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# QUERCETIN INTERACTION WITH THE CHLOROPLAST ATPase COMPLEX

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## Summary

1. Quercetin, a flavonoid which acts as an energy transfer inhibitor in photophosphorylation is shown to inhibit the P-ATP exchange activity of membrane-bound $CF_1$ and the ATPase activity of isolated $CF_1$. Quercetin, affects also the proton uptake in chloroplasts in a manner similar to that of dicyclohexylcarbodiimide.

2. The light-dependent proton uptake in EDTA-treated chloroplasts is stimulated by quercetin. In untreated chloroplasts quercetin has a dual effect: it enhances at pH above 7.5 while at lower pH values it decreases the extent of $H^+$ uptake. Similar effects were obtained with dicyclohexylcarbodiimide.

3. Like quercetin, dicyclohexylcarbodiimide was also found to inhibit the ATPase activity of isolated $CF_1$.

4. Quercetin inhibits uncoupled electron transport induced by either EDTA-treatment of chloroplasts or by addition of uncouplers. Quercetin restores $H^+$ uptake in both types of uncoupled chloroplasts.

5. The mode of action of quercetin and dicyclohexylcarbodiimide in photophosphorylation is discussed, and interaction with both $CF_1$ and $F_0$ is suggested.

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Abbreviations: $CF_1$, chloroplasts coupling factor one; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; EDTA, ethylene diaminetetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; DCIP, dichlorophenol indophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

## Introduction

The flavonoids are a group of metabolites which are widely distributed among plants but their metabolic role is not yet understood. Quercetin and other bioflavonoids have been reported to have toxic effects on enzymes involved in energy conservation [1—3]. In mitochondria quercetin was reported to inhibit the ATPase activity of the coupling factor ($F_1$) without interfering with oxidative phosphorylation [4]. Inhibition of chloroplast coupling factor one ($CF_1$) ATPase activity by quercetin was also reported [5] and the inhibitor binding sites were suggested to be located on the $\alpha$ or $\beta$ subunits of $CF_1$ [6]. Recently it was shown that quercetin acts as an energy transfer inhibitor of photophosphorylation in chloroplasts and that its effect is competitive with the substrate nucleotide, suggesting binding to the active site [7].

The energy-transducting ATPase complex is composed of an hydrophilic component, $CF_1$, containing the active site for ATP hydrolysis and synthesis and a number of more hydrophobic components ($F_0$) involved in the binding of $CF_1$ to the membrane and in the formation of a transmembrane proton gradient [8—10].

Energy transfer inhibitors can be classified on the basis of their inhibitory action. One type interacts directly with $CF_1$ and inhibits the ATPase activity of the membrane-bound as well as of the isolated enzyme, e.g., Dio-9 and phlorizin. The other type interacts apparently with components of $F_0$ blocking proton translocation e.g. dicyclohexylcarbodiimide (DCCD) and triphenylstannic chloride [11—13].

In this communication we show that quercetin interacts with $CF_1$ and with components of $F_0$. It is suggested that both $F_0$ and $CF_1$ are involved in the proton translocation through the thylakoid membranes.

## Materials and Methods

*Materials.* Quercetin was purchased from Sigma Chemical Co and dissolved in dimethyl sulfoxide ($Me_2SO$). DCCD was obtained from Aldrich and dissolved in absolute ethanol. The final concentrations of the solvents in the reaction mixtures were between 0.2 and 1%, and at these concentrations no significant effect on the chloroplast activities was observed; [2,8-$^3$H]ADP was purchased from New England Nuclear. Dithiothreitol, ATP, ADP, CCCP, Tricine, methyl viologen and dichlorophenol indophenol (DCIP) were obtained from Sigma Chemical Co.

*Chloroplast membranes* were prepared from fresh market lettuce or from spinach leaves as previously described [14]. The effects of quercetin on proton uptake and electron transport were tested both with spinach and lettuce chloroplasts and similar results were obtained. Resolved particles were obtained by suspending the chloroplast pellets in a solution of 1 mM EDTA and 1 mM Tricine, pH 8.0, at a concentration of 0.2—0.5 mg chlorophyll/ml [15]. The pellets after centrifugation were resuspended in a solution containing: sucrose, 0.4 M; NaCl, 10 mM and Tris, 50 mM (pH 8.0), to a final chlorophyll concentration of 1—2 mg/ml.

$CF_1$ was isolated by the EDTA or chloroform treatment and purified as

described [16]. $Ca^{2+}$-ATPase activity of the isolated proteins was determined as described [17].

Light-induced incorporation of adenine nucleotide into membrane-bound $CF_1$ [18], P-ATP exchange activity [19], ATP formation [14] and $H^+$ uptake [20] were measured as described.

*Analytical methods.* The content of [$^{32}$P]ATP was determined by the isobutanol-benzene extraction method [14]. Inorganic phosphate was determined as described [21]. Protein concentration was determined according to the method of Lowry et al. [22], and chlorophyll according to the procedure of Arnon [23].

