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THE RELATION OF POST-ILLUMINATION ATP FORMATION CAPACITY (X_E) TO H^+ ACCUMULATION IN CHLOROPLASTS

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SUMMARY

When methylviologen, pyocyanine or low concentrations of phenazine methosulfate serve as electron carriers, the ratio of post-illumination ATP formation (X_E) to H^+ uptake at pH 5–6 is 1/5–1/4. However, under the conditions of the experiments only 50–60 % of the X_E is trapped as ATP. Therefore the true value of the $X_E/\Delta H^+$ ratio probably approaches 1/2. With these electron carriers there is no X_E formation at pH 8. In the presence of high concentrations of phenazine methosulfate ($\geqq$ 0.1 mM) chloroplasts can develop extremely high levels of X_E even at pH 8 although there is little or no H^+ uptake. The highest level of X_E obtained at pH 8 is equivalent to 1 mole ATP trapped per 4 moles of chlorophyll. The value reaches 1 ATP per 3 chlorophyll at pH 6.5 (phenazine methosulfate, 0.3 mM). These phenomena associated with high phenazine methosulfate concentration depend on the presence of 0.05 M NaCl or certain other salts. In the absence of NaCl there is no X_E formation at pH 8 and the familiar pH rise is converted into a light-dependent, reversible pH drop.

INTRODUCTION

Illuminated chloroplasts cause a reversible rise in the pH of the suspending medium[1,2] and the formation of a high energy state "X_E" which is detected as post-illumination ATP formation[3,4]. Chloroplasts exposed to pH 4 media, containing permeant organic acids with appropriate pK's, also produce ATP when transferred to pH 8 buffer containing ADP and P_i (ref. 5). It seems therefore reasonable to equate X_E, both in the preillumination experiments and in the "acid bath" experiments, to the H^+ gradient known to exist. Since the formation of such gradients may play a crucial role in the phosphorylation process[6] any information regarding the quantitative relationships of X_E to H^+ uptake* is important. Preliminary experiments by GALMICHE *et al.*[7] gave a ratio of post-illumination ATP formation to H^+ uptake of 1/5 for pyocyanine systems. However, the theoretical significance of their value is somewhat diminished by the lack of data on the efficiency of X_E capture. A part of this paper represents an extension of the experiments of GALMICHE *et al.*[7]. Efficiency

* The term "H^+ uptake" used in this paper simply refers, for convenience, to a decrease in H^+ concentration in the medium. There is no rigorous proof that direct translocation of H^+ is involved in the light-induced pH-rise phenomenon.

of X_E trapping has been measured and the ratio of X_E to ΔH^+ has been determined under a variety of conditions. At low pH (5–6) the ratio is almost constant over a wide range of conditions, approaching a value of 1/2 if corrected for the efficiency of X_E trapping. In striking contrast X_E at pH 8 ceases to be a function of H^+ uptake, becoming instead dependent on the presence of high concentrations of phenazine methosulfate and NaCl.

MATERIALS AND METHODS

Chloroplasts

Fresh leaves of market spinach (*Spinacia oleracea* L.) were ground for 10 sec in a Waring blendor with a medium containing 0.3 M NaCl, 1 mM $MgCl_2$ and 0.04 M tricine–NaOH (pH 7.6). The homogenate was squeezed through cheesecloth and centrifuged at 2500 × *g* for 4 min. The sedimented chloroplasts were washed once (including the brief centrifugation to remove cell debris) with a medium containing 0.2 M sucrose and 1 mM $MgCl_2$, and finally taken up in a small volume of the same sucrose–$MgCl_2$ medium.

