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ENERGY CHARGE, PHOSPHORYLATION POTENTIAL AND PROTON MOTIVE FORCE IN CHLOROPLASTS

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Summary

Adenylate concentrations were measured in intact chloroplasts under a variety of conditions. Energy charge was significant in the dark and increased in the light, but remained far below values expected from observed phosphorylation potentials in broken chloroplasts, which were 80 000 M^{-1} or more in the light. With nitrite as electron acceptor, phosphorylation potentials in intact chloroplasts were about 80 M^{-1} in the dark and only 300 M^{-1} in the light. Similar phosphorylation potentials were observed, when oxaloacetate, phosphoglycerate or bicarbonate were used as substrates. $\Delta G'_{ATP}$ was —42 kJ/mol in darkened intact chloroplasts, —46 kJ/mol in illuminated intact chloroplasts and —60 kJ/mol in illuminated broken chloroplasts. Uncoupling by NH_4Cl, which stimulated electron transport to nitrite or oxaloacetate and decreased the proton gradient, failed to decrease the phosphorylation potential of intact chloroplasts. Also, it did not increase the quantum requirement of CO_2 reduction. It is concluded that the proton motive force as conventionally measured and phosphorylation potentials are far from equilibrium in intact chloroplasts. The insensitivity of CO_2 reduction and of the phosphorylation potential to a decrease in the proton motive force suggests that intact chloroplasts are over-energized even under low intensity illumination. However, such

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, electrical potential difference.

a conclusion is at variance with available data on the magnitude of the proton motive force.

Introduction

The phosphorylation potential $P = [ATP]/[ADP][P_i]$ is a measure of the energy state of the adenylate system and its ability to transfer phosphate.

Kraayenhof [1] has reported that the phosphorylation potential obtained by chloroplasts under saturating illumination was close to 30 000 M^{-1}. Although it had been assumed that the chloroplasts used in these studies were intact, it is now clear that they had been devoid of functional envelopes. They were able to exchange adenylates with the suspending medium, while the envelope of intact chloroplasts has a low permeability for adenylates [2]. At physiological phosphate concentrations (between 4 and 15 mM [3,4]), a phosphorylation potential of 30 000 M^{-1} corresponds to ATP/ADP ratios between 120 and 450. Such high ratios have never been observed in chloroplasts [5—8]. Measured ATP/ADP ratios were 2 to 4 in the light and about 1 in the dark. Phosphorylation potentials calculated from these ratios range from about 60 to 1000 M^{-1}. One might assume that the differences between observations made in chloroplasts in vivo and in vitro reflect different metabolic flux situations. That this explanation is not valid will be shown in the following.

During illumination of chloroplasts, light energy is used to establish a proton gradient and a membrane potential across thylakoid membranes. It is presently assumed that the proton motive force

$$\Delta G_H^+/F = (1.36/F)\,\Delta pH + \Delta\,\psi \qquad (1)$$

(where ΔG_H^+ is the free energy of intrathylakoid protons and F the Faraday constant) drives endergonic ATP synthesis [9,10]. The phosphorylation potential built up by isolated chloroplasts in the light should in the state 3/state 4 transition, when electron transport is believed to be restricted by the availability of ADP [11], be expected to be not far from equilibrium with the proton motive force. Even a small decrease of the latter should bring about a large decrease of the phosphorylation potential, since theory demands a logarithmic relationship between the proton motive force and the phosphorylation potential. It will be shown that in intact chloroplasts this expectation is not borne out by experiment. Therefore, in intact chloroplasts, the proton motive force and the phosphorylation potential are far from equilibrium even under conditions, where adenylate turnover is slow. This implies that ATP synthesis is not a rate-limiting step in photosynthesis. It will be discussed that such a conclusion is at variance with experimental observations and thermodynamic considerations.

Material and Methods

Chloroplasts capable of photoreducing CO_2 at high rates were prepared from fresh greenhouse or field-grown spinach leaves by a modification [12] of Jensen and Bassham's procedure [13]. Chlorophyll was determined according

to Arnon [14]. Chloroplasts (33 μg/ml if not indicated otherwise) were suspended in a reaction medium containing 0.33 M sorbitol, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4 (if not indicated otherwise), pH 7.6. Concentrations of substrates were usually 1 or 2 mM, the concentration of 9-aminoacridine 5 μM. Catalase (1500 or 2000 I.U./ml) was also added. Oxygen evolution was recorded by a Clark type electrode, 9-aminoacridine fluorescence by a photomultiplier which was protected against actinic and short wavelength light by proper filter combinations as described elsewhere [15]. Short wavelength light for excitation of 9-aminoacridine fluorescence was provided by a 450 W Xenon lamp with a proper filter combination [15]. Red actinic light was provided by a halogen lamp and was filtered through 8 cm water, 1 mm Calflex C (Balzers, Liechtenstein) and 3 mm RG 630 from Schott. For the measurements of quantum requirements, a 674 nm interference filter (from Balzers) was also added. Light absorption by the chloroplast suspension was determined in an Ulbricht sphere. Rates of oxygen evolution were corrected for broken chloroplasts which absorbed light without contributing to oxygen evolution. The percentage of broken chloroplasts contaminating suspensions of intact chloroplasts was determined by the ferricyanide method [5].

