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Effect of Pyridine Homologues on Proton Flux Through the $CF_0 \cdot CF_1$ Complex and Photophosphorylation in Chloroplasts[1]

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Abstract

At concentrations below 1 mM, hydrophobic pyridine homologues decrease the rate of photophosphorylation and light-stimulated hydrolysis of ATP and light-activated exchange of the tightly bound nucleotides in chloroplasts, but increase the rate of the Hill reaction. Unlike uncoupling agents, the presence of the organic base at such low concentrations decreases the rate of light-dependent leakage and has no effect on the efficiency of two-stage photophosphorylation in broken chloroplasts. By assuming that the organic base is bound to independent equivalent sites in the thylakoid membrane, a simple expression can be derived which relates the observed rates of photophosphorylation and light-stimulated hydrolysis of ATP quantitatively to the concentration of the organic base in solution and gives dissociation equilibrium constants which are on the order of the relative hydrophobicities of the pyridine homologues. A possible mechanistic model for the $CF_0 \cdot CF_1$ complex is proposed which could serve as the basis for a unified interpretation of the kinetics of proton translocation in illuminated chloroplasts, the steady-state rate of photophosphorylation, the light-stimulated ATPase activity, and the light-activated exchange of tightly bound adenine nucleotides.

Key Words: Chloroplasts; photophosphorylation; proton flux; CF_1-ATPase; tightly bound nucleotides; pyridine homologues; nucleotide exchange.

Introduction

Permeant bases such as pyridine and imidazole were shown to increase the rate of electron transport (Hind, 1961), the steady-state accumulation of protons, and the yield of two-stage photophosphorylation (Lynn, 1968;

[1]Abbreviations: AMPPNP, adenylylimidodiphosphate; Chl, chlorophyll; $CF_0 \cdot CF_1$, the coupling factor complex of chloroplasts; DCCD, N,N'-dicyclohexylcarbodiimide; DTT, dithiothreitol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; TCA, trichloroacetic acid; Tricine, *N*-tris-(hydroxymethyl)methylglycine.

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Nelson *et al.*, 1971) in illuminated thylakoids, but decrease the rate of phosphorylation under continuous illumination. The relationship between the rate of electron transport, the rate of photophosphorylation, and the buildup of ΔpH across the thylakoid membrane were examined by different methods (Rumberg *et al.*, 1969; Bamberger *et al.*, 1973; Portis and McCarty, 1976). The observed effect of permeant buffer on the initial lag of photophosphorylation shows that phosphorylation can occur in the absence of ΔpH between the bulk aqueous phases (Ort *et al.*, 1976). This observation could be due to the compartmentalization of the internal phase (Dilley and Prochaska, 1978; Prochaska and Dilley, 1978), or due to the fact that the initial photophosphorylation is driven mainly by transmembrane electric potential difference, even if the subsequent steady-state photophosphorylation is driven mainly by ΔpH (Vinkler *et al.*, 1979, 1980). It has been shown that transmembrane electric field can induce conformation change in the $CF_0 \cdot CF_1$ complex as well as ATP synthesis in the absence of ΔpH (Witt *et al.*, 1976; Gräber *et al.*, 1977).

At concentrations below 1 mM, hydrophobic pyridine homologues have been shown to increase the basal rate of electron transport and decrease the observed H^+/e^- ratio in mitochondria (Ho and Wang, 1981a) and chloroplasts (Ho and Wang, 1981b) respectively. In the present work, the effect of pyridine homologues on the kinetics of proton translocation in illuminated broken chloroplasts, the efficiency of 2-stage photophosphorylation, the steady-state rate of photophosphorylation, and the light-stimulated ATPase and light-activated exchange of tightly bound nucleotides have been examined with the aim to gain some information concerning the mechanism of these processes as well as the nature of interaction of the organic base with the thylakoid membrane.

Experimental Procedures

Materials. Chloroplasts were prepared from fresh spinach leaves as described previously (Ho *et al.*, 1979). Chlorophyll concentration was assayed spectrophotometrically (Arnon, 1949). Each chloroplast sample was suspended in STN buffer (sucrose 0.25 M, Tricine 20 mM, pH 7.9, NaCl 20 mM) in a concentration of 3 ~ 4 mg Chl/ml. Broken chloroplasts were obtained by 10 to 100-fold dilution of the original preparation with sucrose-free buffer at 0°C. ADP (sodium salt, grade III), ATP (disodium salt), glutaraldehyde (grade I, 25% aqueous solution), and dithiothreitol were obtained from Sigma Chemical Company. The purity of glutaraldehyde was checked spectrophotometrically before use (Gillet and Gull, 1972). Pyridine, 4-picoline, 4-ethylpyridine, 4-*n*-butylpyridine, 4-*tert*-butylpyridine, pyrazine, and *n*-hexane were purchased from Aldrich Chemical Company. Carrier-free radioactive orthophosphate ($^{32}P_i$) and radioactive ADP (^{14}C-ADP) were

obtained from New England Nuclear Corporation. Radioactive ATP (γ-^{32}P-ATP) was synthesized through photophosphorylation of chloroplasts using ADP and $^{32}P_i$ and subsequently isolated by ion-exchange column chromatography (Nelson, 1980). All radioactive chemicals were found by paper chromatography to contain no detectable amount of radioactive impurity. Pyocyanine was from Schwarz and Mann. Potassium ferricyanide was from Fisher Chemical. All other reagents were of the highest purity available.