Simultaneous measurements of pH changes and X_E

A stirred chloroplast suspension (2 ml) in a small vial (diameter 1.5 cm) was illuminated in the absence of ADP and P_i and the pH changes were monitored using a miniature combination reference–glass electrode (Sargent) and a modified Heath recording pH meter. After shutting off the light with a leaf shutter, a strongly buffered ADP–$^{32}P_i$ mixture (pH 8.0) was quickly injected into the suspension. The [^{32}P]ATP formed was assayed together with appropriate dark controls by the method of AVRON[8]. For details of these and related procedures see each figure. The amount of H^+ moved during the light period was computed from the recorded pH changes, using a conversion factor obtained by determining, in a duplicate run, the volume of 0.001 M HCl necessary to bring the shifted pH back to its original level. The importance of this titration under continuous illumination for deriving ΔH^+ values has been emphasized[9].

It should be made perfectly clear that in all the experiments described in the paper the pH of the reaction mixtures in the post-illumination phosphorylation stage was 8.0. Therefore, whenever a variation in pH is indicated it refers to the pH in the preillumination stage (actually the pH before illumination).

The actinic light (560–700 nm, approx. 500 kergs/sec per cm^2) was provided from a 750-W slide projector. The temperature was 20°.

RESULTS

Time-course of pH changes and X_E formation

Fig. 1 illustrates typical time-courses of X_E formation and decay at pH 6.0 (initial pH) compared with the time-courses of pH changes assayed under identical conditions. Clearly both process have similar kinetics except for the fact that under the conditions employed the pH rise occurring in the first illumination period involves an irreversible (or only very slowly reversible) change which does not seem to be related in any way to X_E. A second illumination given after a 1- or 2-min dark period induces almost the same amount of X_E and a smaller, reversible pH rise superimposed

on the new pH level. Therefore the $X_E/\Delta H^+$ ratio becomes appreciably greater in the second light period. Also, the similarity of the kinetics of the two processes is now even more evident. (Both processes are approximately first order with a decay half-time of about 20 sec under the conditions given in Fig. 1.) For these reasons estimation of the $X_E/\Delta H^+$ ratio was based on the data from the second illumination period when the starting pH was below 6.3. Above this pH the irreversible portion of the pH change becomes smaller, almost disappearing at pH 6.5.

This pH-dependent, irreversible portion of the H^+ uptake (which is probably related with "pH gush" described elsewhere[10]) is independent of the presence of exogenous electron carriers and therefore becomes a quite small proportion of the total when phenazine methosulfate or pyocyanine mediates very large pH changes (Fig. 1A).