Adenylates were determined by the luciferin-luciferase method with an extract from firefly lanterns (Sigma FLE 50) as described in Ref. 16. Calibration was done with known amounts of a freshly prepared ATP solution.

Results and Discussion

I. Energy charge and phosphorylation potential

Intact chloroplasts can reduce a number of substrates such as nitrite, oxaloacetate, phosphoglycerate, glycerate or bicarbonate without the addition of cofactors. Their envelope retains all components necessary for photosynthesis including adenylates and enzymes such as adenylate kinase [2]. When chloroplasts are illuminated with nitrite, oxaloacetate or phosphoglycerate, reduction usually proceeds without a pronounced lag phase and intrachloroplast ATP levels increase to a maximum within a very short time, often less than 3 s [8,17]. With nitrite or oxaloacetate as electron acceptor, the intrachloroplast ATP pool reached its maximum size at very low light intensities (20 $W \cdot m^{-2}$, for nitrite, Fig. 1). These substrates do not consume ATP during reduction. In contrast, glycerate, phosphoglycerate and CO_2 require ATP for reduction. When CO_2 was the substrate, much higher light intensities were needed to saturate ATP pools. With glycerate as substrate, ATP pools remained low even under high light intensities [8]. Steady state conditions for reduction were reached rapidly for all substrates except CO_2. Adenylates were measured under steady state conditions both in the dark and in the light. Fig. 2 shows the intrachloroplast adenylate distribution under a variety of metabolic flux situations as a function of energy charge (EC)

$$EC = (2[ATP] + [ADP])/2([ATP] + [ADP] + [AMP])$$

The concept of energy charge was introduced by Atkinson [18] to describe

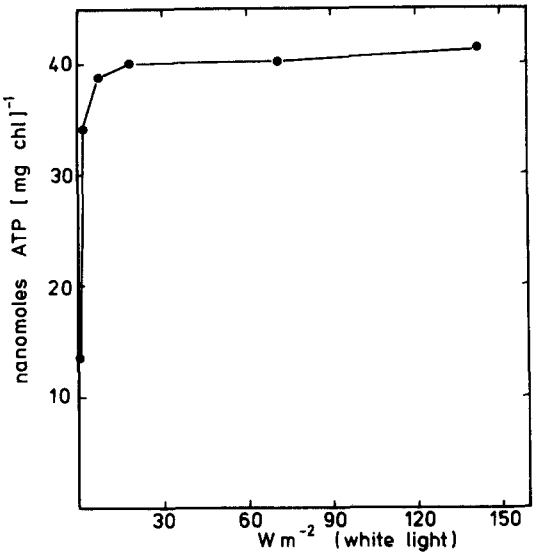

Fig. 1. ATP levels in intact chloroplasts as a function of the intensity of white light. Electron acceptor was 2 mM KNO_2.

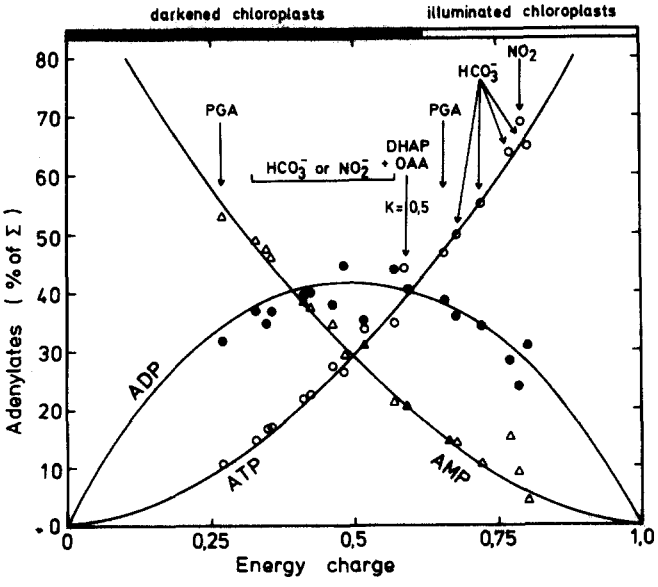

Fig. 2. Relative adenylate concentrations and energy charge in intact chloroplasts in the dark and in the light in the presence of different electron acceptors. Drawn curves show relative adenylate concentrations calculated under the assumption that adenylate kinase equilibrates adenylates according to ATP + AMP $\rightleftarrows$ 2 ADP. The equilibrium constant $[ATP] \cdot [AMP]/[ADP]^2$ used for the calculation is 0.5. Phosphate concentration in the medium was 0.5 mM. PGA, phosphoglycerate; DHAP, dihydroxyacetone phosphate; OAA, oxaloacetate.