## Results

### *Energy transfer inhibition by quercetin*

The results described in Table I confirm that quercetin acts as a typical energy transfer inhibitor of photophosphorylation [7], and further demonstrate that the inhibition does not depend upon the part of the electron transport chain that supports phosphorylation. The formation of ATP coupled to electron transport through both Photosystem II and I (from water to methyl viologen) or through Photosystem I (from reduced dichlorophenol indophenol to methyl viologen or phenazine methosulfate mediated cyclic electron transport), were inhibited by quercetin. The inhibition of cyclic phosphorylation by quercetin was the same when the concentration of phenazine methosulfate was varied between 10—200 μM (not shown). The effect of quercetin on phosphorylation with the electron acceptors, ferricyanide and oxidized dichlorophenol indophenol, was not measured since these compounds react directly with quercetin. No light or dark preincubation of chloroplasts with the inhibitor was necessary for the inhibition to take place.

In chloroplasts several compounds are known to inhibit both ATP formation and hydrolysis [11,13] while others affect only one of the two reactions [2,24,25]. Quercetin however was found to inhibit ATP formation (Table I),

TABLE I

EFFECT OF QUERCETIN ON PHOTOPHOSPHORYLATION

Reaction mixtures for phosphorylation assays contained the following (in μmol) in a final volume of 3 ml; Tricine, pH 8.0, 30; NaCl, 60; $MgCl_2$, 10, $P_i$ (containing $^{32}P$, $2 \cdot 10^6$ cpm) 10, and ADP, 2. Where indicated the following components were added: 0.2 μmol methyl viologen (MV), 0.5 μmol $NaN_3$, 10 μmol ascorbate, pH 8.0, 0.2 μmol 2,6-dichlorophenol indophenol (DCIP), 4 μM DCMU or 0.1 μmol phenazine methosulfate (PMS), and chloroplasts equivalent to 47 μg chlorophyll. Chloroplasts were illuminated for 1 min at 20°C with white light (160 000 lux). Phosphorylation rates are given as μmol ATP formed per mg chlorophyll per h.

| Additions | Phosphorylation | | |
|---|---|---|---|
| | $H_2O \rightarrow MV$ | Ascorbate + DCIP $\xrightarrow{DCMU}$ MV | Cyclic (PMS) |
| None | 289 | 115 | 830 |
| Quercetin, 33 μM | 83 | 58 | 206 |
| Quercetin, 66 μM | 55 | 37 | 19 |

and P-ATP exchange in chloroplasts (Fig. 1). The light-triggered P-ATP exchange reaction was inhibited to the same extent when quercetin was added either before or after the light activation stage, suggesting that it is the exchange activity rather than the light activation step which is sensitive to quercetin. Under the conditions described in Fig. 1, 50% inhibition was obtained by 12 $\mu$M quercetin. The light-triggered $Mg^{2+}$-ATPase activity was equally sensitive to quercetin (not shown). Quercetin was reported to inhibit the ATPase activity of the EDTA-released $CF_1$ that is activated by heat or trypsin treatment [5]. We have checked the effect of quercetin on the ATPase activity of the 4 subunit $CF_1$, lacking the $\delta$ subunit [16,26]. In Fig. 2 the effect of quercetin on the heat activated $Ca^{2+}$-ATPase activity of the 5 and 4 subunit $CF_1$ is given. With both enzymes a similar extent of inhibition was observed suggesting that the $\delta$-subunit is probably not involved in the interaction of quercetin with $CF_1$, in agreement with the results of Deters et al. [5] who showed that quercetin inhibits the ATPase activity of a 2 subunit $CF_1$.

*Restoration of proton uptake in EDTA-treated chloroplasts*

The results presented so far on the interaction of quercetin with $CF_1$ are in agreement with other reports [5—7,27]. However, when the effect of quercetin on the light-induced proton uptake was studied, the following observations were made. It is known that treatment of chloroplasts with EDTA inhibits the