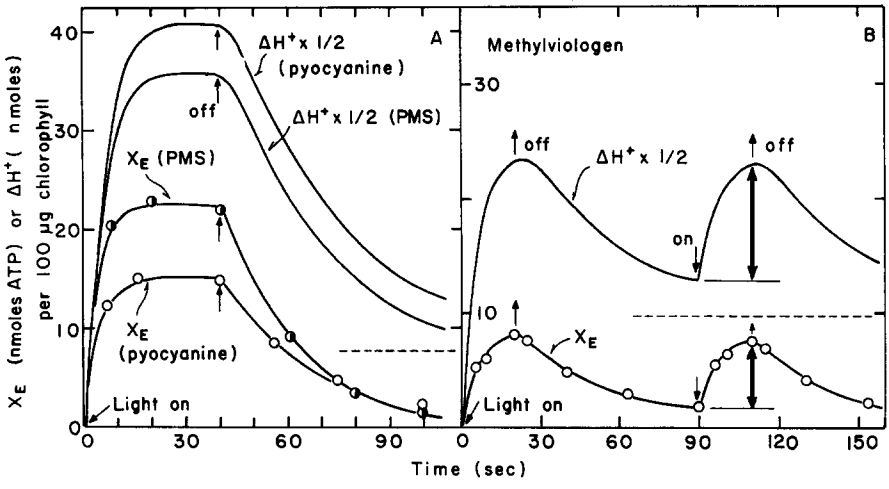

Fig. 1. Time-courses of the pH change and of X_E formation and decay. The broken lines (------) indicate the estimated levels of the "irreversible" pH shift which does not seem to be related to X_E. B, represents an extreme case in which the irreversible pH change is a large portion of the total pH change. The bold vertical arrows (↕) indicate the values of ΔH^+ and X_E taken for the computation of $X_E/\Delta H^+$ ratios. The ΔH^+ curves are reproduced from actual pH tracings to which ΔH^+ scales (determined by titration in the light; see MATERIALS AND METHODS) were applied for easier comparison with X_E. Under these conditions an increase in buffering capacity of 15–20% was observed when the pH rise reached maximum, and therefore the scales are slightly expanded at the beginning of illumination and toward the end of the dark period. The reaction mixture (2 ml) consisted of 0.1 M sucrose, 1 mM $MgCl_2$, 0.05 M NaCl, chloroplasts containing 100 µg chlorophyll and either 5 µM pyocyanine or 0.1 mM phenazine methosulfate or 0.1 mM methylviologen. The initial pH was 6.0. The time-course of X_E formation was determined in a series of identical reaction mixtures by injecting a buffered ADP–$^{32}P_i$ mixture. The buffered ADP–$^{32}P_i$ mixture (1 ml) contained 0.1 M sucrose, 1 mM $MgCl_2$, 0.05 M NaCl, 0.2 M tricine–NaOH (pH 8.0), 20 mM $Na_2H^{32}PO_4$ and 2 mM ADP. The post-illumination phosphorylation was terminated by adding 1 ml of 0.5 M $HClO_4$ 20 sec after the addition of ADP and $^{32}P_i$. For other conditions, see MATERIALS AND METHODS. PMS = phenazine methosulfate.

Estimation of efficiency of X_E capture

There is no technical difficulty in determining the apparent $X_E/\Delta H^+$ ratio. However, the apparent ratio does not mean much unless we know how efficiently we are capturing the X_E as ATP. In the present study an estimate of this efficiency was made as follows: The processes taking place during the period of post-illumination phosphorylation (pH 8) can be considered as a first approximation to be

$$X_{\text{inactive}} \xleftarrow[\text{"decay"}]{k_d} X_{\text{E. total}} \xrightarrow[\text{ADP, } P_i]{k_f} \text{ATP } (= X_{\text{E. obs.}})$$

where k_d and k_f represent the first-order rate constants of the two reactions, decay and ATP formation, competing with each other for the common "substrate", $X_{E,total}$. It is easily seen that if $k_d = k_f$ the efficiency of X_E trapping is 50 %. For a general case, $X_{E.obs.}/X_{E.total} = k_f/(k_d + k_f)$. The combined rate constant $k_d + k_f$ can be calculated from the apparent kinetics of dark ATP formation obtained by adding ADP and P_i (with pH 8 buffer) at zero time in the dark and terminating the reaction after different periods of incubation. The rate constant k_d can be computed directly from the dark decay kinetics obtained by adding pH 8 buffer alone at zero time in the dark and delaying the addition of ADP and P_i. In this latter procedure the term involving k_f (*i.e.* $k_f/(k_d - k_f)$), being common to all the points, is cancelled out. The data obtained conform closely to the expected behavior of the model. From these data the efficiency of X_E capture was estimated to be 56 ± 6 %. (For details, see Fig. 2). An efficiency of a similar order has been suggested by JAGENDORF AND HIND[11] based on more indirect evidence.

It should be noted here that the above treatments assume that the concurrent ATP synthesis does not affect the rate constant (k_d) of the decay process observable