the extent to which the adenylate system is filled with phosphate. The drawn curves in Fig. 2 reflect the adenylate distribution at different energy charge values as calculated under the assumption that adenylates are in adenylate kinase equilibrium (equilibrium constant 0.5, see Refs. 19 and 20). The measured adenylate distribution fits the curves closely indicating that the adenylates were close to adenylate kinase equilibrium both in the dark and in the light. Deviations from equilibrium seen under some conditions in illuminated chloroplasts have been discussed previously [8]. It is also apparent that darkened intact chloroplasts contain significant ATP and that the ATP/ADP ratio in the dark was in different chloroplast preparations between about 0.5 and 1. On illumination, the ratios increased to values not much higher than 2. Energy charge in the dark was between 0.25 and 0.6. It increased in the light to values not much higher than 0.8. Thus the energy charge of illuminated chloroplasts was close to the values reported for chloroplasts in vivo [6,7] and also close to the value assumed to be optimal for the interaction between energy-producing and energy-consuming reactions [18]. In contrast, at adenylate kinase equilibrium the high phosphorylation potential of 30 000 M^{-1} reported for broken illuminated chloroplasts [1] should correspond to an energy charge very close to the maximum value of 1 which energy charge can attain. Since the adenylate data of Fig. 2 were measured under conditions where no efforts were made to minimize adenylate turnover (CO_2 was not rigorously excluded in the nitrite experiment of Fig. 2), it was of interest to see whether intact chloroplasts could increase energy charge to values approaching 1 when ATP consuming reactions were suppressed. To optimize conditions for ATP production, the chloroplasts were illuminated with saturating light and nitrite or oxaloacetate were used as electron acceptors. Reduction of bicarbonate contaminating the chloroplast suspensions was inhibited by 10 mM phosphate [21]. Under these conditions, photosynthetic ATP consumption is minimized. Fig. 3 shows that the maximal energy charge obtained by chloroplasts photoreducing nitrite or oxaloacetate under light saturation was still not significantly higher than 0.8. The energy charge of chloroplasts kept in the dark, with or without added oxaloacetate, was in the presence of a high phosphate concentration between 0.38 and 0.53. Chloroplasts completely uncoupled by a high concentration of CCCP in the light did not decrease energy charge to zero, but maintained it at a value of 0.36.

It must be emphasized that the surprisingly low maximum ATP/ADP ratios and energy charge values calculated from adenylate measurements in extracts from illuminated intact chloroplasts are not an experimental artifact caused by the extraction not only of free but also of bound ADP. If binding of ADP to chloroplast constituents were significant enough to decrease measured ATP/ADP ratios as compared with the corresponding ratios of free adenylates in the chloroplasts, the measured adenylate values should not fit the curves drawn in Figs. 2 and 3 for adenylate kinase equilibrium. Since close fit is observed, it is concluded that binding of adenylates is not considerable and can be neglected [8].

Since energy charge and ATP/ADP ratios in intact chloroplasts were far below the values expected from the reported capacity of chloroplasts to phosphorylate ADP, we wanted to know whether our chloroplasts had a diminished

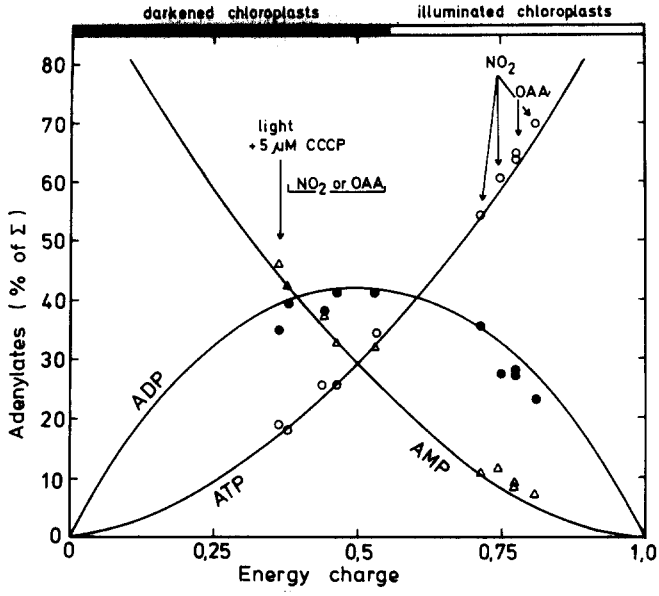

Fig. 3. Relative adenylate concentrations and energy charge in intact chloroplasts. Photosynthetic adenylate turnover was inhibited by 10.5 mM phosphate. See also legend to Fig. 2.

capacity for phosphorylation. When intact chloroplast were osmotically disrupted immediately before the experiment, illumination with saturating light in an isotonic medium containing 5 mM Mg^{2+}, 0.5 mM P_i, 50 µM methyl viologen and proper adenylate concentrations showed that broken chloroplasts can synthesize ATP of a $\Delta G'_{ATP}$ of a least —60 kJ/mol, if the $\Delta G'_{o\,ATP}$ is taken to be —31.4 kJ/mol [22]. These results are very similar to those described by Kraayenhof [1]. The corresponding phosphorylation potential is more than than $8 \cdot 10^4$ M^{-1}. This value is even higher than that reported by Kraayenhof [1]. It should be noted that the rate of phosphorylation did not depend on the phosphorylation potential in the medium for potentials usually observed in intact chloroplasts, whereas is showed a striking dependence on the phosphorylation potential for higher values.

