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PHOTOPHOSPHORYLATION AS A FUNCTION OF ILLUMINATION TIME

II. EFFECTS OF PERMEANT BUFFERS

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SUMMARY

(1) The amounts of orthophosphate, bicarbonate and tris(hydroxymethyl)-aminomethane found inside the thylakoid are almost exactly the amounts predicted by assuming that the buffers equilibrate across the membrane. Since imidazole and pyridine delay the development of post-illumination ATP formation while increasing the maximum amount of ATP formed, it follows that such relatively permeant buffers must also enter the inner aqueous space of the thylakoid.

(2) Photophosphorylation begins abruptly at full steady-state efficiency and full steady-state rate as soon as the illumination time exceeds about 5 ms when permeant ions are absent or as soon as the time exceeds about 50 ms if valinomycin and KCl are present. In either case, permeant buffers have little or no effect on the time of illumination required to initiate phosphorylation. A concentration of bicarbonate which would delay acidification of the bulk of the inner aqueous phase for at least 350 ms has no effect at all on the time of initiation of phosphorylation. In somewhat swollen chloroplasts, the combined buffering by the tris(hydroxymethyl) aminomethane and orthophosphate inside would delay acidification of the inside by 1500 ms but, even in the presence of valinomycin and KCl, the total delay in the initiation of phosphorylation is then only 65 ms. Similar discrepancies occur with all of the other buffers mentioned.

(3) Since these discrepancies between internal acidification and phosphorylation are found in the presence of saturating amounts of valinomycin and KCl, it seems that photophosphorylation can occur when there are no proton concentration gradients and no electrical potential differences across the membranes which separate the medium from the greater part of the internal aqueous phase.

(4) We suggest that the protons produced by electron transport may be used directly for phosphorylation without ever entering the bulk of the inner aqueous phase of the lamellar system. If so, phosphorylation could proceed long before the internal pH reflected the proton activity gradients within the membrane.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; tricine, *N*-tris(hydroxymethyl)methylglycine; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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INTRODUCTION

The proton activity gradient which is associated with photophosphorylation and has been called the "protonmotive force" by Mitchell [1], has two components which are thermodynamically equivalent and may sometimes be interconvertible: a proton concentration gradient and an electrical potential difference. Since electron transport causes an acidification of the thylakoid lumen [2, 3] and the acidified lumen can support ATP synthesis in the absence of electron transport [4], it has been assumed that the proton activity gradient responsible for photophosphorylation involves differences in pH between the inner and outer aqueous phases plus any transmembrane charge differences. The very clearly demonstrated accumulation of protons in the inner aqueous phase of the thylakoid and the somewhat less clearly demonstrated transmembrane electrical potential differences are thought to be essential intermediate energy conservation steps linking electron transport to phosphorylation. However, there has been as yet no formal proof of this proposition. It remains possible that the acidification of the inner aqueous phase is a consequence rather than a cause of events leading to phosphorylation. Thus, during steady-state phosphorylation proton production within the membrane could support phosphorylation directly. Only when electron transport is separated in time from phosphorylation can it be shown unequivocally that protons accumulated in the inner aqueous phase are subsequently used for phosphorylation (e.g. post-illumination ATP synthesis).

In the studies described here, we have attempted to evaluate the role of the thylakoid lumen in phosphorylation by determining the effect of internal hydrogen ion buffers on the time-course of phosphorylation after the beginning of illumination. It is possible to estimate the time-course of proton production with considerable precision since electron transport starts almost immediately and two protons seem to be produced for each electron transferred (e.g. 5, 6). It is also possible to obtain a fairly accurate estimate of the minimum internal buffering at the start of the illumination period, if permeant buffers are present, since it can be shown that these buffers equilibrate across the membrane in the dark. Thus, it is not difficult to obtain a reliable estimate of the illumination time required to protonate the exogenous permeant buffers in the inner aqueous phase. This calculation will give the minimum illumination time required to drive the internal pH below the pK_a of the added buffers.

Our investigations show that acidification of the inner aqueous phase lags far behind the onset of phosphorylation, and therefore it is likely that fully efficient phosphorylation can occur in the absence of any proton concentration differences across the membranes of the thylakoids.

MATERIALS AND METHODS

Materials: $^{32}P_i$ was obtained from ICN. ^3H_2O, tris(hydroxy[^{14}C]-methyl) aminomethane and [*carboxy*-^{14}C]inulin were obtained from New England Nuclear and $NaH^{14}CO_3$ from Amersham/Searle.

The silicone fluids used, (SF-96 (50) and Versilube F-50), were kindly supplied by Dr. J. Frewin of General Electric Corporation in Waterford, N.Y.

Pyridine was purified by distillation at atmospheric pressure. It was collected within one degree of its boiling point.

Methods. The procedures used in this study are for the most part described in the previous paper of the series [7]. Other experimental details are given in the legends of the figures and tables.

RESULTS

1. Determination of the buffering within the thylakoids

It is difficult to measure the internal endogenous buffer of the thylakoid, and therefore it is difficult to calculate the illumination time which would be required to produce enough protons to overcome the endogenous buffering. However, it is not at all difficult to determine the increase in internal buffering due to permeant exogenous buffers. Estimates of the amounts of exogenous buffers within the thylakoid were made in two ways which gave very similar results. First, it was assumed that the more permeant of the buffers would equilibrate across the membrane so that the internal and external concentrations would be almost the same. The total internal buffer was then calculated from the measured internal volume. Next, the amounts of tris(hydroxymethyl)aminomethane, bicarbonate, and orthophosphate actually inside were determined by using these isotopically labeled buffers.

