The H^+/e Ratio in Chloroplasts is 2. Possible Errors in its Determination

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Chloroplasts H^+/e Ratio, Buffering Capacities

The number of H^+ released inside the chloroplast thylakoids per electron flowing to the terminal electron acceptor (the H^+/e ratio) has been investigated using a pH indicator dye technique. The apparent H^+/e ratio was measured by comparing the absorbance change due to H^+-uptake outside the thylakoids in the absence of uncoupling agent, and the absorbance change due to the net H^+-production associated with water-splitting after addition of uncoupling agent in the presence of ferricyanide.

(1) The apparent H^+/e ratio was found to be higher than 2, using methylamine as uncoupling agent (>1 mM). It varied with the concentrations of added buffer.
(2) The apparent H^+/e ratio obtained with methylamine as uncoupling agent (with or without additional buffer) approached the value of 2 with decreasing concentrations of methylamine.
(3) A consistent H^+/e ratio of 2 was also obtained with gramicidin or the nigericin-type ionophore dianemycin over a relatively large concentration range, and did not vary with addition of extra buffer.
(4) The buffering capacity of the chloroplast suspension was estimated and was found to be the same order of magnitude as that of methylamine at the concentrations used.

We suggest that the high H^+/e ratios reported by Fowler and Kok (C. F. Fowler and B. Kok, Biochim. Biophys. Acta **423**, 510–523 [1976]) reflect a failure to take account of the change in buffering capacity of the chloroplast suspension on adding uncoupling concentrations of methylamine. Other possible sources of error in the estimation of H^+/e ratio by different techniques are also discussed.

Introduction

Illumination of a suspension of chloroplasts induces a release of H^+ within the thylakoids due to the splitting of water [1–4] and a translocation of H^+ from the outer phase which is coupled to electron transport to a terminal electron acceptor such as methylviologen or ferricyanide [1–6]. Several attempts have been made to measure the stoichiometry of H^+ translocated from the outer phase or released into the inner phase as one electron flows to the terminal electron acceptor since this value is of fundamental importance in testing hypotheses about the structural and functional organisation of the chloroplast membrane and the mechanism of energy coupling [1, 2, 6–12]. The number of protons released inside the thylakoids per electron flowing through the non-cyclic chain from H_2O to the terminal acceptor (the conventional H^+/e ratio) tending toward 2 was given in most recent works [1, 2, 6–8, 11]. However, values of 4 in the presence of a viologen-type acceptor or 3 in the presence of ferricyanide were given by Fowler and Kok [12] using a fast and sensitive glass electrode technique. These authors showed that the values were similar either in flashing light of short duration or in continuous light at moderate intensity. In order to explain their results, Fowler and Kok [2] had to suggest complicated models of coupling between electron and proton transport, although most of previous interpretations favoured a simple zig zag scheme of electron flow according to Mitchell's chemi-osmotic hypothesis (for details see ref. 13).

In this report we present data suggesting that the true H^+/e ratio is 2 and not 3 or 4 as suggested by Fowler and Kok [12]. We have used a pH indicator dye technique [2] to follow pH changes in a suspension of non dark-adapted broken chloroplasts excited by short saturating flashes. The *apparent* H^+/e ratio was measured by comparing the absorbance change due to H^+-uptake outside the thylakoids in the absence of any uncoupling agent, and the absorbance change due to the net H^+-production accompanying the water-splitting in the presence of ferricyanide after addition of uncoupling agent, as described in ref. 2. We have found that the apparent H^+/e ratio depended on the type and concentration of

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uncoupler used and on the concentration of added buffer, and we have shown that consistent values of 2 were obtained only when the buffering power of the uncoupler added was a small fraction of the total buffering power of the chloroplast suspension. We suggest that the high H^+/e ratios reported by Fowler and Kok [12] reflect a failure to take account of the change in buffering power of the chloroplast suspension on adding uncoupling concentrations of methylamine. Furthermore, we have discussed other possible reasons for a measurement of high values of H^+/e ratios with the glass electrode technique of Fowler and Kok [12].

