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Differential sensitivity to dibucaine of photosynthetic control of electron transport and photophosphorylation in chloroplasts

Henrik Laasch and Engelbert Weis

Botanisches Institut der Universität Düsseldorf, Düsseldorf (F.R.G.)

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The effect of dibucaine on the photosynthetic control of electron transport, as related to acidification of the thylakoid lumen, and photophosphorylation in isolated chloroplasts of *Spinacia oleracea* was studied and compared to that of a typical uncoupler (nigericin) and energy transfer inhibitor (3′-*O*-napthyl-ADP). In some respects, dibucaine resembled an uncoupler: it inhibited photophosphorylation and decreased quenching of 9-aminoacridine fluorescence as well as uptake of [^{14}C]methylamine and inhibited the intrathylakoid accumulation of H^+, as measured by a glass electrode. The preactivated thylakoid ATPase was stimulated by dibucaine, although this stimulatory effect was to some extent superimposed by an inhibition of the enzyme. However, as opposed to what is expected for an uncoupler, dibucaine did not release the pH-dependent control of electron transport. In addition, pH-dependent high-energy quenching of chlorophyll fluorescence, normally inhibited by uncouplers, was not affected by dibucaine. It is concluded that dibucaine selectively reduces the driving force of photophosphorylation, while pH-dependent control of electron transport remains largely unaffected. The data are discussed regarding current models on coupling of photophosphorylation and on regulation of electron transport.

Introduction

During photosynthetic electron transport, protons are pumped into the thylakoid lumen and a pH difference (ΔpH) builds up across the thylakoid membrane. The ΔpH is assumed to serve as a driving force for ATP synthesis [1], but light-induced electrical potentials may also be involved in membrane 'energization' (for a recent review, see e.g., Ref. 2). As a consequence of the uptake of protons, electron transport is regulated: under conditions where the ATP consumption is low and the absorbed light energy is in excess, a strong acidification of the thylakoid lumen will occur. When the H^+ concentration inside the thylakoid vesicles ($[H_i^+]$) exceeds a threshold value, the oxidation of plastohydroquinone ($PQH)_2$) at the cytochrome *b*/*f* complex is assumed to become rate-limiting for electron donation to Photosystem (PS) I [3,4]. As a consequence, the overall electron transport declines. This feed-back regulation was called photosynthetic control of electron transport.

Abbreviations: 9-AA, 9-aminoacridine; Chl, chlorophyll; dibucaine, 2-butoxy-*N*-2-(diethylamino)ethyl-4-quinolinocarboxyamide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; n-ADP, 3′-*O*-naphtyl-ADP; ΔpH, transmembrane pH difference; PAR, photosynthetically active radiation; PMF, proton motive force; PS, Photosystem; q_E, energy-dependent quenching of chlorophyll fluorescence.

Correspondence: H. Laasch, Botanisches Institut der Universität Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf, F.R.G.

There is now ample evidence that PS II is another target of control: PS II photochemistry is regulated by a mechanism of thermal energy dissipation, which is reflected in 'energy quenching' of Chl fluorescence (q_E) [5–10]. Like photosynthetic control, the q_E mechanism is induced by the acidification of the intrathylakoid lumen [11,12]. It is assumed that under such conditions, PS II is converted to a low efficiency state [8]. In the 'high-energy' state of the thylakoid membrane, control of PQH_2 oxidation and of PS II are supposed to act in a synergistic way to adjust light-dependent reactions to the energy requirements of the carbon metabolism.

In this paper, we will study the influence of the agent dibucaine (2-butoxy-*N*-2-(diethylamino)ethyl-4-quinolinecarboxyamide) on photosynthetic control, q_E-quenching and photophosphorylation and we will compare the related actions of dibucaine with those induced by a 'classical' uncoupler, nigericin, and an energy transfer inhibitor 3′-*O*-Naphthyl-ADP (n-ADP) [13]. Dibucaine, a tertiary amine connected to a hydrophobic moiety, is known to uncouple respiratory electron transport from oxidative phosphorylation in mitochondria [14]. In green algae and higher plants, dibucaine has been shown to be an inhibitor of photosynthesis [15,16]. We will demonstrate that dibucaine may act as a 'selective' uncoupler: under appropriate conditions, it reduces the driving force of photophosphorylation, while photosynthetic control and q_E quenching remain largely unaffected.

