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KINETICS OF ACID EFFLUX FROM CHLOROPLASTS FOLLOWING THE ACID–BASE TRANSITION

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SUMMARY

Swollen chloroplasts, suspended in succinic or other acids at pH 4 were mixed with alkali in a stopped-flow apparatus to permit observation of the pH of the medium following an acid–base transition. Immediately after mixing the observed pH values were as much as 0.6 units more alkaline than the final equilibrium pH, then dropped to the equilibrium value over a period of several seconds. Experimental results support the idea that most of the slowly appearing acidity represented diffusional loss from the thylakoids of organic acid molecules, previously absorbed from the initial acid medium. Time-courses for the approach to equilibrium pH showed apparent first-order kinetics, with two distinct components: (a) the major portion of acid loss, having a Q_{10} of 2, and (b) a minor residual portion of the acid loss, having a Q_{10} of 1.2. Component (a) could be distinguished by faster kinetics only at 20° or above. Uncouplers had relatively little effect on (b), but diminished the extent and duration by accelerating the velocity of initial acid loss (a). The combined results of several diverse experiments showed a correlation between the ability to make ATP, and the existence of large and relatively slow initial acid loss (a). Kinetics of this initial phase are similar to those of the post illumination pH drop studied previously, under equivalent conditions.

The very slow response times of the glass pH electrode in unbuffered solutions described previously was found here to limit the observed rate constants for the chloroplast light-induced pH rise, thereby calling into question the accuracy of some previously published kinetic studies.

INTRODUCTION

Subjecting isolated, swollen chloroplasts first to acid, then to an alkaline pH induces a transient high energy state probably due to the gradient of acidity from inside to outside the swollen grana discs[1]. The condition results in the ability to form some ATP[2], and (if sulfhydryl compounds are present) in a change in state of the terminal phosphorylation enzyme so that it becomes active as a Mg^{2+}-dependent ATPase[3] and participates in P_i–ATP[4] and water–ATP[5] exchange reactions.

Abbreviations: DCMU, dichlorophenyldimethylurea; CCCP, chlorocarbonylcyanide phenylhydrazone.

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Maximal expression of these effects requires an organic acid to be present in the initial acid stage of the experiment, presumably to enter and serve as a reservoir of internal protons. Entry of labeled succinic and other acids was indeed observed in the acid stage[6]. When chloroplasts loaded with labeled organic acid were diluted 1:1 with a basic solution so the final pH was 8.5, 85 % of the internal acid molecules apparently diffused out, and 15 % were trapped inside as the relatively non-permeating dianion. It was presumed that the protons previously associated with the trapped dianions had functioned in ATP formation, activating either a proton pump of unspecified nature, or the anisotropic membrane-bound reversible ATPase as postulated by MITCHELL[7]. Evidence was presented to rule out alternative hypotheses, as for instance re-entry of cations[8] or inducing electron transport (ref. 9, in answer to ref. 10) for the direct driving force for ATP synthesis. Hence the efflux of both protons and of succinic acid from chloroplasts after a sudden transition from acid to base represents loss of the potential energy present in the initial pH gradient. In the work reported below the kinetics of efflux of acidity from chloroplasts (protons, succinic acid or both) were studied by means of a stopped-flow apparatus containing a glass pH electrode. The results confirm the existence of a loss of acidity from chloroplast thylakoids following the acid–base transition, and suggest that a very rapid, uncontrolled loss of acid is not consistent with the ability to form ATP.

METHODS

Chloroplasts were isolated from market spinach as described previously[8] and finally resuspended at about 1.0 mg of chlorophyll per ml in 10 mM NaCl. 0.6 ml of these were mixed with 0.6 ml of a solution at pH 4 containing 25 mM succinic acid, and 7.5 μM dichlorophenyldimethylurea (DCMU), in a tube maintained in a water bath at the desired temperature. The acidified chloroplasts were incubated for 25 sec, then rapidly picked up into one of two syringes in the stopped-flow device. The other syringe contained an equal volume (1.0 ml on both sides) of NaOH solution sufficient to neutralize the succinic acid together with 2 mM tricine to bring the final pH to about 8.0. Variations of the temperature, initial and final pH's and reaction mixture components are indicated in the appropriate figures or tables.

The stopped-flow device was constructed following the method described by SIRS[11]. A block diagram is shown in Fig. 1. The entire unit was immersed in a water bath to maintain the temperature constant. The two syringes were depressed simultaneously by a bar, driven by compressed CO_2 at 12–20 lb/inch^2. The two streams of fluid entered a 2-mm-diameter mixing chamber in a lucite block through two jets 1 mm in diameter. The mixed fluids moved 1 cm through the mixing tube, into a larger chamber containing the tip of a Leeds and Northrup miniature pH electrode 124138 held in the chamber by compressed O-rings so that it would not be dislodged by sudden pressure increases. The solution passed through a further tunnel in the lucite block, then on to a receiving syringe which came to a sudden stop at the end of its travel. The moment of stopping was recorded by the event marker on the recorder. A hole was drilled into the lucite block to meet the top of the tunnel at one point after leaving the glass electrode chamber; a stainless steel perforated disk was secured into position over the opening on the top of the tunnel and a

calomel electrode placed into the chamber over the steel disk. In this way electrical contact was maintained between the calomel electrode and the flowing solution, but the reference electrode was protected from pressure changes which affect its response.