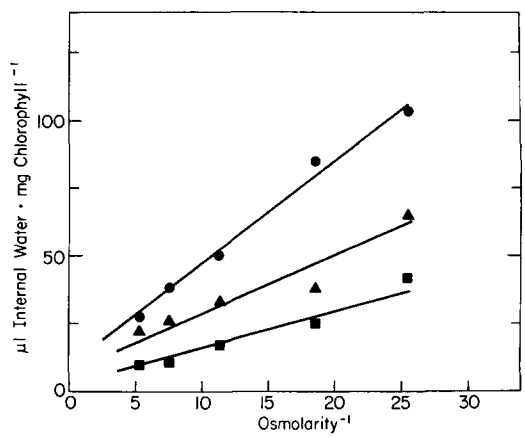

Fig. 1. The dependence of the internal volume of chloroplast lamellae on osmolarity and the effect of permeant amine buffers thereon. Internal volume was measured by centrifugation through silicone fluids as described by Gaensslen and McCarty [3, 8] and in the Methods of the previous paper [7]. The 0.1 ml samples, which were layered on top of the silicone fluid, consisted of: 2 mM $MgCl_2$, chloroplasts containing 100–160 μg chlorophyll, sucrose to attain desired osmolarity and one of the following buffer systems, 20 mM tricine-NaOH (pH 8.0) (■—■), 20 mM tricine-NaOH (pH 8.0) plus 2 mM imidazole (▲—▲), or 20 mM tris(hydroxymethyl) aminomethane (pH 8.0) (●—●). The chloroplast suspensions were allowed to equilibrate for 60 min at 4 °C in the dark. The internal volume was calculated from the uptake of ^3H_2O (1.0 μCi/0.1 ml). The amount of ^3H_2O trapped by the chloroplast membranes and not part of the internal volume was assumed to be the same as the volume occupied by the trapped [^{14}C]inulin (0.5 μCi/0.1 ml). The lower aqueous phase (0.1 ml) contained 10 % sucrose and 1 % Triton X-100. The silicone fluids used were mixtures of Versilube F-50 and SF-96 (50). The mixtures used ranged from 2 : 1 (w/w) (for least dense suspensions) to 4.75 : 0.25 (w/w) (for most dense suspensions). After centrifugation, the tubes were frozen in dry ice and then sliced to separate the upper and lower aqueous phases. The lower sample was bleached for 18–24 h at 45 °C in 1 ml of 30 % H_2O_2 and 5 % sodium lauryl sulfate. Its radioactivity was then determined by liquid scintillation counting using external standard ratios to monitor quenching.

The internal thylakoid volume was calculated from the uptake of ^3H_2O employing the centrifugal filtration technique developed by Gaensslen and McCarty [8]. The ^3H_2O uptake data were corrected for "trapped" suspending medium by measurement of "trapped" [*carboxyl*-^{14}C]inulin (M_r 5000). The amount of inulin trapped was found to vary with osmolarity and with amine concentration.

The dependence of internal water volume on osmolarity is shown in Fig. 1. Osmolarity was varied by the addition of sucrose. The internal volumes of spinach thylakoids suspended in 20 mM tricine-NaOH (pH 8.0), 3 mM $MgCl_2$ and sucrose (full squares) were about the same as those reported by Rottenberg et al. [9] for lettuce chloroplasts determined by the packed cell volume technique when they used KCl to vary osmolarity. The addition of 2 mM imidazole to the suspending medium had a significant influence on chloroplast internal volume and the addition of 20 mM tris(hydroxymethyl)aminomethane had an even more marked influence. Gaensslen and McCarty [3] had previously seen this tendency for amine-induced swelling of chloroplasts in the dark (also see ref. 10).

a) *Tris(hydroxymethyl)aminomethane* (*Tris*). Tris(hydroxymethyl)aminomethane is commercially available with a [^{14}C]hydroxymethyl label and therefore its uptake was studied. As is seen in Fig. 1, the replacement of tricine-NaOH (pH 8.0) in the chloroplast suspending media with 20 mM Tris · HCl (pH 8.0) resulted in an increase of nearly 3-fold in the internal thylakoid volume. The direct measurement of Tris was accomplished using the centrifugal filtration technique used for internal volume determinations. The amount of tris(hydroxymethyl)aminomethane which enters the thylakoid in the dark increases linearly with the thylakoid volume (Fig. 2). In Table I, the internal concentration of Tris has been calculated from the data points of Fig. 2. The calculated amine concentration inside the thylakoid remained roughly constant when the internal volume changed more than 4-fold. This observation suggests that little of the Tris associated with the chloroplasts is bound to negative charges on the membrane as has been suggested in the case of amines such as atebrin [11]. It should be noted, however, that Tris is accumulated by chloroplasts in the

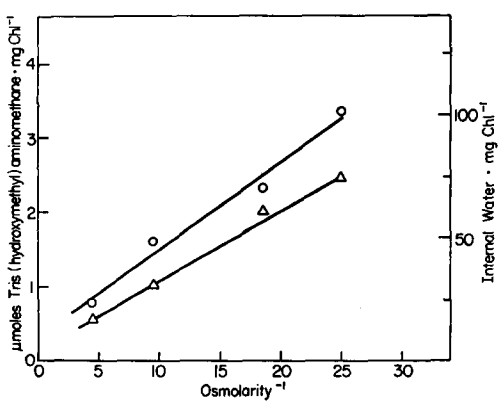

Fig. 2. The uptake of tris(hydroxy[^{14}C]methyl)aminomethane by chloroplast lamellae in the dark and the dependence of the uptake on osmolarity. The techniques and conditions are described in the legend of Fig. 1. The concentration of tris(hydroxymethyl)aminomethane in the suspending medium was 20 mM and the specific activity was 1.0 μCi/0.1 ml. The concentration of Tris inside the thylakoid slightly exceeded the concentration outside. △—△, Tris; ○—○, internal water.