Materials and Methods

Chloroplast preparation. Freshly harvested leaves of *Spinacia oleracea* cv. Monatol were preilluminated for 12 min with white light (300 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR). Subsequently, intact chloroplasts were isolated as described in Ref. 17 and stored in a medium containing 0.33 M sorbitol/50 mM KCl/1 mM $MgCl_2$/1 mM $MnCl_2$/2 mM EDTA/1 mM glutathione/0.8 mM KH_2PO_4/25 mM Hepes, adjusted to pH 7.6 with KOH. The integrity of the chloroplasts was estimated as in Ref. 18. The Chl concentration was determined according to Ref. 19. Unless stated otherwise, chloroplasts were suspended to a Chl concentration of 20 $\mu g \cdot ml^{-1}$. The standard reaction medium contained 0.33 M sorbitol/1 mM $MgCl_2$/1 mM EDTA/10 mM KCl/10 mM Hepes-KOH (pH 8). Broken chloroplasts were prepared from intact chloroplasts immediately before use by osmotic rupture in the presence of 5 mM $MgCl_2$/10 mM KCl//25 mM Hepes-KOH (pH 8). Isosmolarity was restored by addition of a medium with double strength in sorbitol. The measuring temperature was 20 °C.

Electron transport. Photosynthetic electron transport was measured as O_2 consumption in assays using osmotically ruptured chloroplasts. 5 μM methylviologen served as the electron mediator. 0.5 mM NaN_3 was added for suppression of catalase (EC 1.11.1.6) activity. When intact chloroplasts were used, O_2 evolution was measured and 2 mM KNO_2 served as the electron acceptor in the presence of 4 mM KH_2PO_4 and catalase. Chloroplasts were illuminated with red light, filtered through RG 630, KG 4 (Schott, Mainz) and a Calflex C (Balzers) filters.

Proton uptake. Light-induced uptake of H^+ was followed using the 9-aminoacridine (9-AA) technique [20]. 9-AA was used in a final concentration of 5 μM. Its fluorescence was excited with weak measuring light of 400 nm or, concomitant with cyclic phosphorylation, of 366 nm wavelength, and was corrected for weak fluorescence emmited by dibucaine [21]. 9-AA fluorescence quenching was expressed as $q_{AA} = \Delta F/(F - \Delta F)$, where ΔF is light-induced quenching of fluorescence and F is the maximum fluorescence in the dark [12]. Light-dependent accumulation of [^{14}C]methylamine in thylakoid vesicles was assayed following Ref. 22. [^{14}C]Methylamine was labelled with a specific radioactivity of 51.8 mCi/mmol and its final concentration was 1 μM. Light-induced alkalisation of the suspension medium by ruptured chloroplasts was measured with a glass electrode. Here, electron transport was mediated by 50 μM methylviologen. Chloroplasts were suspended to a concentration of 40 $\mu g \cdot ml^{-1}$.

Photophosphorylation. 'Linear' photophosphorylation by ruptured chloroplasts was assayed simultaneously with electron transport and 9-AA fluorescence. The reaction medium, in addition to the standard medium,contained 4 mM $MgCl_2$ and 5 mM KH_2PO_4. Phosphorylation was started in

the light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) by addition of 0.5 mM ADP. ATP was determined enzymatically. 'Cyclic' photophosphorylation was performed in a medium containing 5 μM phenazinemethosulfate/2 mM ADP/2 mM KH_2PO_4/4 mM $MgCl_2$/10 mM KCl/10 μM 9-AA/0.33 M sorbitol- 10 mM Hepes-KOH (pH 7.6). ATP formation was determined following Ref. 23. Hydrolysis of ^{32}P-labelled ATP in the dark by previously activated ATP-ase was performed according to Ref. 24.

Chlorophyll fluorescence. Chl fluorescence was measured using a pulse amplitude-modulated fluorometer (PAM 101, Walz) as described by Schreiber [25]. The intensity of the modulated measuring beam was less than 0.5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (integrated PAR). High-intensity light pulses (2500 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR) of 1 s duration and continuous light were filtered by heat-absorbing and -reflecting and by cut-off filters (OG 590 (Schott), RG 630, KG 4 and Calflex C). For the experiment displayed here, intact chloroplasts were used, with KNO_2 as an acceptor for non-phosphorylating electron transport. Methylviologen could not be used during Chl fluorescence measurements with the PAM fluorometer, since in the presence of this efficient electron acceptor, a complete reduction of the plastoquinone pool necessary for the adjustment of maximum Chl fluorescence during a high-intensity light pulse was not feasible after uncoupling of electron transport, e.g., with nigericin.