At a given phosphorylation potential, ATP/ADP ratios should increase with increasing phosphate concentrations and decrease with decreasing concentrations. In intact chloroplasts, phosphate is taken up by the phosphate translocator in exchange against another transferable anion such as phosphoglycerate. When phosphoglycerate was present in a suspension of intact chloroplasts at a constant concentration and the phosphate concentration was increased, ATP/ADP ratios also increased as expected, because the phosphate concentrations in the stroma could increase at the expense of stromal phosphoglycerate, which was exported in exchange for phosphate. Conversely, when the phosphate concentration in the medium was kept constant and the concentration of phosphoglycerate was increased, the phosphate translocator imported phosphoglycerate into the chloroplast stroma in exchange against phosphate. As expected, ATP/ADP ratios decreased as the increase in the phosphoglycerate concentration caused a decrease in the phosphate concentra-

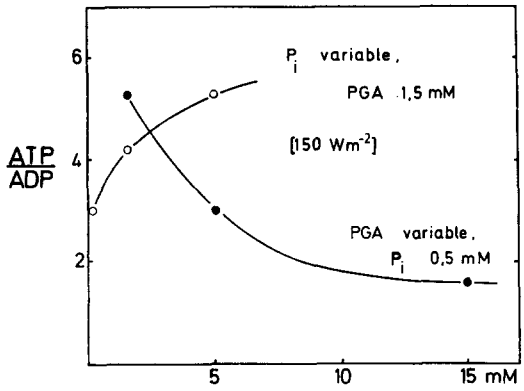

Fig. 4. ATP/ADP ratios in illuminated intact chloroplasts as a function of the concentration of phosphate and phosphoglycerate (PGA) in the medium. The phosphate translocator in the chloroplast envelope exchanges phosphate for phosphoglycerate and vice versa. The total intrachloroplast concentration of phosphate plus phosphate esters is constant [4].

tion (Fig. 4). The equilibrium situation of the phosphoglycerate kinase and glyceraldehydephosphate dehydrogenase reactions may also have been factors in the observed changes of the ATP/ADP ratios. Similar results were obtained with intact chloroplasts photoreducing CO_2 [8]; here, the stromal phosphate concentration was lowered by accumulation of phosphate esters [4].

When, however, phosphate esters such as phosphoglycerate, which can be transfered by the phosphate translocator, were not added, chloroplast ATP/ADP ratios were insensitive to phosphate (Table I). At low concentrations, phosphate is known to be taken up by chloroplasts against its concentration gradient in exchange for internal transferable substrates [23]. Obviously, even low external phosphate concentrations were sufficient to saturate the stromal

TABLE I

ADENYLATES AND PHOSPHORYLATION POTENTIALS IN INTACT CHLOROPLASTS KEPT IN THE DARK OR ILLUMINATED WITH SATURATING LIGHT

Electron acceptor was 2 mM KNO_2.

	Phosphate added to the medium		
	0.6 mM	1.5 mM	10.5 mM
	nanomol (mg Chl)$^{-1}$		
Dark			
ATP	20	21	15
ADP	20	21	14
Light			
ATP	41	43	40
ADP	12	11	13
	Phosphorylation potentials [ATP]/[ADP] · [P_i] * (M^{-1})		
Dark	83	83	90
Light	285	325	257

* Internal phosphate assumed to be 12 mM; see text.

phosphate pool. The maximum concentration of phosphate in the stroma of intact spinach chloroplasts as measured under saturation conditions [4] is about 12 mM. This value was taken to calculate the phosphorylation potential of intact chloroplasts. Table I lists ATP and ADP contents in intact chloroplasts measured in the dark and under saturating light in the presence of different phosphate concentrations. Electron acceptor was nitrite. Calculated phosphorylation potentials were about 80 M^{-1} in the dark and about 300 M^{-1} in the light. Thus the phosphorylation potential of illuminated chloroplasts differed by a factor of only about 4 from that of darkened chloroplasts. The $\Delta G'_{ATP}$ calculated from the phosphorylation potentials of intact chloroplasts in the dark and in the light are —41.8 and —45.6 kJ/mol, respectively. It should be noted that the maximal $\Delta G'_{ATP}$ of broken chloroplasts was 14.6 kJ/mol more negative than that of illuminated intact chloroplasts. The phosphorylation potentials produced by illuminated broken chloroplasts and by intact chloroplasts differed strikingly by more than 2 orders of magnitude even though light intensities used to drive electron transport were comparable in the experiments with intact and broken chloroplasts and the phosphorylation potentials were light-saturated in both cases.

II. Proton motive force and phosphorylation

During electron transport, a proton gradient and a light-dependent membrane potential are formed across thylakoid membranes. When the intrathylakoid pH was increased by adding a suitable uncoupler such as NH_4Cl, electron transport to acceptors such as nitrite or oxaloacetate was stimulated [16]. Interestingly, stromal ATP levels did not decrease [16] and phosphorylation potentials remained largely constant (Table II).