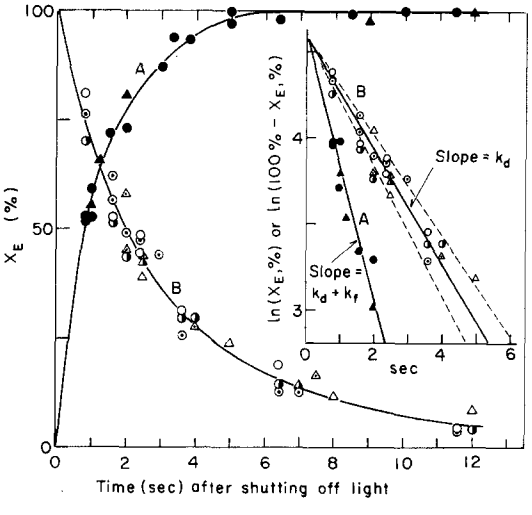

Fig. 2. Apparent kinetics of post-illumination phosphorylation (Curve A) and X_E decay (Curve B) at pH 8.0. Experiments were performed using a series of identical reaction mixtures illuminated at pH 6.3 (unbuffered) for 30 sec which was long enough to establish the steady-state level of X_E. In Curve A, a buffered ADP–$^{32}P_i$ mixture (pH 8.0) was added to the stirred chloroplast suspension immediately after shutting off the light and the [^{32}P]ATP formed was determined after the indicated lengths of time of incubation by terminating the reaction by a quick injection of $HClO_4$. In Curve B, the buffer mixture alone (sucrose, $MgCl_2$, NaCl, tricine; pH 8.0; see Fig. 1) (○ △), or buffer mixture *plus* ADP (⊙ △), or buffer mixture *plus* $^{32}P_i$ (◑ ▲) was added immediately after shutting off the light, and ADP *plus* $^{32}P_i$ or $^{32}P_i$ or ADP was added after the indicated lengths of time of delay to complete the phosphorylation reaction mixture. ATP formation was terminated after 20 sec incubation. Circles, phenazine methosulfate; triangles, pycocyanine. The combined rate constant $k_d + k_f$ obtained from Curve A was 0.76 sec^{-1} and the maximum range of values of k_d estimated from Curve B was 0.34 ± 0.04; hence $k_f = 0.42 \pm 0.04$. The efficiency of trapping of X_E under the present conditions was thus estimated to be $(0.42 \pm 0.04)/0.76 = 56 \pm 6$ %. For explanation, see text. Details of experimental conditions are given under MATERIALS AND METHODS and Fig. 1.

in the absence of ATP formation. There are no crucial experimental data to support this assumption, but neither is there any evidence which suggests that the decay process is mechanistically related to the process of ATP synthesis. The presence of ADP or P_i alone (added at zero time in the dark with pH 8 buffer) did not alter the rate of decay to any significant extent.

A prototype of the above method has been suggested by JAGENDORF AND URIBE[5].

The ratio of X_E formed to H^+ taken up at pH 5–6

At pH 5–6, with any electron carrier tested (pyocyanine, phenazine methosulfate or methylviologen) the ratio of ATP trapped to H^+ accumulated shows a relatively constant value of 1/5–1/4. This is in good agreement with the value of 1/5 for pyocyanine reported by GALMICHE *et al.*[7], and is also close to the value cited for acid-bath phosphorylation ($X_E/\Delta H^+ = 1/3$ with succinic acid as H^+ carrier; ref. 5). If one corrects these ratios for the estimated efficiency of X_E capture (56 %) the values fall near 1/2.5 (Table I). Although there is no compelling reason for assuming that the denominator is an integer, the values seem high enough to suggest that the theoretical value of the ratio may be 1/2.

TABLE I

STOICHIOMETRIC RELATIONSHIP BETWEEN X_E AND H^+ AT pH 5–6

Electron carrier	*Concn. (μM)*	*Initial pH*	*ΔH^+ (nmoles)**	*X_E (nmoles ATP)**	*$X_E/\Delta H^+$*	
					Observed	*Corrected***
Pyocyanine	5	5.0	27.6	6.2	1/4.5	1/2.5
	5	6.0	53.0	11.2	1/4.7	1/2.6
	100	5.8	40.0	9.7	1/4.1	1/2.3
Phenazine methosulfate	7	6.0	46.4	10.7	1/4.3	1/2.4
	100	5.5	53.5	11.2	1/4.8	1/2.7
Methylviologen	100	5.8	30.3	6.9	1/4.4	1/2.5
	100	6.0	30.6	6.7	1/4.6	1/2.6
Endogenous	—	5.9	24.8	6.2	1/4.0	1/2.2

* The values for ΔH^+ and X_E are for the whole reaction mixture (2 ml) containing chloroplasts equivalent to 100 μg chlorophyll. The results shown here were from several sets of experiments with different chloroplast preparations. For conditions, see MATERIALS AND METHODS and Fig. 1.

