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Inhibition of Photophosphorylation in Isolated Spinach Chloroplasts by Lower Aliphatic Straight-Chain Alcohols

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Photophosphorylation in isolated spinach chloroplasts is strongly inhibited by lower aliphatic, saturated, straight-chain alcohols. The effect of methanol, ethanol, propanol, butanol and pentanol was tested and found to be increasing with the chain-length of the alcohol. In spinach chloroplasts the photophosphorylation was inhibited by lower concentrations of alcohols than in "chromatophores" from the photosynthetic bacterium *Rhodospirillum rubrum*. The inhibition in chloroplasts was found to be reversible in the sense that removal or strong dilution of alcohol after the preincubation restored a high photophosphorylation activity.

The inhibitory effect of aliphatic, straight-chain alcohols on photophosphorylation in spinach chloroplasts has been investigated in a search for suitable methods to inactivate photophosphorylation in a controlled and reproducible manner, in connection with attempts to obtain insight into the ultrastructural requirements for this electron transport-linked process. Aliphatic alcohols were selected for trial as comparatively low concentrations of ethanol (in the range of 1 volume %), which has been used as a solvent for certain inhibitors, have been found to slightly decrease the rate of photophosphorylation in isolated chloroplasts[1]. All the alcohols, which were systematically tested, namely methanol, ethanol, propanol, butanol and pentanol, are completely soluble in water in the concentrations used.

EXPERIMENTAL

The alcohols were of analytical grade. Hexokinase (Type IV), ATP, flavin mononucleotide (FMN)** and phenazine methosulfate (PMS) were from Sigma Chemical Company, St. Louis, Missouri, USA.

Spinach was obtained as whole plants from local growers. Unless otherwise mentioned, the chloroplasts were isolated according to the method of Allen, Whatley and Arnon[2], with a change in the pH of the NaCl-Tris medium from 8.0 to 8.3. One washing was performed in the undiluted isolation medium, to secure a fraction containing so-called "whole" chloroplasts.

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** Abbreviations: P_i, orthophosphate; FMN, flavin mononucleotide; PMS, phenazine methosulfate; Tris, tris(hydroxymethyl)aminomethane.

The medium for the phosphorylation experiments contained in 1.0 ml of 0.8 M Tris of pH 8.3, 12 μmoles $K_2H^{32}PO_4$, 30 μmoles $MgCl_2$, 1 μmole ATP, 60 μmoles glucose and an excess of yeast hexokinase. Either FMN or PMS were added as electron carriers in the phosphorylation experiments, in the following amounts: 0.4 μmoles FMN or 0.6 μmoles PMS. The measurements of phosphorylation and chlorophyll content have been described and the same is true for the light source and its intensity[3]. Aerobic conditions were used throughout. The temperature was kept at 20°C. The reaction volume was 3.0 ml.

In the restoration experiments the concentration of alcohol was brought down by either dilution or centrifugation. In the dilution experiments, concentrated suspensions of chloroplasts were preincubated with alcohol and subsequently diluted 15-fold, according to the principle outlined in the following scheme, where + and − indicate whether or not the preincubation medium (preparation medium) and the dilution medium (phosphorylation medium) contain the alcohol in question and 1, 2 and 3 represent absence, presence and 15-fold dilution of alcohol, respectively, in the suspension which after dilution was tested for photophosphorylation activity:

	Preincubation medium	Dilution medium
1	—	—
2	+	+
3	+	—

In the centrifugation experiments, the preincubation mixtures were centrifuged after the incubation period for 7 min at 2 500 × g in a Refrigerated Serwall centrifuge and the chloroplast pellets were resuspended as in the scheme above, thus with centrifugation and resuspension instead of dilution as the sole difference.

The preincubation time was usually 30 min for convenience, and during this time light was prevented from entering the samples. In control experiments not to be described here it was found that a preincubation time of 5–10 min or less was sufficient to allow the alcohol to exert its inhibitory effect to completion. The temperature during preincubation was 0°C. As will be shown below, a very strong effect may indeed be obtained without any preincubation with alcohol.

