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STUDIES ON THE ROLE OF HYDROGEN IONS AND MEMBRANE POTENTIALS IN THE FORMATION OF ATP BY ISOLATED CHLOROPLASTS

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STUDIES ON THE ROLE OF HYDROGEN IONS AND MEMBRANE POTENTIALS IN THE FORMATION OF ATP BY ISOLATED CHLOROPLASTS

By

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ABSTRACT

STUDIES ON THE ROLE OF HYDROGEN IONS AND MEMBRANE POTENTIALS IN THE FORMATION OF ATP BY ISOLATED CHLOROPLASTS

By

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This thesis deals with the role that hydrogen ion concentration differences and internal hydrogen ion reservoirs play in the synthesis of ATP by isolated chloroplasts. The results of the investigation are present in two sections.

The first section reports on the effects of weak amines on the storage of protons by illuminated chloroplasts and the ability of the chloroplasts to use the energy stored in the proton accumulations to make ATP. An attempt was made to correlate the number of stored protons with the amount of ATP formed. The results of experiments performed indicate that there is an exponential decay of the ability of the chloroplast lamellae to make ATP after light extinction.

The second section of the thesis reports on the role of membrane potentials and pH gradients in phosphorylation. It was discovered that very small increases in the pH of medium caused ATP synthesis if an increase in the concentration of Li^+, Na^+ or K^+ is imposed along

with the pH change. This requirement for the cation concentration gradient is almost certainly an expression of a requirement for a diffusion generated charge imbalance across the lamellar membranes, since the provision of a simultaneous gradient of permeant anions prevents ATP synthesis.

The amount of ATP formed is nearly a linear function of the extent of the pH increase. Very little if any ATP synthesis occurs without a pH increase with any membrane potential we have been able to generate. This appears to be true even when the membrane potential by itself should provide enough energy for ATP synthesis. These observations imply that the mechanism of phosphorylation uses membrane potentials and hydrogen ion concentration gradients cooperatively but for different purposes. This in turn implies that the potential and the hydrogen ion gradient are not equivalent contributors to the driving force for ATP synthesis.

The phosphorylation associated with small pH changes shows the same pattern of increased sensitivity to uncouplers and the same dependence on membrane potentials as the phosphorylation which occurs at low light intensities or during brief periods of illumination. Therefore, it seems likely that the energized condition of the systems responsible for ATP synthesis with brief illuminations, low light intensities, or small imposed pH changes are different from the energized condition responsible for steady-state phosphorylation at high light intensities.

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LIST OF ABBREVIATIONS

CCCP	carbonylcyanide M-chlorophenylhydrazone
Chl	chlorophyll
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
FCCP	carbonylcyanide p-trifluromethoxyphenylhydrazone
HEM	N-β-hydroxyethylmorpholine
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
NMM	N-methylmorpholine
SF-6847	3,5-Di-tert-butyl-4-hydroxybenzylidenemalononitrile
TAPS	N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid
Tricine	N-tris(hydroxymethyl)methylglycine

INTRODUCTION

The discovery that ATP synthesis and electron transport in isolated chloroplasts are coupled reactions was made twenty years ago [1,2,3]. Although the exact nature of the linkage is still not understood, enormous progress has been made in this area. In particular, the essential role which the proton plays in this coupling has become increasingly evident.

There are three major theories which attempt to describe the nature of the coupling between the oxidation-reduction reactions of the electron transport carriers and the hydration-dehydration reactions of the enzyme catalyzing ATP synthesis. The fundamental difference in the three theories lies in the description of the intermediate generated by the electron carriers which leads to ATP formation. The theories are:

A. The oldest proposal on the nature of the coupling is known as the chemical intermediate hypothesis. This mechanism was first put forth by Slater in 1953 to explain respiratory chain phosphorylation [4] but it has also been applied to photophosphorylation. The hypothesis states that a low energy covalent bond is formed between an electron carrier and some other molecule. Oxidation of the electron carrier complex results in the transformation of the low energy bond to a high energy bond. A fraction of the free energy change associated with the electron transfer is stored in the high energy bond. The

high energy bond is then used to make ATP. However, the high energy intermediate has never been isolated. This is one of the less important reasons why the chemical intermediate hypothesis is not widely accepted today. Significantly, it fails to explain the role which membrane structures perform in phosphorylation. In addition, it is not at all clear how membrane potentials and ion fluxes can be incorporated into Slater's model, although they are both known to be important in phosphorylation.

B. The chemical intermediate model was essentially unchallenged until Mitchell put forth his chemiosmotic hypothesis in 1961 [5,6,7]. He proposed that the intermediate between electron transport and phosphorylation was a transmembrane electrochemical gradient of hydrogen ions. The many successful predictions accorded by the theory have resulted in its widespread acceptance. Accordingly, the chemiosmotic theory, as well as evidence for and against it, will be discussed in greater detail below.

C. Recently, a group of theories which invoke conformational changes or charge redistributions in the membrane as the intermediate energy storage between the exergonic redox reactions of electron transport and the phosphorylation of ADP has been set forth. In these theories it is the "energized stage" of the membrane itself which the phosphorylating enzyme uses to make ATP. For example, Green proposes that changes in mitochondrial membranes, which are visible with the electron microscope, represent the intermediate energy stage used for ATP synthesis [8]. Boyer has suggested that

conformational changes in proteins embedded in membrane constitute the high energy intermediate [9,10]. A theory advanced by Williams proposed that the localization of hydrogen ions within the membrane could drive ATP synthesis [11]. All of these theories stress that the high energy intermediate should be found within the membrane rather than across the membrane.

The Chemiosmotic Hypothesis

A fundamental requirement of the chemiosmotic model is the existence of an ion-impermeable membrane. This membrane must enclose an inner aqueous space and be almost impermeable to hydrogen ions and hydroxyl ions.