** Corrected for the estimated efficiency of trapping of X_E, 56 % (see text and Fig. 2).

The $X_E/\Delta H^+$ ratio rapidly decreases as the pH exceeds 6, becoming practically zero at pH 7.5 where active H^+ uptake nevertheless continues (see data for pyocyanine in Fig. 3). The picture is completely different, however, if a high concentration of phenazine methosulfate is employed as electron carrier (see below).

Effect of high concentrations of phenazine methosulfate and NaCl

Phenazine methosulfate at $\geqq$ 0.1 mM elicits phenomena not observed with any of the other electron carriers tested, including phenazine methosulfate itself at low concentrations ($<$10 μM). In the presence of such high concentrations of phenazine methosulfate:

(i) An exceptionally high level of X_E is formed. The highest value of X_E gives 1 mole of ATP actually trapped for 3 moles chlorophyll (pH 6.5, 0.3 mM phenazine methosulfate), a value 3 times as high as any heretofore reported for light-induced X_E (Fig. 4). We have not estimated the efficiency of X_E trapping under these extreme

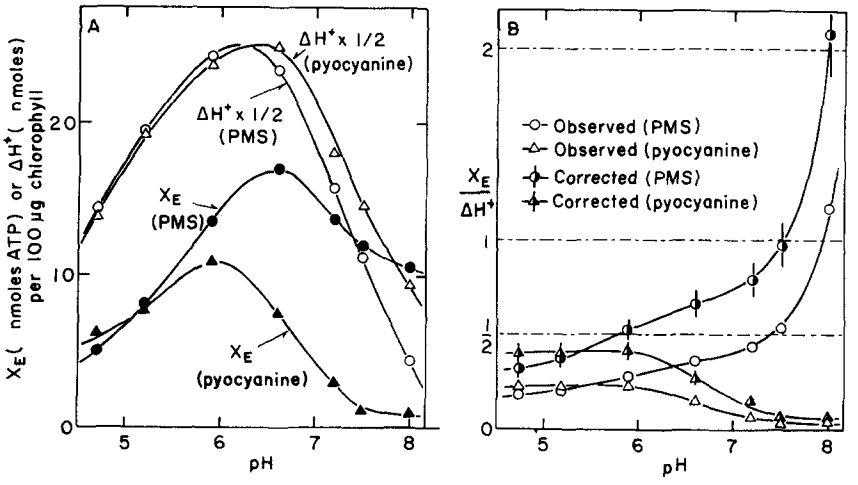

Fig. 3. A. Amounts of H^+ taken up and X_E trapped as ATP at various pH's. Experimental conditions were as in Fig. 1 except for the variations in the initial pH indicated. The pH values refer to the dark pH levels before the illumination period. (In experiments at or below pH 6 the data are for the second illumination period. See the first section of RESULTS.) B. Observed $X_E/\Delta H^+$ ratios and the ratios corrected for the efficiency ($56 \pm 6\%$) of X_E trapping. For the estimation of this X_E efficiency, see text and Fig. 2. PMS = phenazine methosulfate.