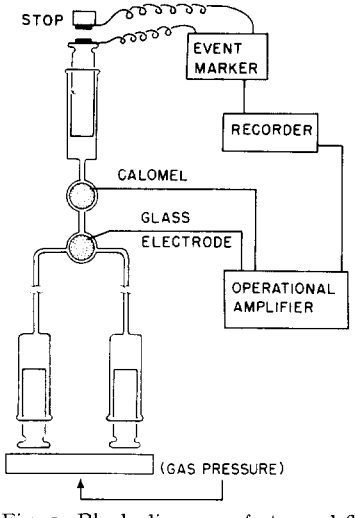

Fig. 1. Block diagram of stopped-flow apparatus. The entire apparatus from one set of syringe barrels to the barrel of the stopping syringe, was built into a water bath. Water at controlled temperatures was circulated through from a Thermatemp controlled-temperature apparatus.

Output from the electrodes was passed through a Model 181 pico-metric amplifier with Model 181A switch box, both from Instrumentation Laboratory, Inc.; then directly to a Bausch and Lomb V. O. M. 7 recorder whose chart was run at 20 inches/min. Although this system had to be calibrated for pH each time, the response time was faster than that found with the pH meters tested. The response time of the electrode system was tested in the stopped-flow apparatus, by switching from a 1 mM buffer (tricine) at pH 8.5 around the electrode, to 1 mM tricine buffer at pH 7.9 in the next depression of the syringes (and *vice versa*). The kinetics of the resulting change were complex; however at 5° the first 50 % of the change was complete in 0.20–0.30 sec, and at 25° in 0.11 –0.18 sec. After the first rapid movement (from one-half to two-thirds of the total) the remaining changes showed half-times varying from 0.35 to 0.83 sec. Large parts of these remaining slower changes showed apparent linear kinetics when plotted on a semi-logarithmic scale.

The total mixing time (*i.e.* from beginning to end of syringe movement) was 0.2 sec. The flow velocity in the mixing chamber was 325 cm/sec, and the time from first entering the mixing chamber to first entering the glass electrode chamber was 2 msec. Due to the large volume of the glass electrode chamber the sensitive tip of the electrode spanned sectors of fluid from those just mixed to those moving past 100 msec after mixing; hence it would report the average pH found in the first 100 msec after mixing. This limitation on the time resolution of the system was not as severe as that imposed by the electrode response time proper (see above), from 0.2–0.8 sec. However neither of these limitations invalidate the observation of events in the time region from 0–15 sec, with which this paper deals.

RESULTS

Fig. 2 shows results from an experiment in which one syringe contained succinate at pH 4.0 either with or without chloroplasts, and the other contained NaOH and tricine so that the final pH after mixing the contents of the two syringes was 7.7. The tracing shows the difference in pH from the final equilibrium value, as a function of time. When no chloroplasts were present the final pH was achieved within 0.1 sec, indicating efficient mixing within the time resolution of the apparatus.

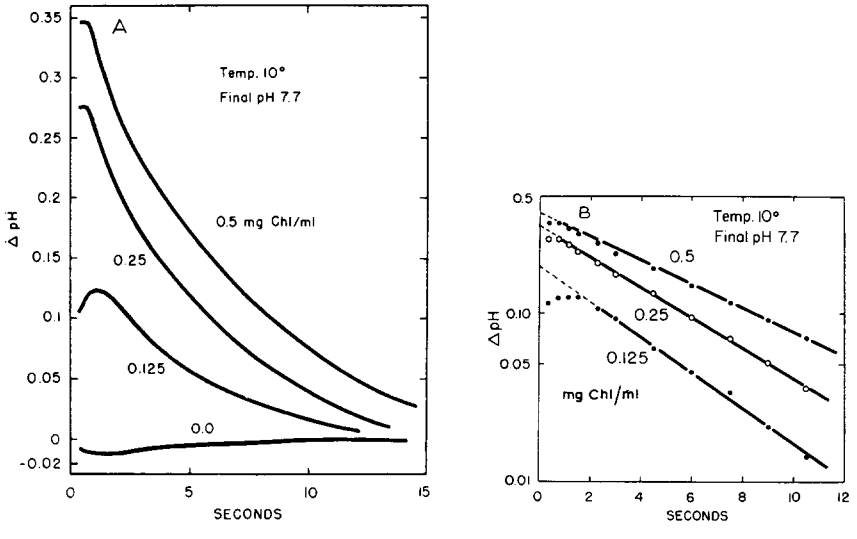

Fig. 2. Overshoot in pH after rapid mixing. A. Recorder tracings of pH at three different chlorophyll concentrations, with and without chloroplasts present. Initial chloroplasts at pH 4.0, with 25 mM succinic acid and 75 μM DCMU. Base solution contained 20 mM NaOH and 2 mM tricine. Note the absence of overshoot when chloroplasts are omitted. B. Re-plot of the data on semi-logarithmic paper.

We assume that mixing was equally rapid whether chloroplasts were present or absent. When chloroplasts were present, however, the pH in this experiment ranged from 0.12 to 0.35 units above the equilibrium value at first, depending on the chlorophyll concentration, then dropped over 10–20 sec to approximate the final equilibrium level. We will designate this phenomenon as a pH "overshoot", in the present manuscript.

The simplest interpretation for the overshoot is that the chloroplast thylakoids absorbed succinic acid from the initial acid mixture, and that when mixed with base either succinic acid or free protons or both leaked out of the thylakoids by relatively slow diffusion, to contribute to the final pH of the medium. This interpretation is supported by the clear relation between height of the pH overshoot and the concentration of chloroplasts used (Fig. 1 and Table I).

Although pH has a logarithmic relation to the concentration of protons in solution, because of the complex series of buffering groups in a crude chloroplast suspension the observed pH will vary in an almost linear fashion with amount of protons added or withdrawn. This was shown previously[12–14] and confirmed for the

TABLE I

EXTENT OF THE pH OVERSHOOT FOLLOWING ACID–BASE TRANSITION

Chlorophyll concn. (mg/ml)	*Buffer capacity (pH/μequiv H^+ per ml)*	*Extent of pH overshoot*		
		pH units	*μequiv H^+/ml*	*μequiv H^+/mg chlorophyll*
0.125	0.142	0.19	0.134	1.07
0.25	0.118	0.33	0.280	1.12
0.50	0.070	0.40	0.573	1.14

present pH ranged (unpublished experiments). Thus the pH tracings can be used without alteration as directly related to the quantity of acidity leaking slowly from the thylakoids, as a function of time, for any one chloroplast suspension.