From this observation, it has been suggested that in intact illuminated chloroplasts the phosphorylation potential is far from equilibrium with the proton motive force. As has been shown above, comparable energization by light produced very different phosphorylation potentials in intact and in broken chloroplasts. Again it may be concluded that in contrast to broken chloroplasts, intact chloroplasts are incapable of approaching equilibrium between the high energy condition produced by illumination in the thylakoids and the phosphorylation potential.

In previous papers, it has been shown that the uncoupler NH_4Cl (or other uncoupling amines) failed to decrease CO_2 reduction by isolated chloroplasts [15] at concentrations which were sufficient for the maximal stimulation of

TABLE II

PHOSPHORYLATION POTENTIALS [ATP]/[ADP] · [P_i] * IN INTACT CHLOROPLASTS ILLUMINATED IN THE PRESENCE OF 1 mM OXALOACETATE AS AFFECTED BY NH_4Cl OR CCCP

External phosphate concentration 10 mM.

	Without uncoupler	1 mM NH_4Cl	5 mM NH_4Cl	5 μM CCCP
Phosphorylation potential (M^{-1})	202	198	200	45

* Internal phosphate concentration assumed to be 12 mM, see text.

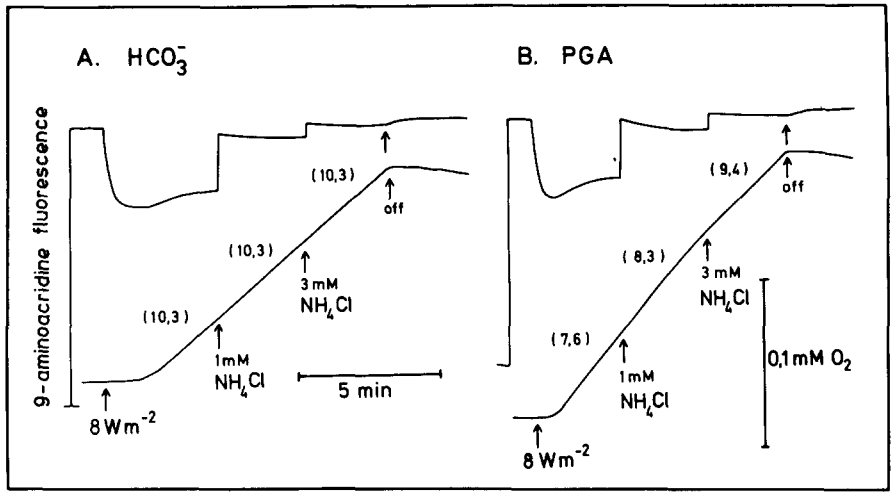

Fig. 5. Simultaneous recording of CO_2- and phosphoglycerate (PGA)-dependent oxygen evolution and of 9-aminoacridine fluorescence in a suspension of intact chloroplasts as influenced by NH_4Cl. Substrate concentration 4 mM, ascorbate 2 mM, catalase $2 \cdot 10^3$ I.U./ml. Illumination with 8 $W \cdot m^{-2}$ 674 nm light. Numbers in bracketts are quanta of red light absorbed during evolution of one molecule of oxygen. Under light saturation the chloroplast preparation photoreduced CO_2 at a rate of 302 μmol (mg Chl)$^{-1}$. h^{-1}.

light-dependent nitrite reduction [16]. Fig. 5 shows an experiment recording simultaneously CO_2 or phosphoglycerate dependent oxygen evolution and 9-aminoacridine fluorescence of a suspension of intact spinach chloroplasts illuminated with a low intensity of red light. After several minutes illumination, first 1 and than 3 mM NH_4Cl were added. The latter concentration is in four-fold excess to that required for maximal stimulation of electron flow in intact chloroplasts which is commonly used as a criterion of the effectiveness of uncoupling. Immediately after addition of NH_4Cl, there was a large increase in 9-aminoacridine fluorescence indicating a considerable decrease of the proton gradient. The rate of oxygen evolution, however, did not change. Even 10 mM NH_4Cl caused only a slight inhibition of oxygen evolution. 1 μM FCCP, however, completely abolished CO_2-dependent oxygen evolution (not shown). Since the decrease in ΔpH caused by NH_4Cl was not compensated by an increase in the membrane potential [15], the proton motive force decreased under the influence of NH_4Cl.

In addition, quantum requirements for CO_2-dependent oxygen evolution were calculated. In the experiment of Fig. 5A, 10 quanta of red light were absorbed per molecule oxygen evolved both before and after addition of 1 or 3 mM NH_4Cl and 13 quanta at 10 mM NH_4Cl (latter result not shown). When phosphoglycerate, which requires less ATP for reduction than CO_2 does, was electron acceptor, the quantum requirement of oxygen evolution was lower than with CO_2 and increased somewhat with increasing NH_4Cl concentration (Fig. 5B). Results of other experiments with either CO_2 or phosphoglycerate as electron acceptor are listed in Table III. It is interesting to note that NH_4Cl always increased the quantum requirement of phosphoglycerate-dependent oxygen evolution, while that of CO_2 reduction was often but not always

TABLE III

QUANTUM REQUIREMENTS OF CO_2- AND PHOSPHOGLYCERATE-DEPENDENT OXYGEN EVOLUTION BY INTACT CHLOROPLASTS

Illumination with 8 or 12 W · m^{-2} 674 nm light. Substrate concentrations 4 mM.

