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THE KINETICS OF THE pH RISE IN ILLUMINATED CHLOROPLAST SUSPENSIONS

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SUMMARY

A flow method applied to a pH-measurement system was able to resolve the initial phase (< 1 sec) of the kinetics of pH changes in illuminated chloroplast suspensions. Immediately upon illumination, a rapid pH rise takes place which stops abruptly when the light is turned off; there is no significant post-illumination pH rise ('overshoot'). The marked overshoot phenomenon observed in a conventional pH recording system was analyzed and shown to be due to an instrument response lag. A graphical treatment is suggested by which ordinary pH change curves can be corrected to remove instrumental artefacts. Flash yield determinations revealed that the initial kinetics of the pH rise are biphasic. The first, rapid phase is selectively suppressed by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and by 2,6-dichlorophenolindophenol. The maximal slope of the second, exponential phase of pH rise with pyocyanine, FMN and ferricyanide as electron acceptors corresponded to about 400, 150 and 100 μequiv H^+/h per mg chlorophyll, respectively (pH 6.2, 5°). Simultaneous measurement of electron transport and pH rise indicated that the maximum stoichiometry of H^+ uptake, the H^+/e_2^- ratio, may be 4.0.

INTRODUCTION

SHEN AND SHEN[1], and HIND AND JAGENDORF[2] discovered that illuminated chloroplasts build up some 'high-energy state' or intermediate which drives ATP formation in a subsequent dark stage. The latter group also found that under similar conditions illuminated chloroplasts induce a marked rise in the pH of their (unbuffered) suspending medium[3,4]. Furthermore, by appropriate rapid alterations of the pH of chloroplast suspensions it was found possible to induce phosphorylation in darkness[5]. Thus pH relationships have become very important in chloroplast biochemistry, and the need for deeper understanding of the kinetic details of the light-induced pH rise and of its quantitation with electron flow is now obvious.

The slow response of the glass electrode commonly used to measure pH presents difficulties in applications where the pH changes rapidly and has led to the suspicion that direct measurement of kinetics in stirred suspensions is of questionable worth,

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Tricine, tris(hydroxymethyl)methylglycine.

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and specifically that the post-illumination pH rise phenomenon ('overshoot')[6] might be an artifact of instrumental origin. This overshoot presents a disturbing anomaly in attempts to interrelate the pH rise, accumulation of the high-energy state (X_E), and electron transport; since the last two are always strictly light dependent under the conditions used to measure pH rise.

Recently CHANCE and co-workers[7,8] have introduced an optical method involving the use of a pH indicator (bromothymol blue) for assaying rapid kinetics of intramembrane pH changes which, if certain problems pointed out by MITCHELL[9] can be eliminated, should become a very useful tool. In this paper, however, we report experiments performed using a constant flow method (applied to a conventional glass-electrode pH-measurement system) which has yielded unequivocal data on the initial kinetics of pH changes in chloroplast suspensions.

It should be added here that the term H^+ uptake will be used in this paper for brevity, where it is necessary to refer to the apparent amount of H^+ transferred. It is by no means established that the pH rise in chloroplast suspensions is caused by a direct uptake or translocation of H^+.

MATERIALS AND METHODS

Chloroplasts were isolated from fresh leaves of field-grown spinach (*Spinacia oleracea* L.). About 600 g of leaves were homogenized in a Waring Blendor for 20 sec at 0° with 1.2 l of a medium containing 0.2 M sucrose, 20 mM Tricine buffer (pH 7.8), 40 mM KCl and 4 mM $MgCl_2$. The pellet obtained by centrifugation (3000 × *g*, 5 min) was resuspended in 500 ml of a buffer-free medium containing the same concentrations of sucrose and salts, and briefly centrifuged to remove cell debris. Chloroplasts were then spun down, washed again, and finally suspended and stored in the buffer-free medium (chlorophyll content 2–3 mg/ml). The pH of this stock suspension was about 6.4. The chloroplasts appeared mostly unfragmented and unswollen.

pH changes were measured with a glass combination electrode type 4858-L60 (A. H. Thomas Co., Philadelphia, Pa., U.S.A.) and a Beckman pH meter model 76; and recorded on a 10-mV strip-chart recorder (Texas Instruments, Inc., type PS01W6A) giving 0.065 pH unit per inch for the flow system and 0.1 pH unit per inch for the 'standard method' (see below). The light source was a 750-W slide projector with the beam passing through $CuSO_4$ solution to eliminate infrared radiation. The illumination period was regulated by means of a calibrated leaf shutter. All experiments were conducted in the cold room at 5°.

For conventional pH measurements in stirred suspensions, the glass electrode was immersed in 5 ml of reaction mixture in a vial (15 mm diameter, 40 mm height) and the apparent time course of the pH changes recorded while the suspension was vigorously stirred with a magnetic stirrer. This form of pH measurement will be referred to in the text as the 'standard method'.