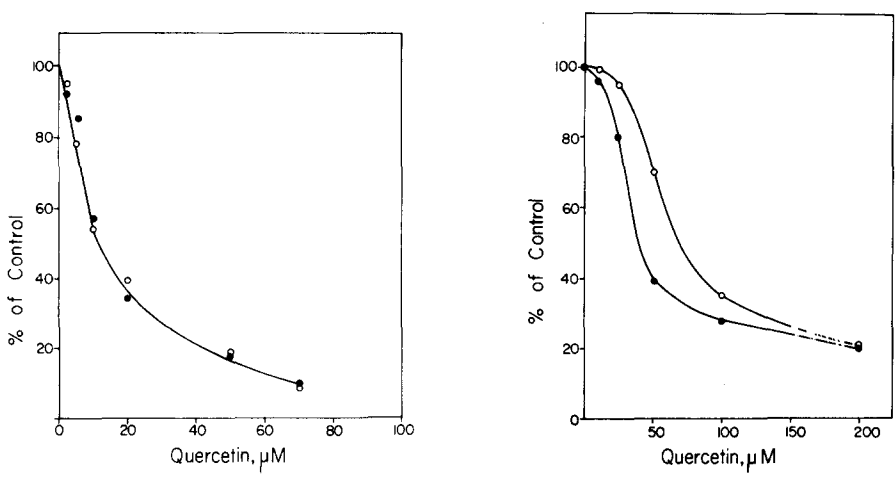


Fig. 1. Inhibition of P-ATP exchange activity by quercetin. The reaction mixtures contained the following (in $\mu$mol) in a final volume of 1 ml at pH 8.0; Tricine, 20; NaCl, 20; dithiothreitol (DTT), 10; $P_i$, 5 (containing [$^{32}$P] $2 \cdot 10^6$ cpm); phenazine methosulfate (PMS) 0.05, and chloroplasts equivalent to 47 $\mu$g chlorophyll, The chloroplasts were illuminated for 2 min in the presence of quercetin (○) or without quercetin which was added after illumination together with 3 $\mu$mol ATP (●). Control activity was: 29.7 $\mu$mol/mg chlorophyll per h.

Fig. 2. Effect of quercetin on the $Ca^{2+}$-ATPase activity of the soluble 5 and 4 subunit $CF_1$. 5 and 4 subunit $CF_1$ preparations were obtained by EDTA or $CHCl_3$ treatment as described [16]. $Ca^{2+}$-ATPase activity was determined as described under Materials and Methods. Heat-activated $CF_1$ (25 $\mu$g protein) was incubated for 8 min at 37°C in 1 ml of a solution containing 40 mM Tricine, pH 8.0, $CaCl_2$ and ATP, 5 mM each and the indicated concentration of quercetin. Control activities for the 5 and 4 subunit $CF_1$ were; 20.3 and 27.3 $\mu$mol $P_i$ released/mg protein per min (●), (○), respectively.

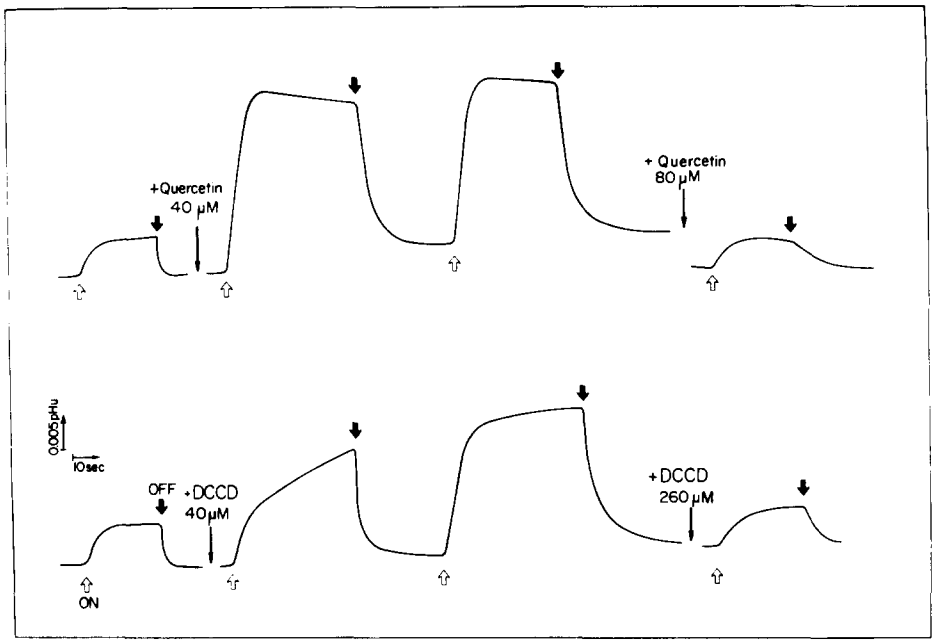


Fig. 3. Restoration of $H^+$ uptake in EDTA-treated chloroplasts by DCCD and quercetin. EDTA-treated chloroplasts were prepared by suspending the isolated chloroplast membranes in 1 mM EDTA, pH 8.0 at a concentration of 230 μg chlorophyll/ml as described in Materials and Methods. Reaction mixtures for the light-induced pH change contained in a final volume of 2 ml; 100 mM NaCl, 20 μM phenazine methosulfate (PMS) and resolved particles, 46 μg chlorophyll, Red light was provided by illumination with a 150-W quartz iodine lamp, through a heat filter and a Corning filter 2304.

net light-dependent proton uptake due to partial removal of $CF_1$ from the thylakoid membranes and the addition of DCCD, which is assumed to interact with the $F_0$ component of the ATPase complex, restores the net proton uptake [8—13]. Fig. 3 shows that like DCCD, quercetin restored proton uptake of EDTA-treated thylakoids. Moreover, the restoration of $H^+$ uptake by quercetin was obtained immediately after its addition while the effect of DCCD was much slower (see also Ref. 12). The effect of DCCD seems also to be light-dependent. Preincubation of the EDTA-treated thylakoids with DCCD for 5 min in the dark did not change the kinetics of the proton uptake as observed during the first illumination period (not shown) while a subsequent illumination period showed an enhanced rate of $H^+$ uptake. The inhibitory effect of high concentrations of both quercetin and DCCD will be discussed later.