Chemical Modification of Chloroplasts. The procedure for cross-linking with glutaraldehyde was described previously (Packer *et al.*, 1968; Ho *et al.*, 1979). Chloroplast samples (~1 mg Chl/ml) with and without 25 mM pyridine were fixed by 50 mM glutaraldehyde for 6 min at 0°C in the dark or under continuous illumination. The reaction was stopped and the excess glutaraldehyde and pyridine were removed by repeated washing with 30-fold dilution of the sample with STN buffer at 0°C.

Hill Reaction. The rate of light-driven electron transport in chloroplasts from water to ferricyanide was monitored with a Clark-type oxygen electrode (Chen and Wang, 1974). The reaction medium (Tricine 20 mM, pH 7.4, 22°C, NaCl 50 mM, sucrose 1.2 mM, and potassium ferricyanide 1 mM) was flushed inside the thermostated glass cell with nitrogen until anaerobiosis was reached before the chloroplast sample was added and incubated anaerobically in the dark for 15 min. Then the actinic red light (250 J m^{-2} sec^{-1} with Edmund Sci. Co. filter No. 823) was turned on and the linear rate of oxygen generation was monitored and recorded with a strip-chart recorder.

Photophosphorylation. Photophosphorylation was assayed as follows. One ml of reaction mixture containing 80 μg chlorophyll, 20 mM Tricine at pH 7.4, 50 mM NaCl, $MgCl_2$ 2 mM, 50 μM pyocyanine, 5 mM ADP, and 5 mM [$^{32}P_i$]P_i was illuminated under red light of constant intensity for 1 min. After the light was turned off, the reaction was stopped with trichloroacetic acid (4%) and the precipitate was centrifuged down (Beckman Airfuge, 78,000 $\times$ *g*, 2 min). A 25-μl aliquot of the supernatant was put on polyethylenimine (PEI) paper for [^{32}P]ATP assay by ascending chromatography (Wang, 1979).

ATPase Activity. Light-activated ATPase activity in chloroplasts was assayed as previously described (Petrack *et al.*, 1965; Carmeli and Avron, 1971). A 0.5-ml dark-adapted chloroplast sample (containing Tricine, 20 mM at pH 7.4, NaCl 50 mM, pyocyanine 50 μM; $MgCl_2$ 2 mM; dithiothreitol (DTT) 5 mM and chlorophyll (~0.1 mg/ml) was illuminated with red light of constant intensity for 3 min at 22°C. After the light was turned off, the activated chloroplast sample was transferred and mixed immediately with 0.5 ml of [γ-^{32}P] ATP solution (5 mM [γ-^{32}P]ATP with 5 $\times$ 10^5 cpm/ml,

Tricine 20 mM at pH 7.4, NaCl 50 mM). The hydrolysis reaction was allowed to proceed for 15 min in the dark and was stopped by the addition of trichloroacetic acid (4%). The concentration of [γ-^{32}P] ATP was assayed with PEI-paper chromatography and the ATPase activities was calculated by the difference of the light-activated and the dark control samples. The ATPase activities in the absence and presence of pyridine homologues were also assayed as a function of time and were found to be constant up to 15 min, which indicated that no significant decay of the activated ATPase activity occurred in the dark during ATP hydrolysis. For heat-activated ATPase activity, the chloroplast sample was incubated at 60°C for 10 min in the presence of 2 mM DTT (Ho and Wang, 1980). The hydrolysis of ATP was monitored by [γ-^{32}P] ATP with subsequent PEI-paper chromatography.

Exchange of Tightly Bound Nucleotides. The light-dependent exchange of tightly bound nucleotides in thylakoid membrane was assayed by the "back exchange" method of Shavit and Strotmann (1980). The preloading of radioactive nucleotides into thylakoid membrane was accomplished by illuminating 5 ml of broken chloroplast sample containing 2 mg Chl/ml, 20 mM Tricine at pH 8.0, 50 μM pyocyanine, 50 mM NaCl, 2 mM $MgCl_2$ and 5 μM [^{14}C]ADP ($\sim 3 \times 10^5$ cpm/μl) at 22°C for 5 min. The sample was then washed 3 times with 30 ml ice cold Tricine buffer (Tricine 20 mM at pH 8.0, NaCl 50 mM) in the dark. The preloaded chloroplast sample was suspended in Tricine buffer in the dark at 0°C. The exchange reaction was carried out by addition of 200 μl of the preloaded chloroplast sample to 250 μl of the reaction medium (Tricine 20 mM at pH 8.0, NaCl 50 mM, pyocyanine 50 μM, $MgCl_2$ 5 mM, and ADP 1 mM) in the dark. The mixture was illuminated with red light of constant intensity for a given period of time at room temperature. The chloroplasts were then pelleted by means of a Beckman Airfuge (78,000 $\times$ *g*, 1 min) and 100 μl of the clear supernatant was taken out for radioactive assay.