TABLE I

CONCENTRATION OF TRIS(HYDROXYMETHYL)AMINOMETHANE INSIDE THE THYLAKOID IN THE DARK

Data collected from Fig. 2. The concentration of Tris in the suspending medium was 20 mM.

Osmolarity^{-1}	Concentration of Tris inside the thylakoid (mM)
4.9	22
9.5	21
18.5	27
25	23

TABLE II

CONCENTRATION OF BICARBONATE/CO_2 INSIDE THE THYLAKOID IN THE DARK

The experimental procedures and conditions are described in the legend of Fig. 2 except that 0.1 M glycine (pH 9.5) was added to the lower aqueous phase of the centrifuge tubes. The concentration of bicarbonate in the suspending medium was 10 mM. The osmolarity^{-1} of the suspending medium was 16.

Determination	Internal H_2O (μl · mg chl^{-1})	Amount of $H[^{14}C]O_3^-$ (μmol · mg chl^{-1})	Concentration of $H[^{14}C]O_3^-$ (mM)
1	26	0.224	8.6
2	22	0.214	9.7
3	24	0.232	9.6

dark to a concentration which is slightly higher than the concentration of the external medium. The reason for this small discrepancy is not clear, since the discrepancy is proportional to the volume and consequently cannot be explained as being due to either a fixed amount of internal acid or a fixed number of ion exchange sites.

b) Bicarbonate/CO_2. The uptake of [^{14}C]bicarbonate by chloroplasts in the dark was measured in the same fashion as was the uptake of Tris. As is shown in Table II, the concentration of HCO_3^- inside the chloroplast is very close to the concentration present in the suspending medium (actually slightly lower). The values obtained for bicarbonate uptake were more variable than were other uptakes (e.g. tris(hydroxymethyl)aminomethane or $^{32}P_i$). Part of this variability may have been due to loss of $^{14}CO_2$ during the bleaching of the samples with H_2O_2; this may also account for the lower computed internal concentration of bicarbonate which we observed.

c) Orthophosphate. The extent and rate of orthophosphate equilibration across the chloroplast membrane is of interest. Orthophosphate ($pK_a = 6.8$) being a reaction substrate for ATP synthesis, was present in the reaction media of all of the phosphorylation experiments described and therefore would in all cases increase the total amount of internal buffering if it were to cross the membrane. A time-course of $^{32}P_i$ uptake by chloroplasts suspended in 20 mM Tris is shown in Fig. 3. The concentration of $^{32}P_i$ present in the suspending medium was 5.0 mM and the internal con-

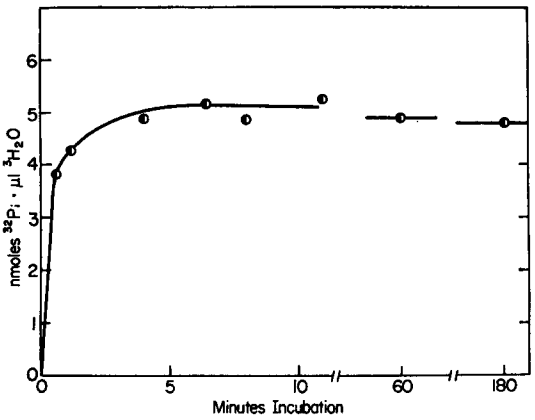

Fig. 3. The time-dependence of orthophosphate uptake by chloroplast lamellae in the dark. The concentration of $Na_2H^{32}PO_4$ (1.0 µCi/0.1 ml) in the suspending medium was 5.0 mM. Procedures used are described in the legend of Fig. 1. The half-time for equilibration of phosphate across the membrane was probably less than 30 s.

centration of orthophosphate attained this value in about 4 min. After only 30 s of incubation, the $^{32}P_i$ had reached an internal concentration of 3.8 mM. Therefore, exogenous orthophosphate should add considerably to the internal buffering of chloroplasts. How much probably depends on the incubation time and the nature of the other ions present [10], but it seems likely that the concentration of phosphate inside the thylakoid usually was not very different from the concentration outside.

2. Effects of internal buffering on the time of illumination required to initiate photophosphorylation

a) The multiple flash technique. As was pointed out in the first paper of this series, the very small amount of ATP formed as a result of a few milliseconds of illumination could not be measured very accurately. Therefore the accumulated product of a number of flashes was measured. The flashes were never separated by less than 15 s of dark, during which time the pH gradients formed in response to light had completely disappeared. This was shown by observing the decay of the pH gradient caused by a single relatively long flash in the presence or absence of valinomycin plus KCl, and in the presence or absence of permeant buffers (e.g. Fig. 4). In no case was there any summation of the pH changes from multiple flashes when the pH was measured using either a glass electrode or the pH-indicating dye cresol red. Many of the conditions employed in this paper (such as pH 8.0, valinomycin+KCl and the weakly uncoupling amine buffers) tend to accelerate the decay of the proton gradient. Therefore the 15 s dark period was almost always far in excess of the time required for complete relaxation of the proton gradient. When the dark period between flashes was increased from 15 to 90 s, there was no change in the amount of ATP synthesized from the flash series or in the length of illumination time required for initiation of photophosphorylation (data not shown). Finally, to make sure that the method of summing the results of a number of flashes was not responsible for some of the observations, we occasionally used single flashes, in which case the results agreed well (except for the scatter of points) with the multiple flash technique (Fig. 8).