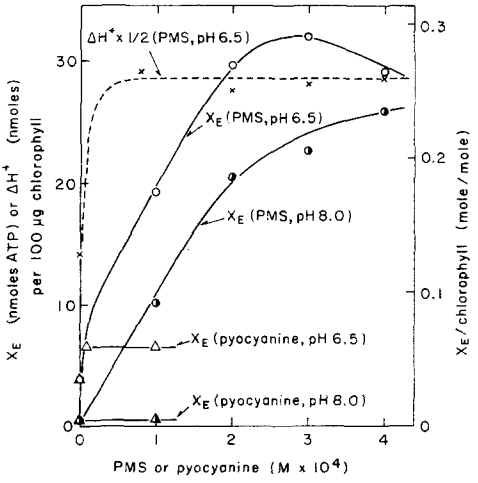

Fig. 4. Dependence of X_E formation on high concentrations of phenazine methosulfate (PMS). Conditions for the experiments at pH 6.5 were the same as in Fig. 1 except for the variations in the concentration of phenazine methosulfate and pyocyanine. This particular set of experiments with phenazine methosulfate involving preillumination at pH 8 were conducted using a buffered system: the reaction mixture for preillumination (2 ml) contained 0.1 M sucrose, 0.05 M NaCl, 1 mM $MgCl_2$, 0.03 M tricine–NaOH (pH 8.0), chloroplasts with 100 µg chlorophyll, and the indicated concentrations of phenazine methosulfate. Illumination time, 30 sec. Conditions for the post-illumination phosphorylation stage were the same as in Fig. 1.

conditions. It would not be surprising if future studies revealed that the size of X_E pool can equal the amount of chlorophyll.

(ii) X_E does not disappear at pH 7.5 or even at pH 8.4. In fact with 0.1 mM phenazine methosulfate an X_E yield of 1 ATP per 10 chlorophylls is routinely observed at pH 8 (Fig. 3) and more than half as much at pH 8.4. The X_E at pH 8 reaches 1 ATP trapped for 4 chlorophylls when the phenazine methosulfate concentration is raised to 0.3 mM (Fig. 4), a value very similar to the highest yield of acid-induced X_E (ref. 5).

(iv) X_E formation at pH 8 is totally dependent on the presence of high concentrations of NaCl. See below.

Effect of NaCl omission on X_E and H^+ uptake

It should be noted that all of the experiments described above were conducted with 0.05 M NaCl present. Omission of this NaCl only mildly depresses (by at most 30%) the pH rise and X_E formation if the electron carrier is pyocyanine or low concentrations of phenazine methosulfate ($<10\ \mu M$). (The $MgCl_2$ which is always present at 1 mM is sufficient to maintain membrane integrity and satisfies the Cl^- requirements of the electron transport system.) However, as indicated above, the phenomena associated with high phenazine methosulfate require high NaCl (optimum 0.05–0.1 M). In the absence of NaCl both X_E formation and H^+ uptake are 80% suppressed at pH 6.5. At pH 8, omission of the NaCl completely suppresses X_E formation and the pH rise in the medium is now converted into a pH drop (Fig. 5). The extent of this reversible pH drop, equivalent to 30–40 nmoles H^+ per 100 μg chlorophyll, is similar in magnitude to the more familiar pH rise at pH 8 with pyocyanine. Re-addition of 0.05 M NaCl obliterates the pH drop, sometimes converting it into a small pH rise, and reactivates X_E formation (Table II and Fig. 5).

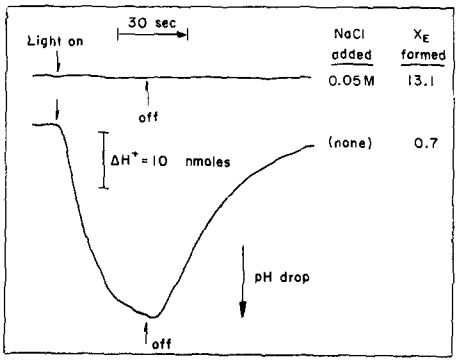

Fig. 5. Light-induced pH drop in the presence of 0.1 mM phenazine methosulfate and its prevention by NaCl. The pH level before illumination was 8.0. The extent of the pH drop was about 0.15 pH unit. The values of X_E (obtained after 30 sec illumination) are for 100 μg chlorophyll. The composition of reaction mixtures for preillumination stage was as in Fig. 1 except for the omission and re-addition of NaCl. Conditions for the post-illumination phosphorylation stage were the same as in Fig. 1.

