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RESTORATION BY SILICOTUNGSTIC ACID OF DCMU-INHIBITED PHOTOREACTIONS IN SPINACH CHLOROPLASTS

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SUMMARY

The restoration by silicotungstic acid of the reversible light-induced pH rise mediated by pyocyanine in EDTA-treated chloroplasts corresponds to an irreversible fixation of the acid. The proton uptake is linearly related to the amount of fixed acid (4 protons per molecule of acid) as long as the amount of silicotungstic acid does not exceed 200 nmoles/mg of chlorophyll.

In the same conditions silicotungstic acid partly restores ferricyanide reduction and O_2 evolution in chloroplasts suspensions supplemented with DCMU. These photoreactions are observed only with chloroplasts and these chloroplasts must have an unimpaired water-splitting mechanism.

Silicotungstic acid does not impair DCMU fixation on the specific sites. More likely in its presence the properties of the membrane change and ferricyanide can accept electrons from a part of the electron transport chain, between the Photosystem II reaction center and the block of the electron flow by DCMU.

Lien and Racker [1] have shown that silicotungstic acid, like EDTA is able to remove the coupling factor CF_1 from the chloroplasts. After coupling factor removal by EDTA, the chloroplasts suspensions do not display any reversible pH change during illumination. We previously found that silicotungstic acid restores that property and that the photoreactions in chloroplast suspensions respond to this acid addition in a different manner depending on the electron acceptor which is used [2]. In this study we intend to report the action of silicotungstic acid on ferricyanide-mediated photoreactions in chloroplast suspensions, especially with respect to their sensitivity to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This study will be done by using the same range of concentrations of silicotungstic acid as was used for restoring the transient pH change mediated by pyocyanine.

Broken and EDTA-treated chloroplasts were prepared as previously indicated [2]. Radioactive silicotungstic acid was synthetized from radioactive sodium[^{185}W] tungstate according to Riad Tourky et al. [3]; instead of being left to crystallize, the

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

acid was neutralized with an excess of pyridine. The pyridinium salt precipitate was removed by centrifugation and the free acid obtained by treatment with Dowex 50 (H^+ form). DCMU, obtained from K and K Laboratories, was purified by recrystallization from benzene. Chloroplasts suspensions were illuminated with white light provided by a 600-W projector (tungsten iodine lamp) and filtered through 15 cm of water and a Balzers filter (Calflex B1). The pH changes, ferricyanide reduction and O_2 evolution were measured as previously described [2]. The DCMU content of the chloroplasts was determined from the difference between the total added DCMU and the amount of DCMU remaining in the solution. Chloroplasts were removed by centrifugation, and the DCMU left in the solution was assayed by adding fresh chloroplasts. This method was called the "chloroplasts removal method" by Izawa and Good [4]. Radioactivities were determined with a low background Tracer-lab flow counter.

When EDTA-treated chloroplasts are mixed with increasing amounts of radioactive silicotungstic acid, two phases can be observed as a function of the amount of added acid. At the beginning, chloroplasts remove all of the acid molecules from the medium (up to 200 nmoles/mg of chlorophyll) but further added silicotungstic acid molecules are only partly removed from the solution. During the first phase, the restored light-induced proton uptake mediated by pyocyanine is quantitatively related to the amount of acid fixed (roughly with a ratio of 4 to 1). During the second phase proton uptake is progressively inhibited (Fig. 1).