The same unbuffered reaction mixture was used both for the constant flow and the standard method. The concentrations of basic ingredients were: chloroplasts equivalent to 100 μg chlorophyll per mg; KCl, 20 mM; $MgCl_2$, 2 mM. In routine experiments 10 μM FMN or 40 μM pyocyanine was added as a cofactor. The initial pH was adjusted with HCl or NaOH to 6.2 ± 0.05, unless otherwise noted.

Tris (hydroxymethyl) methylglycine (Tricine) was purchased from General Bio-

chemicals, Chagrin Falls, Ohio, U.S.A. and (*N*-morpholino)ethanesulfonic acid[10] was a gift from Dr. N. E. GOOD.

RESULTS

pH changes observed by the constant flow method

The essential part of the device employed for the flow method consists of a resin-glass (Plexiglass) block containing a specially coated electrode (Fig. 1). Chloroplast suspension was forced upward through the channel so as to encounter the glass electrode immediately after traversing the illuminated area. The extent of pH changes induced in the suspensions during their travel across the illuminated area could be registered as a shift in the steady state level of pH. In order to improve the time resolution, the sensitive bulb of the electrode was coated, except for an area 3 mm in diameter at the extreme tip, with a layer of Euparal microscopical mounting fluid followed by several layers of white enamel paint. Final calibration of this partially coated electrode was carried out with buffers in the standard assay system, which indicated that the electrode still retained enough sensitivity and a linear response to pH's between 6.0 and 7.0. Careful positioning of the electrode in the channel and proper choice of flow rate were crucial in obtaining a smooth flow. Flow rates between 80 and 140 mm/sec gave reproducible results, but 80 mm/sec was routinely used so as to conserve the suspension.

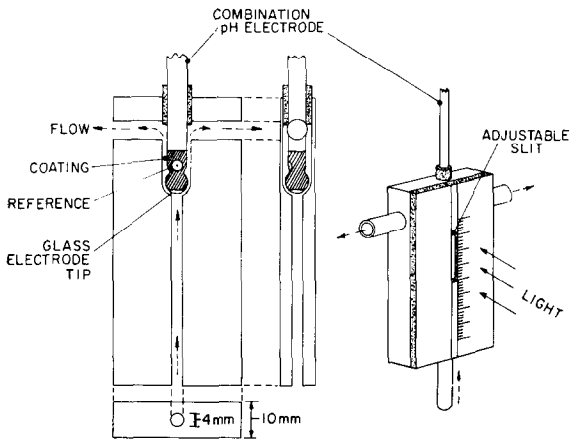


Fig. 1. Illuminated flow cell containing a glass electrode. Chloroplast suspension was forced through the chamber at a constant rate by applying regulated air pressure to a reservoir of suspension. The suspension was used once only.

Exposure times were determined by the flow rates and the slit heights. The light passing through the slit was partially scattered by the chloroplast suspension, giving a glowing area extending about 0.5 mm beyond the slit edges. An attempt was made to determine the effective intensity of this diffused light, with the result shown in Fig. 2. Based on this finding, the effective height of the illuminated area was estimated to be the slit height *plus* a margin of 3 mm at the lower edge of the slit. The correction at the upper edge was neglected since the electrode was very close to this edge (< 1 mm). However, when a dark area (height > 15 mm) was

inserted between the electrode and the light area the same correction was applied to the upper edge. The range of slit heights used was between 5 and 60 mm, effectively 8 and 63 mm, giving an exposure range between 0.1 and 0.8 sec at a fixed flow rate of 80 mm/sec.

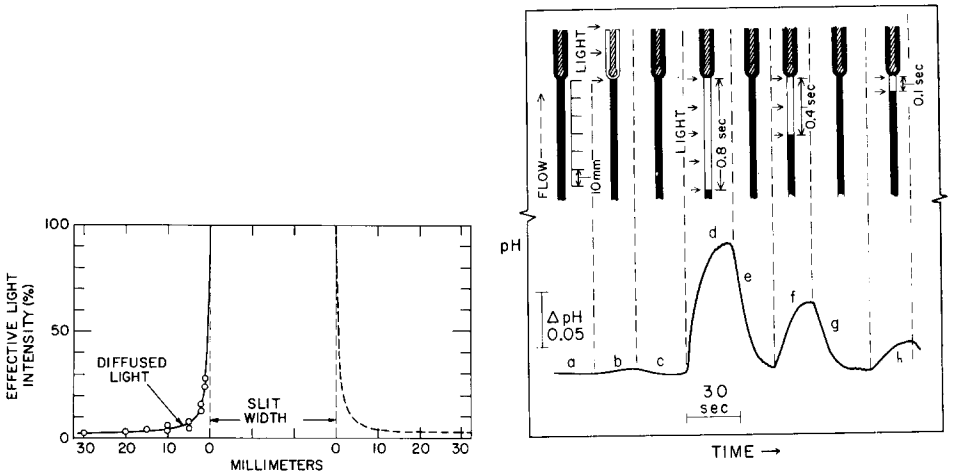


Fig. 2. Distribution of effective light intensity in the illuminated chamber. The cell was filled with a stationary chloroplast suspension (cofactor, FMN) and an area including the electrode chamber was illuminated by light through a slit (height, 30 mm). The maximal slope of the pH rise obtained was taken as representing 100% of the effective light intensity. The suspension in the cell was then renewed and the slit shifted down to permit the light beam to hit an area missing the electrode tip by the distance *d*. The maximal slope of the pH rise was again noted and expressed as a percentage of the above control rate, so giving the effective intensity (%) of diffused light. The figure indicates that the integrated intensity of the diffused light is approximately equivalent to an extension of the slit height by 3 mm in each direction.