Materials and Methods

Chloroplasts preparation and spectroscopic measurements

Chloroplasts were prepared from spinach grown in a greenhouse with supplementary illumination and used immediately after preparation within the next 4 hours. The extraction medium contained 400 mM sucrose, 50 mM sodium phosphate pH 7.8, 10 mM NaCl and 1 mM $MgCl_2$. After removal of cell fragments, and centrifugation at $1500 \times g$, the chloroplast pellet was resuspended in 20 mM KCl and 1 mM $MgCl_2$ to a final chlorophyll concentration of 0.1 – 0.6 mg/ml and resedimented at $5000 \times g$ for 5 – 10 min. The pellet was then finally resuspended in 400 mM sucrose, 10 mM KCl and 1 mM $MgCl_2$ to a concentration of 2 – 4 mg/ml chlorophyll. The "broken" chloroplasts prepared in this way had a very low buffering power and were coupled, since the decay of the electric field across the membrane indicated by the electrochromic band shift at 515 nm [14] was relatively not very fast (half-time $\cong$ 50 – 80 msec) compared with more than a 10 fold acceleration in ATPase-resolved chloroplasts [15].

Spectroscopic measurements were performed as described in a previous paper [16]. A (1×1) cm open cuvette was used and it contained 2.5 ml reaction mixture. Any addition of reagent produced a maximal dilution of 2%. The colour change of the pH indicator cresol red was measured at a wavelength where the background changes (measured in the presence of 10 mM tricine) were negligible (*e. g.* at 572 nm, 575 nm or 577 nm). The absorbance changes indicating the electric field across the membrane [14] were measured at 515 nm under conditions similar to those used for the measurement of the absorbance changes of cresol red. The time constant of the recording apparatus was 50 μsec for the 515 nm change and 5 – 10 msec for the cresol red absorbance change. All traces were obtained from an average of 8 flashes after a pre-illumination by $\cong$ 4 flashes of the sample kept in the weak measuring beam [16]. The time between the flashes was kept to about 11 sec to avoid any significant interfering limiting factors. The standard reaction mixture contained 20 mM KCl, 1 mM $MgCl_2$, 20 μM cresol red (from an ethanol-stock solution), 15 – 25 μg/ml chlorophyll and 0.1 mM ferricyanide with other additions as indicated. The pH was kept constant at pH 7.6 ± 0.1.

Measurement of the apparent H^+/e ratios

The absorbance change due to the change in concentration of the anionic form of cresol red is proportional to the change in concentration of H^+, $\Delta[H^+]$. The latter is a function of the amount of H^+ produced or consumed, ΔH^+, and the buffering capacity of the medium, β, as defined by Eqn (1).

$$\beta = \Delta H^+ / \Delta pH \,. \tag{1}$$

The change in absorbance is proportional to the amount of H^+ produced or consumed, as long as the change in $[H^+]$ is small ($\Delta pH < 10^{-2}$), but only insofar as β remains constant.

In the present paper we have measured the *apparent* uptake of H^+ from the *e*xternal medium (ΔH_e^+), and the *n*et release of H^+ on oxidation of water when translocated protons were allowed to equilibrate rapidly in the presence of an uncoupling agent (ΔH_n^+), by comparison of the colour change in the *a*bsence ($\Delta \%T_a$) and *p*resence ($\Delta \%T_p$) of uncoupler, so that

$$\frac{\Delta \%T_a}{\Delta \%T_p} = \frac{\Delta H_e^+}{\Delta H_n^+} \,. \tag{2}$$

Assuming that the *true* ratio is constant, we would expect the *apparent* ratio as expressed above to vary only if the buffering capacity, β, varied on addition of uncoupling agent.

The ratio of Eqn (2) can be related to more conventional H^+/e ratios (the number of protons released inside the thylakoid per electron flowing through the non-cyclic chain from H_2O to acceptor) by equating ΔH_n^+ with the number of electrons entering the chain [1, 2, 12] and by assuming that the protons taken up from the external medium are subsequently released inside [2, 4]. Then the apparent H^+/e ratio is given by:

$$\frac{H^+}{e} = \frac{\Delta \%T_p - \Delta \%T_a}{\Delta \%T_p} = 1 - \frac{\Delta \%T_a}{\Delta \%T_p} \,. \tag{3}$$

Since the sign of the transmission change is opposite for $\Delta \%T_p$ and ΔT_a (see Results), l.c. the apparent H^+/e ratio is always greater than 1 (the value due

to the stoicheiometric release of protons on oxidation of water), by an amount equal to the ratio of Eqn (2). In the figures, the apparent H^+/e ratio, observed as above, is plotted where indicated.