Partition Coefficients. The partition coefficients of pyridine homologues between *n*-hexane and aqueous buffer were determined and used as an approximate measure of relative hydrophobicities. In each case, a 100-μl sample of the pyridine homologues was dissolved in 25 ml of *n*-hexane. After mixing, 5 μl of the solution was assayed by high-pressure liquid chromatography (HPLC) to determine the initial concentration (C_0) of the pyridine homologue before partition. Subsequently 25 ml of an aqueous STN buffer was added and the mixture was shaken for 30 min and then allowed to settle in a 25°C water bath for 3 hr. A 5-μl sample of the *n*-hexane layer was then assayed again by HPLC for the concentration (C_h) of the pyridine homologue. The partition coefficient was computed as $K' = C_h/(C_0 - C_h)$. For HPLC analysis, a 25 $\times$ 0.45 cm chromatographic column packed with Whatman Partisil-10 PAC (10 μm) was used. An acetonitrile–methanol

mixture (4:1 v/v) was used as the solvent with a flow rate of 1.0 ml/min at a pressure of 900 p.s.i. The elution signal was monitored with a Water Associates 440 absorbance detector at 254 nm at relative sensitivities 1.28 and 0.10 respectively. A Columbia Supergrator III processor connected to the detection unit was used to integrate the area under each elution peak.

Proton Translocation. Proton uptake by chloroplasts under cyclic electron transport conditions was measured by means of a combination pH-electrode (Beckman 39030) fitted to the thermostated glass cell. The light intensity was varied with neutral gray filters, and the duration of illumination was controlled manually by means of a shutter. For the conversion of observed ΔpH to nmol protons taken up, a calibration curve was obtained for each set of measurements by titrating the sample under illumination with 5 mM HCl. Proton uptake by thylakoids in the presence of pyridine exhibited biphasic kinetics for the first two illumination cycles which may be due to the slow equilibration of pyridine base inside and outside the thylakoid. After the third illumination, both proton uptake during illumination and the subsequent leaking out of protons in the dark follow first-order kinetics, and the data were taken for kinetic analysis. The biphasic character of proton uptake can also be removed by incubating the chloroplast sample with pyridine in the dark for 15–20 min.

The kinetic analysis of proton translocation in broken chloroplasts has been described previously (Ho *et al.*, 1979). For chloroplast samples which have been fully equilibrated in the dark with the medium containing a sufficient concentration of the supporting electrolyte (NaCl), both the uptake of protons under constant illumination and the subsequent release of protons in the dark obey first-order kinetics. The proton uptake by illuminated chloroplasts is given by $\ln (1 - \delta/\delta_{ss}) = -k_L t$, where δ and δ_{ss} denote the nmoles H^+ taken up from the medium per milligram chlorophyll at time t and at the steady state respectively, and k_L is the decay constant under light. As shown in Fig. 1, the slope of the plot ($-k_L$) decreases with t when [NaCl] $\leq$ 10 mM. This deviation from linearity is probably due to the slow buildup of membrane potential which retards the diffusion of protons. On the other hand, for [NaCl] $\geq$ 30 mM, k_L is independent of t. The linear $\ln (1 - \delta/\delta_{ss})$ vs. t plot for each of the more concentrated NaCl solutions indicates that a steady-state membrane potential had been reached before the first of the experimental values in the graph was measured. Consequently, in order to simplify the treatment of data, all measurements of proton translocation in this work were made with chloroplast samples containing 50 mM NaCl. Similarly, the subsequent release of protons from the chloroplasts in the dark also obeys the first-order decay equation $\ln (\delta/\delta_{ss}) = -k_D t'$, where k_D represents the leakage constant in the dark.

Differentiation of the equation for proton uptake with respect to t gives

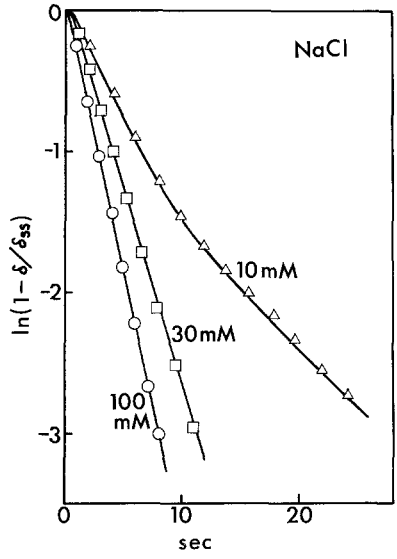

Fig. 1. Effect of supporting electrolyte on the rate of proton uptake by broken chloroplasts. Composition of sample: Chlorophyll, 0.1 mg/ml; [Tricine] = 1 mM, initial pH = 6.65 at 18°C; [pyocyanine] = 50 μM; [NaCl] = 10 mM (△), 30 mM (□), and 100 mM (○).

the rate equation $d\delta/dt = k_L (\delta_{ss} - \delta)$. At $t = 0$ this equation becomes $R_0 = k_L\delta_{ss}$, where R_0 represents the rate of inward pumping of protons. Thus the rate equation may also be written as $d\delta/dt = R_0 - k_L\delta$.