The restoration of $H^+$ uptake depended both upon the type of EDTA-treated thylakoids [15] and upon the quercetin concentration during the assay (Fig. 4). When the chlorophyl concentration was about 0.5 mg chlorophyll/ml, during the EDTA-treatment, the thylakoids retained more of their $H^+$ uptake capability (about 50% in Fig. 4) and quercetin fully restored their activity. On the other hand, in chloroplasts which had lost about 85% or more of their $H^+$ uptake (0.1 mg chlorophyl/ml during EDTA treatment) the restoration by quercetin was less effective. Similar results were obtained with DCCD (Fig. 5).

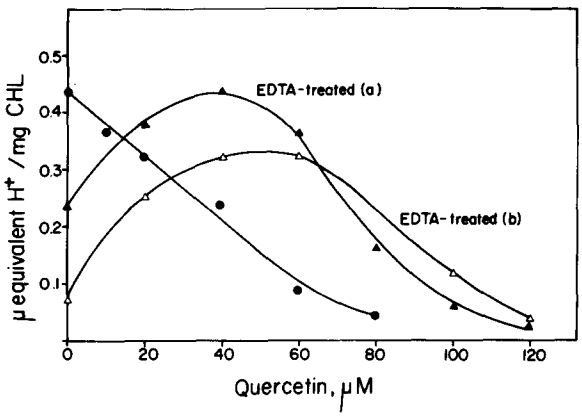


Fig. 4. Concentration dependence of quercetin effect on control and EDTA-treated chloroplasts. Conditions and assays were as described in Fig. 3 except that the resolved particles were obtained by EDTA treatment at chlorophyll concentrations of 500 or 100 μg/ml (a) refers to high and (b) to low chlorophyll concentration, respectively. (•) Untreated chloroplasts.

*Inhibition of $H^+$ uptake by quercetin and DCCD*

Besides the restorative effect of both quercetin and DCCD on proton uptake in EDTA-treated thylakoids, these compounds also inhibited $H^+$ uptake when added at higher concentrations (Figs. 3—5). The inhibition of $H^+$ uptake by quercetin and DCCD was also observed with coupled control chloroplasts, even at low concentrations of the inhibitor. The effectiveness of inhibition seems to correlate with the remaining amount of membrane-bound $CF_1$. When less $CF_1$ was left on the membrane (see (a) and (b) in Fig. 4) higher quercetin concentrations were required. Fifty percent inhibition was obtained by 40, 75 and 90 μM quercetin for control, and EDTA-treated thylakoids (a and b) respectively. It is unlikely that the inhibition of proton uptake in control chloroplasts is due to uncoupling since both DCCD and quercetin do not stimulate but rather slow down the efflux of protons after illumination.

When the inhibition of $H^+$ uptake by quercetin was checked as a function of the pH of the medium (Fig. 6), the strong inhibition observed at pH 6.5 decreased with the increase of the external pH. At pH 7.8—8.0 the extent of $H^+$-uptake in the presence of quercetin was even stimulated by about 30%. Addition of quercetin had no effect on the buffer capacity of the reaction mixture at pH 6.5, but as the pH was increased above 7.0 its buffering capacity increased and was taken into account.

The results shown in Fig. 6 suggest that the inhibitory effect of high concentration of quercetin is restricted to pH values lower than 7.5. We thus repeated the restoration of $H^+$-uptake with EDTA-treated thylakoids at pH 7.7. Fig. 7 clearly demonstrates that at this pH, quercetin does not inhibit the proton uptake. 40 μM quercetin fully restored the proton uptake activity of uncoupled, $CF_1$-deficient thylakoids. As the concentration of quercetin was increased, further stimulation of the extent of $H^+$-uptake in both EDTA-treated and control chloroplasts was obtained. The stimulations of proton uptake by

