H^+ and Electron Poising and Photophosphorylation in Chloroplasts*

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ABSTRACT: Maintenance of both a high redox potential and a high internal concentration of H^+ within chloroplast is required for phosphorylation. Uncoupling agents accelerate rates of postillumination oxidation and deprotonation and thus impair the ability of illuminate chloroplasts to maintain high internal concentrations of reduced electron carriers. Rate of light-induced uptake of H^+ and internal acidification parallels rate of reduction of electron carriers within chloroplasts. At constant rates of electron flow, no uptake of H^+ occurs as long as the internal redox potential is low. The magnitude of H^+ uptake is a function of the type of external buffer present. At constant rates of electron flow and constant internal concentrations of H^+, H^+/chlorophyll ratios vary from 0.15 (sonically treated chloroplasts) to 3.4 (in the presence of imidazole). Alterations in external concentrations of H^+ cause reversible changes in the internal redox potential of chloroplast. The rate of change in redox state of the H^+-sensitive electron carriers with alterations in pH is slowed by organic anions, such as succinate and azide. The above observations suggest that phosphorylation in chloroplasts, whether driven by illumination or changes in external concentration of H^+, is the result of the oxidation of unknown H^+-sensitive electron carriers within chloroplasts. Efficient phosphorylation can occur only when the concentration of these unknown reduced carriers is maintained at relatively high levels by high internal pressures of H^+ and electrons. However, since phosphorylation is associated with oxidative electron flow, it is apparent that rapid phosphorylation can proceed only when the oxidative and reductive flows of electrons are properly poised.

Previous studies (Lynn and Brown, 1967a,b; Jagendorf, 1962; Zweig and Avron, 1965) have indicated that efficient photophosphorylation is associated with conditions in which the electron pressure within chloroplasts is maintained at high levels, *i.e.*, conditions in which the concentration of oxidized terminal electron acceptors, such as ferricyanide, chloranil, PMS^+,[1] or ferredoxin (Ramirez *et al.*, 1968), are maintained at low levels. The studies in this report also indicate that uptake of H^+ by illuminated chloroplasts can occur only when a high redox potential is maintained by electron flow within chloroplasts.

It is well known that the midpoint potential of some oxidoreduction compounds changes as a function of pH whereas that of others does not. Since the electron transport chains in chloroplast and mitochondria are composed of heterogeneous oxidoreductive carriers, some of which require protons for reduction, *i.e.*, quinones, flavins, pyridine nucleotides, while others do not, *i.e.*, cytochromes, it is likely that alterations in internal H^+ concentration will cause a flow of electrons from one carrier to another by disproportionately changing one midpoint potential but not the other. Recent studies (Lynn, 1968) have indicated that electron flow in chloroplasts does cause striking changes in H^+ concentration within chloroplasts, and that any agent which inhibits the internal accumulation of protons inhibits photophosphorylation. The studies in this report demonstrate that a high internal concentration of H^+ is required to maintain the electron transport chain in its reduced state. Low-potential electron acceptors which rapidly oxidize the chain, such as ferricyanide or quinones, even in the presence of rapid electron flow, inhibit the production of internal acidity and phosphorylation. Thus, it is concluded that the driving force for H^+ uptake in chloroplasts is the acquisition of electrons by proton-requiring electron carriers and that a high concentration of H^+ internally is required to maintain high concentrations of reduced carriers within chloroplasts. Since phosphorylation in chloroplasts is associated with oxidative electron flow, *i.e.*, both electron flow (this report) and phosphorylation proceed during the postillumination period (Jagendorf and Uribe, 1966; Lynn and Brown, 1967c), and since both electrons and external protons are required for phosphorylation, it appears that the oxidation and deprotonation of reduced carriers, either during or following illumination, cause phosphorylation.

Materials and Methods

Spinach chloroplasts were prepared as before (Lynn and Brown, 1967a). Sonically treated chloroplasts were prepared as before (Lynn, 1968).

Reduction and protonation of bound neutral red were

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PMS^+, phenazine methyl sulfate; SA, 5-chloro-3-(*p*-chlorophenyl)-2′-chloro-5′-nitrosalicylanilide; TTFB, tetrachlorophenyl-2-trifluoromethylbenzimidazole; CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; TBT, tributyltin chloride.

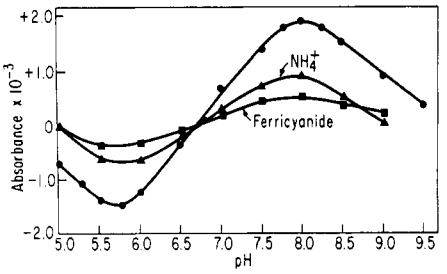

FIGURE 1: Effect of pH on reduction and protonation of neutral red. All experiments were performed at 15° using saturating white light. Time of illumination was 2 sec. Identical spectral changes as those recorded in the figure occurred with prolonged illumination (15 sec). Each experiment was performed in 3 ml of 0.4 M sucrose, containing 0.005 M $MgCl_2$, 0.01 M NaCl, 0.0006 M P_i, 0.007 mM PMS^+, and 0.003 mM neutral red, and 0.065 mg/ml of chlorophyll. The presence of 0.0003 M ADP had no effect on the observed spectral change. The points in the figure represent Δ absorbance, using 570 and 595 mμ as the monitoring wavelengths. Change in absorbance at 450 mμ was also assayed for each experimental point and was shown to decrease at all the above pH values. Calculations of the per cent of the dye undergoing protonation and reduction (see Methods) indicated that at pH 8 only protonation occurred and at pH 5.6 only reducton occurred. At pH 6.8, approximately 50% of the bound dye was reduced and 50% was protonated. Concentation of NH_4Cl was 10^{-3} M, and ferricyanide was 3.5×10^{-4} M. With prolonged illumination (30 sec) (sufficient to reduce all the added ferricyanide), the observed spectral changes in neutral red were the same as those initially observed in the absence of ferricyanide.