Such a membrane will contain the electron transport chain which should include sequences of alternating electron carriers and hydrogen carriers. Electron carriers are compounds such as metal enzymes which require only an electron to be reduced. Hydrogen carriers require both an electron and a proton for their reduction (e.g. quinone). The chemiosmotic theory proposes that the net result of the movement of electrons through a sequence of electron and hydrogen carriers will be the unidirectional movement of hydrogen ions across the membrane. This can be accomplished if the members of the electron transport chain are oriented in the membrane such that a hydrogen carrier can only be oxidized on one side of the membrane and reduced on the opposite side. Reduction of the hydrogen carrier by an electron carrier will require a proton obtained from one aqueous phase (the outside in chloroplasts). Oxidation of the hydrogen carrier at

the other side of the membrane by an electron carrier will release a proton to the nearby aqueous phase (the inside in chloroplasts). Each sequence of alternating electron and hydrogen carriers could be viewed as a site of energy conservation or a "site" of phosphorylation. It should be pointed out that the direction of proton movement described above would be reversed in mitochondrion.

The chemiosmotic theory postulates that the high energy intermediate is the transmembrane electrochemical activity gradient of hydrogen ions resulting from the above described process. This gradient conserves the energy of the electron transfer reactions for a phosphorylating enzyme or "coupling factor". The electrochemical activity gradient of hydrogen ions has been termed the "protonmotive force" by Mitchell. It consists of a hydrogen ion concentration difference and an electric potential difference. Expressed in millivolts the protonmotive force is given by

$$\text{protonmotive force} = \Delta\Psi + \frac{RT}{F}(\Delta\,\text{pH})$$

in which $\Delta\Psi$ is the electric potential difference between two phases, R the gas constant, T the absolute temperature, F the Faraday, and ΔpH the hydrogen ion concentration difference between the inside and outside phases.

If the protonmotive force is the true coupling intermediate then it must be thermodynamically adequate to drive phosphorylation. The free energy stored in a proton activity gradient must be at least equal to the free energy of hydrolysis of the terminal phosphate of ATP (but opposite in sign). The actual energy required for the

phosphorylation of ADP will depend on such things as temperature, pH, Mg^{++} concentration, as well as the concentration of products and reactants. Under common experimental reaction conditions chloroplasts require about 14 kcal/mole [12] to make ATP. This energy must be supplied by the number of protons used per molecule of ATP formed. The driving force providing this amount of energy can exist as a membrane potential, a pH gradient, or some combination of the two. The conversion of calories to volts shows that a proton electrochemical activity gradient (protonmotive force) of 600 mV will yield the required 14 kcal when one mole of protons moves down the gradient. Assuming that two [7] or three [13] protons are used per ATP formed allows each proton to provide only a fraction of the requisite energy (1/2 or 1/3, respectively). Thus two protons will provide the needed energy if a membrane potential of 300 mV or a pH gradient of five units exists. Three protons will provide the requisite energy if a membrane potential of 200 mV or a pH gradient of 3.3 units is present. Since the protonmotive force may exist partly in each form (Δ pH and $\Delta\Psi$), it is always their sum which must meet these energy levels.

A final requirement of the chemiosmotic model is an enzyme which can use the protonmotive force to drive phosphorylation. A membrane-bound enzyme must link the synthesis of ATP to the vectorial movement of hydrogen ions.

Evidence from Research on Chloroplasts Which Supports the Chemiosmotic Hypothesis.

It was reported in 1963 by Jagendorf and Hind that spinach chloroplast would raise the pH of the medium in which they were suspended upon illumination [14]. The pH rise depended on the existence of electron transport, was reversible, and was eliminated by common uncouplers of photophosphorylation [15,16]. These data were easily explained by the chemiosmotic model if the cation and anion changes in the external medium which raised the pH reflected changes in an internal aqueous medium where the pH was lowered. A large body of other evidence solidly supports the statement that the pH inside the chloroplast thylakoid membranes is lowered upon illumination. Thus when the pH is low in a membrane-bound compartment an amine with a high pK will tend to accumulate in this compartment, especially if the uncharged amine freely crosses the membrane and the charged, protonated amine does not. Under these conditions, the ratio of the concentration of protonated amine inside and outside will be equal to the ratio of the concentration of H^+ inside and outside [17]. Many investigators have shown that a wide variety of amines are accumulated in a reversible manner by illuminated chloroplasts [18,19,20,21,22]. The accumulation of amines is sensitive to uncouplers and to the osmolarity of the external medium [21,22]. Amine uptake measurements can be exploited to measure the magnitude of the pH gradients generated, if one also measures the internal volume of the chloroplasts and computes thereby the internal amine concentration. A pH gradient of 3.0 units has been measured using this technique [22].

This evidence indicates that a considerable pH gradient exists across illuminated thylakoid membranes during electron transport. The ability of a thylakoid membrane to use such a pH gradient to make ATP was firmly established by Jagendorf and Uribe in 1966 [23]. In their experiments, chloroplasts that were exposed in the dark to an artificial pH gradient responded by making ATP. The synthesis of ATP was sensitive to standard uncouplers but insensitive to inhibitors of electron transport [23,24]. The formation of ATP was found to be related to the magnitude of the pH change rather than to the absolute pH of either the acidic or the basic stage [23]. A threshold pH increase of approximately three pH units was required before any ATP synthesis was observed [23]. A large number of organic acids were found which greatly increased the yield of ATP if present during the acid stage [23,25,26]. The organic acids presumably increase the supply of internal protons. All of the above data are consistent with the chemiosmotic picture of ATP synthesis. The chemiosmotic theory also predicted that a membrane potential of appropriate polarity (positive inside) would combine with a subthreshold ΔpH to provide enough energy for the synthesis of ATP. This prediction was also borne out by the discovery that an artificially imposed pH increase which was insufficient to drive ATP formation would make ATP if a membrane potential was imposed along with the pH change [27,28]. Indeed, there is also a report which claims that some ATP synthesis can be driven by a membrane potential alone [29].

Isolated chloroplasts are capable of phosphorylating ADP in the dark at the expense of a high energy intermediate formed in an earlier

period of illumination. Shen and Shen were the first workers to demonstrate that ATP synthesis could continue after electron transport stops [30]. There has been a considerable amount of subsequent work which indicates that the high energy intermediate is the pool of internal protons. The ability to form ATP in a subsequent dark period as well as the alkalinization of the external medium are both initiated by illumination and have the same kinetics [31]. The decay of the high energy state shows the same time course as the decay of the pH change in the external medium [32]. Several researchers have shown a correlation between the number of protons taken up in the light and the amount of ATP formed in the dark [32,33]. Compounds which increase the uptake of protons by illuminated chloroplasts also increase post-illumination ATP synthesis [34,35]. In summary, it appears that the illumination-dependent pH increase in the external medium reflects a pH decrease inside the chloroplast and post-illumination ATP formation is driven by the resulting pH gradient across the chloroplast membrane.