The "chromatophores" from the photosynthetic bacterium *Rhodospirillum rubrum* for the comparative experiments were prepared as in Ref.[4], where also growth conditions, harvesting and pertinent details about the photophosphorylation experiments are reported. The "chromatophore" fraction was used and a 30 min preincubation with alcohol in the dark preceded the photophosphorylation experiments.

RESULTS

In Table 1 are given the approximate concentration ranges, where a 50 % inhibition of the photophosphorylation was obtained in isolated spinach chloroplasts under the given conditions*. An increase in the chain-length of the alcohol

Table 1. Approximate alcohol concentration ranges for 50 % inhibition of photophosphorylation.

Alcohol	Concentration in % (v/v)
Methanol	4 – 5
Ethanol	1.5 – 3
Propanol	0.5 – 1
Butanol	0.1 – 0.5
Pentanol	0.04 – 0.1

* In control experiments, performed with the aid of Dr. M. L. Ibanez, Department of Biophysics, University of California, Los Angeles, it was found that neither methanol nor ethanol was metabolized in any significant amount in the studied system.

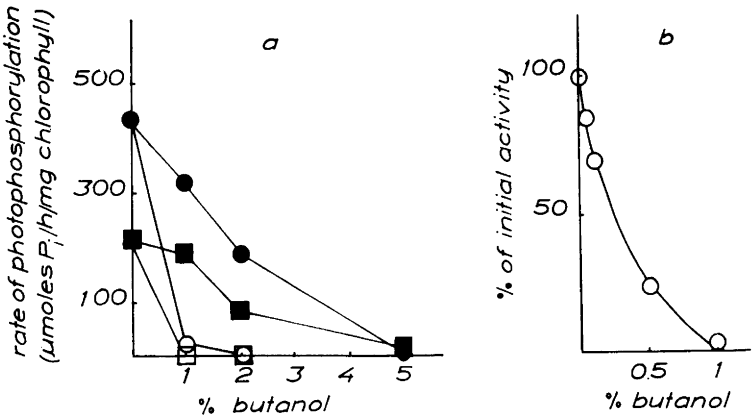

Fig. 1. a) The effect of butanol on photophosphorylation measured as disappearance of P_i. Preincubation with 1 % (v/v) butanol. □ : FMN, undiluted butanol; o : PMS, undiluted butanol; ■ : FMN, 15-fold diluted butanol; • : PMS, 15-fold diluted butanol. Chlorophyll concentration was 0.017 mg/3 ml. b) Inhibition curve for butanol without preincubation of the chloroplasts with the alcohol.

results in a decrease in the concentration which is necessary for inhibition. In similar experiments with isolated "chromatophores" from the photosynthetic bacterium *Rhodospirillum rubrum*, higher alcohol concentrations than with the spinach chloroplasts were found to be required for an inhibition of the photophosphorylation. For example, in this bacterial system a 50 % inhibition was obtained by 7 % ethanol and 0.7 % butanol.

Fig. 1a shows the result of comparisons between photophosphorylation in samples of spinach chloroplasts, where the alcohol concentration in the preincubation medium was retained during a 15-fold dilution of the chloroplasts for the phosphorylation experiment and samples, where a 15-fold dilution of both the chloroplasts and the alcohol was made. Dilution relieved the inhibition within a suitable concentration range of butanol. As was to be expected, this "restoration" of photophosphorylation activity was stronger at lower alcohol concentrations than at higher. A similar picture of reversibility within a certain concentration range was obtained with each of the five alcohols tested.

The curve in Fig. 1b was obtained without preincubation of the chloroplasts with alcohol, also in this case butanol. It is seen that the effect must be a rapid one. Thus no significant structural barriers within the chloroplast prevent the inhibitor from reaching its region or site of action.

Table 2 shows that one may restore photophosphorylation by removing a strongly inhibiting alcohol concentration by centrifugation of the chloroplast suspension and resuspension of the chloroplast pellet in an alcohol-free medium. 1.0 % (v/v) butanol was used in this experiment. An efficient removal of the alcohol and a possibility to virtually completely restore the photophosphorylation after alcohol-treatment of isolated spinach chloroplasts is demonstrated, as the

Table 2. Photophosphorylation in spinach chloroplasts. Its inhibition by addition and restoration by removal of 1 % (v/v) butanol. The final chlorophyll concentration in each tube was roughly 0.01 mg/3.0 ml. In this experiment the medium for chloroplast preparation and preincubation contained a 0.05 M Na_2HPO_4-KH_2PO_4 buffer of pH 7.0 instead of 0.02 M Tris buffer of pH 8.3. The phosphorylation medium was unchanged. Preincubation time: 15 min.