The pH tracings in the experiment of Fig. 2 showed a distinct plateau or even an upward trend during the first fraction of a second. As noted previously[15] phenomena of this sort most likely are artifacts, resulting from a slow electrode system failing to respond to faster changes in actual pH. As noted in METHODS even with the present system the electrode response time was in the range of 0.13–0.8 sec. This electrode system would obviously not be able to report with any accuracy the first 0.5 sec of a rapidly changing pH. The artifact would be expected to be more severe when the height of the overshoot was larger, or when the electrode response might be slower as at lower temperatures. On the other hand the artifact would not be apparent if the kinetics of the overshoot were very fast, so that the true pH dropped rapidly to the point that the electrode system could achieve at an early time. Indeed the initial rise or plateau was not seen under conditions of very fast kinetics for the overshoot (higher temperatures or presence of uncouplers), but it was more prominent at low temperatures when the electrode response might be slower and the total height of the overshoot was larger.

The major part of the downward drift in pH followed a linear time-course when plotted on semi-logarithmic paper, with a slope largely independent of chlorophyll concentration (Fig. 2B). This permitted extrapolation back to 0 sec to estimate the total pH overshoot involved in the particular kinetic stage, disregarding the anomalous early parts of the curve.

In the same experiment the buffer capacity of the final reaction mix was estimated by direct titration with acid and with NaOH. The extent of the pH overshoot in terms of μequiv of acid per ml and per mg chlorophyll could thereby be calculated (Table I). At all three chlorophyll levels, approx. 1.1 μequiv of acidity per mg of chlorophyll were involved in the overshoot. In other experiments the amount of acidity more usually approached 2 0 μequiv/mg of chlorophyll

The nature of the pH curves changed with temperature (Fig. 3A and 3B). The decline in pH towards the final equilibrium value seemed to follow a single straight line on a logarithmic scale at 3.5°, but to a small extent to 10° and to very pronounced degrees at 20 and 30° two distinct components appeared each with apparent first-order kinetics. The apparent first-order rate constants for these two phases at the four temperatures are shown in Table II. The earlier phase had a Q_{10} of 2, the later phase a Q_{10} of 1.2. At the lower temperatures the earlier phase was so slow that

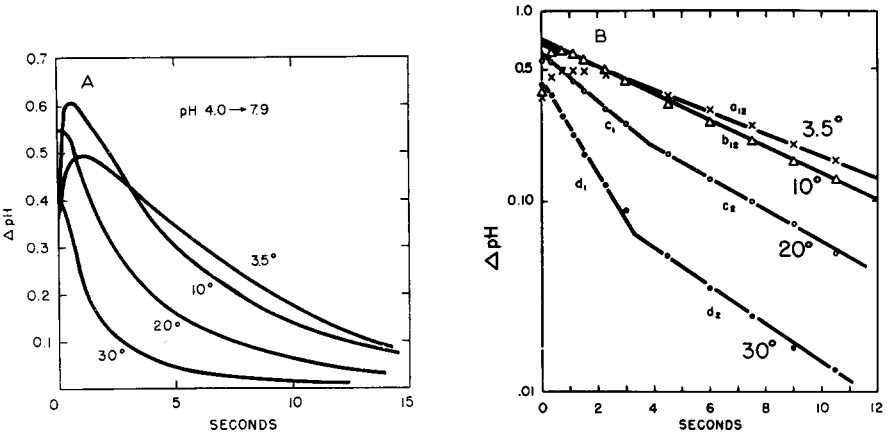

Fig. 3. Overshoot at different temperatures. A. Time-courses. B. Re-plot of data on semi-log paper. Chlorophyll concentration was 0.25 mg/ml after mixing. Temperatures from 3.5 to 30° as indicated. Initial pH, 4.0; final pH, 7.9. Note two distinct phases at the higher temperatures.

TABLE II

TEMPERATURE RELATIONS OF TWO KINETIC PHASES IN THE RETURN OF ACID TO THE MEDIUM

k_1 and k_2 refer to apparent first-order rate constants for the two kinetic phases of pH drift to equilibrium in the pH overshoot, as illustrated in Fig. 3B. At 10° the first phase was not significantly different from the second phase, hence the value for k_1 is an upper limit only at that temperature.

Temperature	k_1 (sec^{-1})	k_2 (sec^{-1})
3.5°	—	0.13
10°	(0.16)	0.16
20°	0.29	0.19
30°	0.58	0.23
Q_{10}, 10–20°	(1.8)	1.2
Q_{10}, 20–30°	2.0	1.2

it merged with the later phase, and only one straight line appeared. "Activation energies" corresponding to these Q_{10} values are 12500 and 3300 cal·degree·mole^{-1}, respectively, for the two phases. The initial more temperature-sensitive phase accounted for 65–85 % of the total extent of the overshoot.