Solutions of methylammoniumhydrochloride and of NaOH used to adjust the pH, were freshly made before each set of measurements. Gramicidin D and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were purchased from Sigma and Boehringer respectively. Dianemycin was kindly supplied by Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis, Indiana (USA).

Results

Fig. 1 (trace A) shows the absorbance change of cresol red indicating an increase of pH (H^+ being taken up) induced by a short flash in the presence of ferricyanide (0.1 mM) and in the absence of any uncoupling agent. In the presence of benzylviologen the extent was nearly doubled due to the protonation

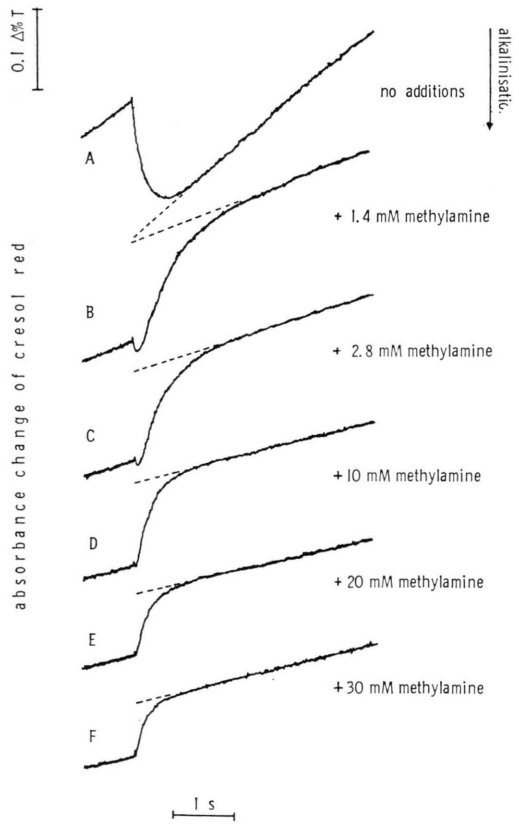

Fig. 1. Absorbance changes of cresol red in the absence and in the presence of methylamine. The measuring wavelength was 577 nm. The chlorophyll concentration was 21 μg/ml. For other details, see Methods.

of reduced viologen (not shown) as previously observed by Junge and Ausländer [2] with the same technique, or by Fowler and Kok [10] with the glass electrode technique. Addition of uncoupling agent allowed a faster exchange of H^+ between the external and internal thylakoid spaces. Thus in the presence of ferricyanide and uncoupling agent a net decrease of pH (H^+ being produced) due to the splitting of water could be observed (Fig. 1, traces B – F). Increasing concentrations of uncoupler such as methylamine [7] seemed to decrease the extent of the pH change induced by the flash. At 1.4 mM, under our conditions, a small H^+-uptake could still be seen followed by a larger H^+-release. At high concentrations of methylamine the H^+-efflux was so fast that the H^+ influx was totally masked. The half-time of the H^+-efflux varied from 500 msec at 1.4 mM methylamine to $\cong$ 80 msec toward higher concentration.