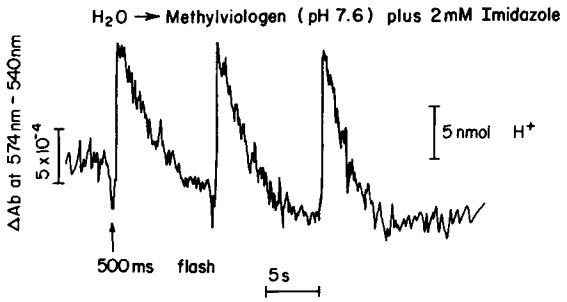

Fig. 4. Light-induced pH change in the presence of imidazole during transport of electrons from H_2O to methylviologen. Changes in pH were monitored optically from the absorbance changes of cresol red (574–540 nm). The 2-ml reaction mixture contained: 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM NaCl, 0.5 mM HEPES-NaOH (pH 7.6), 100 μM methylviologen, 30 μM cresol red and chloroplast lamellae containing 22 μg chlorophyll. The reaction vessel was thermostated at 19 °C and the intensity of the red actinic light (Corning CS 2-64) was 340 Kergs/cm^2 per s. The signal from the Aminco DW-2 was fed into a Nicolet model 1072 instrument computer before replotting on a X-Y recorder. At the end of each experiment the reaction mixture was titrated in the light with 0.001 M HCl to calibrate the absorbance changes in terms of proton flux. Relaxation of the proton gradient seemed to be complete in about 5 s.

b) The effects of permeant buffers on lags in the initiation of photophosphorylation. Imidazole has long been known to uncouple weakly and to inhibit steady-state photophosphorylation (ref. 12 and Table III). It buffers against changes in hydrogen ion concentration best around pH 7.0. Nevertheless, its presence has a barely discernable influence on the lag in the initiation of photophosphorylation. This is true whether or not valinomycin and KCl are present (Fig. 5). The experiments depicted in Fig. 5 were conducted at an osmolarity^{-1} = 6.3. From Fig. 1, the calculated internal volume was 21 μl/mg chlorophyll. Using this figure, it can be calculated that an additional 38 nmol of hydrogen ions per mg chlorophyll would be required to titrate the neutral amine which entered the thylakoid in the dark. Assuming an H^+/e^- ratio of 2.0 (5, 6) and a rate of electron transport 1000 μequiv./mg chlorophyll/h (which is

TABLE III

EFFECT OF PERMEANT AMINE BUFFERS ON THE ATP FORMATION ASSOCIATED WITH CONTINUOUS ILLUMINATION

Reaction conditions are described in Fig. 5. The illumination time was 10 s. Reaction temperature was 15 °C. Additions were as indicated.

Addition	μmol ATP · mg chl^{-1} · h^{-1}	Inhibition (%)
None	170	0
3 mM imidazole	132	22
5 mM pyridine	170	0
20 mM tris(hydroxymethyl)-aminomethane	151	11
0.1 μM valinomycin plus 20 mM KCl and 20 mM tris(hydroxymethyl)-aminomethane	154	10

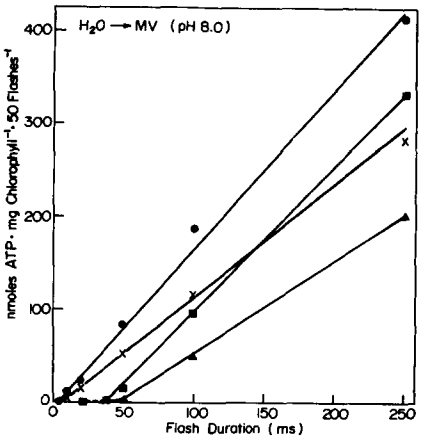

Fig. 5. The effect of imidazole on the ATP formation resulting from short illumination periods in the presence and absence of valinomycin plus KCl. ● — ●, control; ■ — ■, valinomycin + KCl; × — ×, 2 mM imidazole; ▲ — ▲, valinomycin + KCl + 2 mM imidazole. When they were used, the concentration of valinomycin was 0.1 μM and the concentration of KCl was 20 mM. The stirred 2-ml reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM tricine-NaOH buffer (pH 8.0), 5 mM $Na_2H^{32}PO_4$, 1 mM ADP, 100 μM methylviologen, chloroplasts containing 60 μg chlorophyll and the indicated concentration of imidazole. The intensity of heat-filtered (Corning CS 1-69) white light was measured outside the reaction vessel and was found to be 470 Kergs/cm^2 per s there. The reaction vessel was thermostated at 12 °C. ATP formation was determined from a series of repetitive flashes, each separated by a 15 s dark period. Perchloric acid (0.5 ml of 1 M) was injected into each sample immediately after the last flash. The samples were then rapidly frozen in ethanol/solid CO_2.