The effects of a membrane potential on post-illumination ATP synthesis have been examined in experiments analogous to those already described using the acid-base ATP formation system. An imposed cation diffusion potential was found to stimulate the yield of ATP under conditions which generated a sub-threshold ΔpH, e.g. low light intensity or illumination at high pH [27,36]. The imposition of a membrane potential did not affect the yield of ATP if the light-induced pH gradient was thermodynamically sufficient for ATP synthesis by itself. As already noted, the chemiosmotic theory states that a

membrane potential should be able to substitute for or add to a concentration gradient of hydrogen ions since both affect the transmembrane electrochemical activity or leaving tendency of the protons.

Chloroplasts have a latent ability to hydrolyze ATP. Activation of the ATPase requires sulfhydryl compounds plus a "high energy state" (induced by light or an acid-base transition) [37,38]. The chemiosmotic theory states that the hydrolysis of ATP by the coupling factor represents a reversal of electron transport-coupled ATP synthesis, and that ATP hydrolysis should therefore be linked to the inward movement of protons. Such proton accumulation coupled to ATP hydrolysis has been clearly demonstrated by measuring the uptake of amines and the alkalinization of the external medium [19,29,40]. Moreover, McCarty and Racker have shown that the same enzyme is involved in ATP hydrolysis and in steady-state photophosphorylation since both are inhibited by an antibody to the purified coupling factor [41].

The fact that electron transport-coupled ATP synthesis can only be demonstrated in preparations of vesicles (membrane structures which enclose an internal space) fulfills one of the requirements of the chemiosmotic model. The synthesis of ATP that is dependent on electron transport has not yet been demonstrated in a soluble system or in a preparation of "flat" membranes. It should be noted, however, that most biological membranes form vesicles spontaneously in any case. In addition, the ability of a vesicle to couple ATP synthesis to electron transport is related to the membrane's permeability to protons. Compounds and treatments which increase the proton flux through the

membrane also uncouple phosphorylation from electron transport. In fact, most known forms of uncoupling can be explained in terms of effects on the permeability of the membrane to ions.

The known classes of uncouplers are:

a.) Removal of approximately 50% of the coupling factor can completely eliminate phosphorylation although electron transport is stimulated [42,43,44]. The synthesis of ATP can be restored by adding the coupling factor back to the extracted membranes [42]. The formation of ATP can also be restored by the addition of DCCD, chlorotributyltin, or silicotungstate [44,45,46]. These diverse compounds appear to function by plugging up holes in the membrane formed by the removal of some of the coupling factor which then allows the residual coupling factor on the membrane to catalyze phosphorylation.

b.) Detergents uncouple phosphorylation at very low concentrations. The non-ionic detergent Triton-X-100 stimulates electron transport and inhibits phosphorylation while making membranes leaky to protons [47,48].

c.) Certain weak acids are another class of proton uncouplers (e.g. CCCP, FCCP). These compounds apparently function as proton carriers in the membranes since delocalization of the negative charge and the consequent lipid solubility of the anion greatly enhances the ability to uncouple [49,50,51].

d.) Ammonia and aliphatic amines also uncouple photophosphorylation. The mechanism of uncoupling by ammonia is probably as follows: during electron transport the internal free ammonia is protonated by the hydrogen ions produced inside the thylakoid; the resulting ammonium ion is extruded if there are few permeant anions in the medium, and the membrane potential is high. This extrusion of the ammonium cation neutralizes the membrane potential and at the same time carries the hydrogen ion out. Thus, in the absence of permeant anions, the accumulation of internal protons and the development of a membrane potential are nullified by the ammonia and uncoupling is complete, If, however, there are plenty of permeant anions about and the membrane potential is minimal, the ammonium ion tends to accumulate inside and as it builds up the pH goes down and the uncoupling effect of the ammonia diminishes. Ultimately, however, if the ammonia concentration is high enough, the accumulated ammonium ions become so concentrated inside that they diffuse outward even without a driving membrane potential and therefore uncoupling ensues.

e.) Ionophores (ion-carrying lipophilic large molecules) often uncouple, especially if they carry K^+, Na^+ or H^+ and therefore are capable of catalyzing the exchange of these cations across the thylakoid membrane. Nigericin and gramicidin are particularly useful uncouplers for use with chloroplast membranes [52].

Evidence from Research on Chloroplasts Which Does Not Support the Chemiosmotic Hypothesis.

There is evidence for two sites of energy conservation in chloroplasts at which electron flow is coupled to ATP synthesis [53].

One of the sites is on the Photosystem II side of plastoquinone and has been called site II. The other site of energy conservation, site I, is located between plastoquinone and cytochrome f. The two sites of energy conservation can be separated by use of the electron transport inhibitor dibromothymoquinone which blocks the oxidation of reduced plastoquinone [54,55]. Addition of appropriate electron donors and acceptors in the presence of dibromothymoquinone allows the separate examination of the two sites of phosphorylation. In the chemiosmotic model a "site of phosphorylation" is equivalent to a position at which hydrogen ions are produced at the inner surface of the membrane. If the two sites of phosphorylation in chloroplasts are those which send hydrogen ions into a common pool such as the inner aqueous phase of the thylakoid, one would not expect the coupling factor to be able to distinguish the source of the hydrogen ions (site I vs. site II). However, two site-specificities have been discovered. The coupling factor inhibitor $HgCl_2$ is a much less effective inhibitor of site II phosphorylation than it is of site I phosphorylation [56]. Also, the efficiency with which protons are used by the coupling factor can depend on the source of the hydrogen ions. The phosphorylation efficiency (phosphorylation/electron transport) of site II is independent of pH over the range 6-9 whereas the phosphorylation of site I is strongly pH-dependent [57]. At pH 6.5 phosphorylation is coupled with moderate efficiency to proton accumulation if the protons are derived from site II but with negligible efficiency if the proton are derived from site I. These results are inconsistent with any model which proposes a de-localization of the high-energy intermediate as does the chemiosmotic theory.

