous ferredoxin-catalyzed photophosphorylation in isolated chloroplasts by antimycin A, our results suggest that also in vivo such system of photophosphorylation may be working. Parallel to the results in vitro this photophosphorylation in vivo is not dependent on oxygen either. In air as well as in N_2 antimycin A inhibits the DCMU-resistant photophosphorylation.

Our results with antimycin A can be confirmed and extended with another inhibitor, HOQNO. HOQNO in the concentrations which we employed does not inhibit the incorporation of ^{32}P in the light, but in presence of DCMU the remaining DCMU-resistant photophosphorylation is also blocked (table1). The incorporation of ^{32}P in the dark is nearly completely inhibited by HOQNO at the same concentration.

Salicylaldoxime, a copper chelating agent, was employed by Trebst (1963 a) for inhibition of O_2-evolution and cyclic photosynthetic phosphorylation. In accordance to these results we also found in vivo at similar concentrations a complete inhibition of O_2-evolution and of incorporation of ^{32}P in the light in air as well as in N_2 and also in the dark. Lower concentrations ($10^{-3}M$) of salicylaldoxime still inhibited the ^{32}P incorporation in the dark to about 10% of the control, but

in the light we found only an inhibition of ^{32}P incorporation and O_2-evolution to about 50%. Trebst et al. (1963 b) demonstrated that salicylaldoxime in concentrations less than $10^{-3}M$ is not an inhibitor but a cofactor of photophosphorylation in chloroplasts by p-hydroxylation in air. But if salicylaldoxime at $10^{-3}M$ was added together with DCMU, we also observed a strong inhibition of the remaining DCMU-resistant phosphorylation in the light (table 1).

Summarizing, we conclude that in vivo a cyclic photosynthetic phosphorylation takes place besides a non-cyclic photophosphorylation. Perhaps the cyclic photosynthetic phosphorylation in vivo is identical with the endogenous ferredoxin-catalyzed phosphorylation of Tagawa et al. (1963), which is also inhibited by antimycin A. Moreover, our experiments with intact cells should be suitable to demonstrate how these processes may be regulated in the cell. We found a strong inhibition of ^{32}P incorporation in the dark by anaerobiosis (N_2-atmosphere), but no inhibition in the light. Under these conditions no O_2-evolution is measurable in the light, but it might be possible that inside the cell very small amounts of oxygen are still present. But even after addition of the inhibitors of respiration like antimycin A and HOQNO in concentrations, in which they strongly block the ^{32}P incorporation in the dark (table1), no decrease of ^{32}P incorporation in the light was observed. We conclude, therefore, in agreement with our earlier results (Simonis and Urbach 1963) that the oxidative phosphorylation in green cells is inhibited by light. In addition to this, our results suggest a regulation of the different systems of photophosphorylation during the photosynthesis. A more detailed account of this problem will be published separately.

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