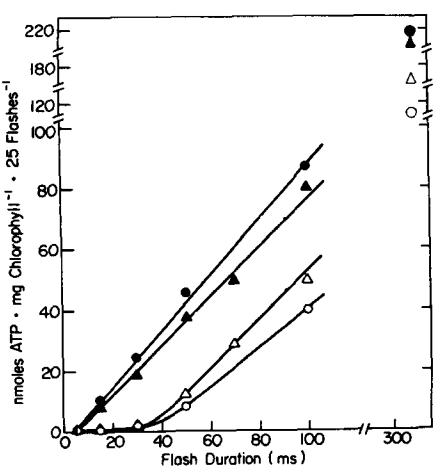

Fig. 6. The effects of HCO_3^-, with or without valinomycin and KCl, on the ATP formation resulting from short illumination periods. The concentration of sodium bicarbonate used was 10 mM. The other reaction conditions were as in Fig. 5. ▲ — ▲, HCO_3^-; △ — △, HCO_3^- plus valinomycin plus KCl; ● — ●, control; ○ — ○, control plus valinomycin plus KCl.

TABLE IV

EFFECT OF INTERNAL BUFFERING ON THE LAG IN INITIATION OF PHOSPHORYLATION

All experiments were conducted in the presence of 20 mM KCl and 0.5 μM valinomycin in order to eliminate transmembrane electrical potentials. Also present were 20 mM Tris buffer (pH 8.0) and 5 mM orthophosphate. Other conditions were as described in Fig. 7. The times reported as the observed lags were estimated by extrapolation to zero ATP yield (for example see Fig. 7). It is assumed that the concentrations of unprotonated Tris and of orthophosphate were the same inside the thylakoid as in the medium (see Table I and Figs. 2 and 3) and that protons were produced inside the thylakoid at a maximum rate of 2000 μmol · mg chlorophyll^{-1} · h^{-1}.

Osmolarity^{-1}	Internal water volume (μl · mg chl^{-1})	Observed phosphorylation lag (ms)	Time required to overcome Tris buffering alone (ΔpH < 1.0) (ms)	Time required to overcome phosphate buffering alone (ΔpH < 2.5) (ms)	Minimum time required to produce ΔpH = 2.5 (ms)
3.4	22	49	265	197	462
8.5	42	56	505	378	883
11.5	52	59	625	467	1092
18.5	80	64	875	655	1530

typical of the initial high rates shown in Fig. 9; see also Fig. 2, ref. 7), acidification of the inside of the thylakoid must have been delayed about 68 extra ms by the presence of the imidazole. But this is a minimum time since the amine would undoubtedly accumulate inside the thylakoid to some extent in response to the developing pH gradient. In fact, if the free amine nearly equilibrated across the membrane during the initial stages of the electron transport, about 680 ms would be required to form a ΔpH of 2 and about 6.8 s to form a ΔpH of 3. These calculations do not even take into account light-inducing swelling!

Bicarbonate ions do not uncouple. In the presence of valinomycin and KCl, they actually increase the rate of phosphorylation slightly. They have no effect at all on the delay in the initiation of phosphorylation in spite of the fact that acidification of the inside of the thylakoid must have been delayed approximately 350 ms. Again this is true whether or not valinomycin and KCl are present (Fig. 6).

Since phosphate is almost certainly inside the thylakoid as well as outside (Fig. 3) and phosphate is of necessity used in all phosphorylation experiments, a good deal of electron transport must be needed to lower the internal pH even in the absence of other permeant buffers. However the lag in the initiation of phosphorylation is almost independent of phosphate concentration. Thus in one experiment, increasing the phosphate concentration from the usual 5 mM to 15 mM only increased the lag

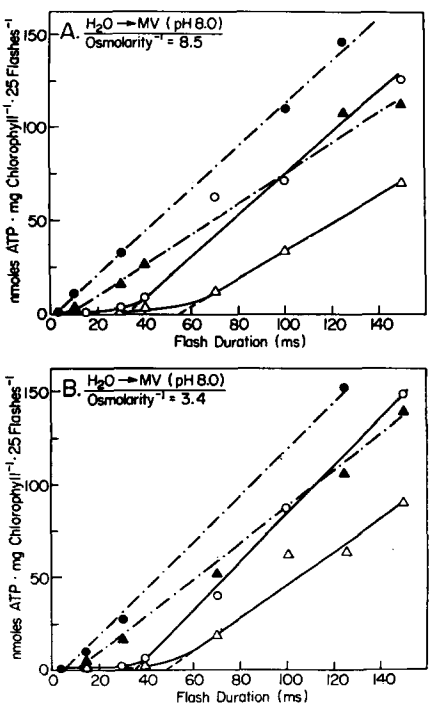

Fig. 7. The effects of tris(hydroxymethyl)aminomethane, with or without valinomycin and KCl, on the ATP formation resulting from short illumination periods. The reaction conditions were as in Fig. 5, except that the valinomycin concentration was 0.2 μM. ●–·–●, tricine (20 mM); ○—○, tricine (20 mM) plus valinomycin plus KCl; ▲–·–▲, Tris (20 mM); △—△, Tris (20 mM) plus valinomycin plus KCl.