	Quanta required/O_2 evolved	
	Without NH_4Cl	With 3 mM NH_4Cl
HCO_3^-		
pH 7.6	15.2	11.6
	12	10.7
	12.8	12.8
pH 8	14	11.8
	13.6	11.8
	11.6	12.4
	11.4	11.4
Phosphoglycerate		
pH 7.6	8	9.2
	8	9.3
	10.9	12.2
pH 8	8.8	11.8
	10.9	12.9

decreased even though the ATP requirement of the latter is higher by a factor of 1.5 than that of the former. In all experiments, NH_4Cl increased 9-aminoacridine fluorescence as drastically as shown in Fig. 5. Moreover, the ΔpH across the thylakoid membranes as measured by the distribution of labelled methylamine and dimethyloxazolidinedione [24] decreased in chloroplasts photoreducing CO_2 in the presence of methylamine from 2.2 to 1.2 (Table IV), whereas the ATP/ADP ratio even increased. This increase of the ATP/ADP ratio

TABLE IV

MEASUREMENT OF THE ΔpH ACROSS THE THYLAKOID MEMBRANE AND THE ATP/ADP RATIO IN INTACT CHLOROPLASTS PERFORMING CO_2 FIXATION WITH VARIOUS CONCENTRATIONS OF METHYLAMINE ADDED

Spinach chloroplasts, 0.1 mg chlorophyll/ml in the standard medium (cf. Material and Methods) containing also 10 mM HCO_3^-, 1 mM phosphate, 0.5 mM dimethyloxazolidinedione, and methylamine as indicated, were illuminated for 5 min with 50 W · m^{-2}. The reaction was terminated by silicon layer filtering centrifugation. The pH in the stromal and in the thylakoid spaces was calculated from the distribution of dimethyl[^{14}C]oxazolidinedione and of [^{14}C]methylamine [24]. For measurement of adenine nucleotides by the luciferin-luciferase method a parallel sample of the chloroplasts was simultaneously deproteinized by perchloric acid.

Methylamine added (mM)	pH stroma	pH thylakoid	ΔpH	$\frac{ATP}{ADP}$
0	7.62	5.38	2.24	0.82
1	7.52	5.46	2.06	1.14
10	7.40	5.72	1.68	1.36
50	7.34	6.13	1.21	1.40

can be explained by a progressive inhibition if CO_2 fixation which was caused by the lowering if the stromal pH [25], not by uncoupling. This inhibition reduced ATP utilization and thereby led to an increase in ATP/ADP ratios. When oxaloacetate which in contrast to CO_2 does not consume ATP during reduction was added as electron acceptor, ATP/ADP ratios were not affected by uncoupling amines (cf. Table II).

Conclusions

A. Energy charge

Three main points emerge from energy charge data:

1. While the high energy charge of chloroplasts in darkened leaves [6,7] may be explained by direct or indirect adenylate exchange between chloroplasts and cytoplasm, it is surprising that even isolated chloroplasts can maintain a significant energy charge in the dark. This results from their ability to synthesize ATP in the dark by substrate phosphorylation [17].

2. In the light, energy charge does not rise to values significantly higher than 0.8 even when ATP consuming reactions are suppressed and electron transport is stimulated by the addition of substrates such as nitrite or oxaloacetate [8].

3. Since intact chloroplasts contain between 50 and 100 nanomoles adenylates per mg chlorophyll [26] and the osmotic volume of isotonic chloroplasts is about 25 μl per mg chlorophyll, the adenylate distribution data of Figs. 2 and 3 indicate that stromal ADP levels are always considerable. Even at very high values of energy charge, they are not much lower than half the maximum concentration which is about 1 mM. ATP levels rarely exceed ADP levels by a factor of more than 2 or 3. Together with other observations [16], this shows that electron transport in intact chloroplasts cannot be under adenylate control. Rather, a low intrathylakoid pH appears to restrict electron transport.

B. Phosphorylation potential

It is unknown why the phosphorylation potentials of broken and intact chloroplasts differ strikingly even under saturating illumination. There is either a restriction on phosphorylation or a decrease in the steady state phosphorylation potential by ATP utilization in intact chloroplasts. Adenylate kinase cannot be responsible for decreased phosphorylation potentials, as in the absence of an AMP generating reaction it can decrease the phosphorylation potential only transiently, not in the steady state. A decrease in the steady state phosphorylation potential by an ATPase such as that known to reside in the chloroplast envelope [27] is compatible with the insensitivity of the phosphorylation potential to ATP consuming electron acceptors like CO_2 or to amine uncoupling only if the apparent equilibrium constant of the ATPase is of the same order as the phosphorylation potential in illuminated intact chloroplasts. It is unlikely that such an ATPase exists. A restriction on phosphorylation in intact chloroplasts is therefore presently considered the most likely explanation for the observed drastic differences in the phosphorylation potentials of intact and broken chloroplasts.