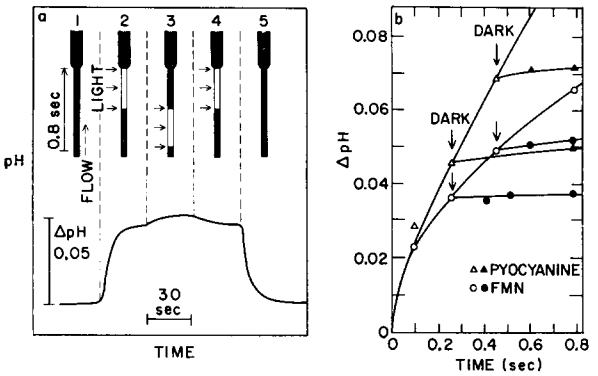
Fig. 3. pH shifts in a chloroplast suspension induced by brief illumination in the constant flow cell. The curve shown is an actual recorder tracing. Cofactor: pyocyanine. For experimental conditions see METHODS.

The curve shown in Fig. 3 is a reproduction of a recorder tracing obtained by the flow method. The steady state pH level shifted to various extents depending on the exposure time (curves d–h). The apparent rate of pH shift had a half-time of about 5 sec; approximately the response time of the glass electrode. The experiment shown by curves a–c ensured that chloroplast suspension flowed quickly enough over the electrode surface to leave no cumulative effect of light.

It was surprising to find that an illumination time of only 0.1 sec was sufficient to induce a pH shift of 0.02 units (curve h, Fig. 3). This prompted us to examine the post-illumination pH rise, which has been reported to be maximal following brief light exposures[6]. The procedure was simply to insert a variable unilluminated area between the electrode and the illuminated section, and ascertain whether or not the resulting steady state pH was significantly higher than the control having no dark interval. Insertion and removal of the dark phase was manipulated quickly, by simply sliding a slit unit (an opaque strip with a window, 4 mm × 30 mm or 4 mm × 15 mm).

Fig. 4a shows that insertion of a dark interval (curve 3) gave only a small additional pH shift above the level elicited by the light period alone. The difference

was small enough to cast doubt on the reality of the overshoot phenomenon. Correction of these small increments for the diffused light effect in the inserted dark section practically reduced them to zero (Fig. 4b). The pH rise (Fig. 4b) is very



Figs. 4a and b. Lack of post-illumination pH rise (overshoot) as shown by the constant flow method. Fig. 4a illustrates the experimental procedures and an actual recorded pH response curve (cofactor, FMN). Fig. 4b summarizes the data so obtained. For details of procedure, see text. For experimental conditions, see METHODS.

rapid at the beginning of illumination, after which follows an exponential slower rise commencing at about the 100 msec point (see curves f and h, Fig. 3). The pH rise stops almost instantaneously when the light is turned off, so that even if the slight upward slopes in the dark are significant, the post-illumination pH rise could not continue for more than about 50 msec with the same slope as in the light period. However, it is also true that there is no pH decrease within 0.5 sec after turning off the light. As will be shown later on a longer time scale, the dark reversion after a small pH rise around pH 6.2 is indeed extremely slow.

Analysis of post-illumination pH rise observed by standard method

The failure to demonstrate a measurable post-illumination pH rise by the flow method led to the conclusion that the overshoot phenomenon observable in a standard pH assay system is due to a response lag inherent in the instrument. The kinetics of the overshoot must then reflect the response characteristics of the pH assay system used, as is shown in Figs. 5a–c.

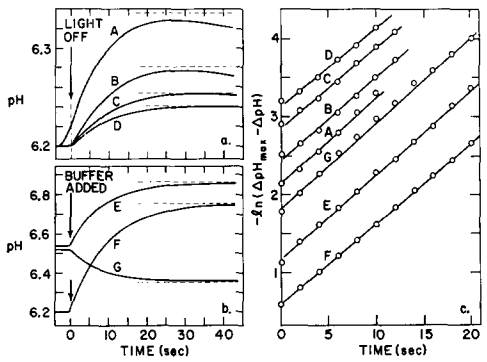
The measuring system used in this study responded to abrupt upward or downward changes of pH showing first-order kinetics with a rate constant of 0.11 (sec^{-1}) and a half-response time of about 6 sec. However, the kinetics of the overshoot phenomenon in chloroplast suspensions diverges from first order in the later stages where the dark reversion process (pH drop) is no longer negligible. When, as after a brief illumination, this dark reversion is slow the overshoot may continue for as long as 20 sec.

The pH meter and recorder responded rapidly to test voltages, therefore the response lag must be introduced mainly by the glass electrode.