Several possibilities for the origin of two distinct kinetic phases were envisaged and tested. For instance, it was thought that the larger, more temperature-sensitive first phase might be due to the efflux of associated succinic acid, while the second phase could be due to protons exiting at a rate controlled by the counter inward movement of cations. However identical kinetics for the overshoot were found when the strong mineral acid, HCl, was used in the initial acidification in place of succinic acid (Fig. 4A). The extent of the overshoot with HCl was less than 10 % that found with succinate, and also the initial electrode artifact at the lower temperature was missing with HCl. The smaller concentration of internal acidity when using HCl, inferred from the smaller overshoot, is consistent with the previous observations[2]

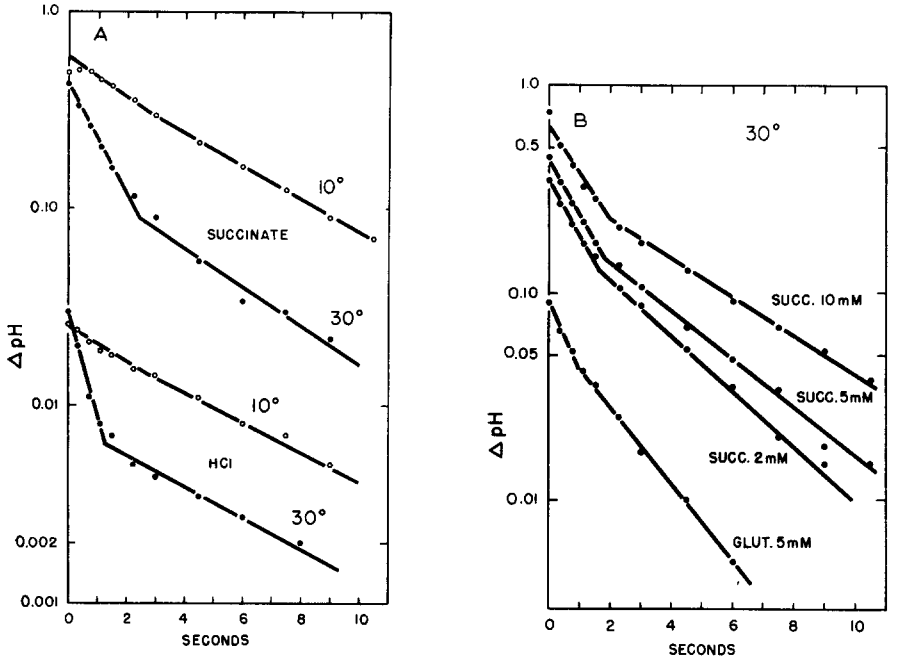

Fig. 4. Comparison between different acids. A. Succinic acid 25 mM at pH 4.0; base solution 20 mM NaOH and 2 mM tricine. (Note that these concentrations are cut in half due to mixing.) HCl, approx. 0.1 mM (pH adjusted to 4.0); base solution, 1.6 mM NaOH and 2 mM tricine. Temperature as indicated. B. Succinic or glutamic acids, at concentrations (after mixing) as indicated. Concentrations in the acid stage, which is most relevant for pool size, are twice those shown. 2 mM tricine in all base solutions, together with NaOH at 12, 17 or 30 mM for increasing concentrations of succinate, and 9 mM for the glutamic acid. Initial pH, 4.0; final pH, 7.9.

of a smaller amount of ATP formed in an acid–base experiment when using HCl. Fig. 4A also indicates that the quantity of protonatable groups either in or on the thylakoid membranes is less than the amount of succinate which enters from an external concentration of 25 mM.

Similarly the amount of the overshoot depends on succinate concentration in the acid stage (Fig. 4B) and is much smaller when glutamate is used. These observations are consistent with the previously postulated role for succinate (and for other acids which penetrate at pH 4) as internal proton reservoirs. Failure of labeled glutamate to penetrate to any large extent was noted earlier[6]. Another factor here is a slight buffering action of glutamate at pH 8; thus the buffer capacity with 5 mM glutamate resulted in a 0.46 pH unit change per μequiv of H^+ added per 2 ml, whereas with 5 mM succinate the pH change was 0.545 unit.

The initial chloroplast acid pH, then the final alkaline pH, were shifted independently (Figs. 5A and 5B). When the acid pH was raised from 3.8 to 4.7 the extent of the pH overshoot was much reduced, and the first phase became more distinct as its velocity was increased (Fig. 5A). The smaller extent of the overshoot is consistent with the previous demonstration of lesser entry of succinate as the pH in the acid stage is raised[6].