assayed spectrally, using a Britton-Chance Aminco dual-wavelength spectrophotofluorimeter with attached xenon light source, as before (Lynn and Brown, 1967c). Light intensity was measured as before (Lynn and Brown, 1967b). Protonation and deprotonation of neutral red was followed by quantitating the reciprocal changes that occur at 450 and 540 or 570 mμ. The control wavelength used was 595 mμ. Reduction of neutral red was associated with parallel decreases in absorption at 540 and 450 mμ. Although the extinction coefficients of neutral red at 450 and 540 mμ are different (at pH 7.0, E_{450} 8.4×10^3, E_{570} 5.9×10^3), changes in internal pH result in reciprocal and linear, although unequal, change in absorbance of the two wavelengths. Changes in the oxidoreductive state of the dye result in parallel and linear, although unequal, changes in absorbance of the two wavelengths. The concentration of the dye undergoing pH change can therefore be calculated as follows: $[A_{570}][E_{570}] - [\Delta A_{450}$ observed $- A_{450}$ calculated$][E_{450}]$ = concentration of dye undergoing pH change. ΔA_{450} calculated = expected change in absorbance at 450 mμ in association with change in absorbance at 570 mμ. The calculations and observations on the changes in spectral properties of neutral red were based on the assumption that no changes occurred as a result of changes in the dielectric environment of the dye. The spectral characteristics are identical in water and in 90% ethanol. However, in absolute ethanol oxidized neutral red is colorless. To avoid spectral interference due to changes in absorbance of the cytochromes, 570 mμ rather than 540 mμ was generally used as the monitoring wavelength for neutral red. Changes in redox state of cytochrome *b* were followed using light at 559 and 575 mμ, 553 and 575 mμ were used for cytochrome *f*, 550 and 575 mμ for cytochrome *c*, and 703 and 722 mμ for P_{700}. The reference wavelength for all the cytochromes was 535 mμ. The reference wavelength for P_{700} was also 575 mμ. Negligibly small amounts of cytochrome$_{563}$ were observed in these preparations of chloroplasts (Hind, 1968; Cramer and Butler, 1967). Changes in light scattering were monitored under all the conditions of this report, using light at 574 and 592 mμ. The changes in absorbance due to changes in light scattering were negligibly small in all experiments.

All spectral experiments were performed at 15° in 3 ml of 0.4 M sucrose, containing 0.005 M $MgCl_3$, 0.01 M NaCl, 0.0005 M sodium phosphate, 0.0002 M ADP, and chloroplasts, unless otherwise stated. These conditions (low temperature, high concentration of Mg^{2+}, and hypertonic sucrose) were used to decrease rates of oxidoreduction and thus facilitate the measurements (Cramer and Butler, 1967).

Rate and extent of H^+ uptake were assayed titrimetrically in 20 ml of the above solution, as before (Lynn and Brown, 1967b). Rate of phosphorylation was assayed titrimetrically and enzymatically, as above (Lynn and Brown, 1967b). All materials used in these studies were reagent grade commerical products. Nigericin was obtained from Mr. R. L. Harnod, Commerical Solvents, Inc., Terre Haute, Ind. SA was obtained from Dr. P. C. Hamm, Monsonto Chemicals, Inc., St. Louis, Mo.

Results

Protonation and Reduction of Neutral Red. Neutral red, an oxidoreduction pH indicator dye, is protonated by illuminated chloroplasts at pH values above its pK_a, but reduced at pH values below its pK_a (Figure 1) (Lynn, 1968). Both protonation and reduction of neutral red are inhibited by uncoupling agents, such as NH_4^+, quinacrine, organometals, benzimidazoles, salicylanilide, and nigericin, or by high concentrations of electron acceptors such as ferricyanide, PMS^+, benzoquinone, and chloranil. As previously indicated (Lynn, 1968), these spectral changes of neutral red represent changes within chloroplasts, for neutral red is bound to the chloroplasts and these spectral changes are observed under conditions in which no change in external pH occurs (Lynn, 1968). At an acidic pH, protonation of neutral red can also be observed. This is so, however, only at very low concentrations of the dye (Table I), or in the presence of low concentrations of uncoupling agents or low light intensities.

As indicated in Table I, uncoupling ions inhibit reduction of neutral red at concentrations which do not alter rates of electron flow. (Electron flow was measured titrimetrically, as before (Lynn and Brown, 1967a), using ferricyanide as electron acceptor.) This inhibition of reduction of neutral red by ions is associated with markedly accelerated rates of oxidation (Table I) and deprotonation of neutral red (Table II) following illumination, as well as with impairment of rates of H^+ uptake (Table I).

Reduction of other low potential electron acceptors,

TABLE I: Effect of Uncouplers on Reduction and Protonation of Neutral Red and H^+ Uptake at Various Concentrations of Neutral Red.[a]

Additions		Neutral Red			
Neutral Red (M)	Uncouplers (M)	Protonated (μmoles, $\times 10^{-3}$)	Reduced (μmoles, $\times 10^{-3}$)	Oxidized (μmoles/min)	H^+ Uptake (μmoles/min)
1. 0	0	0	0	0	3.3
2. 1×10^{-6}	0	10.0	0	0	4.2
3. 4×10^{-6}	0		44	0.11	5.0
4. 1×10^{-5}	0		104	0.15	4.0
5. 4×10^{-5}	0		150	0.25	2.5
6. 7×10^{-5}	0		131	0.24	1.8
7. 4×10^{-6}	TBT, 7×10^{-8}	7.5	0	0	1.4
8. 4×10^{-6}	NH_4^+, 1.5×10^{-3}		11	0.47	1.9
9. 4×10^{-6}	TTFB, 1×10^{-7}	8.0	0	0	1.2
10. 4×10^{-6}	SA, 5×10^{-9}	5.0	0	0	1.3
11. 4×10^{-5}	TBT, 7×10^{-8}		35	1.05	0.4
12. 4×10^{-5}	NH_4^+, 1.5×10^{-3}		51	0.81	0.5

[a] Experimental conditions as in Figure 1. Initial pH 6.20. Δ changes in absorbance at the end of 10-sec illumination using 569, 450, and 594 mμ as monitoring light were recorded and expressed as net amount of neutral red that was either reduced or protonated by illumination. H^+ uptake was assayed in 20 ml and spectral studies were done in 3-ml aliquots. Calculations of amounts of dye oxidized and reduced were all based on the original 20-ml volume. Initial rates of oxidation of neutral red following illumination are also tabulated. Initial rates of H^+ uptake were recorded titrimetrically (see Methods). Uncoupling agents were added 1 min prior to illumination and at least three consecutive light–dark cycles were recorded for each experimental determination. Rate of electron flow was assayed titrimetrically, using ferricyanide as terminal electron acceptor under all of the above conditions. The uncoupling agents, at the above concentrations, had no effect on rate of electron flow. Data not shown.