C. Phosphorylation

Mitchell's widely accepted chemiosmotic theory, which has received impressive experimental support [28—30], holds that the proton motive force drives ATP synthesis. In intact chloroplasts the phosphorylation potential, which, together with the NADPH/NADP ratio, is the driving force of photosynthesis, is far from equilibrium with the proton motive force as conventionally measured. There are two main arguments for this statement:

1. Comparable energization of intact and broken chloroplasts by light-dependent electron transport results in steady state phosphorylation potentials which are lower by a factor of about 100 in intact chloroplasts compared with broken chloroplasts.

2. Amine uncoupling, which lowers the proton motive force, has little effect on the phosphorylation potential of intact chloroplasts and on CO_2 reduction. That the proton motive force is really decreased under the influence of NH_4Cl or other amines is indicated by measurements of electron transport, of 9-aminoacridine fluorescence, of the distribution of ^{14}C-labelled methylamine and dimethyloxazolidinedione, of chlorophyll fluorescence, of 518 nm absorption [15] and of Mg^{2+}-transport across the thylakoid membranes (Krause, G.H., private communication). If during electron transport to CO_2 via NADPH the chloroplasts are energized more than actually necessary to produce the low phosphorylation potentials generally observed in intact chloroplasts and sufficient to drive photosynthesis it must be concluded that electron transport to NADP should be able to generate more ATP than actually required for photosynthetic CO_2 reduction.

Unfortunately, this conclusion is in conflict with a number of experimental observations. When the light intensity was suddenly reduced during CO_2 reduction of intact chloroplasts, the ATP level dropped while the NADPH level did not respond [12]. This suggests that ATP rather than NADPH limits CO_2 reduction under rate-limiting light. Measurements of the quantum yield of oxygen evolution by intact chloroplasts are also suggestive of a rate-limitation by ATP: The quantum yield of CO_2 dependent oxygen evolution is lower than that of phosphoglycerate-dependent oxygen evolution (Fig. 5). Bioenergetically, the two reactions differ only by the higher ATP requirement of CO_2 reduction.

From equilibrium thermodynamics it follows that

$$1.36\, n\, (\Delta pH + \Delta\psi/59) = \Delta G'_{o\,ATP} + 1.36 \log([ATP]/[ADP][P_i]) \qquad (2)$$

n corresponds to the number of protons cooperating in the synthesis of an ATP molecule. Values for n reaching from 2 to 4 have been reported in the literature. Table V lists values of phosphorylation potential $[ATP]/[ADP][P_i]$ expected from Eqn. 2 for different values of n and $(\Delta pH + \Delta\psi/59)$. To accomodate experimentally observed phosphorylation potentials between $3 \cdot 10^4$ and $6 \cdot 10^4\ M^{-1}$ in broken chloroplasts under the assumption of equilibrium, $(\Delta pH + \Delta\psi/59)$ should be 5, 3.5 or 2.5, if 2, 3 or 4 protons cooperate in the synthesis of ATP, i.e. if $n = 2$, 3 or 4. Steady-state values for $\Delta\psi$ are below 30 mV [31], which leaves proton gradients of about 4.5, 3 or 2 pH units with corresponding n-values of 2, 3 or 4 as minimal requirements for ATP synthesis. Maximum reported ΔpH values for broken chloroplasts were not

TABLE V

PHOSPHORYLATION POTENTIALS (P) AND THEIR RELATIVE ALTERATIONS (Q_p) CALCULATED FOR DIFFERENT VALUES OF THE PROTON MOTIVE FORCE (pmf) UNDER THE ASSUMPTION OF EQUILIBRIUM BETWEEN P AND pmf

$\Delta G'_{0ATP} + 1.36 \log P = n \cdot 1.36$ pmf, $P = [ATP]/[ADP][P_i]$, pmf = $\Delta pH + \Delta\psi/59$ (n is the number of protons assumed to cooperate in the synthesis of ATP, $\Delta\psi$ the membrane potential in mV, $\Delta G'_{0ATP}$ = −31.4 kJ/mol, Ref. 22). The ratio of the phosphorylation potential at a given proton motive force to that resulting from an alteration of the proton motive force by 10% can be expressed as $Q_p = P_{(pmf)}/P_{(pmf\pm10\%)} = 10 \pm 0.1n$ pmf. The equilibrium phosphorylation potentials P given in the table have to be multiplied (divided) by Q_p if the proton motive force is increased (decreased) by 10%. It should be noted that the $\Delta G'_{ATP}$ value represented by the left part of the above equation is valid for pH 7 while the pH of the stroma phase of intact chloroplasts is close to 8 in the light [33]. The phosphorylation potentials of the table are therefore overestimate rather than underestimate.