Kinetics of initial phase of pH rise as observed by the flow method and flash experiments

Since the post-illumination pH rise simply expresses a response lag inherent

in the pH measuring system, it follows that the maximal extent of an overshoot represents the pH level already achieved during the preceding illumination (provided that the dark reversion rate is negligible). Consequently it is possible to estimate the earlier time course of the pH rise simply by plotting the maximal extents of the overshoots observed in a series of samples illuminated briefly for different periods of time. We shall refer to this as the 'flash method' for observing initial kinetics.



Figs. 5a–c. Analysis of the post-illumination pH rise observed by the standard method, and of the response characteristics of the measuring system used. For details of the standard assay using stirred suspensions, see METHODS.

Fig. 5a. Overshoot observed by the standard method. The duration of illumination and cofactors used were: 3 sec, FMN (curve A); 1 sec, FMN (curve B); 0.2 sec, pyocyanine (curve C); 0.1 sec, pyocyanine (curve D). The apparent half-rise of the overshoot (after turning off the light) was 6.0 to 7.0 sec.

Fig. 5b. Responses of the pH assay system to abrupt shifts in pH induced by the addition of buffers. The buffer used was 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, of which the apparent pK_a (= 6.18) is known to be little affected by dilution[10]. Experimental procedures were the same as in Fig. 5a except that the illumination was replaced by the quick injection of buffers (0.2 M, 0.5 ml).

Fig. 5c. Agreement between the apparent first-order kinetics of the postillumination pH rise (A–D) and of the response lag inherent in the pH assay system used (E–G). The straight lines are derived from corresponding curves in Figs. 5a and 5b. The apparent first-order reaction constant for the response lag (E–G) showed a slight deviation from assay to assay; *i.e.*, 0.105 to 0.120 (sec^{-1}), or a half-response time of 5.8 to 6.6 sec.

Time courses obtained with the flash method agree well with those given by the flow method (Fig. 6). It is even more evident that the pH rise at the beginning of illumination exhibits a very rapid 'burst' phase with a half-rise time of less than 50 msec. The burst phase seems kinetically independent of exogenous cofactors (see also Fig. 4b). The extent of the burst was 0.025–0.04 pH units. Estimation from the titration curves (Fig. 7) showed that these values correspond to 25–40 mμequiv H^+/mg chlorophyll, or about one H^+ for 30–40 chlorophyll molecules. The initial slope of the subsequent exponential phase is dependent on the cofactor added, typical rates of pH change being (μequiv H^+/mg chlorophyll): 300–400 with pyocyanine, 100–150 with FMN and 30–50 without cofactor.

It should be noted that since titration curves were essentially linear between pH 6.2 and 7.0 due to buffering characteristics of chloroplasts (see also ref. 6), a pH scale in this range could be directly converted into a linear scale denoting absolute

amount of H^+ (see Fig. 6). The preceding and following calculations in terms of the equivalents of H^+ were made in this way.

The burst phase is not repeatable within 60 sec; *i.e.* once induced by a flash, subsequent flashes at shorter intervals than this cause pH rises only to the extent corresponding with the slower exponential phase. The non-repetitive nature of the burst is not, however, due to the initial pH having been shifted as a result of the first flash. The experiments depicted in Fig. 8a show that the extent of the burst is almost the same between initial pH values of 6.1 and 6.6. The burst is more sensitive to 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) than is the exponential phase. Thus,

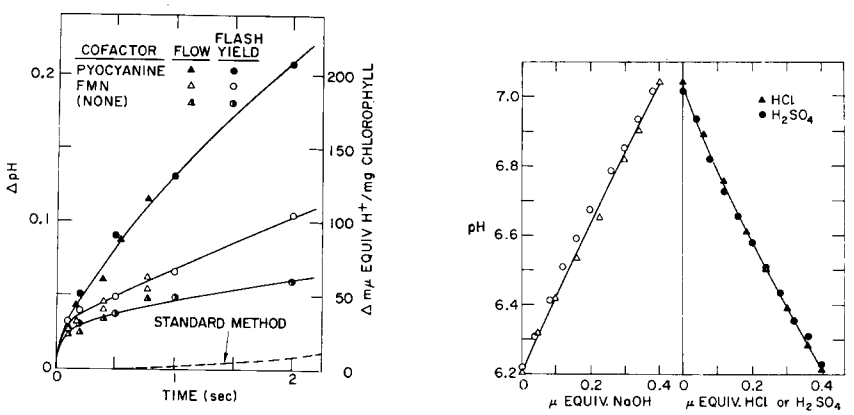
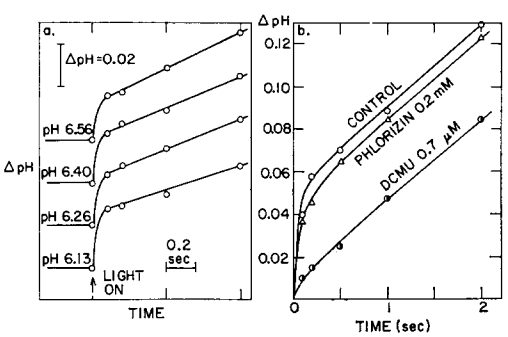


Fig. 6. Comparison of the early pH-rise kinetics observed by the constant flow and the flash methods. The standard assay system was used for the flash experiments: a series of samples was illuminated for various periods (0.1–2.0 sec, only once per sample) and the net extents of the pH rise including overshoot were measured. The principle of this method is discussed in the text.