The observed first-order kinetics of proton uptake was first explained by Karlish and Avron (1968) by assuming that the rate of inward pumping of protons is proportional to the concentration of internal buffer base. Such an assumption seems difficult to justify, because thylakoids are quite permeable to chloride ions, and the rate of inward pumping of protons should be determined by the rate of electron transport, not by the concentration of internal buffer base which receives the protons. It is true that when all the internal basic groups are protonated, the outward proton leakage rate will be very high. But since the enhanced leakage rate has already been represented by the leakage term in their kinetic equation, it should not be included again in the pumping term. For this reason, Ho *et al.* (1979) proposed an alternative model, and showed that the term $-k_L\delta$ represents the rate of outward leakage of accumulated protons, which gradually becomes equal to the inward pumping rate R_0 as the system approaches the steady state.

During each proton translocation measurement, the medium pH changed from 7.4 to 7.5 or 7.6. At these pH values, pyridine homologues, with pK_a between 5.2 and 6.0, exist mainly in the unprotonated base form. Therefore the uptake of pyridine base from the medium would hardly affect the [pyridine base]/[pyridine ion] ratio in the medium. As expected, we found by titration of the chloroplast sample with standard HCl or NaOH both under illumination and in the dark that the buffer capacity of the sample

remain invariant within a random error of ±0.5% of the observed ΔpH. In actual experiments, each measurement was followed by titration of the sample with standard HCl under illumination after the steady state had been reached.

Results and Discussion

Proton Flux through the $CF_0 \cdot CF_1$ Complex. Previous data on proton translocation in illuminated broken chloroplasts under different light intensities showed that under illumination the accumulated protons can leak out via two paths with an overall first-order rate constant given by

$$k_L = k_D + mR_0 \tag{1}$$

where the initial rate of proton pumping (R_0) normally increases with light intensity, but the parameter m is independent of light intensity (Ho *et al.*, 1979). Consequently, the rate of proton uptake by illuminated thylakoids may be written as

$$d\delta/dt = R_0 - mR_0\delta - k_d\delta \tag{2}$$

The term $mR_0\delta$ in Eq. (2) could represent either the slowing down of the rate of proton pumping (R_0) due to the accumulation of more protons at higher light intensity which decreases the rate of electron transport, or the increase in rate constant for the leakage of accumulated protons at higher light intensity. The first possibility is ruled out by the following observations: (a) Simultaneous measurements of proton uptake and oxygen generation by illuminated broken chloroplasts with 1,4-benzoquinone as the Hill reaction acceptor showed that the electron transport rate remained constant when δ gradually approached its steady-state value (Ho and Wang, 1981b); (b) valinomycin and K^+, which decrease membrane potential by dissipating the space charge, were found to increase k_D but have no effect on the value of m.

On the other hand, DCCD (70 μm) or AMPPNP (0.5 mM) + Mg^{2+} (5 mM), which at low concentrations are known to be bound specifically to CF_0 and CF_1 respectively, has no effect on k_D but decreases the value of m at pH 8. Therefore we may conclude that both the second and third terms on the right-hand side of Eq. (2) are due to the leakage of accumulated protons; mR_0 ($=k_L - k_D$) represents the light-dependent rate constant for proton leakage through the $CF_0 \cdot CF_1$ complex because at pH 8 its value is decreased by DCCD or AMPPNP + Mg^{2+}; k_D represents the rate constant for light-independent general proton leakage. The light dependence of $k_L - k_D$ suggests that under illumination the proton channel in $CF_0 \cdot CF_1$ may switch from a partially blocked to a fully open conformation, and that the fraction of

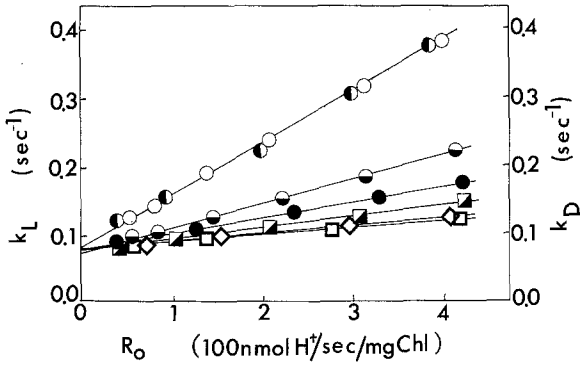

Fig. 2. Effect of 1 mM pyridine homologues on the kinetics of proton translocation by broken chloroplasts. Composition of chloroplast sample: chlorophyll concentration = 80 μg/ml. [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1 mM at initial pH 7.4 at 18°C. The kinetic analysis has been described previously (Ho *et al.*, 1979) and also summarized in Experimental Procedures. The experimental points represent k_L values for different chloroplast samples: ○, control chloroplasts; ◒ chloroplasts with 1 mM pyridine; ● chloroplasts with 1 mM 4-picoline; ◪, with 1 mM 4-ethylpyridine; ◇, with 1 mM 4-*n*-butylpyridine; □, with 1 mM 4-*t*-butylpyridine; and ◑ with 30 mM pyrazine. The dark decay constant k_D of the six chloroplast samples in the above order are 0.081, 0.072, 0.079, 0.081, and 0.081 sec^{-1}, respectively, which were found to be independent of light intensity of prior illumination. Experimental points of the highest R_0 values in each set were measured under the same light intensity (250 J m^{-2} sec^{-1}).

$CF_0 \cdot CF_1$ complexes with their proton channels fully open is proportional to R_0 which increases with light intensity until a saturation value is reached.