such as methylene blue (Table III), is also impaired by these uncoupling ions. This inhibitory effect is most marked at an alkaline pH. However, rate of postillumination oxidation of methylene blue is not affected by these uncoupling agents. Rates of autoxidation of methylene blue are much slower than that of neutral red (compare Table I and Table II). Under anaerobic conditions, the rate of autoxidation of methylene blue following reductive illumination was shown to be essentially zero (data not shown). This indicates that methylene blue, unlike neutral red, is unable to be ozidized following illumination by oxidized electron carriers within the chloroplast, but is oxidized by molecular oxygen.

Uncoupling agents also inhibit reduction of internal electron carriers, such as cytochromes *b* and *f* (Figure 2), as well as accelerate the rate of oxidation of the cytochromes in the dark. Rate of oxidation of cytochrome *b* by ferricyanide in the dark is more rapid than rate of oxidation of cytochrome *f* (Avron and Chance, 1966).

Other organic ions which inhibit internal acidification and photophosphorylation are tabulated in Table IV. These agents also strikingly accelerate rate of deprotonation of neutral red following illumination. All agents which inhibit internal acidification also inhibit in a parallel manner both photophosphorylation and re-

TABLE II: Effect of Salt on Protonation of Neutral Red.[a]

Additions (M)	Neutral Red	
	Protonated	Deprotonated
None	32	41
KCl, 5×10^{-3}	30	64
TBT, 6×10^{-8}	16	65
TBT + KCl, 5×10^{-3}	14	154
Nigericin, 5×10^{-9}	27	77
Nigericin + NaCl, 5×10^{-3}	17	168
Nigericin + KCl, 5×10^{-3}	11	251
NH_4Cl, 1.5×10^{-3}	14	191
NH_4Cl + nigericin, 5×10^{-9}	9	235

[a] Experimental conditions as in Figure 1, except no NaCl was added and the initial pH was 7.15. Extent of protonation of neutral red at the end of 10-sec illumination and initial rate of deprotonation of neutral red following illumination were monitored as in Figure 1. The absorbance changes are expressed as Δmμmoles of protonated neutral red formed and mμmoles/min of neutral red deprotonated following illumination.

TABLE III: Effect of Uncoupling Agents on Reduction of Methylene Blue.[a]

Additions (M)	pH 7.4 Reduced (μmoles, $\times 10^{-3}$)	pH 7.4 Oxidized (μmoles/min, $\times 10^{-3}$)	pH 5.9 Reduced (μmoles, $\times 10^{-3}$)	pH 5.9 Oxidized (μmoles/min, $\times 10^{-3}$)
None	2.2	8.1	3.5	12.1
TBT, 10^{-7}	0.9	7.5	2.7	10.5
NH_4^+, 10^{-3}	1.1	9.0	3.2	14.1
TTFB, 6×10^{-8}	0.6	8.3	2.9	12.5
CBZ, HVE, 10^{-5}	0.7	8.3	3.0	13.0
PMS^+, 10^{-5}	0	0	0	0
TBT, 6×10^{-7}	0.0	0	1.4	11.1
NH_4^+, 2×10^{-3}	0.4	7.5	2.1	12.7
Chlorpromazine, 3×10^{-6}	1.2	7.5	2.2	12.5

[a] Experimental conditions as in Figure 1, except no neutral red was added. Illumination time was 1 sec, using saturating light at 650 mμ. Reduction and oxidation of methylene blue (8×10^{-6} M) was followed, using 690 and 712 mμ as monitoring wavelengths. Initial pH was adjusted with dilute HCl or NaOH.

duction of neutral red. Two of the agents, *i.e.*, carbodiimide and tetraphenylphosphonium chloride, inhibit phosphorylation without affecting internal acidification (Table IV). The concentrations of the inhibitors required for inhibition of reduction are in most cases the same as those required for inhibition of protonation. Nigericin and TTFB, anions with pK_a's of less than 6.0, inhibit protonation and reduction of neutral red most effectively at an acidic pH. This is probably a reflection of the fact that these two anions in their un-ionized state are more insoluble in water, and thus are probably more tightly bound to the hydrophobic membranes of chloroplasts at an acidic pH.

Oxidoreductive Poising. As indicated in this report and previously (Lynn and Brown, 1967a,b), efficient phosphorylation can occur only under conditions in which the electron transport chain of chloropolasts is maintained in a reduced state during electron flow. Similarly, protonation of neutral red and H^+ uptake require

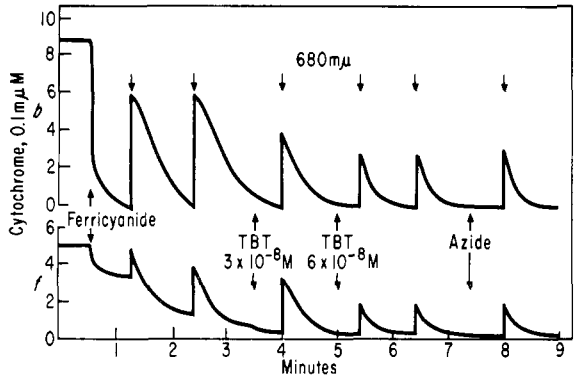

FIGURE 2: Inhibition of reduction of internal cytochromes by uncoupling agents in the presence of excess ferricyanide. Experimental conditions as in Figure 1, except no neutral red was added. pH 6.8. Rates of reduction and oxidation of cytochrome b_{559} and cytochrome f_{552} were followed. Illumination (680 mμ, 5.6 μEinsteins/min) was 2 sec in duration, as indicated by the arrows in the figure. Sodium ferricyanide (0.5 μmole) was added at 0.5 min. Uncoupling agents (*i.e.*, TBT, TTFB, SA, and tributylgermanium chloride) were added at 3.5 min, after two light–dark cycles. The data shown are for TBT only. However, similar traces were obtained using the above uncoupling agents at the concentations listed in Table IV. The data are expressed as amounts of the cytochromes either reduced or oxidized with time, using known extinction coefficients for chloroplast cytochromes *f* and *b*. To ensure that the changes in absorbance at 552 and 559 mμ were due to changes in oxidoreduction of cytochromes, changes in absorbance at 547, 555, and 564 mμ were also observed in these experiments. Maximal spectral changes occurred at 552 and 559 mμ.