pmf	$n = 2$		$n = 3$		$n = 4$	
	P	Q_p	P	Q_p	P	Q_p
2.0	$3 \cdot 10^{-2}$	2.51	$3 \cdot 10^{0}$	3.98	$3 \cdot 10^{2}$	6.31
2.5	$3 \cdot 10^{-1}$	3.16	$1 \cdot 10^{2}$	5.62	$3 \cdot 10^{4}$	10.0
3.0	$3 \cdot 10^{0}$	3.98	$3 \cdot 10^{3}$	7.94	$3 \cdot 10^{6}$	15.8
3.5	$3 \cdot 10^{1}$	5.01	$1 \cdot 10^{5}$	11.2	$3 \cdot 10^{8}$	25.1
4.0	$3 \cdot 10^{2}$	6.31	$3 \cdot 10^{6}$	15.9	$3 \cdot 10^{10}$	39.8
5.0	$3 \cdot 10^{4}$	10.0	$3 \cdot 10^{9}$	31.6	$3 \cdot 10^{14}$	100.0

much above 3 [32], which appears to rule out n-values of 2 and, consequently, ATP/NADP ratios as high as 2. Only a n-value of 2 would overenergize spinach chloroplasts and yield more ATP during electron transport to NADP than needed for CO_2 reduction.

In illuminated intact chloroplasts, Heldt et al. [24] have measured ΔpH values across thylakoid membranes between 2.2 and 2.8 with methylamine and dimethyloxazolidinedione. Using data from Heldt's group [33] for calibration of the 9-aminoacridine fluorescence quenching data we have calculated that 3 mM NH_4Cl lowers ΔpH by as much as 0.8 to 1 pH units. The methylamine distribution indicates a somewhat smaller decrease of the proton gradient (Table IV). From Table V it can be estimated which dramatic effect even a much smaller decrease in ΔpH would have on the phosphorylation potential under the assumption that adenylates are in equilibrium with the proton motive force. At $n = 2$, a decrease in ΔpH from 3.0 to 2.7 should reduce the phosphorylation potential to about one forth of the original value. Expected changes are even larger at higher n-values. The observation that both the phosphorylation potential and CO_2 reduction are rather insensitive to a decrease in ΔpH by NH_4Cl must therefore be regarded as striking evidence of a large deviation from equilibrium.

After the addition of NH_4Cl has collapsed the proton gradient of intact chloroplasts to values close to 2, observed phosphorylation potentials of about 300 M^{-1} are just compatible with $H^+/ATP = 3$ (or $n = 3$, Table V), if steady-state membrane potentials in photosynthesizing chloroplasts are below 30 mV (Ref. 34 and Enser, U. and Heber, U., unpublished). Again it can be seen from Table V that a H^+/ATP ratio of 2 and $ATP/2e = 2$ are in conflict with observed phosphorylation potentials.

Final comments

We would not hesitate to admit that bioenergetics should be discussed in terms of irreversible thermodynamics rather than in terms of equilibria. As to equilibration between the proton motive force and the phosphorylation potential, however, the arguments considered are not affected; they are indeed intensified: in a flux system the proton motive force has been suggested to exceed the maximal phosphorylation potential by a factor of about 2 (calculated from Ref. 35 with the data from Ref. 36). Since phosphorylation potentials of −60 kJ/mol have been observed this would require values of ($\Delta pH + \Delta\psi/59$) of 10.3, 6.9 and 5.5 for $n = 2$, 3 and 4, respectively. Even the lowest figure of 5.5, which would be needed at an ATP/2*e* ratio as low as 1, is considerably higher than available experimental data permit to calculate. In view of this it is tempting to speculate that the proton activity within the thylakoid membrane is much higher than that in the bulk phases in which measurements are performed, and that there is no full equilibration between the proton activity in the membrane phase and in the intrathylakoid space. It appears that a 'micro-chemiosmotic' theory (Refs. 37, 38, but see Ref. 39) might describe the situation better than the 'macro-chemiosmotic' theory which deals with bulk phases. However, proton movement within a hydrophobic membrane phase is necessarily restricted. This requires that sites of proton pumping should be in close proximity to sites of ATP synthesis. In contrast, the coupling enzyme believed to be responsible for ATP synthesis is unequally distributed in the thylakoid membrane and is inserted into the membrane only where the thylakoid faces the stroma region of the chloroplast (Berzborn, R.J., 1979, personal communication). Other evidence against a micro-chemiosmotic theory has recently been presented by Junge [40]. We regret that our data do not make understanding of membrane-bound phosphorylation easier. They rather complicate the problem. Impressive experimental support and conceptual simplicity have won the chemiosmotic theory wide acclaim. From our data we wonder whether reality is a simple as theory demands.

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The intrathylakoid space of intact chloroplasts has been observed to be more acidic than the stroma by about one pH unit even in the dark (Enser and Heber [41]). This pH gradient is insensitive to the addition of FCCP and therefore cannot contribute to energy conservation. Hence, the insufficiency of the proton motive force to account for observed phosphorylation potentials is even more drastic than reported in this contribution.

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