Butanol		Activity μmoles P_i esterified/h/mg chlorophyll	
In incubation medium	In phosphorylation medium	Added co-factor FMN	PMS
—	—	290	570
+	+	30	110
+	—	320	560

phosphorylation rates are, within the experimental error, the same in the samples, where butanol has been added and removed, as in the alcohol-free controls.

DISCUSSION

The fact that an increasing chain-length in the series from methanol to pentanol gives the alcohol a stronger inhibitory capacity against the structure-dependent photophosphorylation in isolated spinach chloroplasts may well be partially or completely dependent on an increasingly lipophilic character of the alcohols in question. As these compounds are asymmetrical due to their possession of a hydrocarbon-part and a polar alcohol-group, they may be expected to become oriented in a determined way in the complex lipid-protein double-membrane matrix of the photosynthetic apparatus, be it localized on both the grana and the stroma membranes within the spinach chloroplast or only on the former. The well-known possibility that the chlorophyll molecules are oriented with their porphyrin-discs at the boundaries between the lipid and protein layers of the grana discs and "anchored" in the lipid phases by means of the lipid-soluble phytyl-chain should be considered in connection with the inhibitory effect of the alcohols on photophosphorylation. If the system for the primary photoreactions and the subsequent dark electron transport and energy transfer reactions for the production of ATP is localized on or in immediate vicinity of lipid-protein interphases, the interference of added alcohols with the photophosphorylation may well be a reflection of surface-active properties of the alcohols at the interphase. Then the enrichment of added alcohol in the region where photophosphorylation occurs may cause inhibition by changing through mass action the three-dimensional geometrical structure of the photosynthetic apparatus.

A comparison of the amounts of alcohol necessary to inhibit photophosphorylation with the amounts of chlorophyll in the inhibited suspensions tends to support the concept that a mass action of alcohol on the gross membrane structure may

occur and cause the effect. From the data in Table 2 one can calculate that the molar ratio butanol/chlorophyll is about 30/1 and the weight ratio about 2.5/1 in this experiment.

What effects can one expect if one goes further in the alcohol series from pentanol to the higher alcohols, which are more lipophilic and more insoluble in water? Practically nothing appears to be known about how such agents may influence sensitive photosynthetic functions. Krogmann and Jagendorf[5] have reported, in connection with a study of fatty acid inhibition of the Hill reaction in spinach chloroplasts, that octadecanol was not inhibitory, but the tested concentration range was not given. On the other hand, we tested in a single experiment whether 0.1 and 3.0 % heptanol affected photophosphorylation in spinach chloroplasts and obtained after a preincubation which included shaking of the material a complete inhibition. It is planned to study the effect of higher alcohols further.

The finding that the inhibition of photophosphorylation may be even completely reversed by removal of the alcohol provides a system where various parameters of photosynthetic structure and function may be investigated in a well-controlled manner. It would appear to be worthwhile to test if the alcohols also influence in isolated chloroplasts more stable reactions than photophosphorylation, such as the Hill reaction and more primary reactions such as the photoreactions, and also if both an effect and its reversal can be demonstrated on the energy transfer-linked electron transport control mechanism and on the carbon dioxide fixation reactions. By such a continuation of this approach it may become possible to obtain further information about what the relationships are between various photosynthetic functions and about their dependency on ultrastructural integrity within the chloroplasts.

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REFERENCES

1. Baltscheffsky, H. *Unpublished observations.*
2. Allen, M. B., Whatley, F. R. and Arnon, D. I. *Biochim. Biophys. Acta* **27** (1958) 16.
3. Baltscheffsky, H. *Acta Chem. Scand.* **14** (1960) 264.
4. Baltscheffsky, H. *Biochim. Biophys. Acta* **40** (1960) 1.
5. Krogmann, D. W. and Jagendorf, A. T. *Arch. Biochem. Biophys.* **80** (1959) 421.

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