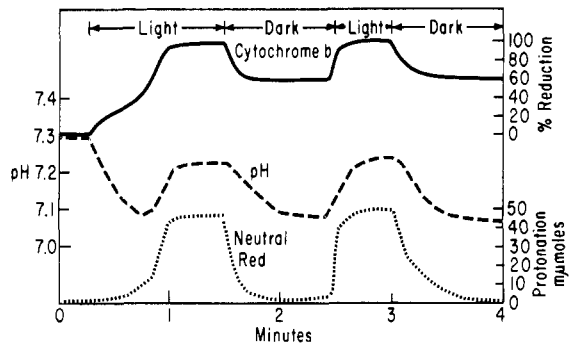

FIGURE 3: Kinetic relationship between protonation of neutral red, H^+ uptake, and the internal redox potential. Experimental conditions as in Figure 1, except that ADP was omitted and 0.5 μmole of sodium ferricyanide was added. Illumination (680 mμ, 5.6 μEinsteins/min) was supplied as indicated in the figure. Rate of reduction of cytochrome *b*, H^+ uptake, and protonation of internal neutral red were monitored (see Methods). Rate of reduction of ferricyanide is indicated by the first portion of the pH trace. Rate of reducton of cytochrome *b* was assayed in the absence of neutral red.

TABLE IV: Inhibition by Organometals and Other Organic Ions.[a]

	Concentration Required for 50% Inhibition		
	Internal Acidification pH 7.2 (M)	Reduction pH 6.2 (M)	Phosphorylation pH 7.2 (M)
Tributyltin chloride	7×10^{-8}	8×10^{-8}	8×10^{-8}
Dibutyltin dichloride	5×10^{-7}	5×10^{-7}	5×10^{-7}
Monobutyltin trichloride	2×10^{-5}	2×10^{-5}	2×10^{-5}
Trimethyltin chloride	7×10^{-7}	9×10^{-7}	8×10^{-7}
Tetramethyltin	No effect	No effect	No effect
Tri-*N*-propyltin oxide	2×10^{-7}	1.5×10^{-7}	2×10^{-7}
Tetrabutyltin	No effect	No effect	No effect
Tributylgermanium chloride	2×10^{-6}	2×10^{-6}	2×10^{-6}
Diphenylgermanium dichloride	10^{-5}	8×10^{-6}	10^{-5}
Triethylgermanium bromide	2×10^{-5}	2×10^{-5}	3×10^{-5}
Tetraphenyllead chloride	4×10^{-7}	4×10^{-7}	5×10^{-7}
Triphenylantimony chloride	3×10^{-6}	2×10^{-6}	3×10^{-6}
Tetraphenylantimony chloride	7×10^{-7}	5×10^{-7}	5×10^{-7}
Triphenylselenium chloride	2×10^{-6}	2×10^{-6}	2×10^{-6}
Tetraphenylphosphonium chloride	No effect	No effect	5×10^{-6}
Imidazole	2×10^{-3}	3×10^{-3}	5×10^{-3}
TTFB	3×10^{-7}	8×10^{-8}	3×10^{-7}
CBZ-L-histidyl-L-valine benzyl ester	1.5×10^{-5}	1×10^{-5}	1.5×10^{-5}
Histidine	No effect	No effect	No effect
Polyhistidine	No effect	No effect	No effect
Guanidine	No effect	No effect	No effect
Chlorpromazine	4×10^{-6}	3×10^{-6}	4×10^{-6}
Salicylanilide	3×10^{-8}	3×10^{-8}	4×10^{-8}
Carbodiimide	No effect	No effect	5×10^{-7}
Desoxycholate	10^{-4}	$\times 10^{-5}$	10^{-4}
Nigericin[b]	5×10^{-9}	2×10^{-9}	5×10^{-9}
Polylysine (mol wt = 32,000)	3×10^{-7}	4×10^{-7}	3×10^{-7}

[a] Experimental conditions as in Figure 1. Extent of protonation of neutral red (pH 7.2), rate of phosphorylation (pH 7.2), and extent of reduction of neutral red (pH 6.2) were assayed, as in Figure 1, with various concentrations of uncoupling agents. The concentrations of each agent required to inhibit the above light-dependent reactions by 50% are listed in the table. All agents were added 1 min before illumination and at least three light–dark cycles were observed under each condition. [b] 5×10^{-3} M KCl was added.

that the electron carriers be maintained during electron flow in a reduced state (Figure 3). In the presence of ferricyanide, which oxidizes all the internal cytochromes *b* and *f*, as well as P_{700} (Avron, 1967), and with low light intensities, H^+ uptake, reduction of cytochrome *b*, and internal acidification do not occur with illumination until most of the ferricyanide is photochemically reduced (Figure 3). Although not shown in Figure 3, cytochrome *f* and P_{700} are also not reduced until most of the added ferricyanide is reduced. Similar effects are observed with high concentrations of other terminal electron acceptors, such as chloranil and benzoquinone. Since rate of over-all electron flow is constant under all the above conditions, it appears that neither phosphorylation nor H^+ uptake can occur until a high concentration of reductants is established by electron flow in chloroplasts.