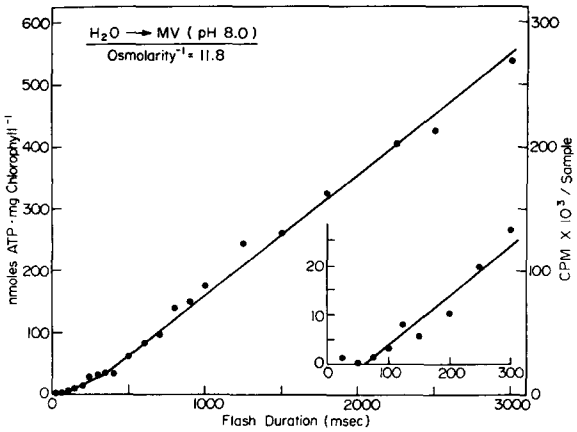

Fig. 8. The effects of tris(hydroxymethyl)aminomethane with valinomycin and KCl on the ATP formation resulting from single short flashes. The 2-ml reaction mixture was composed of: 20 mM Tris · HCl (pH 8.0), 15 mM KCl, 5 mM NaN_3, 2 mM $MgCl_2$, 1 mM ADP, 0.75 mM $Na_2H^{32}PO_4$, 2 μM valinomycin and chloroplasts containing 80 μg chlorophyll. The reaction temperature was 24 °C and the light intensity was 680 Kergs/cm^2 per s of heat filtered white light. The lower phosphate concentration was used in order to obtain a higher specific activity without a proportional increase in unextractable radioactive polyphosphates.

about 7 ms, when the increase in the delay in the internal acidification must have been about 400 ms.

The discrepancy between the onset of phosphorylation and the acidification of the inside of the thylakoid is particularly marked when tris(hydroxymethyl) aminomethane is used as the permeant buffer and for this reason the Tris system was thoroughly investigated (Figs. 7–9 and Table IV). Tris lowers both the efficiency and the rate of steady-state phosphorylation somewhat (Table III and Fig. 9B). It also causes the chloroplasts to swell in the dark (Fig. 1), which not only increases the amount of Tris inside but must also increase the amount of phosphate inside. It should be noticed that Tris does extend the lag in the initiation of phosphorylation in the presence of valinomycin and KCl (Fig. 7) but the effect is not at all commensurate with the computed delay in internal acidification. Furthermore, more basic amines which uncouple but do not buffer, such as methylamine, also extend the lag in the presence of valinomycin and K^+ while trace amounts of carbonylcyanide phenylhydrazone uncouplers extend the lag for very long periods (data not shown). Therefore it seems likely that the slight extension of the lag caused by Tris is a reflection of its well-known uncoupling action, not of buffering. It should also be noted that the lag observed in the presence of Tris is not very dependent on osmolarity and is therefore not very dependent on internal volume or on the amount of internal Tris (Fig. 7 and Table IV).

Finally it should be carefully noted that phosphorylation, when it does start, does so abruptly at the steady-state efficiency and steady-state rate. This is true in the presence of any combination of Tris, Tris with valinomycin and KCl, or valinomycin and K^+ alone, or for that matter in the absence of either (Fig. 9). In view of these observations, it seems unlikely there is any great heterogeneity within the membranes with respect to the characteristics responsible for the delays.

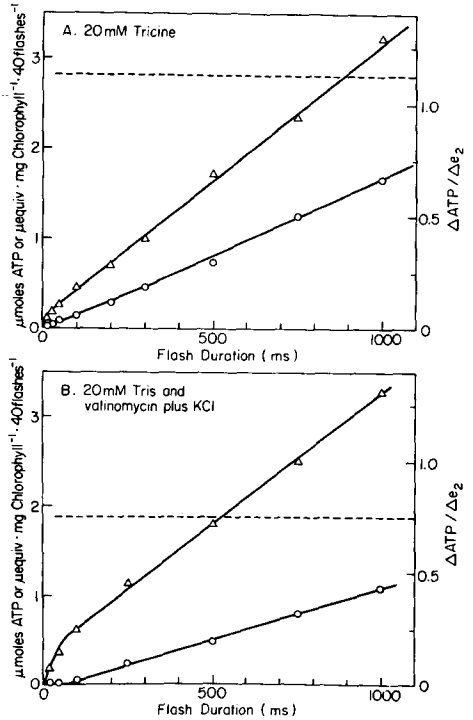

Fig. 9. Electron transport and ATP formation resulting from short illumination periods. When they were used the concentration of valinomycin was 0.2 μM and the concentration of KCl was 20 mM. The stirred 2 ml reaction mixture, contained 0.1 M sucrose, 2 mM $MgCl_2$, 1 mM ADP, 5 mM $Na_2H^{32}PO_4$, 100 μM potassium ferricyanide and chloroplasts containing 60 μg chlorophyll and either 20 mM tricine-NaOH, pH 8.0 (Fig. 9A) or 20 mM Tris-HCl, pH 8.0 (Fig. 9B). The amount of ferrocyanide formed due to the photochemical reduction of ferricyanide was determined by reacting the ferrocyanide ions with bathophenanthroline sulfonate. The procedure employed was similar to that of Jagendorf and Hind [26]. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (0.1 ml of 10 mM) was injected into each sample immediately after the last flash. Chloroplasts were then removed by forcing the reaction media through a Millipore filter (0.22 μm pore size) and a 1-ml sample was assayed for ferrocyanide concentration. 5 ml of developing solution consisting of: 100 mM formate (pH 3.0), 0.4 mM *N*-(2-hydroxyethyl)ethylenediamine triacetic acid, 0.56 mM ferric nitrate and 1.3 mM bathophenanthroline sulfonate which were added to the 1 ml reaction sample. After mixing the color in the samples was allowed to develop for 30 min in the dark at 21 °C and then the absorbance at 535 nm was determined. The millmolar extinction coefficient for this complex was determined to be 23.2 ± 1.6 (from more than 100 determinations) for concentrations of ferrocyanide ranging from 0.1 to 10.0 μM. $\Delta ATP/\Delta e_2$ (– – –) was computed from the slope of the ATP formation (○ — ○) divided by $\frac{1}{2}$ the slope of electron transport (△ — △).