Since the pH changes were relatively slow ($t\frac{1}{2} \cong 80 - 500$ msec), values extrapolated to zero time after the flash were used for calculations, as shown by the dotted line in Fig. 1. The apparent H^+/e ratio from Fig. 1 seemed to increase from nearly 2 at low concentrations to 3 – 4 at higher concentrations of methylamine (see also Fig. 2 B). Two possible explanations could account for this finding. (1) Methylamine at relatively high concentrations (5 – 30 mM) may inhibit the electron transport from water to ferricyanide, so that less than 1 H^+ is released per flash and electron transport chain. (2) Methylamine does not significantly inhibit the release of H^+ but acts as a buffer and consequently changes the buffering capacity of the medium. The first possibility was excluded by measuring the initial amplitude of the electrochromic band shift at 515 nm [14]. Since water is the main electron donor to the electron transport chain, inhibition of water-splitting also inhibits the charge separation at the reactive centres under repetitive conditions. Fig. 2 A shows that at concentrations of methylamine up to 20 – 30 mM the initial amplitude of the 515 nm shift was hardly affected (but see also ref. 18). The second possibility that methylamine acts as a buffer could be tested by increasing the buffering capacity of the chloroplast suspension, so that the change in buffering capacity on adding methylamine would be less significant. This was achieved in two ways: (i) addition of low concentration of tricine as an overall buffer, and (ii) ad-

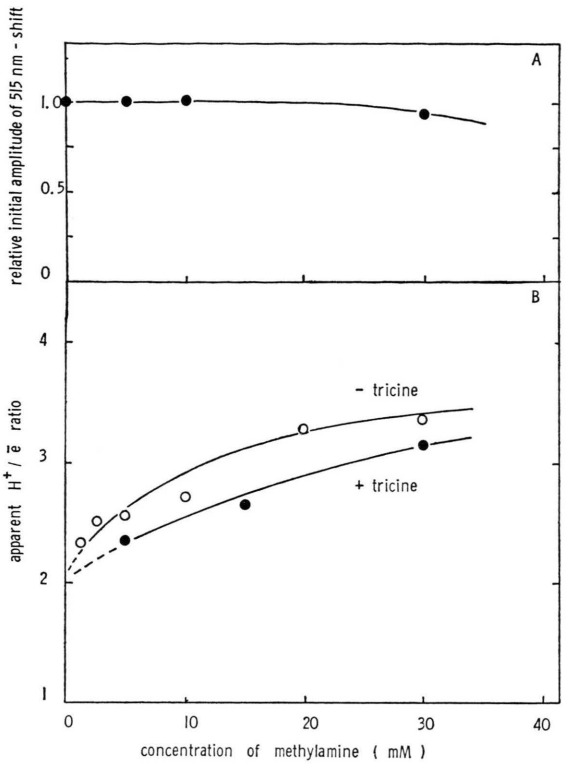

Fig. 2. Relative flash-induced amplitude of the 515 nm-shift (A), and the apparent H^+/e ratio (B), as a function of the concentration of methylamine. The 515 nm absorbance changes were measured in the presence of 10 mM tricine pH 7.6. The data in (B) were taken from Figs 1 and 3 and calculated as described in Methods. The tricine concentration in (B) was 25 μM, pH 7.6.

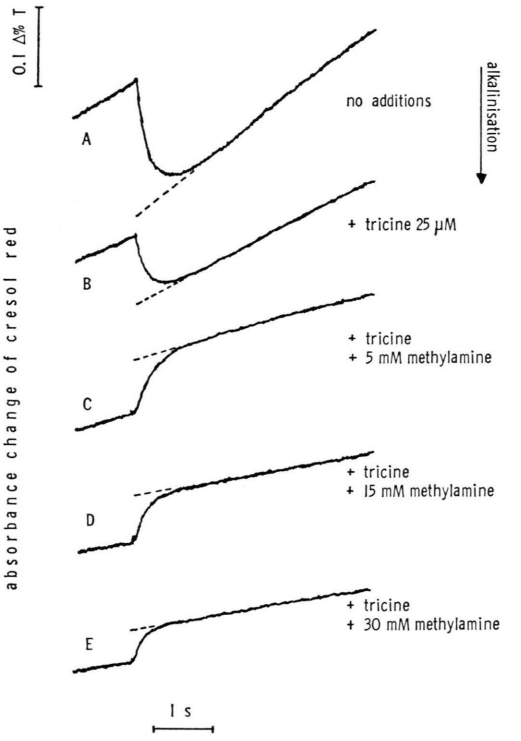

Fig. 3. Absorbance changes of cresol red in the absence and presence of methylamine with supplementary addition of tricine. The traces were obtained using the same chloroplast preparation as in Figs 1 and 2. Conditions similar to those described in Fig. 1 and Methods.