The effects of pyridine homologues at 1 mM concentration on the kinetics of proton translocation in illuminated broken chloroplasts are summarized in Fig. 2. As an approximate measure of their relative hydrophobicities, the partition coefficients (K') for the equilibrium distribution of pyridine homologues between *n*-hexane and aqueous buffer have been determined. The values are listed in Table I together with a number of energy-transducing characteristics of chloroplasts measured in the presence of pyridine homologues. Figure 2 shows that at 1 mM all pyridine homologues decrease the slope m without increasing the intercept k_D. This is contrary to the property of uncouplers which generally increase k_D without affecting the value of m (see Fig. 3). Thus the hydrophobic pyridine homologues do not behave like most uncouplers which increase the rate of proton leakage across the membrane, although they also increase the rate of electron transport and decrease the rate of photophosphorylation.

Further support for the conclusion that pyrimidine homologues are not

Table I. Effect of Pyridine Homologues on Proton Uptake, Dark Leakage Constant, and Rate of Photophosphorylation in Broken Chloroplasts[a]

Pyridine homologue	pK_a	Partition coefficient (K')	Concentration (mM)	Steady-state proton uptake, δ_{ss} (μmol H^+/mg Chl)	Proton leakage constant in the dark, k_D (sec^{-1})[b]	Rate of steady-state cyclic photophosphorylation (μmol ATP/mg Chl/hr)
Control	—	—	—	1.05	0.081	740
Pyridine	5.2	0.58	10	3.98	0.048	693
			50	6.28	0.042	340
4-Picoline	6.1	1.23	10	4.52	0.052	316
			25	4.61	0.051	174
4-Ethyl-pyridine	6.02	5.05	1.0	2.79	0.079	656
			10	4.69	0.050	299
4-*n*-Butyl-pyridine	6.0	27.1	0.1	1.66	0.082	584
			1.0	2.82	0.083	276
4-*t*-Butyl-pyridine	5.99	85.6	0.1	1.72	0.080	578
			1.0	3.20	0.082	270

[a] Experimental conditions of each measurement are described under Experimental Procedures. Constant intensity of red light (250 J m-2 sec^{-1}) was used. Experiments run at pH 7.4 and 18°C.

[b] k_D is the first-order proton leakage constant in the dark. The kinetic analysis is described under Experimental Procedure.

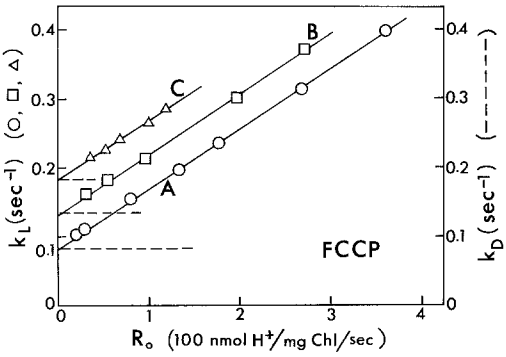

Fig. 3. Effect of FCCP on proton translocation in broken chloroplasts. Composition of medium: 1 mM Tricine (at pH 6.68, 18°C), 50 mM NaCl, 25 μM pyocyanine. [FCCP] = 0 for control (○), 1×10^{-7} M (□) and 2×10^{-7} M (△). At [FCCP] = 5×10^{-7} M, the chloroplasts became so leaky and δ_{ss} became so small that the values of R_0, k_L, and k_D computed from the experimental trace were quite unreliable.

uncouplers for chloroplasts has been obtained from measurements of the efficiency of two-stage photophosphorylation by broken chloroplasts in the presence of the organic base. The procedure used to determine the yield of two-stage photophosphorylation was essentially the same as reported previously (Ho *et al.*, 1979), except that illumination in the light stage was 3 min and the pH of both light and dark stage media was kept at 7.4. The experimental results are listed in Table II. If the pyridine homologues were uncouplers, we would expect them to decrease the ATP/H^+ ratio. But Table I shows that in spite of the increased δ_{ss} and two-stage photophosphorylation yield in the presence of these organic bases, the phosphorylation efficiency as measured by the ATP/H^+ ratio remained constant.

The effect of pyridine at 1 mM and 25 mM respectively on the leakage rate of accumulated protons from thylakoids is illuminated in Fig. 4A. Apparently at 25 mM, pyridine decreases both k_D because of the trapping of protons in thylakoids as impermeable pyridinium ions and mR_0 by hindering the opening of the $CF_0 \cdot CF_1$ channel under illumination. In order to separate these two effects from each other, additional experiments were conducted with glutaraldehyde-fixed thylakoids. Previous studies showed that the proton leakage constant of thylakoids under illumination (k_L) can be fixed by glutaraldehyde (Packer *et al.*, 1968; Ho and Wang, 1979). After the thylakoids had been treated with glutaraldehyde, k_L was no longer sensitive to light, i.e., $m = 0$, hence $k_L = k_D$ and both were equal to the value k_L of the control thylakoids under the same light intensity. Since pyridine can be removed by washing the treated thylakoids but the cross-linking by glutaraldehyde cannot, the problem can be simplified by cross-linking thylakoids with

Table II. Effect of Pyridine Homologues on Two-Stage Photophosphorylation and Heat-Activated ATPase Activity in Broken Chloroplasts[a]