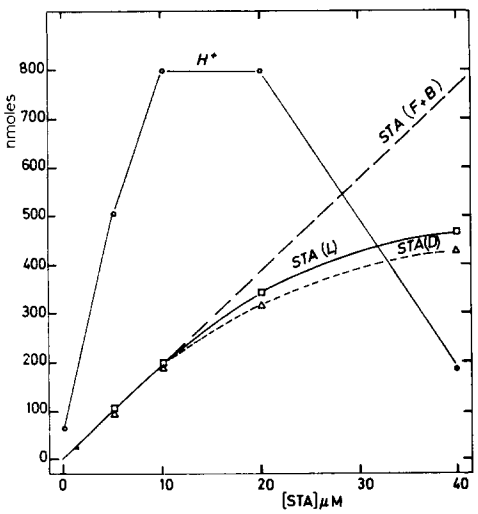

Fig. 1. Extent of the proton uptake (H^+) amounts of bound silicotungstic acid (STA) in light (L) and in darkness (D), and amounts of free and bound acid (F+B) as a function of the acid concentration. The quantities are given in nmoles of HCl or silicotungstic acid per mg of chlorophyll. EDTA-treated chloroplasts corresponding to 50 μg of chlorophyll per ml are in a medium consisting of 1.5 mM tricine–maleate, (pH 6), 10 mM $MgCl_2$, 50 μM pyocyanine. One part of the suspension is illuminated (white light 40 000 lux) at 5 °C, pH is recorded and proton uptake is calculated knowing the buffer capacity of the medium; suspension is then spun down and the radioactivity is determined in the pellet and the supernatant .The second part is allowed to stay the same time (10 min) but in darkness and then treated as the first one.

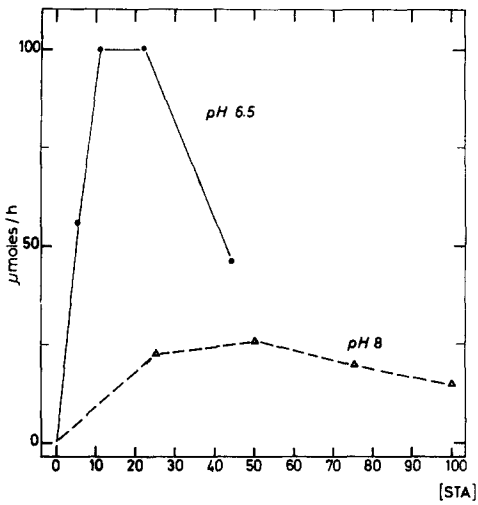

Fig. 2. Light-dependent ferricyanide reduction by chloroplasts in the presence of 5 μM DCMU, as a function of the concentration of silicotungstic acid (STA), in μmoles/h and per mg of chlorophyll. EDTA-treated chloroplasts (80 μg of chlorophyll per ml) in a medium consisting of 1 mM Tris (△---△, pH 8) or tricine–maleate (●–●, pH 6.5), 20 mM KCl, 10 mM $MgCl_2$, 0.5 mM ferricyanide, 5 μM DCMU, are illuminated at 20 °C with white light (40 000 lux). The rate of the control, without DCMU and silicotungstic acid, is 490 nmoles · h^{-1} per mg of chlorophyll at pH 6.5 and 380 nmoles · h^{-1} at pH 8.

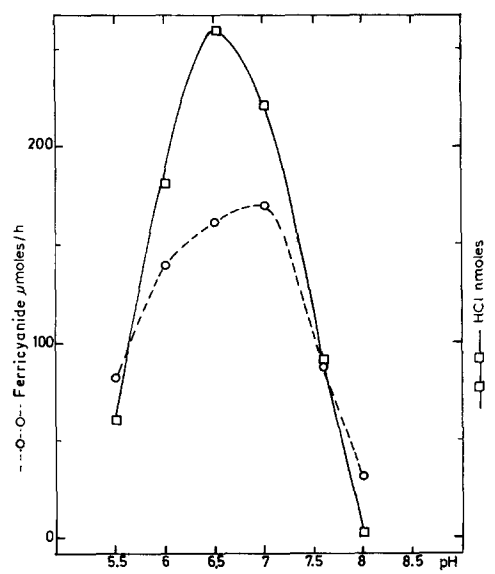

Fig. 3. Light-dependent ferricyanide reduction (μmoles/h per mg of chlorophyll) and pyocyanine-mediated proton uptake (HCl nmoles/mg of chlorophyll) by EDTA-treated chloroplasts in the presence of silicotungstic acid as a function of the pH. For ferricyanide reduction, the medium is 1 mM tricine–maleate, 20 mM KCl, 10 mM $MgCl_2$, 0.5 mM ferricyanide, 5 μM DCMU, 10 μM silicotungstic acid and EDTA-treated chloroplasts correspond to 20 μg of chlorophyll per ml. For proton uptake, the medium is 1.5 mM Tris–maleate, 10 mM $MgCl_2$, 50 μM pyocyanine, 10 μM silicotungstic acid and EDTA-treated chloroplasts correspond to 50 μg of chlorophyll per ml.