Under conditions which should abolish a membrane potential, ATP synthesis by chloroplasts does not require acidification of the internal aqueous space [58]. The time of illumination required to lower the internal pH a given number of units could be determined if one knew the rate of proton accumulation and the amount of internal buffering. Since the amount of internal buffering would be difficult to measure accurately, Ort and co-workers examined the delays in the onset of phosphorylation resulting from exogenous buffers (which were shown to equilibrate across the membrane). In all cases permeant buffers had little or no effect on the length of illumination required to initiate phosphorylation. Thus, the synthesis of ATP appears to proceed in the absence of either a transmembrane pH gradient or a membrane potential. These results and the site-specificities described immediately above suggest the need for a modification of the chemiosmotic hypothesis in which the protons produced by electron transport are used by the coupling factor without ever leaving the membrane [58]. An intramembrane proton activity gradient may be driving phosphorylation instead of Mitchell's transmembrane proton activity gradient. Clearly hydrogen ion concentration differences and membrane potentials are involved in ATP synthesis but we have no idea where these gradients are situated or how the two driving forces cooperate.

Data will be presented in this thesis which suggests that a proton concentration gradient is an absolute requirement for ATP synthesis in chloroplasts. The formation of ATP can be driven by a pH gradient of sufficient magnitude or by the combination of a sub-threshold pH gradient and a membrane potential (positive inside).

However, very little if any ATP synthesis is observed in the absence of a pH gradient, regardless of the magnitude of the membrane potential imposed. It appears that a pH gradient is able to provide something, other than a thermodynamically adequate driving force, that a membrane potential is not able to provide. That is to say, the formation of ATP from ADP and phosphate by the coupling factor seems to require more than protons and an adequate protonmotive force. A proton gradient may fulfill some mechanistic requirement of the phosphorylation reaction which the chemiosmotic theory has not accounted for.

MATERIALS AND METHODS

Isolation of Chloroplasts

Chloroplasts were isolated from commercial spinach (Spinacia oleracea L.) using a modification of the procedure described by Jensen and Bassham [59]. The isolation was carried out in a coldroom at 5 °C. Approximately 40g of spinach were homogenized with a Waring Blendor for 5 sec in 90 ml of the following solution: 0.4M sorbitol; 50mM MES-NaOH, pH 6.1; 2mM EDTA; 3mM $MgCl_2$; 1mM $MnCl_2$; 2mM ascorbic acid; 1mM dithiothreitol; and 8 mg/ml bovine serum albumin. The ascorbic acid, dithiothreitol, and bovine serum albumin were added to the chilled grinding medium immediately before use. After homogenization the resulting slurry was squeezed through 16 layers of well-washed cheesecloth. The filtrate was centrifuged at 2500 X g for 2 minutes. After pouring off the supernatant solution and allowing the inverted centrifuge tubes to drain briefly, the chloroplast pellet was resuspended for 60 sec in about 4 ml of distilled water. An equal volume of double strength resuspension medium was then added to the suspension of chloroplast lamellar vesicles ("broken chloroplasts") to achieve the following final concentration: 0.2M sorbitol; 2 mg/ml bovine serum albumin, pH 6.1; and 4mM $MgCl_2$. For the proton uptake and post-illumination ATP synthesis experiments the "broken chloroplasts" were resuspended in the following solution: 0.2M sorbitol; 5mM MES-NaOH, pH 6.7; 4mM $MgCl_2$; 4 mg/ml bovine serum albumin.

Preparations of chloroplast lamellar vesicles were regularly used during the 2 hours following isolation.

Chlorophyll concentration was determined essentially as described by Arnon [60]. A 0.1 ml aliquot of the chloroplast suspension was diluted into 10 ml of 80% acetone. The acetone extract was centrifuged at 2500 X g for 5 min to remove precipitated protein. Absorbance was measured at 645 nm, 663 nm, and 710 nm. Any "absorbance" at 710 nm was believed to result from light scattering and therefore was subtracted from the measurements of chlorophyll a (667 nm) and chlorophyll b (645 nm). The total chlorophyll concentration of the acetone extract was determined using the following equation: $(A_{645} - A_{710})\ 20.2 + (A_{663} - A_{710})\ 8.02 =$ μg chlorophyll per ml.

Determination of ATP Formation

Incorporation of radioactive orthophosphate into ATP was measured by two different procedures. The post-illumination ATP formation experiments and the initial acid-base ATP synthesis experiments used a procedure in which the unreacted orthophosphate ($^{32}P_i$) was converted to phosphomolybdic acid and extracted with organic solvent. The extraction was performed as follows: A 3.7 ml aliquot removed from each chloroplast reaction was added to a test tube containing ten ml of 10% perchloric acid saturated with hexanol. One ml of 20% ammonium molybdate was added and briefly mixed with each sample. The molybdate was allowed to combine with the unreacted orthophosphate for 5 min before extraction of the phosphomolybdate with 15 ml of hexanol (saturated with 10% $HClO_4$). For extraction the

phases were mixed for 40 sec with a glass plunger. The phases were then allowed to separate and the phosphomolybdate containing organic phase was removed by suction. The aqueous phase containing the ATP was filtered through wet Whatman #4 paper to remove any droplets of the organic phase. Unlabeled phosphate (0.1 ml of 1.0M) was added to each tube followed by two extractions with 15 ml volumes of hexanol in order to remove the excess molybdic acid which was found to interfere with the measurement of radioactivity. A 5.0 ml aliquot from each sample was then added to a scintillation vial with 10 ml of water. Radioactivity was determined by measuring Cerenkov radiation in a Packard Tri-Carb scintillation counter. The number of counts expected from the incorporation of 1.0 μmole of $^{32}P_i$ into ATP was determined by placing a 1.0 μmole aliquot from the $^{32}P_i$ stock solution in a scintillation vial along with 10 ml of water and 5 ml of one of the hexanol-extracted perchloric acid solutions. A standard containing 1 μmole of ^{32}P labeled orthophosphate was counted in the same manner along with the samples from each experiment.