Fig. 7. pH-titration curves for the chloroplast suspension (reaction mixture) used in the pH shift experiments. The data are from two different chloroplast preparations (o,o and Δ,Δ). 5 ml of the standard suspension (chlorophyll, 100 μg/ml) was titrated with 0.002 M NaOH and back-titrated with 0.002 M HCl or 0.001 M H_2SO_4. Titrant was added in 0.02–0.03 ml aliquots at 30-sec intervals through a fine hypodermic needle. The suspension was stirred and the pH changes continuously recorded. A pH change of 0.1 units corresponded to production or consumption of 50 mμequiv H^+ in the total reaction mixture, or to 100 mμequiv H^+/mg chlorophyll.



Figs. 8a and b. The effect of varied pH level, DCMU and phlorizin on the kinetics of the initial phase of the pH rise. These time courses were obtained by the flash technique (see text and Fig. 6). Fig. 8a shows that the response is not critically dependent on the starting pH level (cofactor, none). Fig. 8b illustrates that DCMU suppresses the burst (cofactor, FMN). The concentration of phlorizin used gives 50% inhibition of photophosphorylation[11].

in Fig. 8b, 0.7 μM DCMU suppresses the former without noticeably affecting the exponential phase (cofactor, FMN). Similarly, 40 μM 2,6-dichlorophenolindophenol (DCIP) inhibits the burst much more than the overall pH rise (see Fig. 11). At the concentrations used here, neither DCMU nor DCIP affects the titration curves: their specific effect is not due to an alteration of the buffering capacity of the chloroplast suspensions. Phlorizin, an inhibitor of photophosphorylation interfering with the terminal steps of ATP formation[11,12], is without significant effect on the pH rise.

Estimation of the real time course of overall pH changes

Estimation by computation. As shown in Figs. 5b and 5c the glass-electrode pH-recording system responds to abrupt shifts in pH in a manner characteristic of an apparent first-order reaction. It follows that the observable rate of pH change, $d(\Delta pH_{obs})/dt$ at any given moment is proportional to the difference between the real pH change of the suspension (ΔpH_{real}) and the observed pH change (ΔpH_{obs}).

$$d(\Delta pH_{obs})/dt = k_c(\Delta pH_{real} - \Delta pH_{obs}) \quad (1)$$

where k_c is the apparent first-order reaction-constant characteristic of the instrument (in this case, $k_c = 0.11$ sec^{-1}; (see Fig. 5c). In this treatment, ΔpH will be used rather than the more formal $\Delta[H^+]$.

If the time course of the real pH change is also close to having apparent first-order kinetics it follows that

$$d(\Delta pH_{real})/dt = k(\Delta pH_{max} - \Delta pH_{real}) \quad (2)$$

where ΔpH_{max} is the maximal extent of the real pH change under the given conditions and k is the reaction constant. Integration of Eqn. (2) yields

$$\Delta pH_{real} = \Delta pH_{max}(1 - e^{-kt}) \quad (3)$$

and substitution of Eqn. (3) in Eqn. (1) gives

$$d(\Delta pH_{obs})/dt = k_c\{\Delta pH_{max}(1 - e^{-kt}) - \Delta pH_{obs}\} \quad (4)$$

yielding on integration

$$\Delta pH_{obs} = \Delta pH_{max}\{1 - (k_c e^{-kt} - k e^{-k_c t})/(k_c - k)\} \quad (5)$$

Eqn. (5) predicts a lag at the onset of illumination and also allows one to solve for the value of k, the rate constant for the real pH changes. It should be pointed out that if $k_c \gg k$, this equation becomes identical with Eqn. (3). Computer analysis indicated that in order to fit Eqn. (5) to the actual recorded curve (curve B, Fig. 9a) k could not be a constant, but rather

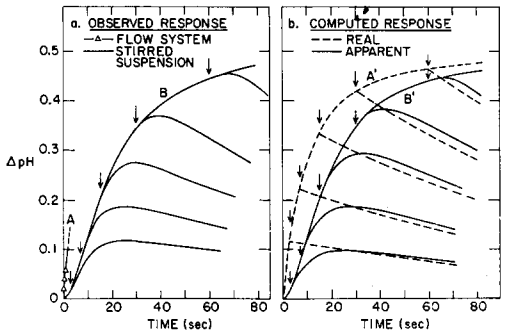
$$k = 0.07e^{-0.021t} + 0.02 \quad (6)$$

In other words the real kinetics are not truly first order. The apparent pH rise curve reconstituted from Eqns. (5) and (6) is very close to the actually observed response in stirred suspensions (see curve B' in Fig. 9b and B in Fig. 9a). The real time course can be easily computed by combining Eqns. (3) and (6) (see curve A' in Fig. 9b and A in Fig. 9a).