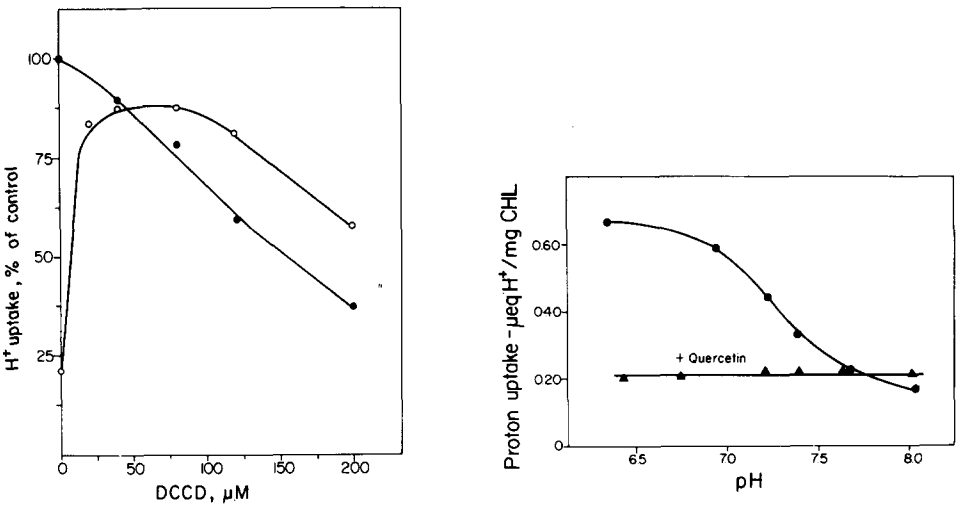


Fig. 5. Concentration dependence of DCCD effect on control and EDTA-treated chloroplasts. Conditions were as in Fig. 4 except that the values for $H^+$ uptake were calculated for the second illumination, where the effect of DCCD was maximal. The % of control activity was related to the activity of untreated chloroplasts in the presence of equal volumes of ethanol as in samples with DCCD. (●), (○) are for untreated and EDTA-treated chloroplasts, respectively.

Fig. 6. Dependence of the inhibition of $H^+$ uptake by quercetin on pH. $H^+$ uptake was measured as in Fig. 3, except that the chloroplasts (containing 30 μg chlorophyll) were illuminated with white light from a heat filtered 300-W tungsten lamp. Other conditions were as described [20].

quercetin at high pH and the inhibition at low pH were also observed with methyl viologen, as the electron carrier, only in this case the extent of $H^+$ uptake was smaller than that observed with phenazine methosulfate.

*The effect of quercetin on electron transport*

In view of the results presented above the effect of quercetin on electron transport was reinvestigated under conditions where it affected proton uptake,

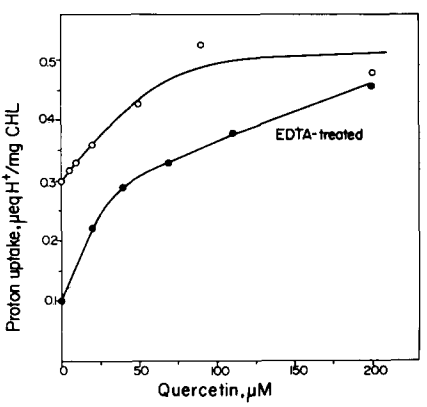


Fig. 7. Quercetin stimulation of $H^+$ uptake by control and EDTA-treated chloroplasts at pH 7.7. Conditions were as described in Fig. 6. EDTA-treated chloroplasts were obtained as described in Materials and Methods and the chlorophyll concentration during the EDTA treatment was 500 μg/ml. (○), (●): are for untreated and EDTA-treated chloroplasts, respectively.

namely, at two different external pH values with coupled and uncoupled chloroplasts. Fig. 8A shows the effect of quercetin on basal electron flow in control and EDTA-treated chloroplasts at pH 6.9 and 7.9. While the control rates were hardly affected (besides some stimulation at pH 7.9 by concentrations lower than 40 μM), the uncoupled rates were inhibited by quercetin.

It is possible that the apparent recoupling of electron transport in EDTA-treated chloroplasts is due to the interaction of quercetin with components of the ATPase complex, otherwise unaccessible to quercetin. However, a similar inhibition of electron transport by quercetin was observed if uncoupling occurred by the addition of an uncoupler. The rate of $NH_4Cl$-uncoupled electron transport was even slightly stimulated by quercetin at concentrations lower than 40 μM and markedly inhibited by higher concentrations (Fig. 8B).

*Effect of quercetin on $H^+$ uptake in the presence of uncouplers*

The similar sensitivity of electron transport to quercetin in different types of uncoupled chloroplasts might be correlated with the effect of quercetin on proton permeability.