Energy-dependent quenching of Chl fluorescence (q_E) was determined during a steady-state level of electron transport and 9-AA fluorescence. The procedure of q_E determination was basically as described in refs. 9 and 26. A saturating light pulse was shone into the sample to induce maximum variable fluorescence $(F_v)_s$. During this light pulse, photochemical quenching was absent, since the primary acceptor of PS II (Q_A) was completely reduced. A subsequent addition of 0.3 μM nigericin dissipates light-induced acidification of the thylakoid lumen and q_E quenching [12]. When a new steady-state of electron transport, 9-AA and variable Chl fluorescence had been established, a further saturating light pulse was triggered for determination of maximum Chl fluorescence in the uncoupled state $(F_v)_{su}$. A coefficient $1 - ((F_v)_s/(F_v)_{su})$ was used for quantification of q_E. Under our conditions, we found only small changes in basic fluorescence (F_0) and a correction of q_E for F_0 quenching did not have a significant effect on q_E.

Results

In Fig. 1, we compared the effects of dibucaine on photosynthetic electron transport with those of the uncoupler nigericin and the energy-transfer inhibitor, 3′-*O*-naphtyl-ADP (n-ADP, see Ref. 13). In the presence of nigericin, both phosphorylating (+ADP +P_i) and non-phosphorylating electron transport (−ADP) were stimulated to a maximal rate (Fig. 1a). This stimulation indicates a complete release of photosynthetic control. In the same concentration range, photophosphorylation was inhibited (Fig. 2a). In contrast, the n-ADP-induced inhibition of phosphorylation was accompanied by a decline of phosphorylating electron transport to the low level, observed in the absence of ADP (Figs. 1b and 2b). n-ADP had no effect on electron transport previously uncoupled by nigericin.

Light-induced H^+ uptake by chloroplasts was estimated by 9-AA fluorescence quenching (q_{AA}). Photophosphorylation caused a considerable decline in q_{AA} due to H^+ consumption by the phos-

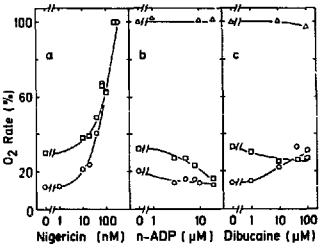

Fig. 1. Effects of nigericin (a), n-ADP (b) and dibucaine (c) on non-phosphorylating (○), phosphorylating (□) and uncoupled (△) electron transport ($H_2O \rightarrow$ methylviologen) in broken chloroplasts, measured as O_2 consumption. The irradiance was 600 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR. Electron transport rates in the presence of 0.3 μM nigericin and absence of n-ADP or dibucaine were set as 100%. Activity of 100% was 230 (a), 217 (b) and 242 (c) μmol O_2/mg Chl per h.

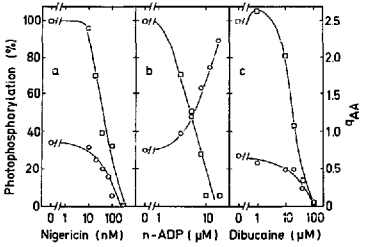

Fig. 2. Effects of nigericin (a), n-ADP (b) and dibucaine (c) on 'linear' photophosphorylation (□) and 9-AA fluorescence quenching (q_{AA}) (○). Phosphorylation and q_{AA} were measured simultaneously with electron transport, as displayed in Fig. 1. 100% activities of phosphorylation were 152 (a), 171 (b) and 192 (c) μmol ATP/mg Chl per h.

phorylating reaction (not shown). As could be expected, q_{AA}, measured in the presence of ADP and P_i, was decreased by nigericin but increased by n-ADP (Fig. 2a, b). These observations completely agree with those expected for the effects of classical uncouplers or energy transfer inhibitors [27]. In some respects, dibucaine resembled an energy-transfer inhibitor rather than an uncoupler: electron transport in the absence of ADP was only slightly stimulated. Under phosphorylating conditions, electron transport was even slightly inhibited (Fig. 1c). However, similar to the effect of nigericin, the dibucaine-induced inhibition of photophosphorylation was correlated to a decline in q_{AA} (Fig. 2c). Dibucaine had no effect on electron transport in the presence of nigericin, i.e., it did not act as an inhibitor of electron transport.