The overshoot curves shown in Fig. 9b were derived by assuming that the real pH decreases upon turning off the light, following first-order kinetics

$$\Delta pH_{real} = \Delta pH^{\circ}_{real} \cdot e^{-k't} \quad (7)$$

where $\Delta pH°_{real}$ is the real extent of the pH rise at the moment illumination ceases (which can be determined using Eqns. (3) and (6)); and k' is the rate constant, estimated to be 0.008 (sec^{-1}) from the slopes of the observed off-response curves in Fig. 9a. The term t in Eqns. (7)–(9) is the time elapsed since turning off the light.



Figs. 9a and b. Computation of real time courses from apparent time courses obtained by the standard pH-assay method. The vertical arrows indicate light cut-off points. Cofactor, FMN.

If the observed extent of the pH rise at the moment illumination ends is denoted by $\Delta pH°_{obs}$, it follows from Eqns. (1) and (7) that the observed reversion rate is

$$d(\Delta pH_{obs})/dt = k_c(\Delta pH°_{real} \cdot e^{-k't} - \Delta pH°_{obs}) \quad (8)$$

yielding, on integration,

$$\Delta pH_{obs} = \Delta pH°_{real}(e^{-k't} - e^{-k_c t})/(1 - k'/k_c) + \Delta pH°_{obs} \cdot e^{-k_c t} \quad (9)$$

Eqn. (9), in conjunction with Eqns. (3) and (6), can be used to predict the amount of overshoot following the end of illumination at any point throughout a time course. A set of computer solutions for the overshoot equations is shown in Fig. 9b, and should be compared with the observed data (Fig. 9a).

Estimation by a graphical method. In practice the above computations, which are often very complicated, can be replaced by a simple graphical method. We found it relatively easy to estimate graphically the slope, $d(\Delta pH_{obs})/dt$, of a recorded pH-change curve at any given point. This value, when divided by the apparatus constant k_c (Eqn. (1)), yields the difference between the real and the apparent pH values at the point where the slope has been determined; hence, the real pH value at that point. A series of such determinations should give the same curve as the one computed and, in fact, does so (Fig. 10). By this graphical method it is even easier to derive the real post-illumination kinetics from analysis of observed overshoot curves, since there is an indisputable index, *viz.*, the fact that the real off-response curves always cross the observed overshoot curves at their peak (see Fig. 9b). This is necessarily so as indicated by Eqn. (1). Whatever the shape, an observed curve momentarily registers the real pH value at its peak or base, where $d(\Delta pH_{obs})/dt = 0$.

It has thus become possible to extend the flash method (described in the preceding section) to the general case where dark reversion processes are not negligible. This modified flash method is important for documenting initial kinetics because straightforward analysis and correction of an entire observed pH rise curve tends to become unreliable at a very early stage where the ΔpH is small. Details of initial

kinetics such as the burst phenomenon are only accessible by the constant flow method or by this modified flash treatment.

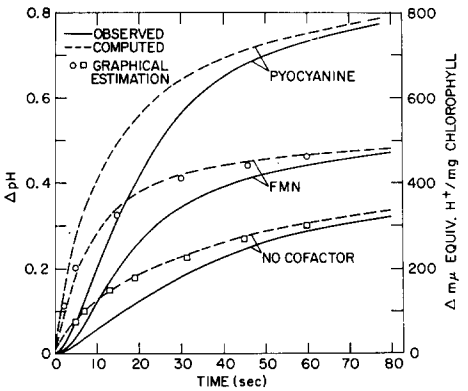


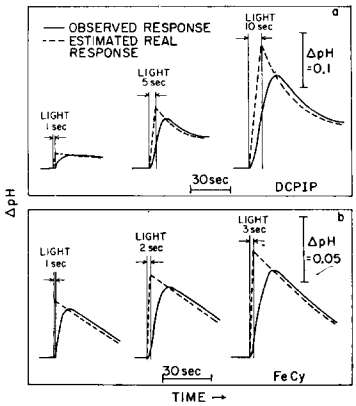
Fig. 10. Comparison of apparent pH rise curves and the real time courses obtained by the graphical method and by computation. For details of these treatments see text. For experimental procedures see METHODS.

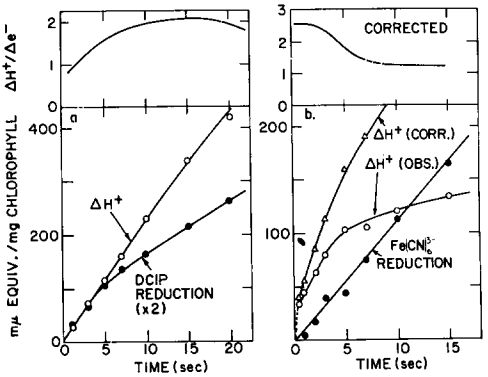
The overall real time course curves obtained by these methods appear to be complex and to depart substantially from first-order kinetics, which gives the closest fit to the data (Fig. 10). The maximal extents of the pH rise with pyocyanine, FMN and without cofactor averaged 0.8, 0.5 and 0.3 pH units, respectively, using suspensions containing chloroplasts equivalent to 100 μg chlorophyll/ml. These values, roughly corresponding to 0.9, 0.6 and 0.3 equiv H^+/mole chlorophyll respectively, coincide very closely with the data obtained by JAGENDORF AND NEUMANN[6] under comparable conditions with 'broken' chloroplasts.