An alternative method of measuring the incorporation of $^{32}P_i$ into ATP was used for most of those experiments in which chloroplasts made ATP in the dark as a result of a pH increase in the external medium. This procedure for measuring ATP formation was developed by Boyer's laboratory and is described below [61]. In order to determine the recovery of ATP from each reaction, 1.5 umoles of unlabeled ATP was added at the end of each reaction. A 3.7 ml aliquot from the reaction was centrifuged for 3 min at about 900 X g to remove precipitated protein. Following the centrifugation, 0.4 ml of a

solution containing orthophosphate and pyrophosphate (0.5M NaH_2PO_4, 0.125M $Na_4P_2O_7$) and 0.7 ml of a charcoal suspension (100 mg/ml) were added to the supernatant solution. The mixture was allowed to sit for 5 min. The charcoal with adsorbed nucleotides was collected on a filter paper, washed with 5.0 ml of a solution of 0.025M $Na_4P_2O_7$, 0.3N H_3PO_4 and 0.3N $HClO_4$, and then washed with 5.0 ml of distilled water. The adsorbed nucleotides were eluted from the charcoal by placing the charcoal and filter paper in a 125 ml flask with 10 ml of 0.6N ammonia in 40% ethanol and shaking for 30 min. The charcoal was removed by filtration through a 0.2 μm Millipore filter. The filtrate was applied to a 2.0 ml column consisting of quaternary amine resin (Dowex AG1-X4, 200-400 mesh) which had been previously washed with 1.0 N HCl and then with distilled water until the column eluate was netural. After the filtrate had passed over the column, the column was washed with 2.0 ml of distilled water followed by 5.0 ml of 0.2M Tris·HCl pH 8.0. AMP and ADP were removed from the column by extensive washing with 0.060 N HCl (35ml). The ATP was eluted from the column in 15 ml of 1.0N HCl. Absorption at 260nm was measured for each sample and recovery of the added unlabeled ATP was calculated using a molar extinction coefficient of 1.47×10^4 [62]. The recovery of ATP routinely varied between 50% and 60%. Using this procedure the background level of ^{32}P in the ATP fraction when no $AT^{32}P$ was formed (i.e. no chloroplasts added to the reaction or $HClO_4$ added to the chloroplasts before the addition of $^{32}P_i$) was found to be less than 0.05 nmole ATP·mg chl^{-1}. Radioactivity was again determined by measuring Cerenkov radiation in a scintillation counter. An aliquot

of the $^{32}P_i$ stock solution containing 1.0 nmole $^{32}P_i$ was placed in a scintillation vial with 15 ml of 1.0N HCl and counted along with the samples from each experiment.

The charcoal used in the above procedure was prepared as described by Boyer [61] to facilitate the recovery of adsorbed nucleotides. Ten grams of acid-washed charcoal was suspended in 100 ml of 0.3N $HClO_4$, 0.05 M $Na_4P_2O_7$, 0.1 M NaH_2PO_4, 2.0mM AMP and stirred for 15 min. The charcoal was collected on filter paper in a Buchner funnel, washed with distilled water, and resuspended in 200 ml of 0.6N ammonia in 40% ethanol. After stirring for 15 min the charcoal was collected again on filter paper in a Buchner funnel and finally resuspended in 100 ml water. This "AMP-treated" charcoal suspension was stored in a refrigerator.

Measurement of Light-Induced pH Changes

Light-dependent pH changes in the external medium were measured with a Corning semi-micro combination pH electrode connected to a Heath-Schlumberger EU 200-30 pH electrometer. The output from the electrometer was amplified and then recorded using a Heath/Schlumberger strip chart recorder with accompanying Heath/Schlumberger EU 200-02 DC offset module. The response half-time for the system was approximately one sec. The 3.0 ml reactions were run in a thermostated water bath at 19 °C and stirred continously. The actinic light (broad band red light>590 nm) was provided by a 500W slide projector. At the end of the experiment the pH change was converted to H^+ equivalents by back-titrating the reaction mixture in the light with aliquots of HCl.

Measurement of Amine Uptake

The concentration of radioactive compounds was continuously monitored using a technique which measured the rate of dialysis [63,64]. A diagram of the apparatus is shown in Figure 1. The upper chamber has a volume of 5 ml in which the chloroplasts and radioactive compound are placed. The lower chamber has a capacity of 0.2 ml, is completely filled and constantly flushed with water. The chambers are separated by a dialysis membrane (Spectrapor 2, Spectrum Medical Industries, Inc.). The contents of both chambers are mixed with magnetic stirring bars. The rate at which the labeled compound passes through the dialysis membrane and into the lower chamber is proportional to its concentration in the upper chamber. This rate can be determined by measuring the concentration of radioisotope in the effluent from the lower chamber. After a sufficient volume of water has passed through the lower chamber to permit a steady-state to be reached (i.e. isotope entering the lower chamber equals isotope leaving the lower chamber) the concentration of labeled compound in the effluent becomes a true measure of the concentration of unbound radioisotope in the upper chamber. The half-time for reaching the steady-state in Figure 16 was approximately 5 sec. The dialysis cell was made of plexiglass and illuminated on two sides with broad band red light (> 590 nm). Light was obtained from two 500W slide projectors. An aliquot from each fraction of the effluent was diluted in Tritosol for liquid scintillation counting [65].