The family of curves for different base stage pH's is shown in Fig. 5B. The

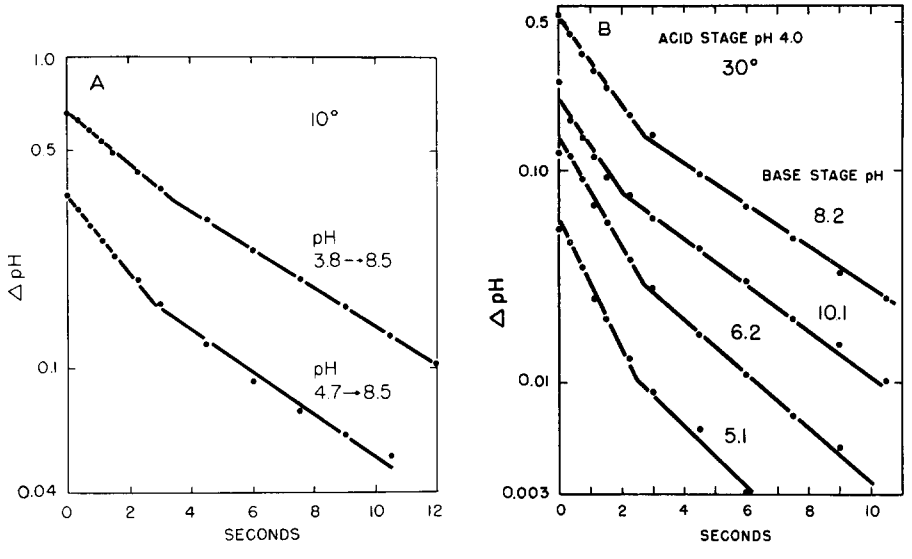

Fig. 5. Variations in acid or base stage pH. A. Two different acid pH's, 3.8 and 4.7, as shown; final pH, 8.5; temperature, 10°. Acid solution contained 25 mM succinic acid; chlorophyll concentration 0.50 mg/ml (0.25 mg/ml when in base stage). Rate constants for initial phase: 0.16 sec^{-1} for pH 3.8, 0.29 sec^{-1} for pH 4.7. Base solution contained 2 mM tricine, and 22 mM NaOH to neutralize the pH 3.8 acid stage, 14 mM NaOH to neutralize the pH 4.7 acid stage. B. Four different alkaline pH's: Acid solution contained 25 mM succinic acid and 0.50 mg chlorophyll per ml (final concentration in base stage 0.25 mg chlorophyll per ml). NaOH used to neutralize the succinic acid was 8, 16, 20 and 22 mM, respectively, for final pH of 5.1, 6.2, 8.2 and 10.1 as shown. Temperature, 30°.

extent of the apparent pH overshoot is highest at pH 8.2, dropping off at pH 10.1 as well as at the lower pH's (see DISCUSSION). All curves showed two phases with first-order kinetics, as before.

Addition of uncouplers to the medium caused both a marked acceleration and a shorter duration of the initial kinetic phase of acid efflux. This is shown for increasing concentrations of NH_4Cl (Fig. 6), which made the initial phase very distinct even at 10°. Results from experiments with chlorpromazine, chlorocarbonyl-

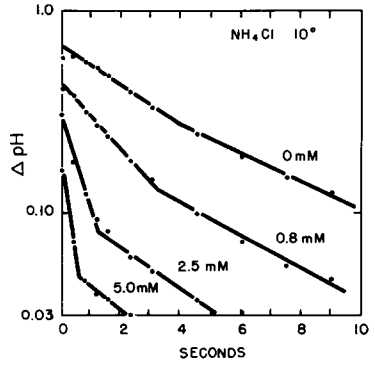

Fig. 6. Effect of NH_4Cl on kinetics of acid efflux. NH_4Cl concentrations in the acid stage as indicated; other conditions as described in METHODS.

TABLE III

UNCOUPLER EFFECTS ON ACID RETURN AND AMOUNT OF ATP FORMED

The temperature was 10° except where noted. ATP formation was assayed as described previously for the acid–base experiment[24] with final concentrations in the base phase of phosphate, 0.5 mM; $MgCl_2$, 5.0 mM; and ADP, 1.0 mM.

Expt.	*Reagent added*	*Acid stage concn. (mM)*	*First phase rate constant (sec^{-1})*	*ATP formed (nmoles/mg chlorophyll)*
1	None	—	0.22	32
	NH_4Cl	2.5	0.41	4.1
		5	2.01	1.9
	Chlorpromazine	0.08	0.24	24.8
		0.8	0.29	0
3	None	—	0.27	29.8
	CCCP	0.05	0.34	8.2
		0.5	0.42	1.7
	Chloroquine phosphate	0.6	0.50	1.6
		1.2	0.72	0.2
3	None (5°)	—	0.18	35.9
	(15°)	—	0.28	29.7
	(25°)	—	0.39	16.4
4	None (30°)	—	0.49	—
	Triton (30°)	—	2.0	—

cyanide phenylhydrazone (CCCP), chloroquine phosphate (an analog of atebrin) and Triton are shown in Table III. In these experiments ADP, $MgCl_2$ and radioactive phosphate were present in the alkaline mixture so that ATP synthesis due to the acid–base transition could be measured in the same reaction. (In other experiments the phosphorylation reagents were found to have no effect on the kinetics of acid return to the medium, at the concentrations used here.) Since previous experiments (Fig. 3A and 3B) had shown an acceleration of the first kinetic phase due to higher temperatures, an experiment at three different temperatures is included in Table III. A general correlation can be seen, with higher rate constants for the first phase of acid return associated with lower yields of ATP. This correlation is apparent in Fig. 7 where the numbers for ATP formation are plotted against the rate constants

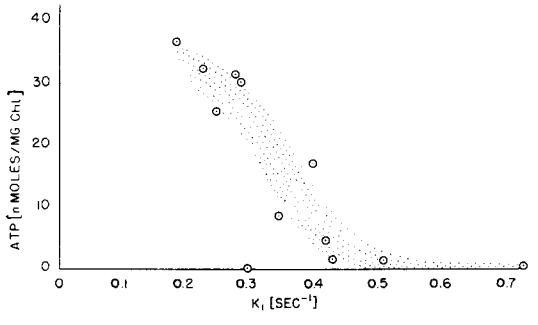

Fig. 7. Correlation between rate constant for initial phase of acid efflux, and amount of ATP synthesized in acid–base experiments.

listed in Table III. Even though the results of Fig. 7 came from different experiments using a variety of reagents as well as different temperatures to vary the rate constants, a consistent general correlation between the two can be seen. Reasonable amounts of ATP were formed only when rate constants were 0.35 sec^{-1} or less (half-time of 2 sec or greater). Only chlorpromazine inhibited phosphorylation without accelerating acid efflux.

Finally a comparison was made between the kinetics of acidity efflux in these acid–base experiments, with that to be seen during the light- and electron transport-driven ion movements of chloroplasts. As is known[12,16] chloroplasts in the light apparently remove acidity from the medium, then return it after the light is turned off. The kinetics of this return of acidity (post-illumination pH drop) are first order[17–19] and were reexamined at this point.