Since it is likely that the electron transport chain of chloroplasts is composed of oxidoreductive couples whose oxidoreductive potentials are variably sensitive to hydrogen ion concentrations, alterations in external pH should also cause a flow of electrons between these electron carriers. That this is true is indicated in Figure 4. In the presence of a reduced electron acceptor of system I, such as cytochrome *c*, addition of protons (pH 7.4–5.2) in the dark causes oxidation of cytochrome *c* and a concomitant reduction of cytochrome *b*. Addition of OH^- reverses this process. The changes in redox state with change in pH (Figures 4 and 5) (data not shown) are not inhibited by CMU. Uncoupling agents, such as NH_4^+, TBT, and TTFB, at the concentrations indicated in Table IV, partially but not completely inhibit rate and extent of these pH-sensitive changes in redox state (data not shown). That these spectral changes do represent changes in the redox state of the cytochrome, rather than changes in light scattering of the chloroplast, was proven by showing that no spectral changes with alterations in external pH, using 570- and 585-mμ radiations as control wavelengths, occur. Also,

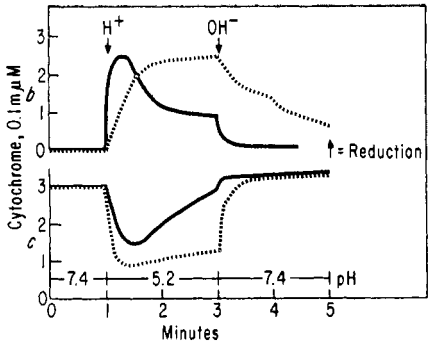

FIGURE 4: Effect of alterations of external pH on the redox potential of bound cytochromes. Experimental conditions as in Figure 1. Neutral red was omitted and 9 μg of reduced cytochrome *c* was added. Change in redox potential of cytochromes *b* and *c* was monitored (see Methods) in separate but identical experiements, as in Figure 2. Sufficient amounts (0.03 ml) of HCl and NaOH to alter the external pH as indicated in the figure were added in the dark. Concentration of CMU where added was 3×10^{-5} M. (——) +CMU; (- - -) −CMU. These changes in redox state, as a consequence of changes in pH, were not observed in disrupted chloroplasts (prepared by heating to 80° for 5 min or by prolonged sonic treatment).

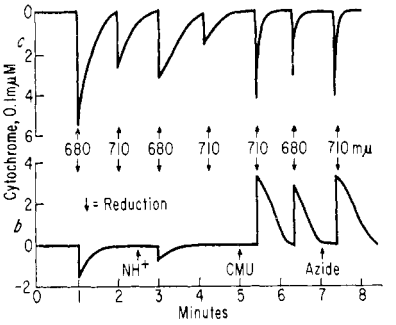

FIGURE 6: Effect of NH_4^+, CMU, and azide on changes of redox potential of cytochromes by illuminated chloroplasts. Experimental conditions as in Figure 4. Rates of oxidoreduction of cytochromes *b* and *c* were monitored as in Figure 4. Illumination (10 μEinsteins/min, 2-sec duration) was supplied, as indicated. NH_4Cl (0.001 M), CMU (2×10^{-5} M), and sodium azide (2×10^{-4} M) were added where indicated. Cytochrome *b* was 81% reduced initially in these chloroplasts (assayed by addition of excess ferricyanide in the dark). Other uncoupling agents, such as TBT, TTFB, and SA, produced effects very similar to those shown for NH_4^+, under these conditions.

alterations in external pH in the presence of excess low-potential terminal electron acceptors, such as ferricyanide which causes complete oxidation of the above electron carriers, result in no change of the redox state of the cytochromes (data not shown). Thus, it is concluded that an increase in external H^+ ion concentration does cause a reverse flow of electrons within chloroplasts. A further indication that the observed spectral changes with alterations in external pH do indeed represent electron flow is shown in Figure 5. When cytochrome *c* is fully reduced with ascorbic acid at pH 7.4, light causes oxidation of some of cytochrome *c*. Addition of protons oxidizes a portion of the cytochrome and subsequent illumination reduces the now oxidized cytochrome *c*. Prolonged sonic treatment (15 sec) or heating (80° for 5 min) of chloroplasts cause complete inhibition of the spectral changes depicted in Figures 4 and 5.

It is not clear from these data whether the observed

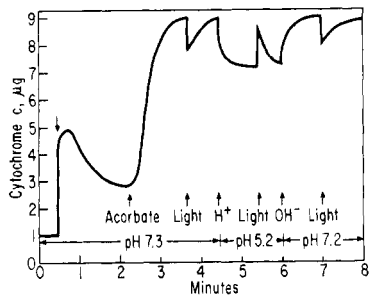

FIGURE 5: Effect of alterations of external pH on the redox potential in the presence of ascorbate. Experimental conditions as in Figure 4. Change in oxidoreduction of cytochrome *c* was followed and expressed in micrograms. Illumination (680 mμ, 10 μEinsteins/min, 2-sec duration) was added at the indicated intervals. Sodium ascorbate (1.0 μmole) was added at 2.2 min. HCl (0.03 ml) and NaOH (0.03 ml), sufficient to alter the external pH as indicated, were added at 4.4 and 6 min, respectively.

changes in redox state of the cytochromes with changes in pH are coupled to electron carriers of system I or II or of both. However, since system I, when illuminated, can cause a flow of electrons from cytochrome *b* to cytochrome *c* (Figure 6), and since CMU does not inhibit either hydrogen ion sensitive redox changes or the changes in oxidoreduction of these cytochromes which is associated with activation of system I (Figure 6), it is possible that these pH-sensitive changes in redox state are the result of changes in redox state of the pH-sensitive electron carriers associated only with system I.

Relation of H^+ Uptake to Internal Acidification. Previous studies have indicated that illuminated chloroplasts extract approximately 1 mole of protons/mole of chlorophyll from the exterior (Lynn, 1968; Jagendorf and Uribe, 1966; Avron, 1967; Newmann and Jagendorf, 1964; Dilley and Vernon, 1965). Other studies have indicated that some uncoupling agents inhibit phosphorylation without inhibiting the magnitude of H^+ uptake (Kahn, 1968). The studies in this report, however, indicate that most of the known inhibitors of phosphorylation inhibit the rate of formation of internal acidification as well as the rate of H^+ uptake by illuminated chloroplasts. As indicated in Figure 7 the rate and extent of H^+ uptake are dependent upon the ionic content of the external environment. Imidazole (3×10^{-4} M) markedly increases the extent of H^+ uptake, *i.e.*, over 3 μmoles of H^+/μmole of chlorophyll are taken up in the presence of imidazole. This effect of imidazole on H^+ uptake is greatest when the suspending medium is 0.1 M NaCl rather than 0.4 M sucrose. Since longer periods of illumination are required for maximum H^+ uptake in the presence of imidazole (Figure 7), and since the initial rates of H^+ uptake in the presence and absence of imidazole are almost equal, imidazole does not appear to increase appreciably the efficiency of H^+ uptake. This effect of imidazole is inhibited by NH_4^+ and other uncoupling agents (Figure 7) and is not associated with any increase in rate of electron flow, as measured with