EDTA-treated chloroplast suspensions (20–50 μg of chlorophyll per ml) lose the ability to reduce ferricyanide with fairly low concentrations of DCMU, around 0.5 μM. Silicotungstic acid can partly restore that ability (Fig. 2). By comparing Figs. 1 and 2 it can be seen that the same range of acid concentration, at least at pH 6.5, is needed for the recovery of ferricyanide reduction in the presence of DCMU and of proton uptake mediated by pyocyanine in EDTA-treated chloroplasts. The pH requirements for these two effects are similar as shown in Fig. 3. The same effect can be obtained by pretreating chloroplasts with silicotungstic acid before adding them to the reacting medium. For that purpose, broken or EDTA-treated chloroplasts were suspended in 1 mM tricine–maleate at pH 6, with 200 nmoles of silicotungstic acid per mg of chlorophyll. After standing for 10 min at 5 °C, the suspension was spun down. The pellets which had exhausted all the acid from the medium (Fig. 1) were resuspended in 0.4 M sucrose and kept at 0 °C. These pretreated chloroplasts do not respond any more to DCMU addition and reduced ferricyanide at the same rate as the EDTA-treated chloroplasts in the presence of silicotungstic acid, at the optimum concentration, with or without DCMU. That is, the fixation of silicotungstic acid to the membrane induces the insensitivity of the ferricyanide photoreduction to DCMU.

The rate of the light-dependent ferricyanide reduction is optimum at a white light intensity of 20 000 lux in the absence of silicotungstic acid, but in its presence the light requirement is much higher (Fig. 4). This discrepancy may question the biological nature of the photoreaction in the presence of that acid. The light-dependent ferricyanide reduction in the presence of silicotungstic, with and without DCMU,

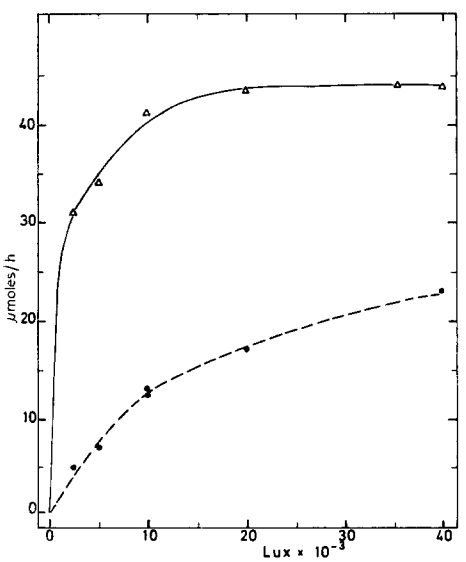

Fig. 4. Light-dependent ferricyanide reduction with broken chloroplasts suspension as a function of the white light intensity, in μmoles/h and per mg of chlorophyll. Broken chloroplasts (65 μg of chlorophyll per ml) are suspended in a medium consisting of 1 mM tricine–maleate (pH 6.5), 20 mM KCl, 10 mM $MgCl_2$, 0.5 mM ferricyanide (△–△). In the treated sample, 50 μM DCMU and 20 μM silicotungstic acid are both present (●- - -●). Temperature is 20 °C.

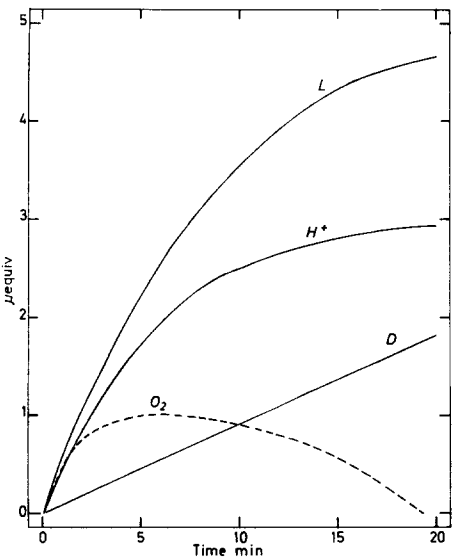

Fig. 5. Ferricyanide reduction in darkness (D) and in light (L) expressed as μmoles, acidification of the medium (H^+) expressed as μmoles of HCl and O_2 content changes (O_2) expressed as electron μequiv, in the presence of DCMU and silicotungstic acid. Amounts are given per mg of chlorophyll. Reacting medium is 2 mM tricine–maleate (pH 6.5), 20 mM KCl, 10 mM $MgCl_2$, 0.5 mM ferricyanide, 10 μM DCMU and 25 μM silicotungstic acid. Broken chloroplasts are illuminated in the medium (85 μg of chlorophyll per ml) at 20 °C. White light intensity is 40 000 lux.