Alternative to the 9-AA approach, the intrathylakoid H^+ accumulation was assayed by following the light-induced uptake of ^{14}C-labelled methylamine [28]. In Table I, the latter is compared to the quenching of 9-AA fluorescence. Methylamine uptake was quantified in analogy to 9-AA fluorescence quenching. The data indicate a simultaneous decline of q_{AA} and methylamine uptake with rising concentrations of dibucaine. It may be concluded that the decline of q_{AA} is not based on a specific interaction of dibucaine with 9-AA, but rather on a decrease of the intrathylakoid H^+ concentration.

TABLE I

COMPARISON OF THE EFFECT OF DIBUCAINE ON 9-AA FLUORESCENCE QUENCHING (q_{AA}) AND UPTAKE OF [^{14}C]METHYLAMINE

The uptake of [^{14}C]methylamine and quenching of 9-AA fluorescence were measured simultaneous at a light flux of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ and a Chl concentration of 20 $\mu g \cdot ml^{-1}$. The uptake of [^{14}C]methylamine, in analogy to the quantification of q_{AA}, was defined as the quotient of methylamine associated with thylakoid membranes (a_i) over free methylamine in the medium (a_0). 100% q_{AA} corresponded to a value of 2.35 and 100% a_i/a_0 to 0.115.

Dibucaine (μM)	9-AA fluorescence q_{AA}; $F/(F-\Delta F)$ (%)	[^{14}C]Methylamine a_i/a_0 (%)
0	100	100
1	89	91
10	63	58
40	40	33
100	8	5

Comparable effects of dibucaine and nigericin are indicated by Fig. 3, where the efficiency of phosphorylation (ATP/e_2 ratio) was plotted versus q_{AA}. With both dibucaine and nigericin, a decline of the ATP/e_2 ratio was correlated to a decline in Q_{AA}. A slight stimulation of phosphorylation (Fig. 2c) and of the ATP/e_2 ratio (Fig. 3), observed at low dibucaine concentrations ($< 10^{-8}$ M), has already been described by Giersch [29] for amine-type uncouplers. In the case of n-ADP, on

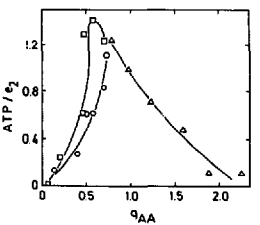

Fig. 3. Dependence of the efficiency of 'linear' phosphorylation (ATP/e_2 ratio) on 9-AA fluorescence quenching (q_{AA}). The ATP/e_2 ratios were calculated from the rates of phosphorylating electron transport (Fig. 1) and ATP synthesis (Fig. 2). q_{AA} was varied by additions of nigericin (○), n-ADP (△) and dibucaine (□).

TABLE II

EFFECTS OF DIBUCAINE ON LIGHT-INDUCED NET UPTAKE AND DARK EFFLUX OF H^+, MEASURED WITH A GLASS ELECTRODE

Broken chloroplasts were suspended in a low-buffer medium. Net uptake of H^+, as induced by light-driven electron transport (methylviologen → H_2O), was calculated from the change of pH in the suspension medium. The half-time ($t_{1/2}$) of dark efflux of H^+ was determined after the light-dark transition. The irradiance was 150 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR.

Dibucaine (μM)	Net H^+ uptake (neq·mg^{-1} Chl)	$t_{1/2}$ (s)
0	390	12
1	380	11
10	280	9
80	140	2

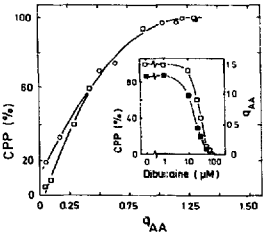

Fig. 4. Relationship of simultaneously measured 'cyclic' photophosphorylation and 9-AA fluorescence quenching (q_{AA}), at an irradiance of 1100 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR, in the presence of various concentrations of nigericin (○) and dibucaine (□). The inset shows the original data of cyclic phosphorylation (□) and q_{AA} (■) in dependence of the dibucaine concentration.

the other hand, a decline of the ATP/2_2 ratio was correlated to an increase of q_{AA}.