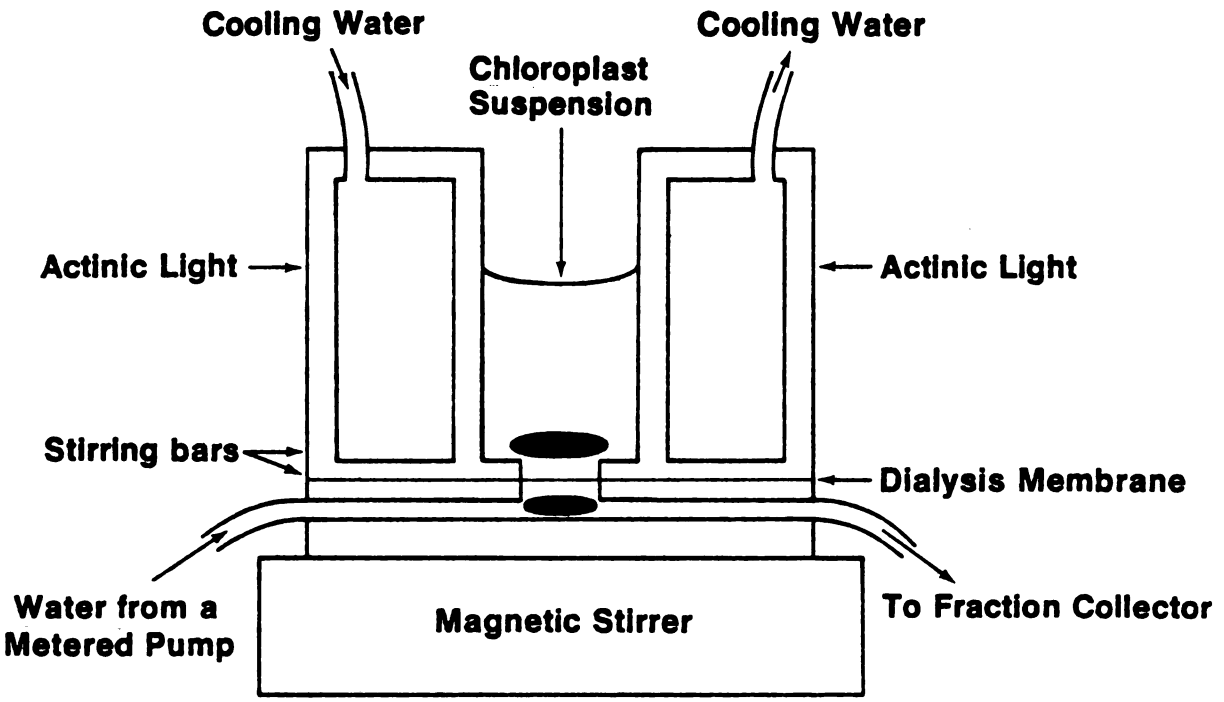


Figure 1.

Diagram of the apparatus used for measuring changes in the concentration of amine by measuring changes in the rate of dialysis. See Materials and Methods for details.

Post-Illumination ATP Formation and Acid-Base ATP Formation

The post-illumination ATP formation experiments were performed in the same apparatus used for the amine uptake measurements with the following modifications and additions. The lower chamber and dialysis membrane were replaced by a piece of solid plexiglass. An opaque plastic sleeve was used as a shutter to turn off the light from both sources at the same time. The shutter closing activated a programmable digital timing device which controlled the injection of the solutions which started and terminated the phosphorylation reaction. The timing circuit opened solenoid valves which allowed compressed air at very low pressure to blow the solutions into the reaction chamber. The timing device allowed the interval between the injections to be varied with excellent reproducibility and accuracy down to about 10 msec. An aliquot was removed from each reaction and assayed for $AT^{32}P$ using the organic extraction of phosphomolybdate described previously.

Mixing times were measured by determining the absorbance changes resulting from the injection of limiting amounts of acid into a solution containing a pH indicator. The absorbance changes were detected and recorded with a photocell connected to a storage oscilloscope. Full mixing of the injected solution with the reaction mixture was achieved within 200 msec (data not shown).

The acid-base ATP formation experiments were also performed in the apparatus used for the amine uptake measurements with solid plexiglass in place of the lower chamber. The chloroplasts were

incubated for 60 sec in a 2.0 ml stirred "acid-stage" solution. The pH of the "acid-stage" was usually adjusted with small additions of HCl or KOH prior to the addition of the chloroplasts and the pH of each reaction mixture was determined after the addition of the chloroplasts. Following the "acid-stage" incubation, 1.0 ml of a strongly buffered "basic" solution was injected. The reaction was terminated 10 sec later by the addition of 1.0 ml of a 2 N $HClO_4$ solution containing 10mM EDTA. An aliquot was taken from each reaction and assayed for $AT^{32}P$ by Boyer's procedure as described previously. Further details of reaction conditions are given in the legends of the figures and tables.

RESULTS

Section I The Effects of Weak Amines on Proton Accumulation and ATP Synthesis by Isolated Chloroplasts

Illumination of a suspension of chloroplasts in the presence of an electron acceptor causes the pH of the external medium to increase [14]. This alkalinization is a result of electron transport reactions which also acidify the internal aqueous space of the chloroplast. Many workers have shown that a wide variety of weak amines will stimulate proton uptake by illuminated chloroplasts [34,35,66]. In these experiments the buffer presumably crosses the chloroplast membrane in the unprotonated form and increases the internal buffering capacity of the organelle. Examples of proton uptake stimulated by weak amines as affected by pH are shown in Figures 2 and 3. The stimulation of proton uptake can be very large. A 30-fold increase was observed with imidazole at pH 8.0 (see Figure 2). The maximum proton uptake was seen when the pH was 0.4 to 1.0 units above the pK_a of the amine tested. This is in agreement with previous reports [35,66].

Chloroplasts illuminated in the presence of an electron acceptor can make ATP in a subsequent dark period [30,67]. The high-energy intermediate responsible for the post-illumination ATP formation is thought to be the pH gradient across the chloroplast membrane. It is no surprise, therefore, to find that the same weak amines which

Figure 2.

Effects of weak amines on light-dependent proton uptake as a function of the pH of the external medium, I. The 3.0 ml reaction mixture contained the following: 0.2M sorbitol; 100mM NaCl; 2mM $MgCl_2$; 50 µM methylviologen; chloroplast lamellae containing 100 µg of chlorophyll and 1mM buffer (MES, pH 5.0 to pH 7.0; MOPS, pH 7.0 to pH 8.4; Tricine, pH 8.4 to pH 9.4). All other conditions as described in Materials and Methods.

Figure 2.