During the course of this work it became apparent that most of the previous studies of the light-induced pH change (see ref. 16, for instance) had been hampered by undetermined slow electrode responses, to a greater extent than was true in our present work. In one paper or another these limitations have included both the use of a slowly responding ordinary pH meter, and the absence of buffers from the medium which are needed to facilitate rapid response on the part of the glass electrode[20]. Table IV illustrates the discrepancies that can be found between apparent rates in the absence of buffer, and either the true rate or something closer to it observed in the presence of buffer. In this table 2-(*N*-morpholino)ethanesulfonic

TABLE IV

EFFECTS OF BUFFER, TEMPERATURE, AND PRIOR EXPOSURE TO ACID ON KINETICS OF THE LIGHT-INDUCED pH RISE

The numbers shown are the apparent first-order rate constants, in sec^{-1}, for the pH rise induced by light (Light On) and its decrease when the light is turned off (Light Off). The reaction mixtures contained 50 μg chlorophyll per ml, 10 μM pyocyanine and 10 mM NaCl; total volume 4.0 ml. When chloroplasts were acidified before illumination they were brought to pH 4.0 for 20 sec by the addition of either HCl (pH 6.2 series) or 10 mM succinate (pH 7.5 series). The acid was neutralized by adding an appropriate amount of NaOH. The buffer used at pH 6.2 was 2-(*N*-morpholino)ethanesulfonic acid, that at 7.5 was tricine, both at 1 mM when added. Plots of ongoing pH *minus* final pH on semi-log paper showed straight line kinetics for the entire time-course. The two numbers shown under each heading are from two successive illuminations in each case, and give some indication of the variability in the measurement. In general when buffer was present the variability between replicates fell within 25%; without buffer greater variations were encountered. In all cases rate constants without buffer present were lower than those with buffer present.

Previous pH 4 stage	*Light*	*Temp.*	*pH 6.2 buffer*				*pH 7.5 buffer*	
			−		+		+	
(A) None	On	10°	0.033	0.033	0.143	0.174	0.20	0.25
		30°	0.055	0.083	0.46	0.57	0.35	0.41
	Off	10°	0.032	0.035	0.054	0.066	0.082	0.097
		30°	0.055	0.068	0.23	0.28	0.25	0.30
(B) 20 sec	On	10°	0.053	0.067	0.144	0.182	0.79	—
		30°	0.079	0.121	0.58	0.68	2.12	2.43
	Off	10°	0.064	0.077	0.139	0.174	0.50	0.69
		30°	0.087	0.096	0.42	0.65	1.20	1.27

acid was used at pH 6.2 and tricine at pH 7.5. The actual extent of the discrepancy is greatest under conditions where the true rate is very fast, and is minimal where the true rate is slower. Thus in the "Light Off" (post-illumination pH drop) kinetics in Table IVA, comparing the rates with and without buffer at 10° (pH 6.2) the rate with buffer added is only twice as fast as that without. However at 30°, the rate constant with buffer is 4–5 times as fast as that without buffer. Similar accelerations were demonstrated using the following buffers at pH 6.2: pyrophosphate, arsenate, malonate, bicarbonate or glucose 1-phosphate. The acceleration was not seen when using formate, whose pK is very far from 6.2. The accelerating effect of these buffers saturated at 0.5 mM, with the exception of bicarbonate whose complex effects at higher concentrations will be described elsewhere.

The question that we originally asked was whether the speed of acidity loss from chloroplasts after an acid–base transition (rate constants between 0.2 and 0.8 sec^{-1}, see Tables II and III) was intrinsically different from that seen in the post-illumination pH drop in which rate constants of the order of 0.2–0.03 sec^{-1} (half-times of 3–20 sec) had usually been recorded. However the light-driven pH changes have been studied most often at pH 6.2, between 5 and 15°, and without a buffer in the medium. Thus the "Light Off" rate constants in Table IV at 10° rise from 0.033 to 0.06 due to having buffer present, then further to the range of 0.3 when the temperature is raised to 30°. These kinetics at 30° are closely comparable to those found in the acid–base experiments.

A further difference between previous light-induced pH change studies and the present ones, is that our chloroplasts have been held at pH 4.0 for 20 sec before their acidity loss is examined. This prior to exposure to acid could, and as is seen in Table IVB indeed does, have a profound effect on the kinetics of ion movements subsequently induced by light. Both the initial light-driven uptake of protons, and the post-illumination loss of acidity, are increased from 2- to 6-fold because the chloroplasts had been previously acidified. The accelerations are much more pronounced when the light-driven experiment is performed at pH 7.5, and are less extensive at pH 6.2. This activation of the speed of ion fluxes represents a new phenomenon which requires further investigation. Given the results shown in Table IV, however, it is at least possible to say that there are no very serious discrepancies in the rates of loss of acidity to the medium after an acid–base transition and that seen after illumination, and there is as yet no need to postulate the occurrence of different processes in the two types of experiment.

DISCUSSION

The present experiments, showing a slow return to equilibrium pH after adding base, are in many respects equivalent to earlier ones by MITCHELL AND MOYLE[20,21] in which mitochondria were subjected to pulses of acid or base. As before, the rational explanation for the overshoot phenomenon lies in the slow return to the medium of internal acidity requiring diffusion of protons or other molecules through the membranes of the organelle, in this case the swollen thylakoid disks. Our experiments differ in using a stopped-flow device for very rapid mixing, in using pre-soaking in a penetrating acid (succinic) to increase the internal buffering capacity greatly, and in use of an extremely large pH difference rather than the much smaller

ones employed by MITCHELL AND MOYLE[20,21]. It should be noted that the use of a stopped-flow apparatus permits the study of the larger pH difference. The electrode is originally at or close to the final pH; all one asks is that it records the initial overshoot (*i.e.* the pH of the medium only, when neutralized by base and buffer in the incoming solution) and its decay as acid leaks out of the vesicles.