Below pH 6, however, both X_E formation and H^+ uptake in the presence of high phenazine methosulfate becomes less dependent on NaCl and the correlation between X_E and H^+ uptake becomes more evident. At pH 5 the high-phenazine

TABLE II

THE YIELDS OF X_E AND ΔH^+ AT pH 8.0 UNDER VARIOUS CONDITIONS

Electron carrier	*Concn.* (μM)	*NaCl* (0.05 *M*)	ΔH^+ (*nmoles*)*	X_E (*nmoles ATP*)*	$X_E/\Delta H^+$
Phenazine methosulfate	5	—	24.2	0.5	0.02
	5	+	21.2	0.9	0.04
	100	—	−42.5**	0.7	—
	100	+	0.1***	10.2	(∞)
Pyocyanine	5	—	23.4	0.2	0.01
	5	+	31.5	0.2	0.01
	100	—	26.0	0.3	0.01
	100	+	37.0	0.4	0.01
Methylviologen	100	—	6.0	0.3	0.05
	100	+	8.6	0.2	0.02
Ferricyanide	100	—	§	0.8	—
	100	+	§	0.4	—

* The values are for the whole reaction mixture (2 ml) containing chloroplasts equivalent to 100 μg chlorophyll. Preillumination time, 30 sec. For experimental conditions, see MATERIALS AND METHODS and Fig. 1.

** A light-induced reversible pH drop (see text).

*** This value is highly variable but is consistently very small (< 10 nmoles).

§ An irreversible pH drop due to ferricyanide reduction.

methosulfate systems have a NaCl dependence which is not very different from the dependence of pyocyanine or low-phenazine methosulfate systems.

In their earlier observations SHEN AND SHEN[3] did, in fact, detect a significant amount of X_E at pH 7.8. These observations, which until now had not been confirmed in other laboratories, can probably be explained in terms of the reaction mixture used. Their system contained fairly high phenazine methosulfate concentration (50 μM) and 0.3 M NaCl.

DISCUSSION

At pH's between 5 and 6 illuminated chloroplasts attain the capacity (X_E) to form 1 ATP molecule for every 4 or 5 H^+ accumulated, regardless of the electron acceptor used. Moreover the kinetics of the decay of the H^+ gradient and X_E are very similar. These observations strongly suggest that, at least at these pH's, X_E formation and the pH rise in the medium are two manifestations of a single phenomenon, development of an ion gradient across the lamellar membranes. This possibility has been discussed in detail[5].

When the efficiency of X_E capture is taken into account it is seen that the true value of the $X_E/\Delta H^+$ ratio is very likely 1/2. This is the stoichiometry predicted by the chemiosmotic hypothesis[6]. Although the reported values of the ratio of H^+ taken up to electron transported are diverse[10,12–16], all of the data agree that the H^+/e^- is certainly not less than 1.0 even at pH 8. Thus, if we assume that the mechanism of photophosphorylation consists of an electron transport-driven H^+ accumulation followed by a utilization of the ion gradient for ATP formation, a realistic

stoichiometry of photophosphorylation ($P/e_2^- = 1$, at least) can be computed from the data for these two components of the reaction. Unfortunately this computation applies the value of the ratio $X_E/\Delta H^+ = 1/2$ determined at pH 6 to the optimal photophosphorylation conditions (pH 8). Actually the ratio is far too low ($<1/20$) at pH 8 and, indeed, at this pH the relation of the barely detectable X_E to H^+ uptake is obscure (Table II).

A very large amount of X_E is formed at pH 8 under special conditions, that is, in the presence of high concentrations of both phenazine methosulfate and NaCl. (A brief screening indicated that NaCl, KCl, NaBr, KNO_3, sodium citrate, *etc.*, are similarly effective.) There is little or no H^+ uptake under these conditions. Recent experiments have shown that under the same conditions illumination of chloroplasts causes a rapid, salt-dependent uptake of phenazine methosulfate and a net reduction of a portion of phenazine methosulfate. If this reduction takes place inside the lamellar membranes, an internal acidification could occur: $H_2O + PMS^+ \rightarrow 1/2\ O_2 + PMSH + H^+$ (PMS: phenazine methosulfate) and a large H^+ gradient might therefore result without any change in the pH of the external medium. This hypothesis is now being examined.

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