TABLE V: Effect of Imidazole on Phosphorylation, H^+ Uptake, and Protonation of Neutral Red.[a]

Additions (M)		ATP Formation	H^+ Uptake		
P_i	Imidazole	(μmoles/min)	μmoles/min	μmoles	Internal pH
6×10^{-5}		0.66	2.0	0.27	−0.75
6×10^{-5}	1.5×10^{-4}	0.89	4.1	1.05	−0.49
6×10^{-5}	3×10^{-4}	0.75	4.3	1.20	−0.28
6×10^{-5}	10^{-3}	0.68	4.0	1.15	−0.26
1×10^{-3}		1.50	1.9	0.19	−0.69
1×10^{-3}	1.5×10^{-4}	1.48	2.6	0.65	−0.31
1×10^{-3}	3×10^{-4}	1.55	3.4	0.90	−0.24
1×10^{-3}	10^{-3}	1.42	3.6	0.92	−0.20

[a] Experimental conditions as in Figure 1, except the suspending medium was 0.1 M NaCl rather than 0.4 M sucrose Concentrations of P_i and imidazole are indicated in the table. Illumination was saturating white light. Formation of ATP and internal acidification were assayed in the presence of 0.0004 M ADP. Rate and extent of H^+ uptake was measured in the absence of ADP. Initial pH 7.55. Chlorophyll content, 0.41 μmole/20 ml.

ferricyanide as the terminal acceptor, nor does imidazole at these concentrations inhibit phosphorylation at low or high concentrations of P_i (Table V). Internal acidification, as measured with neutral red, is, however, inhibited by imidazole (Table V). This apparent inhibition of internal acidification by imidazole is probably the result of competition between neutral red and imidazole for entrance into the chloroplast, rather than a true measure of internal pH. Imidazole, under the conditions of Table V, was shown to inhibit the binding of neutral red by chloroplasts [assays of binding, as before (Lynn, 1968)]. As indicated in Table VI, organic ions other than imidazole whose pK_a's are approximately 7, such as brom thymol blue and neutral red, also increase the extent of H^+ uptake. The stimulatory effect of imidazole on H^+ uptake is observed only at pH values above its pK_a (Figure 8). Brom thymol blue and neutral red also stimulate extent of H^+ uptake, but only at a pH above their pK_a's. Arsenate plus ADP have also been shown to increase H^+ uptake at an alkaline pH (Karlish and Avron, 1967). Thus, both neutral red and imidazole appear to enter chloroplast in their un-ionized state and act as internal buffers or trapping agents for incoming protons.

In sonically treated chloroplast, imidazole has very little effect on extent of H^+ uptake, nor does it appreciably inhibit internal acidification (Table VII). Since illuminated sonically treated chloroplasts extract very few protons (Table VII) although internal acidification does occur, it appears that protonated imidazole and neutral red (protonated as a consequence of illumination) become trapped within intact chloroplast but not within disrupted chloroplasts, and thus serve as effective internal buffers. Sonic treatment of chloroplasts also apparently causes a loss of native internal buffers, since disrupted chloroplasts require very few external protons to establish a low internal pH.

TABLE VI: Effect of Bromothymol Blue, Imidazole, and Neutral Red on H^+ Uptake.[a]

Additions (M)	H^+ Uptake μmoles/min	μmole
	0.35	0.09
BTB (4×10^{-6})	1.01	0.18
BTB + PMS^+ (2×10^{-6})	1.98	0.42
NR (5×10^{-6})	1.45	0.23
NR + PMS^+ (2×10^{-6})	2.15	0.42
PMS^+ (2×10^{-6})	2.00	0.28
Imidazole (3×10^{-4})	0.48	0.19
Imidazole + PMS^+ (2×10^{-6})	2.35	0.72

[a] Experimental conditions as in Table V, except suspending medium was 0.4 M sucrose. PMS^+ was added where indicated. Initial pH 7.55. Chlorophyll content, 0.45 μmole/20 ml. Rate and extent of H^+ uptake assayed as in Table V.

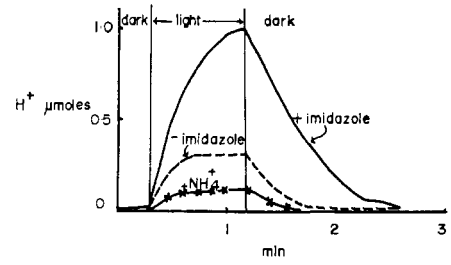

FIGURE 7: Effect of imidazole on H^+ uptake by illuminated chloroplasts. Experimental conditions as in Figure 1, except no ADP was added and the suspending medium was 20 ml of 0.1 M NaCl. Initial pH 7.5. Chlorophyll content, 0.4 μmole. H^+ uptake was followed titrametrically (see Methods) in the presence of saturating white light, as indicated. Either imidazole (3×10^{-4} M) or NH_4Cl (0.001 M) was added, as indicated in the figure.

TABLE VII: Effect of Imidazole on Sonically Treated Chloroplast.[a]

Imidazole (M)	ATP Formation (μmoles/min)		H^+ Uptake (μmoles/min)		ΔH^+ Uptake (μmole)		Internal ΔpH	
	1.39	0.99[b]	1.41	0.85	0.38	0.12	−0.81	−0.64
2.5×10^{-4}	1.48	0.81	2.25	0.98	0.80	0.17	−0.86	−0.55
7×10^{-4}	1.34	0.68	2.55	0.79	0.83	0.14	−0.76	−0.43

[a] Experimental conditions as in Table VI. Sonically treated chloroplasts (chlorophyll content 0.35 μmole) were prepared as before (6). Initial pH 7.5. H^+ uptake, phosphorylation, and internal acidification (using neutral red) were assayed, as in Table V, in the presence and absence of imidazole. Internal acidification is expressed as Δ change in internal pH with saturating illumination (Lynn, 1968). [b] The first set of numbers under each column was obtained with intact chloroplasts and the second set of numbers with sonicated chloroplasts.