The effect of quercetin on the extent of proton uptake was measured in the presence of several uncouplers and the results are summarized in Table II. At pH 6.5 both quercetin and the uncoupler CCCP, alone or together, inhibited net $H^+$ uptake (Table II, Expt. 1). At pH 7.8 quercetin alone had no inhibitory

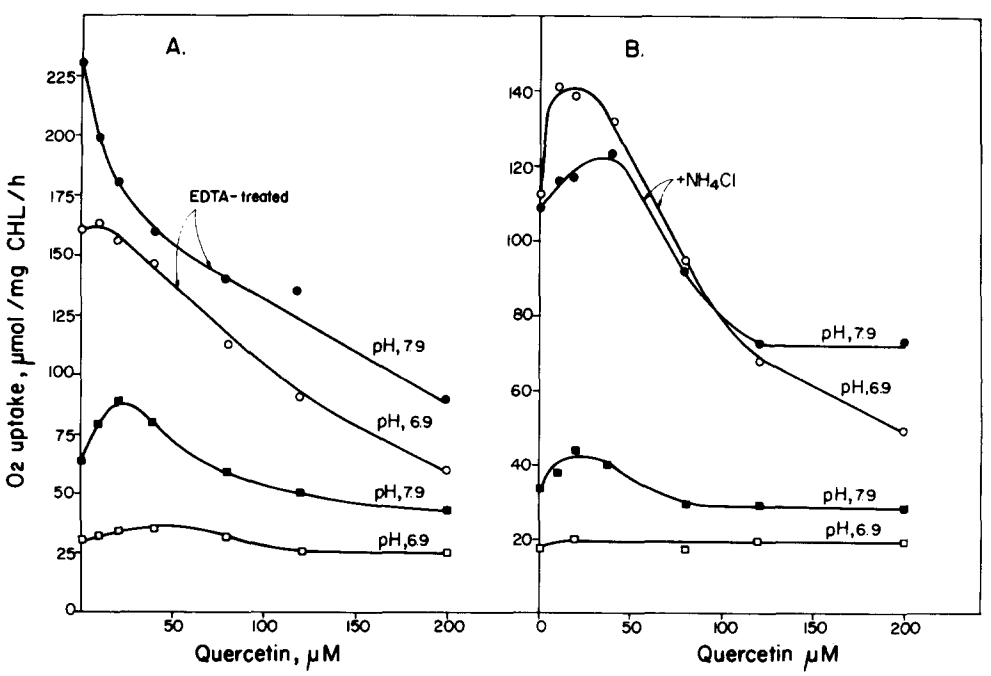


**Fig. 8. Effect of quercetin on uncoupled electron transport. EDTA-treated chloroplasts were obtained as in Fig. 3 except that the chlorophyll concentration during EDTA treatment was 266 μg/ml. Reaction conditions were as desribed in Table II except for the substitution of Tricine by Mes when the reaction was measured at pH 6.9. $NH_4Cl$ concentration was 3.3 mM.**

effect (see also Fig. 7). While in the presence of CCCP quercetin stimulated the extent of $H^+$ uptake markedly. The order of addition of the two compounds or the prior incubation of the chloroplasts with quercetin had no effect on the final result. Quercetin also stimulated the $NH_4Cl$ or gramicidin-inhibited proton uptake (Table II). The restoration of the proton impermeability by quercetin in uncoupled thylakoids was dependent on the uncoupler concentration. At higher uncoupler concentration the leak in proton permeability was not repaired by quercetin addition.

*Effect of quercetin and DCCD on nucleotide exchange*

Illumination of chloroplasts is known to induce the exchange of bound and free nucleotides. This light-dependent exchange was reported to be sensitive to DCCD and triphenyltin chloride but not to Dio-9 or phlorizin [28]. Table III shows that quercetin inhibited the nucleotide exchange as well. Furthermore, while the effect of DCCD required a rather long preincubation, maximal inhibition by quercetin was obtained a few seconds after its addition.

*Interaction of quercetin and DCCD with soluble $CF_1$*

In view of the similar effects of DCCD and quercetin in thylakoid membranes, we decided to reexamine the DCCD effect on the soluble enzyme since quercetin inhibited the $Ca^{2+}$-dependent ATPase. Indeed, under certain conditions the inhibition of the ATPase activity by DCCD could be demonstrated (Table IV; Shoshan, V. and Selman, B.R., unpublished results). These conditions included prior incubation of the activated $CF_1$ or DCCD at 37°C for 30 min at pH 7 or lower. Table IV shows that when the activated $CF_1$ was not incubated with the inhibitor before the ATPase assay, quercetin inhibited while DCCD hardly affected the reaction (column a). When the activated enzyme was

TABLE II

EFFECT OF QUERCETIN ON UNCOUPLED $H^+$ UPTAKE

Reaction mixtures contained in a volume of 2 ml: 100 mM NaCl, 20 μM PMS and chloroplasts containing 36 μg chlorophyll. Each sample contained also 0.15% $Me_2SO$. Concentrations of quercetin, CCCP, $NH_4Cl$ and gramicidin were 0.13 mM, 5 μM, 0.67 mM and 0.5 μM, respectively.