Pyridine homologues	Concentration (mM)	Steady-state proton uptake, δ_{ss} (μmole H^+/mg Chl)	Two-stage photophosphorylation yield (nm ATP/mg Chl)	Two-stage photophosphorylation efficiency (ATP/H^+)[b]	Heat-activated ATPase (μmol ATP/mg Chl/hr)
Control	—	1.05	126	0.120	310
Pyridine	10	3.98	480	0.121	—
	50	6.28	738	0.118	296
4-Picoline	10	4.52	520	0.115	—
	25	4.61	548	0.119	298
4-Ethylpyridine	1.0	2.79	332	0.119	—
	10	4.69	550	0.117	308
4-*n*-Butyl-	0.1	1.66	197	0.119	—
pyridine	1.0	2.82	328	0.116	301
4-*t*-Butyl-	0.1	1.72	200	0.116	—
pyridine	1.0	3.20	376	0.118	294

[a] Experimental conditions are the same as those for Table I.

[b] Expressed as ratio of nmol ATP produced by two-stage photophosphorylation to nmol H^+ taken up when the light was turned off.

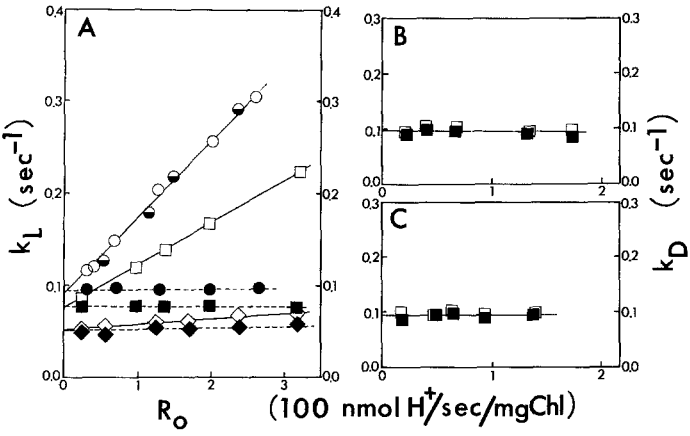

Fig. 4. Effect of pyridine and glutaraldehyde treatment on the kinetics of proton translocation by broken chloroplasts. Composition of chloroplast samples: chlorophyll concentration = 80 μg/ml; [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1 mM at initial pH 7.4 at 18°C. Glutaraldehyde fixation was described in Experimental Procedures. (A) ○ and ● represent k_L and k_D values respectively for control chloroplasts; □ and ■ represent k_L and k_D values for samples in the presence of 1 mM pyridine; ◊ and ♦ represent k_L and k_D values for samples in the presence of 25 mM pyridine; ◒ represents k_L values for chloroplast samples which contained 25 mM pyridine initially, but the pyridine was subsequently removed by repeated washing with STN buffer. (B) □ and ■ represent k_L and k_D values of chloroplast samples fixed by glutaraldehyde in the presence of 25 mM pyridine and under saturating light intensity (250 J · M^{-2} sec^{-1}). Both pyridine and excess glutaraldehyde were subsequently removed by washing with STN buffer. (C) □ and ■ represents k_L and k_D values respectively of chloroplast samples fixed by glutaraldehyde in the dark in the presence of 25 mM pyridine. Both pyridine and excess glutaraldehyde were subsequently removed before kinetic data were taken.

glutaraldehyde under different light intensities in the presence of various concentrations of pyridine, subsequently washing away the pyridine and studying the proton translocation in the resulting cross-linked thylakoids under different light intensities.

The data in Figs. 4B and 4C show that the first-order rate constants for proton leakage (k_L or k_D) of thylakoids fixed in the presence of 25 mM pyridine under illumination is only slightly higher than that for a similar sample fixed in the dark. Therefore we may conclude that pyridine homologues in general can hinder the opening of the $CF_0 \cdot CF_1$ channels in response to light and consequently reduce the steady-state rate of photophosphorylation.

Steady-State Photophosphorylation. Pyridine homologues decrease the rate of steady-state photophosphorylation. Since this inhibitory effect increases with their hydrophobicity, we may treat the problem as a membrane

phenomenon, as in the case of mitochondria (Ho and Wang, 1981a), by assuming that the organic base in solution is in equilibrium with that bound to equivalent specific sites in the thylakoid membrane.

Let R be the observed steady-state rate of photophosphorylation under a specified set of experimental conditions; R_1, the steady-state photophosphorylation rate under similar experimental conditions but in the absence of the organic base B; R_2, the photophosphorylation rate when these specific sites are completely occupied by B; K, the intrinsic equilibrium constant for the dissociation of B from its binding sites in the membrane. We may, as a first approximation, neglect the interaction between the bound ligands and consider K as the true equilibrium constant for the essentially independent binding sites to obtain

$$R = R_1\left(\frac{K}{K + [B]}\right) + R_2\left(\frac{[B]}{K + [B]}\right) \tag{3}$$

where [B] is the molar concentration of the free base of the pyridine homologue in solution. This equation can be written in a more convenient form as

$$\frac{1}{R_1 - R} = \frac{1}{R_1 - R_2}\left(1 + \frac{K}{[B]}\right) \tag{4}$$

In agreement with Eq. (4), all plots of the observed values of $1/(R_1 - R)$ versus the corresponding values of $1/[B]$ as shown in Fig. 5A are linear with a common intercept at 0.00129 mg chlorophyll · hr/mol ATP. The dissociation equilibrium constants calculated by dividing the corresponding slopes of the straight lines in Fig. 5A by their common intercept are also qualitatively consistent with the relative hydrophobicities of the pyridine homologues: 88 mM for pyridine, 18 mM for 4-picoline, 9.4 mM for 4-ethylpyridine, and 1.6 mM for 4-*n*-butylpyridine. The observed larger decrease in phosphorylation rate by the more hydrophobic pyridine homologues also supports our conclusion from Fig. 2 that they inhibit more effectively the opening of the $CF_0 \cdot CF_1$ channel under illumination.