Role of Anions in Maintaining Internal Redox States. The previous acid bath experiments of Jagendorf and Uribe (1966) indicated that large anions were required for phosphorylation. As indicated in this report, the addition of OH^- to acidic chloroplasts causes changes in the redox state of some of the electron carriers in chloroplast. If alkali is added to chloroplasts which contain large amounts of un-ionized anions, which anions can serve as internal reservoirs of protons, the time required for oxidation of the electron chain should be prolonged and thus allow more time for movement of P_i, ADP, and ATP on and off the oxidative coupling sites. That succinate and other anions do slow the rate of oxidation following reduction of cytochromes *c*, *f*, and *b* by illumination is shown in Figure 9. Since oxidation of proton-sensitive electron carriers of chloroplast also requires deprotonation, rates of oxidation following illumination are slowest at lower pH values (Figure 9). In the presence of CMU, which blocks system II, cytochrome *b*, *f*, and P_{700} are oxidized upon illumination of system I, while cytochrome *c* is reduced (Figure 7) (Cramer and Butler, 1967; Avron, 1967). Following illumination, the rate of oxidation of reduced cytochrome *c* is accelerated, since the concentration of oxidants within the electron chain is, in the presence of CMU, high (Figure 9). Succinate also slows the rate of oxidation of reduced cytochrome *c* in the presence of CMU. In the presence of uncoupling agents (Table IV), anions were shown to have no effect on the dark rates of electron flow under the conditions of Figure 9. Thus, it is concluded that the redox potential within chloroplasts is controlled by internal H^+ ion concentration and that agents, such as impermeable anions or permeable cations, which control H^+ ion concentration by serving either as internal buffers, or as exchange cations for H^+, exert striking effects on rate and direction of electron flow within chloroplasts.

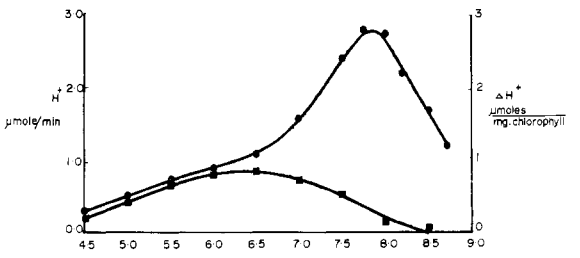

FIGURE 8: Effect of hydrogen ion concentration on H^+ uptake in the presence and absence of imidazole. Experimental conditions as in Figure 7. Initial pH as indicated. Chlorophyll content, 0.61 μmole. The points on the curves represent maximal uptake of H^+ using saturating white light after 1-min illumination in the presence and absence of 0.0003 M imidazole. (●) +imidazole; (■) −imidazole.

Discussion

The data in this report, indicating that rate of phosphorylation is linearly related to both the reducing potential and the proton potential within chloroplasts, suggest that phosphorylation is a consequence of the oxidation of some protonated reduced electron carriers within chloroplasts. That phosphorylation is linearly associated with oxidative electron flow seems indicated

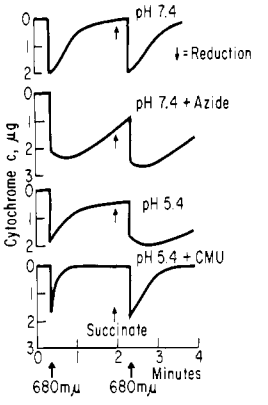

FIGURE 9: Effect of anions on rate of oxidation of cytochromes following illumination. Experimental conditions as in Figure 6. Cytochrome *c* (9.0 μg) was present in all experiments. Rates of oxidation or reduction of cytochromes *c* and *b* were monitored (see Methods) following illumination (680 mμ, 10.1 μEinsteins/min for 2 sec). Sodium succinate (3×10^{-3} M), adjusted to the appropriate pH, was added where indicated. Concentrations of sodium azide and CMU were 6×10^{-4} and 3×10^{-5} M, respectively. Data are shown for cytochrome *c* only. However, similar decreases in rate of oxidation of cytochrome *b* in the presence of anions following illumination were also observed.

by the observation that rate of phosphorylation (Lynn, 1968) and oxidative electron flow (data in this report) decline in a similar manner in the postillumination period. It appears, therefore, that the rate of phosphorylation in chloroplasts is dependent upon the concentration of some reduced protonated unknown electron carrier whose oxidation results in phosphorylation. High concentrations of this unknown carrier with subsequent high turnover rates can be maintained only under conditions which result in the establishment of both a high redox potential and a high proton potential within chloroplasts. Previous studies have also indicated that optimal phosphorylation in chromatophores (Cusanovitch and Kamen, 1968) requires that the over-all redox potential of the system be poised at a relatively high redox potential.

The data in this report also indicate that the uptake of H^+ by chloroplasts occurs under conditions which result in the establishment of a high internal redox potential within chloroplasts. Rate of H^+ uptake parallels rate of reduction of internal electron carriers. It appears, therefore, that H^+ uptake is a reductive process, *i.e.*, that H^+ uptake is a consequence of the trapping of electrons by high potential redox substances within chloroplasts. Net H^+ uptake ceases once the system is saturated with electrons.

The cycling of electrons on and off various carriers, some of which require protons for reduction while others do not, should lead to marked fluctuations in local H^+ activity within chloroplasts. It seems likely that this cyclic alteration in localized internal H^+ activity is used to promote the observed H^+–cation exchange across these proton-impermeable membranes. The extent of the observed H^+ uptake, as shown in this report, simply reflects the availability of *internal* buffers whose ionized and un-ionized species have different rates of diffusion across these semipermeable membranes. Alternatively, the formation of reduced protonated carriers could in some manner be used to drive a H^+–cation-exchange pump, as proposed by Dilley and Vernon (1967). The studies in this report do not clearly distinguish between the above two proposals. However, it is clear that neither H^+ uptake nor phosphorylation can occur as long as the internal electron carriers are maintained in their oxidized states.