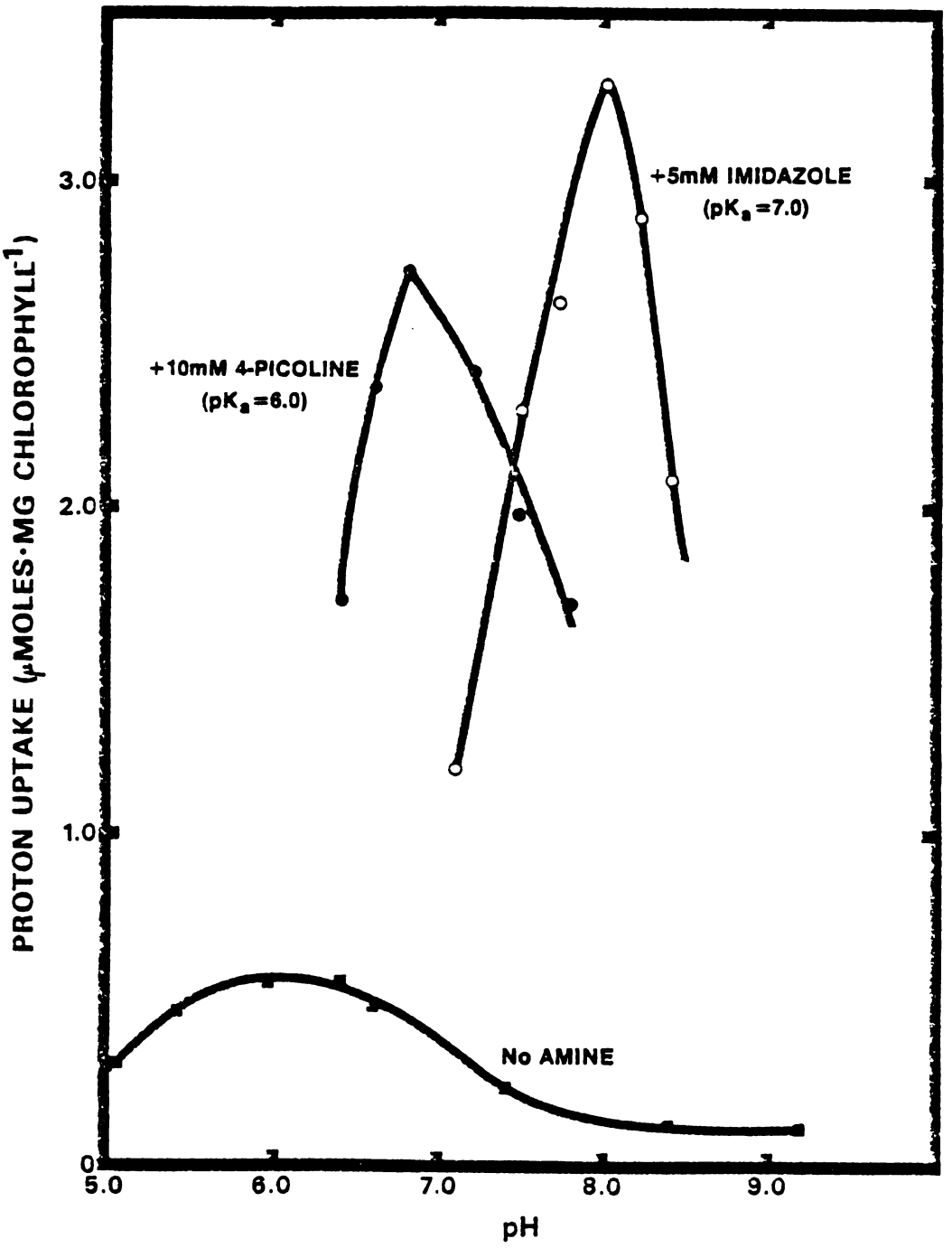
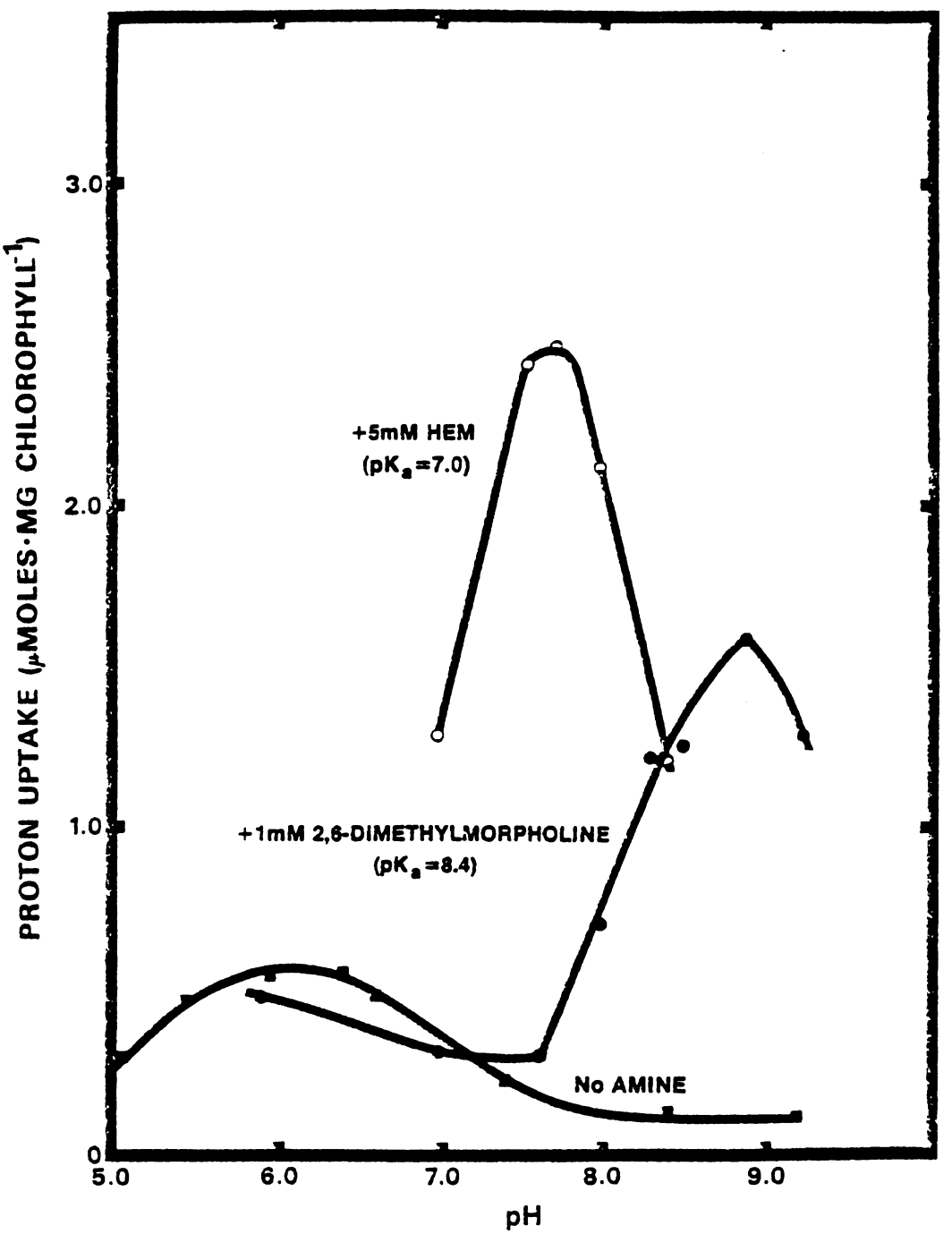