dition of bovine serum albumin as a buffer for the external phase [19]. Fig. 3 shows the effect of methylamine on the absorbance changes of cresol red obtained in the presence of low concentration of tricine. In the chloroplast preparation used, 25 μM tricine was enough to inhibit the flash-induced pH increase by approximately 50%. Comparison with the traces in Fig. 1 shows that higher concentrations of methylamine were needed to obtain a similar apparent H^+/e ratio (Fig. 2 B). Thus by increasing the buffering capacity of the chloroplast suspension, the buffering by methylamine was less and less apparent. A similar effect could be seen in the presence of bovine serum albumin. Fig. 4 A shows the decrease of the flash-induced increase of pH on increasing the bovine serum albumin concentration, reflecting the change in buffering power of the chloroplast suspension. In this preparation, at 20 mM methylamine and in the absence of serum albumin the apparent H^+/e ratio was nearly 3. The ratio approached a value of 2 as increasing amounts of bovine serum albumin were added.

We also tested the effect of the nigericin-type ionophore dianemycin. As is shown in Fig. 5 the H^+/e ratio was nearly constant ($\cong 2$) from 1 μg/ml dianemycin up to $4-5$ times this concentration, though at higher concentrations the ionophore probably also showed a buffering effect. The buffering power of methylamine could once more be demonstrated when both dianemycin and methylamine were present. The pH decrease in the presence of dianemycin at 1 μg/ml was strongly inhibited if methylamine (20 mM) was added, so that the apparent H^+/e ratio changed from 2 to $\cong 3$ (not shown).

Finally we wish to present some results obtained with the uncouplers gramicidin and CCCP. Gramicidin, when carefully used, gave results similar to

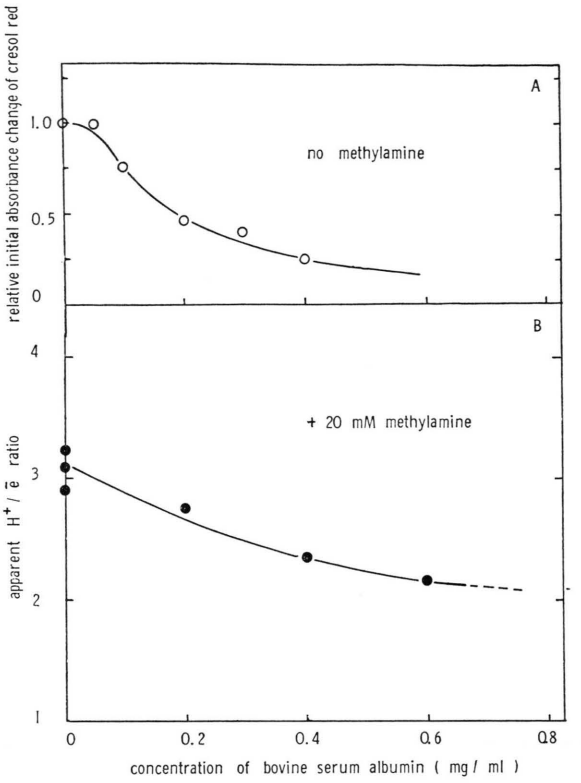

Fig. 4. Relative initial absorbance changes of cresol red in the absence of methylamine (A) and the apparent H^+/e ratio (B) as a function of the concentration of bovine serum albumin. The data were obtained using the same chloroplast preparation as in Figs 1—3. Conditions similar to those described in Fig. 1.