The chemical nature of the redox carriers which are required for H^+ uptake and phosphorylation is unknown. However, fluorometric data of Joliot *et al.* (1966) and Malkin and Kok (1966) indicate several unknown couples associated with system II, and at least one must exist for systems I (Chance *et al.*, 1965).

The studies in this report also seem to offer an alternative explanation for the proposal of Mitchell (1966) and Jagendorf (1966). The demonstration that phosphorylation in the acid bath experiment is associated with electron flow of electrons suggests that the energy for phosphorylation may be derived, not from a pH gradient, but rather from an oxidative reaction, as in glycolysis or in the system of Wang (1967). This suggestion is substantiated by the following observations: (1) the existence of postillumination and acid–base-induced electron flow, both of which are temporally related, and result in phosphorylation; (2) effect of uncoupling agents on the redox state of the carriers, with an associated effect of uncoupling agents on internal pH; (3) effect of anions in causing delay in the dissipation of the redox changes, and the associated delay in dissipation of the pH gradient; (4) the yield of "Xe," which is possibly low enough to be accounted for on the basis of one ATP formed for every free electron in the system (Lynn and Brown, 1967a,b). Unpublished experiments from this laboratory indicate that approximately 0.26 μmole of reducing equivalents (as measured with ferricyanide) per mg of chlorophyll is present in freshly isolated chloroplasts. The half-life of "Xe" is also temporally related to the half-life of the OH^--driven electron flow.

Thus, the data in this report seem to fit the notion that phosphorylation in chloroplast is the result of an oxidation and that H^+ uptake is the result of the reduction of proton-requiring redox substances in a system which is surrounded by membranes which are differentially permeable to H^+, OH^-, and other anions. Since phosphorylation in the above formulation requires both a reductive process (to establish a high concentration of reductants) and an oxidative process, it is clear that optimal phosphorylation can proceed only when the above two processes are electronically poised. Phosphorylation, a process which consumes H^+, and uncoupling agents (substances which cause a loss of H^+ from chloroplasts) were previously shown to decrease the over-all redox potential of chromatophores (Cusanovitch and Kamen, 1968). Thus, it appears that the chief role of internal H^+ in oxidative photophorylation is to facilitate the establishment of a high concentration of high redox potential reductants, the oxidation of which supplies the energy for esterification of P_i.

The data in this report suggest but do not conclusively prove that electron flow is the cause of phosphorylation in the postillumination period or in the acid bath experiment. The chief objection to the above concept concerns the question of stoichiometry. Jagendorf and Uribe (1966) originally concluded that more ATP was made in the acid bath experiment than could be accounted for on the basis of the known availability of reducing equivalents within chloroplast. Since it is likely that efficiency of phosphorylation is poor under the severe conditions of the acid bath experiment, the above conclusion is probably correct. However, since chloroplast readily carry out cyclic phosphorylation, using protonated mobile electron carriers, such as PMS or endogenous carriers, it appears likely that the imposed pH gradient, in the presence of large anions which prolong the pH-induced flow of electrons, is also capable of causing cyclic phosphorylation. During the flux of H^+, and OH^-, and anions across the membranes of the chloroplast, mobile electron and H^+ carriers would also flux and this could lead to reduction and oxidation of various portions of the electron chain, depending upon the local internal pH. This concept is consistent with the observation that the half-life of "Xe" is longer than the half-life for ATP formation (Jagendorf and Uribe, 1966). The formation of ATP, *i.e.*, the production of OH^- internally, like uncoupling agents should lead to a more rapid dissipation of imposed pH gradients.

Validation of the above suggestion requires further experimentation. Experiments designed to ascertain more precisely the kinetic relationships between post-illumination and acid–base-induced electron flow, phosphorylation, and changes in internal pH are in progress.

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References

Avron, M. (1967), *in* Current Topics in Bioenergetics, Sanadi, D. R., Ed., New York, N. Y., Academic, p 1.

Avron, M., and Chance, B. (1966), *in* Currents in Photosynthesis, Thomas, J. B., and Goedheer, J. C., Ed., Rotterdam, Donker, p 455.

Chance, B., San-Pietro, A., Avron, M., and Hildreth, W. W. (1965), *in* Non-heme Iron Proteins, San-Pietro, A., Ed., Yellow Springs, Ohio, Antioch, p 225.

Cramer, W. A., and Butler, W. L. (1967), *Biochim. Biophys. Acta 143*, 332.

Cusanovitch, M. A., and Kamen, M. D. (1968), *Biochim. Biophys. Acta 153*, 418.

Dilley, R. A., and Vernon, L. P. (1965), *Arch. Biochem. Biophys. 11*, 365.

Dilley, R. A., and Vernon, L. P. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 395.

Hind, G. (1968), *Biochim. Biophys. Acta 153*, 235.

Jagendorf, A. T. (1962), *Surv. Biol. Progr. 4*, 181.

Jagendorf, A. T., and Uribe, E. (1966), *Brookhaven Symp. Biol. 19*, 215.

Joliot, P., Delsome, R., and Joliot, A., Jr. (1966), *in* Currents in Photosynthesis, Thomas, J. B., and Goedheer, J. C., Ed., Rotterdam, Donker, p 359.

Kahn, J. S. (1968), *Biochim. Biophys. Acta 153*, 203.

Karlish, S. J. D., and Avron, M. (1967), *Nature 216*, 1107.

Lynn, W. S. (1968), *J. Biol. Chem. 243*, 1060.

Lynn, W. S., and Brown, R. H. (1967a), *J. Biol. Chem. 242*, 412.

Lynn, W. S., and Brown, R. H. (1967b), *J. Biol. Chem. 242*, 418.

Lynn, W. S., and Brown, R. H. (1967c), *J. Biol. Chem. 242*, 426.

Malkin, S., and Kok, B. (1966), *Biochim. Biophys. Acta 126*, 413.

Mitchell, P. (1966), *Biol. Rev. 41*, 445.

Newmann, J., and Jagendorf, A. T. (1964), *Arch. Biochem. Biophys. 107*, 109.

Ramirez, J. M., Del Camp, F. F., and Arnon, D. I. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 606.

Wang, J. H. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 37.

Zweig, S., and Avron, M. (1965), *Nature 208*, 190.