The suggestion that dibucaine had effects comparable to those of uncouplers is further confirmed by direct measurements of H^+ uptake with a glass electrode (Table II): in the concentration range where dibucaine inhibited phosphorylation, the net uptake of H^+ was inhibited and the rate of passive H^+ release, observed upon light-dark transition, was enhanced.

Phenazinemethosulfate-dependent, 'cyclic' phosphorylation was also inhibited by dibucaine (Fig. 4). This inhibition was closely correlated to a decline in q_{AA}. The data are derived from simultaneous measurements of q_{AA} and phosphorylation in the presence of increasing concentrations of dibucaine (Fig. 4, inset). A similar correlation was found when nigericin was used instead of dibucaine (Fig. 4). Only at concentration above 40 μM dibucaine was phosphorylation slightly more inhibited, relative to q_{AA}, than that observed with nigericin. This might be explained by assuming that 'uncoupling' by dibucaine is superimposed by an additional inhibitory effect on the phosphorylating enzyme itself.

Actually, we found that the hydrolytic activity of preactivated ATPase, measured in the presence of 0.3 μM nigericin, was 30% inhibited by 40 μM dibucaine compared to that measured in the presence of only nigericin. This indicates an inhibitory effect of dibucaine (Table III, b and d). In the absence of nigericin, however, dibucaine even stimulated the rate of ATP hydrolysis (Table III, a and c). After addition of 40 μM dibucaine, hydrolysis was increased by more than 100% relative to control (Table III). Most likely, this stimulation is caused by a removal of the thermodynamic constraints imposed by the reversed H^+ pumping, coupled to ATP hydrolysis. It is a characteristic effect, expected for uncouplers [27].

A comparison of the effect of the uncoupler nigericin on photosynthetic control with that of dibucaine is displayed in Fig. 5. In both intact

TABLE III

ACTION OF NIGERICIN AND DIBUCAINE ON [^{32}P]ATP HYDROLYSIS BY BROKEN CHLOROPLASTS

Hydrolysis of ^{32}P-labelled ATP by preactivated chloroplast ATPase. Labelled ATP was added to a concentration of 0.8 mM, 10 s after addition of nigericin and/or dibucaine. Hydrolysis in the presence of 0.5 μM nigericin was taken as 100% activity. The rates of ATP hydrolysis were constant over a period of at least 2 min.

Addition	ATP hydrolysis (μmol/mg Chl per h)	Activity (%)
(a) no uncoupler	15	19
(b) 0.5 μM nigericin	77	100
(c) 40 μM dibucaine	32	42
(d) 0.5 μM nigericin +40 μM dibucaine	52	68

(Fig. 5a) and broken chloroplasts (Fig. 5b) the rates of electron transport increased with decreasing q_{AA} when nigericin was added. This indicates a release of control. Contrarily, electron transport remained almost unaffected, i.e., the photosynthetic control was almost preserved when the q_{AA} was lowered by dibucaine. With the amine-type uncoupler, NH_4Cl, an intermediate pattern was observed: the decline in q_{AA} was correlated to a stimulation of electron transport, but the correlation was not linear. Although dibucaine alone had a minor stimulatory effect, the basic response of basal electron transport (−ADP) on nigericin was not effected, when 50 μM dibucaine was present (Fig. 6).

Chl fluorescence had been shown to be sensitive to light-induced H^+ uptake: acidification of the inner thylakoid space causes fluorescence decline [12]. This pH-dependent quenching of Chl fluorescence has been shown to be sensitive to uncouplers. Actually, with increasing concentrations of uncouplers like nigericin or gramicidin D,

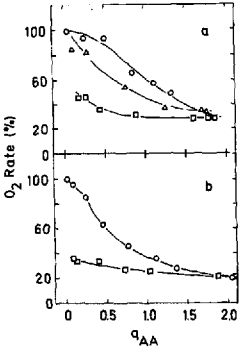

Fig. 5. Non-phosphorylating electron transport dependence on q_{AA}, under constant irradience (a) in intact chloroplasts ($H_2O \rightarrow NO^{2-}$), measured as O_2 evolution, and (b) in broken chloroplasts ($H_2O \rightarrow$ methylviologen), measured as O_2 consumption. O_2 evolution/consumption in the presence of 0.3 μM nigericin was taken as 100% activity. q_{AA} was influenced by either nigericin (○), dibucaine (□) or NH_4Cl (△). Using intact chloroplasts, 100% activity was 60–110 and in broken chloroplasts, 230–260 μmol O_2/mg Chl per h.