c) Effects of permeant buffers on the development of the capacity for ATP formation during preillumination. It has long been known that chloroplasts illuminated in the absence of ADP and phosphate develop the ability to produce ATP when they are transferred in the dark to a medium containing these missing components of the phosphorylation system [3]. It has also long been known that the amount of ATP formed can be greatly increased if permeant buffers with appropriate dissociation constants such as pyridine are added to the medium during the light phase of the

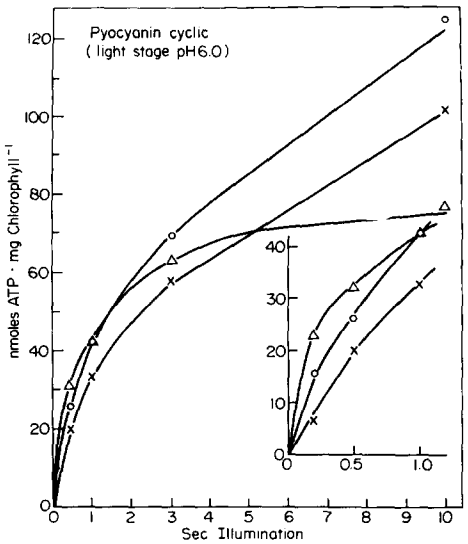

Fig. 10. The effect of imidazole and pyridine on the capacity of chloroplast to make ATP in the dark after an illumination period. Chloroplasts containing 68 μg of chlorophyll were illuminated with white light (600 Kergs/cm^2 per s) for the indicated amount of time in a continuously stirred reaction mixture (2 ml) containing 0.1 M sucrose, 50 mM NaCl, 2 mM $MgCl_2$, 10 mM MES-NaOH (pH 6.0), 10 μM pyocyanine and the following concentrations of the permeant buffers: ○ — ○, 5 mM pyridine; × — ×, 3 mM imidazole; △ — △, control.

reaction [13]. We have shown that the buffers imidazole and pyridine slow the development of the phosphorylation capacity during preillumination even though they increase the ultimate capacity if the illumination is sufficiently prolonged (Fig. 10). As expected [14], an increase of internal osmotic volume further enhances the imidazole-stimulated two-stage ATP yield (Ort, D., unpublished). These observations are in complete accord with the conventional picture of the chemiosmotic process. Permeant buffers can indeed be accumulated within the thylakoids in their protonated forms where they can serve as reservoirs of protons to drive phosphorylation. But the observations also illustrate the fact that the two-stage mechanism operative in the preillumination experiments is fundamentally different, in regard to the requirement for a transmembrane proton gradient, from the mechanism operative in normal single-stage experiments. Different reservoirs must be involved in the two different reactions.

DISCUSSION

The observations described in this paper and in previous work [7] imply that photophosphorylation can proceed rapidly and at full efficiency when there are no hydrogen ion gradients and probably no electrical potential differences across the membranes which separate the bulk of the inner aqueous phase from the suspending medium. The data are far more accurate than is needed to establish the conclusions. Amounts of buffer inside in the dark have been estimated in two ways which agree closely. Electron transport has also been determined in two ways which agree. ATP

formation has been measured directly, and the measurements are probably correct to within a few percent. Rates of acidification of the inside of the thylakoid have been deduced from electron transport rates on the basis of assumptions which are widely accepted and could hardly be in error by as much as a factor of 2. Yet the discrepancy between the time of illumination required to initiate phosphorylation and the minimum time of illumination required to acidify the inside of the thylakoid in the presence of permeant buffers is never less than one or two orders of magnitude. In fact, it seems very probable that added internal buffering does not delay the initiation of phosphorylation at all.

It is true that the concentration of permeant buffer inside the thylakoid in the light may not be the same as the concentration in the dark. However, this hardly seems pertinent. In the first place, we are dealing here with the early stages of the transition from dark to light, and the dark concentration probably should be applied in the calculations. In the second place, the mechanisms which could cause uptake or extrusion of buffers in the light almost certainly would themselves involve the consumption of protons, being but extensions of the buffering act [15]. Thus the extrusion of bicarbonate-CO_2, which probably takes place in response to electron transport, would consume exactly the same number of protons as would be consumed by the buffering action of the bicarbonate if the CO_2 did not move. Amine buffers would probably accumulate in the light, in which case the buffering effect would be actively augmented. There is no reason to believe that phosphate would move at all in response to a growing pH gradient but, even if it did, the remarks made about bicarbonate would apply.