| Additions | pH 6.5 | | pH 7.8 | |
|---|---|---|---|---|
| | μequiv. $H^+$/mg Chl | % | μequiv. $H^+$/mg Chl | % |
| Exp. I | | | | |
| None | 0.462 | 100 | 0.178 | 100 |
| Quercetin | 0.193 | 42 | 0.196 | 110 |
| CCCP | 0.113 | 24 | 0.031 | 17 |
| Quercetin + CCCP | 0.063 | 14 | 0.182 | 102 |
| $NH_4Cl$ | — | — | 0.037 | 21 |
| Quercetin + $NH_4Cl$ | — | — | 0.355 | 199 |
| Exp. II | | | | |
| None | — | — | 0.119 | 100 |
| Quercetin | — | — | 0.190 | 160 |
| Gramicidin | — | — | 0.040 | 34 |
| Quercetin + gramicidin | — | — | 0.131 | 110 |

TABLE III

INFLUENCE OF QUERCETIN AND DCCD ON ENERGY-DEPENDENT ADENINE NUCLEOTIDE EXCHANGE

Nucleotide binding to thylakoid membranes was assayed at 22°C as described [18]. Chloroplasts were washed three times in a medium which contained: 50 mM NaCl and 2 mM Tricine-NaOH (pH 8.0) and then resuspended in a solution of 50 mM NaCl; 20 mM Tricine (pH 8) and 1 mM $MgCl_2$. Reaction mixtures contained in a total volume of 0.5 ml; 25 mM, Tricine (pH 8.0); 50 mM, NaCl; 5 mM, $MgCl_2$; 20 μM phenazine methosulfate; 10 μM, [$^3$H]ADP (containing $3 \cdot 10^5$ cpm/nmol) and chloroplast equivalent to 0.2 mg of chlorophyll/ml. Reaction mixtures were stirred for 10 s then illuminated with white light $3 \cdot 10^5$ erg $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$ for 30 s. The light-induced nucleotide exchange was quenched by the addition of 0.2 ml of a solution containing 50 μM CCCP and 20 mM ADP at pH 8.0. The [$^3$H]ADP content of the chloroplast membranes was determined as described [29]. (a) and (b) are for chloroplasts preincubated with the inhibitors at room temperature for 10 s and 20 min, respectively. Dark control (without illumination) was 0.056 nmol/mg chlorophyll.

| Inhibitor | μmol [$^3$H]ADP bound/mg Chl | |
|---|---|---|
| | a | b |
| None | 0.381 | 0.201 |
| DCCD, 2 mM | 0.330 | 0.115 |
| Quercetin, 1 mM | 0.085 | 0.093 |

incubated with DCCD, the ATPase activity was inhibited even when DCCD was omitted from the final assay medium (column b). In contrast, when quercetin was omitted from the assay medium, no inhibition was obtained. These results suggest that DCCD binds covalently while quercetin does not. Also, the addition of DCCD did not affect quercetin inhibition and the addition of quercetin did not affect DCCD inhibition, which suggests that DCCD and quercetin may not interact with the same sites.

TABLE IV

INACTIVATION OF $CF_1$ ATPase BY QUERCETIN AND DCCD

Chloroform released $CF_1$ was heat activated as indicated (Fig. 2). Then the pH was adjusted to 7.0 by 100 mM Mops, and DCCD and/or quercetin were added to give the concentrations indicated in parentheses. Protein concentration at this stage was 1.3 mg/ml. The mixtures were then incubated for 30 min at 37°C. Aliquots of 5 μl were assayed for ATPase activity either before (a) or after (b) the incubation. In (a) but not in (b) the inhibitors were also added to the assay medium.

| Inhibitor (μM) | ATPase activity (μmol $P_i$ released/mg protein/min) | |
|---|---|---|
| | a | b |
| None | 15.3 | 14.5 |
| DCCD (40) | 14.7 | 5.8 |
| DCCD (160) | 13.7 | 2.0 |
| Quercetin (50) | 10.3 | 14.3 |
| Quercetin (200) | 1.1 | 13.9 |
| DCCD (160) + Quercetin (50) | 10.1 | 1.7 |
| DCCD (160) + Quercetin (200) | 1.3 | 1.3 |