But how can we be sure that the observed decrease in steady-state rate of photophosphorylation by pyridine homologues is due to their effect on the $CF_0 \cdot CF_1$ channel rather than due to uptake of the organic base which would increase the internal buffer capacity of the thylakoid loculus space? The data in Table I show the increase in internal buffer capacity due to the uptake of the organic base as indicated by δ_{ss} is on the order 50 mM pyridine > 10 mM 4-ethylpyridine > 1 mM 4-*t*-butylpyridine, whereas the inhibition of cyclic photophosphorylation is in the reverse order 1 mM 4-*t*-butylpyridine > 10 mM 4-ethylpyridine > 50 mM pyridine. Therefore the observed decrease of phosphorylation rate by low concentrations of pyridine homologues cannot be accounted for by the increase in internal buffer capacity.

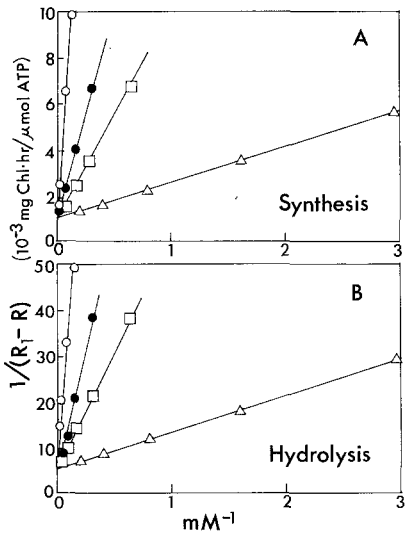

Fig. 5. Dependence of one-stage photophosphorylation and light-activated ATPase activity on the concentration of pyridine homologues. ○ represents value in the presence of pyridine; ●, 4-picoline; □, 4-ethylpyridine; △, 4-n-butylpyridine. Analysis of the data is according to Eq. (2). (A) Effect of pyridine homologues on cyclic photophosphorylation. The assay conditions are described under Experimental Procedures. The phosphorylation rate in the absence of pyridine homologues is R_0 = 740 μmol ATP/mg Chl/hr. (B) Effect of pyridine homologues on the light-activated ATPase activity. The procedure for light-activation and the assay of ATP hydrolysis is as follows. A 0.5-ml dark-adapted chloroplast sample (containing Tricine, 20 mM at pH 7.4; NaCl, 50 mM; pyocyanine, 50 μM $MgCl_2$, 2 mM; DTT, 5 mM; and chlorophyll, ~0.1 mg/ml) was illuminated with red light of constant light intensity for 3 min at 22°C, and immediately mixed with 0.5 ml of [γ – ^{32}P]ATP in the same buffer solution (5×10^5 cpm/ml). Hydrolysis was allowed to proceed for 15 min in the dark, stopped with TCA (4%), and assayed by chromatography on PEI paper. The ATPase activity was computed as the difference between the measured value of the light-activated sample and that of the dark control sample. The ATPase activity in the absence of pyridine homologues is R_0 = 160 μmol ATP hydrolyzed/mg Chl/hr.

Table I also shows that the observed values of the dark leakage constant k_D in the presence of 1 mM butylpyridines are equal to that for the control sample in the absence of the organic base. This latter observation also shows that the observed decrease of the photophosphorylation rate by 1 mM butylpyridine was not due to the trapping of protons by the organic base.

Light-Stimulated ATPase. The pyridine homologues were also found to inhibit the light-stimulated ATPase activity of thylakoids. The concentration dependence of their inhibitory effect can also be described by Eq. (4) except that for the present case R, R_1, and R_2 represent the V_{max} value for light-stimulated ATP hydrolysis as observed under a given set of experimental conditions, that observed under similar experimental conditions but in the absence of pyridine homologue, and that when the specific binding sites are completely occupied by the organic base respectively. The experimental results are summarized in Fig. 5B.

The dissociation equilibrium constants of pyridine homologues calculated by dividing the straight lines in Fig. 5B by their common intercept are 79 mM for pyridine, 21.2 mM for 4-picoline, 12.5 mM for 4-ethylpyridine, and 2.0 mM for 4-*n*-butylpyridine. These values are within experimental uncertainties, in agreement with those determined from the photophosphorylation data in Fig. 5A. The agreement is consistent with the widely accepted conclusion that both photophosphorylation and light-stimulated ATPase involve the same proton channel in the $CF_0 \cdot CF_1$ complex.