Since acidification of the bulk of the inner aqueous phase is not required for phosphorylation and does not even add anything to the efficiency or rate of phosphorylation, the relevance of the lumen's pH to the mechanism of phosphorylation needs to be reconsidered. Preillumination studies, in which electron transport and phosphorylation are separated in time, show us that protons accumulated in the inner aqueous phase can be used for phosphorylation [3, 13, 14] with a fairly high efficiency [16]. Furthermore, ATP hydrolysis in the dark acidifies the inner space [3, 17] and ATP synthesis in the light decreases the acidification [18, 19]. Thus it seems obvious that the inner aqueous phase is in communication with the phosphorylation machinery. The pH of the inner aqueous phase may therefore be an indicator of events leading to phosphorylation. Unfortunately, there is no way of knowing whether or not it is a quantitatively reliable indicator. The high proton activities which seem to be required for phosphorylation must reside elsewhere and we cannot be sure that the proton activities at the two locations ever attain the same level.

It is easier to decide what is not involved in ATP synthesis than to decide what is involved. The lag in the onset of phosphorylation which we have observed is implicit in the data of Graber and Witt [20] who also found that ATP synthesis per flash of light fell to nothing as the time separating very brief flashes was increased. The fact that the length of the lag is inversely proportional to light intensity suggests that a definite amount of electron transport is required before phosphorylation can begin, which in turn suggests that some sort of pool must be filled to a critical level before phosphorylation starts. This is consistent with Junge and Witt's observation that one molecule of the ionophore gramicidin D can uncouple the phosphorylation driven by about 10^5 chlorophyll molecules [21], from which they concluded that more than 100

functional units of the electron transport system must pool their products for ATP synthesis. However, if the functional units of phosphorylation are the individual thylakoids and if phosphorylation is driven by an accumulation of protons therein as Junge and Witt propose, these same thylakoids must exclude almost all of the permeant buffers which are certainly somewhere inside the lamellar system.

There could be a good deal of heterogeneity with regard to the volume of the vesicles in our chloroplast preparations and we may be oversimplifying by treating the system as though it were made up of uniform vesicles. It is true that electron microscopy has shown that there is a tendency for the end membrane of a grana stack to swell more than the rest of the membranes. However, despite the non-uniformity it is quite clear that all the vesicles swell (if Fig. 2, ref. 7; Fig. 2, ref. 28; and Fig. 4A, ref. 29). This is to be expected since the structure of a stack of grana discs is in fact not a stack of discrete, independent osmotic units, but a folded structure with an internal space which constitutes an osmotic continuum (compare Figs. 2 and 3 of ref. 26 and see review of Park and Sane, ref. 30), a fact which is difficult to reconcile with the Junge and Witt identification of the "thylakoid" as the functional unit discussed above [21]. Since only a very gross heterogeneity of vesicle size could account for the discrepancy between internal acidification and the onset of phosphorylation, the oversimplification of assuming uniform vesicle size seems justified. If our results are to be explained by heterogeneity of vesicle size, this heterogeneity must be of a very special kind, since phosphorylation starts very abruptly at full steady-state rates and efficiency as soon as the still unexplained short lag is over and internal buffering has no effect at all on the lag. A few very large vesicles would have to contain virtually all of the buffer and be responsible for almost none of the prompt photophosphorylation. However these same putative large vesicles would have to be responsible for almost all of the post-illumination phosphorylation. In contrast, most of the phosphorylating membranes would have to form vesicles so small as to contain almost none of the permeant anionic or cationic buffers. Again there is absolutely no evidence of such a distribution of vesicle sizes.

For these reasons we believe that most of the protons used for phosphorylation do not enter the lumen of the thylakoid vesicles at all but, in the presence of ADP and phosphate, are used directly for phosphorylation. We consider this conclusion inescapable, even though we realize that it leaves obscure the nature of the Junge-Witt functional unit of phosphorylation with its presumed pool. Almost certainly a unit and a pool do exist but their nature eludes us. We have no suggestion to offer concerning the physical location of the proton accumulation which seems to be necessary for phosphorylation. Neither have we any suggestion concerning the location of the intramembrane potential gradient, which produces the much studied electrochromic absorbance changes and seemingly also contributes to phosphorylation. However, placing the proton activity gradient within the membrane itself helps to solve an embarrassing thermodynamic problem. Steady-state phosphorylation almost certainly can proceed without a transmembrane potential [22, 23]. Therefore, if the gradient responsible for phosphorylation were across the membrane in its entirety, there would either have to be an inside-outside pH difference of over 5 units, or 3 protons instead of 2 would have to be used for each ATP molecule formed. Neither alternative appeals to us. Amine distribution data point to transmembrane pH differences of 2.7–3.0 [18] while both comparative biochemistry and some actual measurements (for a review,

see ref. 24) argue for an H^+ : ATP ratio of 2. The problem disappears if the protons which are used for phosphorylation are produced and almost immediately utilized in the largely hydrophobic environment of the membrane where very great proton activities might be tolerated.

Placing the proton activity gradient within the membrane helps to solve another problem, an apparent site specific inhibition of phosphorylation by low pH. Low pH inhibits Photosystem I-driven phosphorylation very much more than it inhibits Photosystem II-driven phosphorylation. However, low pH does not inhibit the accumulation of protons inside the thylakoid in either case [25]. Clearly a differential inhibition of the utilization of protons for phosphorylation would be impossible if the protons produced by the two photoacts were pooled in a common reservoir such as the internal aqueous phase of the thylakoid. However, it is important to realize that our alternative model of phosphorylation involving intramembrane events in no way requires that each proton-producing reaction be spatially associated with a coupling factor. For instance, Robertson and Boardman [26] have recently suggested that small, rapidly diffusing lipophilic molecules might carry protons laterally through fluid regions of the membrane, ultimately delivering them to the coupling factor. Or protons could be transferred through the membrane along a chain of fixed negative charges.

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