The relationship between the rate of apparent H^+ uptake and of electron transport

Attempts were made to examine the quantitative relationship between the H^+ uptake and electron transport at earlier states of illumination with DCIP and ferricyanide as electron acceptors. The pH rise was observed by using a series of samples illuminated briefly for different periods of time (flash method). The apparent post-illumination pH rise (overshoot) obtained for each sample was analyzed by the graphical method described in the preceding section, and the real pH value at the end of each light period so determined (Fig. 11). By plotting these values against the corresponding illumination periods the true time course of the on-response was obtained. Reduction of DCIP and ferricyanide was assayed in a similar series of samples. The results are summarized in Figs. 12a and 12b and Table I.

As seen in Fig. 12a, the pH rise starts linearly in the presence of DCIP without showing the burst phenomenon. For the first several seconds the apparent H^+ uptake and electron transport proceed linearly after which the latter declines to a lower rate. The ratio of the rates of these two processes is shown in the upper portion of the figure, which indicates a maximum value of about 2.0 or a H^+/e_2^- ratio (the preferability of e_2^- rather than $2e^-$ is discussed in ref. 13) of 4.0. However, in the presence of DCIP a fairly rapid dark reversion process takes place, presumably also coexisting and competing with the forward reaction in the light. Therefore, correction of the





Figs. 11a and b. Graphical estimation of real pH values at the end of an illumination period. A series of samples (each 5 ml) was illuminated for different durations and the overshoots analyzed graphically (see text) to determine the real extent of the pH rise at each light cut-off point. Plotting of these data gave the real pH-rise curves shown in Figs. 12a and b. Concentrations of DCIP and $K_3Fe(CN)_6$, 40 μM and 0.2 mM respectively. Assay conditions are described in METHODS. FeCy stands for $K_3Fe(CN)_6$.

Figs. 12a and b. Relationship between H^+ uptake and electron transport. The pH-rise curves were determined experimentally and analyzed graphically as shown in Figs. 11a and b. The 5-ml samples were filtered, immediately after illumination, through a Millipore filter (pore size 0.6 μ; Millipore Filter Corp., Bedford, Mass., U.S.A.) and the absorbance of DCIP in the clear filtrate determined at 600 mμ. The extinction coefficient of DCIP between pH 6.2 and 6.4 was taken as $1.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ (see ref. 22). Similarly, the reduction of ferricyanide was estimated from the amount of ferrocyanide detected in the assay procedure described elsewhere[23] (except that Millipore filtration made acid-deproteinization unnecessary). For other experimental procedures, see Figs. 11a and b. "Corrected" or "corr." in the figure indicates correction for H^+ production due to ferricyanide reduction (see Table I).

TABLE I

MAXIMAL RATIOS BETWEEN THE RATES OF H^+ UPTAKE AND OF ELECTRON TRANSPORT

*Expt. No.**	*Electron acceptor*	*Time range after light-on (sec)*	*H^+ uptake***			*Electron flow***	*H^+/e_2^-*		
			With electron acceptor		*Without electron acceptor (endogenous)*		*Obs.*	*Corr.*	*Corr. for endogenous H^+ uptake*
			Obs.	*Corr.*					
1	DCIP	10–20	69	79***	20	35	4.0	4.5***	3.4
2	DCIP	5–15	72	83***	27	34	4.2	4.9***	3.3
3	$Fe(CN)_6^{3-}$	0.5–3.0	64	104§	35	40	3.2	5.2§	3.4
4	$Fe(CN)_6^{3-}$	0.5–3.0	50	86§	28	36	2.4	4.4§	3.2

* A different chloroplast preparation was used for each experiment.

** μequiv H^+ (or e^-)/h per mg chlorophyll.

*** Values were corrected for the dark reversion process assuming this takes place during illumination. From Figs. 11a and 12a it was deduced that the kinetics of the H^+ uptake, if corrected for the back reaction, could be regarded as zero order (reaction constant, k) between 0 and 20 sec. The back reaction was practically first order with rate constant 0.025 (sec^{-1}). The value of k was hence estimated from Fig. 12a to be 0.026. This value is 15% higher than the slope of the observed pH rise, hence a correction factor of 1.15 has been applied here.

§ Values were corrected for H^+ production equivalent to ferricyanide reduction on the basis of the formula:

$$1/2H_2O + Fe(CN)_6^{3-} = 1/4O_2 + Fe(CN)_6^{4-} + H^+.$$

H^+-uptake rates is necessary. It was estimated that the corrected rate of the forward reaction was about 15 % higher than the uncorrected, thus raising the H^+/e_2^- ratio up to 4.9 (Table I).