presents the same kinetics as the medium acidification (related to the water-splitting reaction) and, at least during the first 2 min, as O_2 evolution. During that period there is a constant ratio of 4 between the number of HCl equivalents and of molecules of evolved oxygen. There is always a ratio of 1 between the ferricyanide photoreduction (light minus dark reduction) and medium acidification expressed in HCl equivalents (Fig. 5). These photoreactions are observed only if the chloroplasts are present and if their water-splitting mechanism is unimpaired : ferricyanide reduction and O_2 evolution are not restored by silicotungstic acid addition in heat- or Tris-treated chloroplasts which are not able to evolve oxygen [5, 6]. It is reasonable to assume that these photoreactions in the presence of silicotungstic acid are directly related to the photosynthetic electron transport.

The lack of inhibitory effect of DCMU on the photoreduction of ferricyanide by chloroplast suspensions in the presence of silicotungstic acid, is not due to the non fixation of DCMU on its specific sites. Chloroplasts pretreated with EDTA or silicotungstic acid exhibit the same affinity for DCMU (Table I) in the different conditions where the DCMU concentrations in the chloroplasts vary from $1.6 \cdot 10^{-5}$ to $6.4 \cdot 10^{-5}$ M and the inhibition of the Hill reaction from 0 to 80 %, as Izawa and Good [4] have shown. Therefore, we must conclude that in the presence of silicotungstic acid either the DCMU inhibition is not effective or the ferricyanide can accept electrons from a part of the electron chain between the Photosystem II reaction center and the site of DCMU action, which should be located as proposed by Duysens and Sweers [7]. In that last case ferricyanide could react in this unusual way because

TABLE I

DCMU FIXATION BY EDTA-TREATED CHLOROPLASTS BEFORE AND AFTER PRETREATMENT WITH SILICOTUNGSTIC ACID

EDTA-treated chloroplasts, pretreated (+ STA) or not (− STA) with silicotungstic acid (200 nmoles of acid per mg of chlorophyll), are suspended in 5 ml of 1 mM tricine–maleate (pH 6.5), 20 mM KCl and 10 mM $MgCl_2$. DCMU is added to the chloroplast suspension (4.5 mg of chlorophyll in 5 ml) at the indicated concentration. After centrifugation DCMU is determined in the supernatant. Pellets are suspended in 10 ml of the same buffer without DCMU and DCMU content of the washing medium is measured by testing its inhibitory properties on the photoreduction of ferricyanide by fresh chloroplasts. Assuming a chloroplasts volume of $6.2 \cdot 10^{-2}$ ml/mg of chlorophyll [4], 1 nmole of DCMU fixed per mg of chlorophyll corresponds to a DCMU concentration of $1.6 \cdot 10^{-5}$ M in the chloroplasts.

DCMU (μM)	Amounts of DCMU (nmoles/mg of chlorophyll)					
	Removed by the chloroplasts		Left in the medium		Removed by the first wash	
	− STA	+ STA	− STA	+ STA	− STA	+ STA
1	0.96	0.94	0.06	0.08	0.08	0.08
2	1.93	1.91	0.14	0.15	0.14	0.14
4	3	3	0.78	0.78	0.67	0.67

of the change of the membrane properties. That change could also provide a good explanation for the restoration of the light-driven proton uptake in EDTA-treated chloroplasts by silicotungstic acid. The fact that dichlorophenol–indophenol is not reduced in the presence of DCMU and silicotungstic acid, favors that last interpretation and seems to rule out the release of the block by DCMU of the electron chain.

NOTE ADDED IN PROOF (Received January 10th, 1974)

Methylviologen has a very low redox potential and mediates a light-dependent oxygen uptake which requires the operation of the two photosystems. This photoreaction is insensitive to the addition of silicotungstic acid and is inhibited by DCMU, even in the presence of silicotungstic acid. So, in the presence of silicotungstic acid, DCMU really inhibits the electron flow between the two photosystems.

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