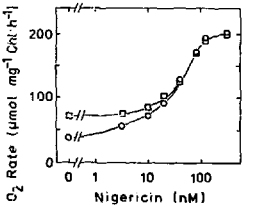

Fig. 6. Uncoupling of non-phosphorylating electron transport ($H_2O \rightarrow$ methylviologen) by increasing concentrations of nigericin (○) and nigericin plus 40 μM dibucaine (□). The irradiance was 1000 μE·m^{-2}·s^{-1}, PAR.

both, q_{AA} and high-energy quenching, the latter quantified by the coefficient q_E, decreased. The correlation of the two parameters was almost linear. However, q_E quenching proved to be almost insensitive against dibucaine in the concentration range where it causes a decline in q_{AA}. In the presence of 10^{-4} M dibucaine, i.e., when q_{AA} was almost zero, q_E was not significantly lower than in its absence. At concentrations below 10^{-4} M, q_E even slightly increased. This effect of dibucaine on the relationship between q_E and q_{AA} has already been reported in a previous paper [9]. A comparable although much weaker effect was observed when NH_4Cl was used for uncoupling.

Discussion

In this study, we related photophosphorylation to the quenching of 9-AA fluorescence. In the literature, there is still some controversy about the interpretation of 9-AA fluorescence quenching as an indicator for light-dependent energization of thylakoid membranes. Briefly, it was proposed that unprotonated, fluorescing aminoacridine pentrates the thylakoid membrane and is subsequently protonated and trapped, dependent on the transmembrane ΔpH [20,30]. Since the latter is thought to be a driving force of photophosphorylation [1], q_{AA} would represent a measure for H^+-related membrane energization.

An alternative interpretation had been that negative charges become surface-exposed during membrane energization on the outer side of the

thylakoid membrane, and that 9-AA would electrostatically bind to these charges. Since these negative charges are thought to be related to a driving force of phosphorylation, again 9-AA would be a suitable measure for energization [31,32]. Besides all discrepancies on the mechanism of quenching, Strotmann and Lohse [33] found a strong empirical relationship between the phosphorylation potential under equilibrium conditions and quenching of 9-AA fluorescence. It therefore appears reasonable to use q_{AA} as a relative measure for the proton motive force (PMF), created in the light.

Under the particular conditions used in our study, the 9-AA approach was further probed by (i) a comparison of q_{AA} with the uptake of [^{14}C]methylamine in the presence of dibucaine (Table I) and (ii) by control experiments with a well-characterized uncoupler (nigericin) and energy-transfer inhibitor (n-ADP). In agreement to what is expected for the PMF, q_{AA} declines upon uncoupling with nigericin (Fig. 2a) and rises upon inhibition of the ATPase with n-ADP (Fig. 2b). In the presence of nigericin, the thylakoid membrane may become unspecifically leaky for H^+ and the driving force of phosphorylation, and the photosynthetic control may collapse. In contrast, neither a decline in the PMF (and related q_{AA}) nor in photosynthetic control is expected when inhibition of phosphorylation is caused by energy-transfer inhibition.

With respect to its effects on the PMF, there is evidence for an uncoupler-like action of dibucaine. Inhibition of phosphorylation and the decline of the ATP/e_2 ratio were correlated to: (i) a decrease in q_{AA} (Figs. 2 and 5) and methylamine uptake (Table I): (ii) a decrease of the apparent net uptake of H^+ in the light and accelerated release of H^+ after light-dark transition (Table II) and (iii) a stimulation of ATP hydrolysis in the dark (Table III). Apparently, this uncoupling effect is superimposed by some direct inhibition of the ATPase (Table III, b and d).

Since electron transport per se is not affected in the concentration range studied (under 10^{-4} M, Fig. 1c), a considerable increase of basal electron transport would be expected if dibucaine were an uncoupler, according to the classical definition [27]. However, electron transport remained in a fairly controlled state (Fig. 5). In addition, the pH-dependent quenching of Chl fluorescence, q_E, was not inhibited by dibucaine (Fig. 7). Since both the photosynthetic control [3,4] and q_E quenching [12] are clearly related to the acidification of the thylakoid lumen, the results provide strong evidence that dibucaine does not cause unspecific leakiness for H^+ in the thylakoid membranes.