## Discussion

*The interaction of quercetin with the ATPase complex*

The experiments reported above illustrate some new aspects of the interaction of quercetin with the energy conservation system in chloroplasts. As reported by others [7] we find that quercetin is an effective inhibitor of photophosphorylation (Table I), as well as P-ATP exchange activities in chloroplasts (Fig. 1). Quercetin inhibits also the ATPase activity of soluble $CF_1$ (Fig. 2). These results are compatible with the suggestion that quercetin is an energy transfer inhibitor which, like Dio-9 or phlorizin, exerts its effect through interaction with $CF_1$ [9,10,11]. However, further investigations lead to the conclusion that the interaction of quercetin with the chloroplast membranes is not limited to $CF_1$ only. Like DCCD and tributylstannic chloride, quercetin restored $H^+$-uptake activity in EDTA-treated chloroplasts (Figs. 4 and 5). Moreover, the interaction of quercetin with these membranes was much faster than that of DCCD and did not require preillumination (Fig. 3). Treatment of chloroplasts with EDTA is known to release $CF_1$ from the membranes with the possible exposure of components of $F_0$, resulting in an increased leak of protons out of the thylakoids. The addition of compounds like DCCD and triphenylstannic chloride is assumed to block the flow of protons outwards restoring the proton uptake. As shown here, quercetin acts similarly and might therefore interact with a component of $F_0$. Another reaction reported to be sensitive to inhibitors like DCCD but not to Dio-9, is the light-dependent nucleotide exchange, also sensitive to quercetin (Table III). Since both quercetin and DCCD also inhibited the $CF_1$-ATPase activity (Table IV), we suggests that these inhibitors interact both with $CF_1$ and $F_0$. The inhibition of mitochondrial $F_1$-ATPase by DCCD was recently reported [29]. DCCD and quercetin differ in their mode of interaction with the membrane-bound and the isolated $CF_1$ protein. While the effect of quercetin is immediate upon its addition, maximal expression of the effect of DCCD required preillumination or rather long preincubation. DCCD has been suggested to bind covalently to carboxyl groups of $F_0$ and $F_1$ [29], while quercetin has been assumed to interact with sulfhydryl and amino groups of the protein [1]. However, quercetin was found to bind rapidly to isolated $CF_1$ [6] and to the membrane-bound enzyme (Fig. 1), even in the presence of dithiothreitol, which suggests that it might not interact with sulfhydryl groups. Moreover, since the inhibition can be reversed by washing the membranes, it appears not to bind covalently to functional groups on the protein. Interaction of inhibitors, substrates and chemical modifiers with $CF_1$ or its subunits was shown to affect the proton permeability of the membrane. Known energy transfer inhibitors such as Dio-9 and phlorizin interact with $CF_1$ and stimulate the $H^+$-uptake [30]. Chemical modification of the $\gamma$-subunits of $CF_1$ by bifunctional maleimides results in an increased proton permeability and uncoupling [31]. The three smaller subunits of $TF_1$, $\gamma$, $\delta$ and $\epsilon$, from a thermophilic bacterium, were required to restore the proton impermeability of $F_0$ containing liposomes [32]. Interaction of $CF_1$ with nucleotides affected also the proton permeability of thylakoid membranes [30]. Our results show that quercetin interacts with both $CF_1$ and $F_0$ and thereby affects the $H^+$ permeability of the membranes.

*pH dependence of quercetin interaction with the ATPase complex*

The effect of quercetin on proton uptake was found to be both concentration and pH dependent. In untreated chloroplasts quercetin inhibited at pH values lower than 7.5 while it stimulated at higher pH (Figs. 6 and 7). In uncoupled chloroplasts quercetin restored the proton uptake at low concentration while higher concentrations were inhibitory (Fig. 4). However, at pH 7.5 and above, no inhibition was observed and all concentrations of quercetin tested stimulated the net proton uptake capacity (Fig. 7). The inhibition of $H^+$-uptake by DCCD at low pH and the stimulation at high pH was reported [12]. The mode of inhibition at low pH is not clear but it does not seem to be caused by an increased proton permeability of the membrane since addition of quercetin slowed down the dark efflux of protons and did not stimulate the rate of electron transport at these low pH values (Fig. 8). The enhancement of proton uptake by quercetin at the higher pH range could be due to the blocking of a specific leak (i.e. through the ATPase complex) that occurs in isolated chloroplasts.

*Effect of quercetin in the presence of uncouplers*

Most uncouplers are thought to act through ion diffusion, increasing the membrane permeability to protons. It is also assumed that most uncouplers do not interact directly with membrane proteins involved in the transport of protons. However, quercetin was found to inhibit equally well the electron transport in chloroplasts uncoupled by physical removal of $CF_1$ or that induced 'non-specifically' by an uncoupling agent (Fig. 8). Moreover, quercetin restored the $H^+$ uptake in chloroplasts uncoupled by addition of different uncouplers (Table II). The fact that quercetin affects the membrane permeability immaterial of the way uncoupling was achieved suggests that the leak of protons through the ATPase complex is blocked in its presence. Uncouplers may increase preferentially the specific proton leak through the ATPase complex at low uncoupler concentration, while at higher concentrations the additional 'non-specific' leakage becomes predominant. The blocking effect of the specific proton leaks by quercetin is more effective at low uncoupler concentration. If uncouplers were to react with specific proteins in the membrane and enhance the proton permeability due to this type of interaction, then quercetin might be assumed to interact with these membrane components and thus prevent the uncoupler induced permeability change.

High affinity binding sites for uncoulers were recently suggested in mitochondria. Using photoaffinity labeling techniques, the interaction of uncouplers with a few membrane-bound proteins including the $\beta$ subunit of $F_1$ and $F_0$ proteolipid was reported [33,34].

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