A drawback of measuring pH only is that it does not distinguish between loss of protons and that of singly charged or uncharged succinic acid from the thylakoid interiors. Indeed it seems certain from the quantities involved in the overshoot that succinic acid was the major contributor. Thus in the comparable earlier study of labeled succinic acid uptake at pH 4 and loss at pH 8.5 (ref. 6), 1.0 μmole of succinic per mg of chlorophyll entered at pH 4, of which 0.1–0.15 μmole was retained after dilution and raising the pH to 8.5. At this alkaline pH presumably 0.85 μmole of protonated succinic acid diffused out rapidly, probably carrying about 1.35 μequiv of protons with them. The remaining 0.25 μequiv of protons would have exited alone (leaving the corresponding 0.15 μmole of succinate dianion trapped behind). Thus in the present experiments only the exit of succinic acid could have accounted for the 1.0–2.0 μequiv/mg chlorophyll observed leaving the swollen thylakoids.

When either the non-penetrating amino acid glutamate, or HCl were used in the acid part of the experiment not nearly as much acidity was involved in the overshoot, and the observed pH drop must have been due to the exchange of internal protons for external cations. (In this discussion it is assumed that in the absence of light no membrane potential is involved in these reactions; and that if it were it would be quantitatively negligible compared to the volume of protons and acid molecules moving.) Nevertheless it is interesting that the kinetics of the approach to equilibrium pH are very similar no matter whether HCl, succinic or glutamic acids are used (Fig. 4).

A reasonable reconstruction can now be made of events at pH 8.5, as chloroplasts come in loaded with internal succinic acid at pH 4. Some protons are lost from the inside. This leads to dissociation of the internal succinic acid. More protons leak out, some of these now derived from succinic acid molecules that had entered in the uncharged form. At the same time, and probable somewhat faster, uncharged succinic acid and half-charged succinic acid may be exiting down their diffusion gradient. Once outside the high pH causes their immediate dissociation to succinate anions so that the external succinic acid concentration is maintained at or close to zero, and more succinic acid keeps exiting. The only succinate molecules retained inside would be those whose protons had been able to leave fast enough. This accounts for the loss of 85 % of the internal succinic label[6] at pH 8.5, even though the actual dilution of total succinate molecules (both charged and uncharged) going from pH 4 to 8.5 was only one-half. The sum of protonated succinic acid and separate protons returned to the medium would cause the observed return of the external pH to its equilibrium value.

At lower pH values for the acid stage (pH 5.1 and 6.2 in Fig. 5B) the extent of the overshoot is less. Assuming approximately equal buffering capacity throughout (refs. 13, 14, 16; but see ref. 22) the smaller overshoot would result from the fact that succinic acid in the external medium is not completely dissociated at these pH's. Thus although succinic acid would exit down its concentration gradient

initially, the gradient would not be maintained as succinic acid accumulated in the outside solution. Less succinic acid will come out at equilibrium in the first place, and in the second place the undissociated part of the succinic acid which does come out will not contribute acidity to the final pH.

The finding of two quite distinct, kinetically separable components in acidity loss from chloroplasts after the acid–base transition was unexpected. The first phase is much more sensitive to (speeded up by) higher temperatures (Figs. 3A and 3B), and by uncouplers (Fig. 6, Table III). The fact that its Q_{10} is 2.0 suggests that it represents diffusion through a membrane whose structure and permeability change as the temperature is varied. These considerations are consistent with exit of protons and/or succinic acid through the thylakoid membranes. This component accounts for the majority of acidity loss after the acid–base transition.

The second kinetic component with a much slower rate constant is difficult to interpret. It lasted for 10–15 sec or longer, and had a Q_{10} of 1.2 as might be expected from simple diffusion or some other physical process. Ordinarily it accounted for no more than 20–30 % of the total pH overshoot. Any suggestion as to the origin of this component would be purely speculative at the present time.

If loss of acidity from chloroplasts to the medium always represents efflux of succinic acid it might seem strange for all uncouplers to increase the rate of this flow (Fig. 6, Table III). However there is no guarantee that the ratio of succinic acid to free protons in the efflux remains the same once an uncoupler is introduced. Uncouplers which act primarily by increasing permeability to protons or other ions (perhaps CCCP at least, among those tested) might permit faster rates of proton efflux and cation entry in the base stage, without changing the rate of permeation of free succinic acid. In that case a much larger part of the acidity loss would be due to the now more efficient proton transport; and one would predict a greater retention of succinic molecules as the sodium salt of the dianion. In the case of NH_4Cl, the free base NH_3 group may enter very rapidly, and the observed increase of acidity would result from dissociation of NH_4^+, replacing the equilibrium amount of free NH_3 on the outside[23,25]. The flux in this case would be that of NH_3 from out to inside the thylakoid membranes, causing neutralization of internal acidity and increase in external acid. Again one would predict a greater retention of internal succinate than usual, this time as the ammonium salt. In the case of Triton and other detergents, with a generally damaging effect on the membrane, permeability to succinate might very well be affected and the free acid may be diffusing out more rapidly than usual. Smaller than usual amounts of succinate would be retained. These predictions concerning succinate retention have yet to be tested. It is interesting to note that Triton and NH_3 have the most drastic effects on the rate of the first stage of acidity loss (Table III).

There are a number of similarities between the loss of acidity from chloroplasts after an acid–base jump, and that seen after the light-induced pH rise (Fig. 7). The ability to synthesize some ATP is associated with both of them[1]. Both rates are speeded up by uncouplers. The initial phase after the acid–base transition has a Q_{10} of 2 (Table II), and the post-illumination pH drop has a Q_{10} of about 1.8.