If we assume that the catalytic hydrolysis of ATP by broken chloroplasts requires the removal of product proton through the $CF_0 \cdot CF_1$ channel, then we expect the latent ATPase to be activated when the $CF_0 \cdot CF_1$ channel is opened by light. Since pyridine homologues inhibit the light-driven opening of $CF_0 \cdot CF_1$ channels, they are expected to inhibit the light-stimulated ATPase as observed.

On the other hand, if some of the $CF_0 \cdot CF_1$ channels are already changed to the fully open conformation by thermal or chemical activation of the ATPase in the dark, we will not expect the pyridine homologues to have any effect on the ATPase activity. The last column in Table II shows that the heat-activated ATPase of thylakoids are indeed unaffected by pyridine homologues.

Light-Activated Exchange of Tightly Bound Nucleotides. The "back exchange" method (Shavit and Strotmann, 1980) was used to monitor the release of preloaded [^{14}C]adenine nucleotides under illumination. Data on the effect of pyridine homologues on the light-stimulated exchange of tightly bound nucleotides are summarized in Fig. 6. In general, pyridine homologues inhibit the light-stimulated exchange of tightly bound nucleotides. As before, the effect is larger for the more hydrophobic molecules. Since these organic bases also inhibit the light-activated opening of the proton channel of the $CF_0 \cdot CF_1$ complex, it is quite possible that the observed rapid exchange of tightly bound nucleotides is not stimulated directly by light or light-driven electron transport, but by the resulting proton flux through the opened $CF_0 \cdot CF_1$ channel (Gräber *et al.*, 1977).

A Possible Mechanistic Model. The above observations on the regulation of proton flux through the $CF_0 \cdot CF_1$ complex, the rate of photophosphorylation, and the light-stimulated ATPase and light-activated exchange of tightly bound nucleotides could be interpreted in the following way: Let us assume that in resting chloroplasts the proton channel through CF_0 and the γ-subunit of CF_1 is oriented toward the α-subunit with a tightly bound ADP. The protons which arrive through the channel could trigger the dissociation of this tightly bound ADP with concomitant protein conformational change. This conformational change could connect the proton channel to a neighboring β-subunit. In photophosphorylation, the proton flux could then be directed toward the bound P_i at the catalytic site to facilitate the nucleophilic attack

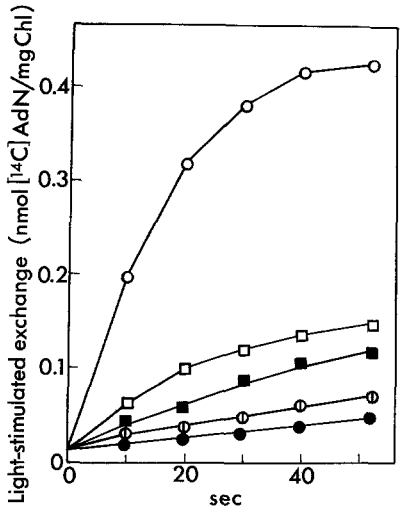

Fig. 6. Effect of pyridine homologues on the light-activated exchange of tightly bound nucleotide in broken chloroplasts. A 5-ml sample of broken chloroplasts (containing 2 mg Chl/ml, 20 mM Tricine at pH 8.0, 50 μM pyocyanine, 50 mM NaCl, 2 mM $MgCl_2$, and 5 μM [^{14}C]ADP (~3 × 10^5 cpm/μl) was illuminated for 5 min at 22°C. After washing as described under Experimental Procedures, a 200 μl-sample of the preloaded chloroplasts was mixed with 250 μl of 1 mM ADP solution (also containing 20 mM Tricine at pH 8.0, 50 μM pyocyanine, 50 mM NaCl, and 5 mM $MgCl_2$), and the mixture was illuminated with red light of constant intensity for a given period of time at room temperature. The chloroplasts were then centrifuged and the supernatant was assayed for radioactivity. ○, value taken as function of time in the absence of pyridine homologues; □, in the presence of 10 mM pyridine; ■, 2.5 mM 4-ethylpyridine; ◑, 1 mM 4-*n*-butylpyridine; ●, the dark control in the absence of pyridine homologues.

by the nearby unprotonated ADP (Ho and Wang, 1979; Mitchell, 1977). The subsequent rebinding of ADP to one of the α-subunits with concomitant conformational change could be synchronized with the departure of product ATP from the catalytic site. The conformational change could again disconnect the proton channel from the catalytic site and reorient it toward the neighboring ADP-ligated α-subunit to start the next reaction cycle. In this way, each reaction cycle could also involve the light-activated exchange of an ADP bound to one of the α-subunits. In light-stimulated ATP hydrolysis, none of the α-subunits of the activated $CF_0 \cdot CF_1$ complex would be ligated by ADP, and hence this proton channel would remain connected to the catalytic site to facilitate the rapid escape of protons in the opposite direction during the catalyzed nucleophilic attack by water at the γ-phosphate group of the bound ATP (Ting and Wang, 1980). Such a model would not only depend on proton flux as the immediate driving force, but also incorporate some features of the alternating site hypothesis (Slater, 1974; Boyer, 1974; Adolfsen and Moudrianakis, 1976; Kayalar *et al.*, 1977) in its gating of the

proton flux controlled by the stimulated exchange of tightly bound adenine nucleotides on the α-subunits.

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