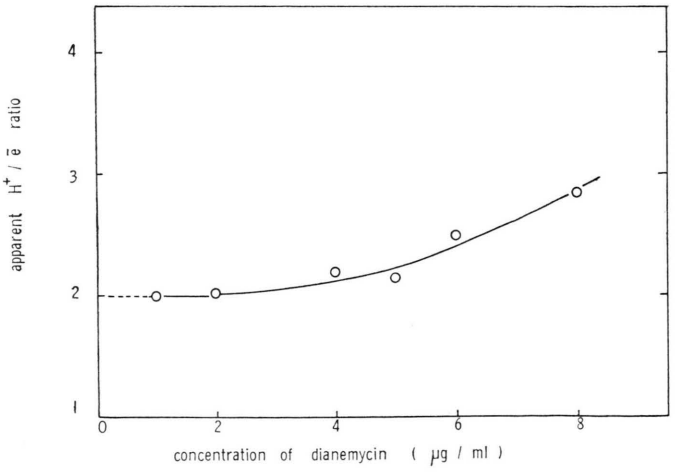

Fig. 5. The apparent H^+/e ratio as a function of the concentration of dianemycin. The data were obtained using the same chloroplast preparation as in Figs 1—4. Conditions similar to those described in Fig. 1.

those with dianemycin, yielding an apparent $H^+/e \cong 2$ at relatively low concentration ($< 3\ \mu M$ with 15 μg/ml chlorophyll in the preparation tested) (not shown). With CCCP the result were more complicated. The H^+-efflux observed at relatively low concentration (0.5 μM) was slower than with other uncouplers (half-time $\cong$ 1 sec) and at the same time the extent of the pH-decrease was relatively small, resulting in a high value of the apparent H^+/e ($\gg 2$). The H^+-efflux was accelerated at higher concentrations of CCCP but the extent of the pH decrease seemed to be even more inhibited. Two effects might compete here, the buffering effect of the uncoupler and its inhibitory effect on the water-splitting enzyme [20, 21]. Since CCCP appears to be the least suitable uncoupling agent, we have not yet investigated these effects further.

Discussion

The results obtained from Figs 1 – 5 show that the apparent H^+/e ratio approaches the *true* value of 2 when account is taken of the buffering effect of the uncoupler used. The buffering capacity of the chloroplast suspension depends obviously on the preparation procedure, and on the amount of buffer in the preparation medium. The chloroplast preparations used here contained very low concentration of additional buffers (see Methods), but if extra buffers were added (100 μM tricine), methylamine at high concentration (30 mM) could still be seen to have a significant buffering effect.

We shall see now that the conclusions drawn from the experiments in this work are consistent with some theoretical calculations. The buffering capacity β as defined above [Eqn (1)] can be calculated according to the equation derived by Van Slyke [22] for groups of known pK. For the chloroplast suspension it can be estimated in two ways:

(1) The amount of H^+ taken up or released may be estimated by calibration with a known amount of acid, and flash-induced change of pH may be measured by comparing the flash-induced change of transmission with the change of transmission per pH unit of the pH indicator (obtained from the extinction coefficient).

(2) By titration of the chloroplast suspension with a known amount of acid and observation of the pH change with a glass electrode and a sensitive pH meter.

The second method gives the total buffering capacity due to both the external and the internal phase of the chloroplast. The first method gives the buffering capacities either of the external phase alone or of the external and internal phases. The changes of pH induced by the flash in the absence of added buffers in the preparations shown in Figs 1 – 5 were $0.5 - 1 \times 10^{-2}$ pH unit (compare with pH changes given by Fowler and Kok [12] $\cong 0.1 - 0.2 \times 10^{-2}$ unit). With $\cong 3 \times 10^{-8}$ mol H^+ (per liter) taken up after the flash obtained by calibration with a known amount of HCl, or by calculations assuming 1 H^+ per $\cong 600$ chlorophylls [23] and with the concentration of chlorophyll indicated in Methods, the buffering capacity of the chloroplast suspension used was estimated to be $0.3 - 0.6 \times 10^{-5}$. Similar values were found when the 2nd method mentioned above was used. When chloroplast preparations showing a less marked effect of methylamine in increasing the H^+/e ratio were used, the estimated buffering capacity of the chloroplast suspension was correspondingly higher ($\cong 1 - 3 \times 10^{-5}$). Similar high values for the chloroplast suspension could be estimated using the data given by other authors (2×10^{-5} by Ausländer and Junge [19], 3×10^{-5} by Fowler and Kok [12] assuming a similar amount of H^+-uptake ($\cong 3 \times 10^{-8}$ mol per liter) for a similar chlorophyll concentration). These authors usually had low concentration of additional buffer in their reaction mixture ($\cong 10^{-4}$ M tricine for example). In Table I the calculated buffering capacities (β) of tricine and methylamine are listed at different concentrations and values of pH. The values shown are of the same order as those of the chloroplasts preparations. 200 μM tricine has a similar buffering power as 30 mM methylamine at pH 7. If the buffering capacity for the chloroplast suspension before addition of methylamine was less than or equal to this value (*e. g.* $\beta \cong 10^{-5}$) an apparent H^+/e ratio of 2.5 – 3 could be expected. These are probably the conditions under which Fowler and Kok [12] did their flash experiments. Fowler and Kok [12] also observed high H^+/e ratios by using the tangent method introduced by Schwartz [1], though these ratios varied, depending on the light intensity or frequency of flashing. In considering these results other sources of error in the estimation of H^+/e ratios should also be discussed.