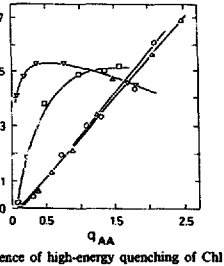

Fig. 7. Dependence of high-energy quenching of Chl fluorescence (q_E) on quenching of 9-AA fluorescence (q_{AA}). In the presence of a constant irradiance (150 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR); q_{AA} was varied by additions of nigericin (○), gramicidin D (△), NH_4Cl (□) and dibucaine (▽).

Therefore, dibucaine appears to be a substance which selectively lowers the PMF, leaving the pH-dependent control intact. The results cannot simply be explained by a 'mixed-type' action of dibucaine: unspecific leakiness for H^+ of the membranes would result in a release of photosynthetic control and q_E, even if it were superimposed by energy-transfer inhibition or by another blockage of the ATPase. Obviously, dibucaine and, to a certain extent, also NH_4Cl (Figs. 5 and 7) represent a new class of effectors. We propose to term them 'selective uncouplers'.

We observed that in the presence of dibucaine, when the PMF is low, the same concentration of nigericin is required to induce complete release of photosynthetic control as in its absence (Fig. 6). This suggests that control is connected to a 'proton potential' which does not directly equilibrate with the potentials related to the PMF. There are already theories assuming that the H^+ gradient between the inner and the outer bulk phases is of minor importance for the PMF. Kraayenhof and

co-workers [31], e.g., developed a model of energy transduction which is based on the assumption that negative charges created in the light produce a potential in the vicinity of the ATPase-synthase complex, feeding H^+ to the enzyme. In the framework of this model, dibucaine might excert its action by screening these charges, leaving the bulk ΔpH in the thylakoid lumen unaffected. It would account for a dibucaine-induced decline in q_{AA} and photophosphorylation, since neutralizing this surface potential would inhibit 9-AA binding and lower the PMF.

Dibucaine might also behave similarly to what Rottenberg and co-workers [34] have characterized as 'decouplers', which selectively discharge so-called 'energy-transducing H^+ potentials', leaving the 'bulk-to-bulk' potential intact. Adopting this hypothesis, the energy-transducing potential would be the target of dibucaine action without effects on the 'bulk-to-bulk' ΔpH. On the other hand, assuming that methylamine equilibrates between the bulk phases, the results shown in Table I suggest that dibucaine lowers a bulk-to-bulk ΔpH like a classical uncoupler. However it may not necessarily affect sequestered H^+ domains within the membranes. Dilley and co-workers discussed the occurrence of 'localised' proton gradients connected to sequestered H^+ pools at the thylakoid membrane [35,36] (for a review, see Ref. 37). Dibucaine could affect an 'H^+ pool' related to photophosphorylation, leaving others involved in control mechanisms, possibly more localised H^+ pools, less affected.

The idea of a possible separation between different H^+ domains by means of dibucaine is further supported by the observation that the degree of 'selectivity' of dibucaine apparently varies with conditions. It has been suggested that preferably in low-salt media, localized ion domains are separated from the bulk phases, while high-salt conditions may favor an equilibration between different domains [38]. Interestingly, we have observed that in chloroplasts suspended in high-salt media, q_E quenching was less resistant to dibucaine than in low-salt media (results not shown). A general variability in the relationship between q_E quenching and ΔpH has recently been discussed [9,17]. We shall not rigorously discuss different mechanisms of energy transduction here, but evidently the postulate of a direct connection between control mechanisms (photosynthetic control and q_E) and the PMF, mediated by a uniform bulk-to-bulk ΔpH, is difficult to explain by the data presented.

The relationship between energy-conserving reactions and control mechanisms in chloroplasts is of general importance with respect to regulation. During light-limited CO_2 fixation, the electron transport chain in leaves remains highly oxidized and there is little pH-mediated feed-back control consisting of the photosynthetic control and q_E mechanisms. When photosynthesis is limited by the flux of carbon, strict pH-mediated feed-back control comes into effect [8–10]. On the other hand, no significant increase in the $ATP/ADP + P_i$ ratio is observed going from light-limited assimilation to conditions where assimilation is limited by carbon metabolism [39]. It suggests that pH-dependent feed-back control operates in a way which does not require a large increase in the phosphate potential and, thus, in the driving force for photophosphorylation. Possibly, these observations support the idea presented in the present study that distinct H^+ pools may relate to photophosphorylation and control mechanisms and that, under certain conditions, these pools do not equilibrate with each other.

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