Ferricyanide does not suppress the burst phase, but with this electron acceptor a linear pH rise continues only for several seconds, tapers off, and is finally overcome by H^+ production due to ferricyanide reduction. The time course of ferricyanide reduction, on the other hand, is linear for at least 20 sec. If one regards the initial burst phase as unrelated to ferricyanide reduction, the apparent maximum ratio is about 1.5, or a H^+/e_2^- ratio of 3.0. Obviously this value needs a correction for H^+ production arising from ferricyanide reduction, regardless of the destination of this H^+—inside or outside of the membranes. Correction raises the H^+/e_2^- ratio to 5.0 (Table I). The rate of the dark back reaction in this experiment was also considerable, but within the critical time period of 0–3 sec was negligible compared to the on-response rate (Fig. 11b). An indication that the H^+/e_2^- ratio for the ferricyanide system is more than 2.0 has already been obtained by NEUMANN AND JAGENDORF[4].

There is still another factor which may have to be considered in assessing the H^+/e_2^- ratio, namely, an appreciable pH rise which takes place in the absence of exogenous electron acceptors. It is not likely that this (pseudo-)cyclic process, catalyzed by endogenous redox substances of low (possibly negative) potential, can continue in the presence of the strong oxidant ferricyanide (known to suppress cyclic or pseudocyclic X_E formation and photophosphorylation completely)[14–16]. Its fate in suspensions containing added DCIP is more questionable. At any rate, the H^+/e_2^- ratios corrected to exclude this endogenous process still remain high—3.2 to 3.4 (Table I). We may tentatively conclude, therefore, that the H^+/e_2^- ratio is most likely 4.0.

DISCUSSION

The present study was initiated to resolve the issue of the post-illumination pH rise in chloroplast suspensions. Our failure to find significant overshoot would seem to add weight to the viewpoint that X_E and the pH gradient across the membrane are fundamentally the same thing. Furthermore, our treatment reveals that the reported kinetics of X_E are fairly similar to those of the pH changes. For instance, taking original data of HIND AND JAGENDORF[2] on X_E obtained with added pyocyanine we find a half-rise time of 7 to 13 sec and a half-decay time of 30 to 44 sec (0°, pH 6.0; chlorophyll, 125 μg/ml). In our experiments, conducted under comparable conditions (5°, pH 6.2; chlorophyll, 100 μg/ml), the half-rise time for the pH shift with pyocyanine was about 10 sec and the half-decay time, 40 to 60 sec. A precise comparison is impracticable, but the similarities are nevertheless quite evident.

Our observations seem suggestive from the standpoint of the chemiosmotic coupling hypothesis[9]. Thus, the observed initial rates of H^+ uptake at 5° with pyocyanine and FMN as cofactors were respectively 300–400 and 100–150 μequiv H^+/h per mg chlorophyll. Extrapolating these values to 20° using a reported temperature coefficient of 1.4 (ref. 4) and assuming that two H^+ are necessary to form one molecule of ATP (see ref. 9), it may be seen that the H^+ flux could support photophosphorylation rates of almost 400 and 150 μmoles of ATP/h per mg chlorophyll for pyocyanine

and FMN, respectively. These values lie well within an amply documented range of normal phosphorylation rates.

Again, if one extrapolates to actual phosphorylation conditions, the most probable stoichiometry, $H^+/e_2^- = 4$, would be just enough to support the stoichiometry of phosphorylation, $P/e_2^- = 2$. The maximum stoichiometry of photophosphorylation actually observed is $P/e_2^- = 1.3$ (ferricyanide system)[16] which, if corrected for the basal, non-phosphorylating electron transport, becomes indeed 2.0 (see ref. 11). The present observations thus tend to support the theory of chemiosmotic coupling as applied to the mechanism of photophosphorylation.

The very rapid pH rise (burst) observed at the beginning of illumination may reflect a shift in the redox state of some of the components of the electron-transport chain. The characteristics of the burst—practical independence of exogenous cofactors (except for the inhibitory DCIP), high sensitivity to DCMU, and magnitude equivalent to $2H^+$ per 60 to 80 chlorophyll molecules—all suggest a component of considerable pool size located close to photo-system II of DUYSENS, AMESZ AND KAMP[4]. As is well known, illumination of chloroplasts with white (or broad-band red) light causes a rise in the reduction level of plastoquinones. Originally CRANE, EHRLICH AND KEGEL[18] reported an extensive reduction of endogenous quinones in chloroplasts illuminated for 5 min. However, FRIEND AND REDFEARN[19,20] later reported that the reduction saturates within 1 min and the amount of quinones accounting for the reduction is 10 to 30 % of the total plastoquinones. They also showed that the reduction is DCMU-sensitive[19] and that addition of DCIP before illumination causes the total oxidation of quinones and suppresses the shift in the light[20]. Similarly, RUMBERG *et al.*[21] estimated the amount of their 'photoactive quinone' to be about 10 % of the total chloroplasts quinones. These are amounts which could well be equated with the observed extent of the pH burst. If the pH rise phenomenon indeed represents translocation of H^+ across the lamellar membranes, it seems probable that at least one site of H^+ translocation is associated with a plastoquinone pool involved in the electron-transport chain close to photo-system II.

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