Table I. Calculated buffering capacities of tricine, methylamine and the chloroplasts suspension.

Components	pH	Buffering capacity $\beta/10^{-5}$
Tricine (pK 8.15)		
25 μM	7	0.15
200 μM	7	1.20
Methylamine (pK 10.6)		
3 mM	7	0.17
30 mM	7	1.70
30 mM	6	0.17
Chloroplasts		
no additional buffer	7.6	0.3 – 0.6 *
with additional buffer	6 – 8	1 – 3 **

* This work (Figs 1 – 5), at a chlorophyll concentration of 15 – 21 μg/ml.

** This work and calculated from data given in refs 12 and 19.

(a) Polya and Jagendorf [24] noted the possibility that the buffering capacity of the chloroplast suspension may change within the time of recording of the traces. As mentioned in an earlier paper [16], rapid changes of external H^+-concentration are buffered mainly by the external phase, while slower changes (or changes in the presence of uncoupling agent) would be buffered by internal as well as external buffering groups. Under the conditions of our experiments (with flashing light at low frequency and with the type of preparation used) it seems that the internal buffering capacity was a small fraction of the total buffering capacity, since with dianemycin or gramicidin at low concentrations, a nearly constant H^+/e ratio ($\cong 2$) could be obtained, which did not vary with addition of extra buffer. However, as noted by Polya and Jagendorf [24] and Walz *et al.* [25] for the total buffering capacity of the chloroplasts, with flashing light at higher frequency or with continuous light at moderate intensity, the internal buffering capacity increases compared with the external one, because of the change of internal pH towards lower values. The tangent method introduced by Schwartz [1] to determine the H^+/e ratio might be prone to errors, if this effect was not taken into account, since the rate of electron transport as measured from the rate of H^+-production due to water-splitting in the presence of ferricyanide, and the steady-state of H^+-transport as measured by the initial rate of H^+-efflux on switching off the light, are measured over different time scales.

(b) The reverse diffusion potential arising from the fast H^+-efflux and the slower flux of counterions, means that the true H^+-influx in the steady-

state (the initial H^+-efflux upon darkening) can be measured only with difficulty, unless steps are taken to ensure that the flux of counter-ions is fast enough to prevent a reverse potential from building up. This is particularly true at relatively high intensity of illumination light (*cf* refs 26 and 12). Aspects of this problem have been discussed at length elsewhere [11, 26, 27].

(c) The relative rates of diffusion of protons to the glass electrode, and into the bulk buffering phase are not known for the electrode arrangement used by Fowler and Kok [3, 12]. They suspended chloroplasts in the glass electrode chamber in such a way that there was a layer of chloroplasts on the very thin glass membrane, and on the top of this was the sucrose containing medium. This is almost equivalent to having two chambers, the one containing chloroplasts, and the other containing medium, the two phases being separated from each other by a diffusion barrier.

We feel that any of these effects may account for the apparent change in H^+/e ratio observed by Fowler and Kok [12]. Our present experiments do not provide any basis for discriminating between these potential artifacts, if indeed they were involved. However, we may note that, if the stoicheiometries measured by the experiments at slow flash frequencies were in error, as indicated by our experiments, then the corresponding stoicheiometries measured under other conditions may also be suspect.

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