Considerable attention has been paid to the kinetics of light-induced pH changes in chloroplasts (see for instance refs. 14–16, 21, among others). It is unfortunate that the light-induced pH rise experiments were initially performed without

any added buffers[16] and without an appreciation of the kinetic limitations of glass electrode measurements. Even more unfortunate is the fact that this example was followed in later papers attempting to deal with kinetics. As clearly shown by MITCHELL AND MOYLE[20] and demonstrated again in Table IV, the slow electrode response due to use of an unbuffered solution can be limiting for the apparent speed of pH changes under many, if not most, circumstances. Similarly most ordinary pH meters respond very slowly to input changes. It is not possible to evaluate published reports of kinetic values since the seriousness of the electrode lags would depend on the temperature, on any inherent lags in the pH meter if one was used, on the inadvertent presence of buffers left over from chloroplast preparation procedures or added in the form of reagents designed to represent biochemical modifiers, *etc.* Nevertheless results from previous experiments performed without the stated presence of a buffer, or when using an unmodified pH meter, must be interpreted with great caution at this point. These reservations do not apply, of course, to pH measurements made in a flowing system[15] or to those made by optical methods using a pH-indicating dye in solution.

One of the more significant implications of the changes of pH around chloroplast thylakoid vesicles is the possibility that the gradient from inside to outside drives ATP formation[1,7]. If the pH gradient is the only controlling factor, then good correlations should be found between the kinetics of the pH changes, and those of the ability to make ATP. Whether following an acid–base transition[2] or in a post-illumination period[24] ATP formation in these storage experiments is largely finished by 3 or 4 sec indicating a probable half-time of less than 1 sec. Also, experiments designed to show the loss of the ability to make ATP (delayed additions of radioactive phosphate and of ADP) show kinetics of the same order of magnitude. On the other hand, even in the presence of a buffer so that electrode lags are considerably less than 1 sec, the half-time for the post-illumination pH drop at pH 7.5 is 7 sec at 10° (a temperature at least 5° higher than those used for most post-illumination ATP synthesis experiments) and 2.5–3 sec even at 30° (Table IV). These more leisurely kinetics for the post-illumination pH drop suggest that the pH difference is not the sole factor required for ATP formation after illumination; perhaps an additional, more labile intermediate or condition is formed in the light and its decay limits the ability to form more ATP even if the pH gradient remains. A similar tentative conclusion was reached previously[18] on the basis of the pH-insensitivity of dark decay kinetics of the pH rise, compared to extreme pH-sensitivity of dark decay for the post-illumination ATP synthesis.

For acid–base ATP synthesis the pH gradient must be the initial driving force whether or not its decay is the sole limiting factor for continuing synthesis. The loss of internal acidity in an uncontrolled, excessively rapid pathway would thus be expected to result in a decrease of ATP formation. The actual duration of the initial phase of acidity efflux appears to be 4 sec or more (Figs. 2, 3, 6) under conditions (low temperature, absence of uncouplers) which are optimum for ATP formation. At 30°, or in the presence of uncouplers, it is clear that this phase is shortened to 2 sec or less. At the same time the ability to form ATP decreases. A half-time for pH drop of 2 sec or longer seems required for reasonable ATP synthesis (Fig. 7). These results are consistent with continued maintenance of an adequate acidity gradient as a necessary prerequisite for continued ATP synthesis.

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REFERENCES

1 A. T. JAGENDORF, *Federation Proc.*, 26 (1967) 1361.
2 A. T. JAGENDORF AND E. URIBE, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 170.
3 J. H. KAPLAN, E. URIBE AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 120 (1967) 365.
4 R. BACHOFEN AND I. SPECHT-JURGENSEN, *Z. Naturforsch.*, 22b (1967) 1051.
5 N. SHAVIT, G. E. SKYE AND P. D. BOYER, *J. Biol. Chem.*, 242 (1967) 5125.
6 E. URIBE AND A. T. JAGENDORF, *Plant Physiol.*, 42 (1967) 697.
7 P. MITCHELL, *Biol. Rev. Cambridge Phil. Soc.*, 41 (1966) 445.
8 Y. NISHIZAKI AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 133 (1969) 225.
9 C. D. MILES AND A. T. JAGENDORF, *Biochemistry*, 9 (1970) 429.
10 W. S. LYNN, *Biochemistry*, 7 (1968) 3811.
11 J. A. SIRS, *Trans. Faraday Soc.*, 54 (1958) 207.
12 A. T. JAGENDORF AND J. NEUMANN, *J. Biol. Chem.*, 240 (1965) 3210.
13 P. RUMBERG, E. REINWALD, H. SCHRODER AND U. SIGGEL, *Naturwissenschaften*, 55 (1968) 77.
14 M. SCHWARTZ, *Nature*, 219 (1968) 915.
15 S. IZAWA AND G. HIND, *Biochim. Biophys. Acta*, 143 (1967) 377.
16 J. NEUMANN AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 107 (1964) 109.
17 R. A. DILLEY AND L. P. VERNON, *Arch. Biochem. Biophys.*, 111 (1965) 365.
18 S. J. D. KARLISH AND M. AVRON, *Biochim. Biophys. Acta*, 153 (1968) 878.
19 R. E. MCCARTY AND E. RACKER, *J. Biol. Chem.*, 242 (1967) 3435.
20 P. MITCHELL AND J. MOYLE, *Biochem. J.*, 104 (1967) 588.
21 P. MITCHELL AND J. MOYLE, *Biochem. J.*, 105 (1967) 1147.
22 G. M. POLYA AND A. T. JAGENDORF, *Biochem. Biophys. Res. Commun.*, 36 (1969) 696.
23 A. R. CROFTS, *Biochem. Biophys. Res. Commun.*, 24 (1966) 127.
24 G. HIND AND A. T. JAGENDORF, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 715.
25 A. R. CROFTS, *Biochem. Biophys. Res. Commun.*, 24 (1966) 725.