PROTON UPTAKE (μMOLES·MG CHLOROPHYLL^{-1})
3.0
2.0
1.0
0
5.0
6.0
7.0
8.0
9.0
pH
+5mM IMIDAZOLE
(pK$_a$=7.0)
+10mM 4-PICOLINE
(pK$_a$=6.0)
No AMINE

Figure 3.

Effects of weak amines on light-dependent proton uptake as a function of the pH of the external medium, II. Reaction conditions as described for Figure 2.

Figure 3.


PROTON UPTAKE (μMOLES·MG CHLOROPHYLL^{-1})
3.0
2.0
1.0
0
5.0
6.0
7.0
8.0
9.0
pH
+5mM HEM
(pK$_a$=7.0)
+1mM 2,6-DIMETHYLMORPHOLINE
(pK$_a$=8.4)
No AMINE

stimulate proton uptake also stimulate post-illumination ATP synthesis if they are present during the illumination [34,35]. These amines are believed to act by increasing the supply of protons which can be used by the phosphorylating enzyme after the light is shut off. Stimulation of post-illumination ATP formation by various weak amines is shown in Figures 4, 5 and 6.

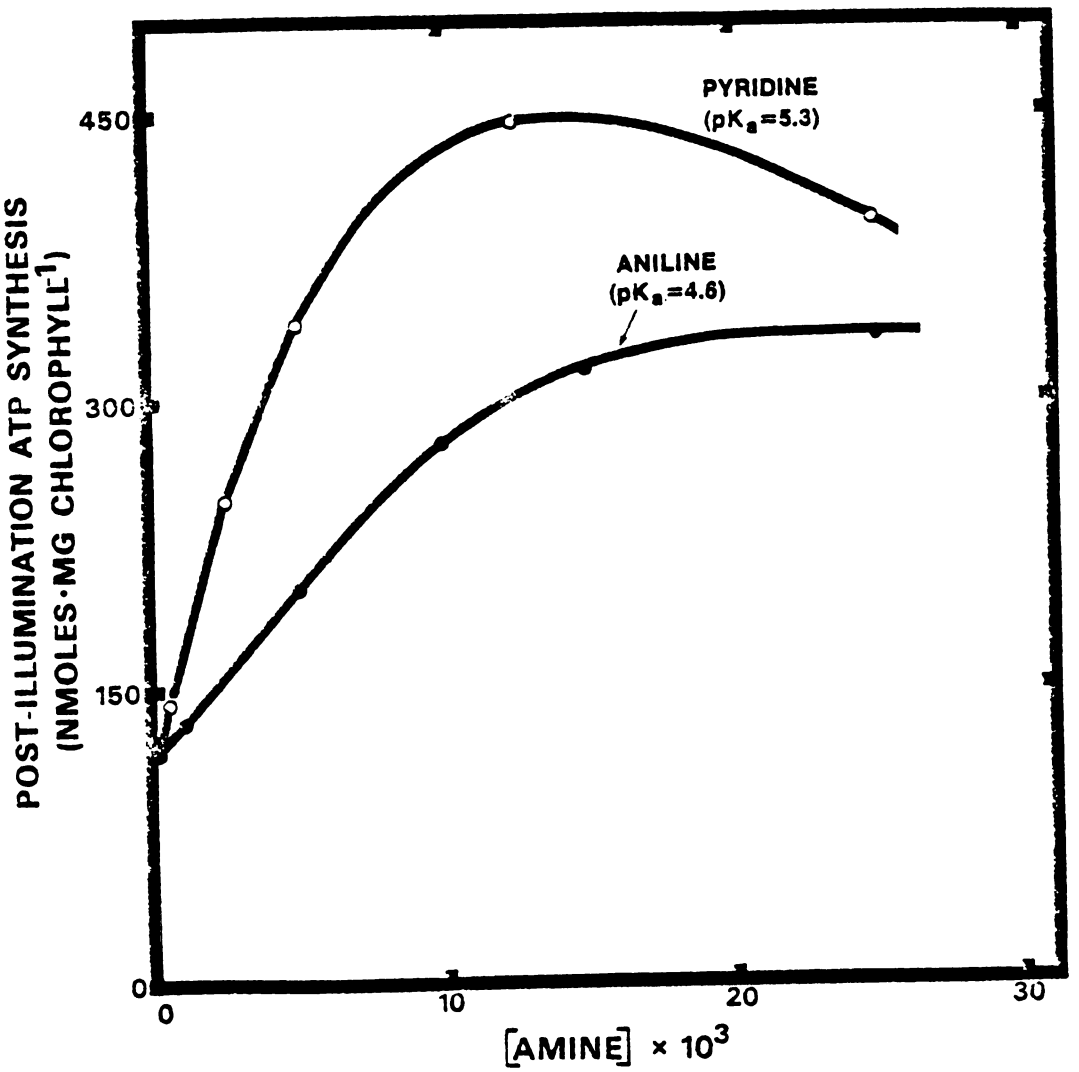
Stimulation of post-illumination ATP synthesis by a weak amine requires the presence of a permeant anion. Thus, HEM during illumination was largely without effect unless Cl^- was also present (see Figure 7). These results are consistent with the idea that an accumulation of protonated amine is responsible for the stimulation of ATP synthesis by the amine, since a large membrane potential seems to develop in the absence of permeant anions and the protonated amine is extruded. In any event, there is no amine accumulation in the absence of permeant anions (Izawa, S. and Good, N.E., personal communication).

The original aim of this research was to attempt to determine the ratio of the number of protons used by the coupling factor to the number of molecules of ATP formed. By using weak amines we were able to increase the storage of protons by the chloroplasts and also to increase the post-illumination synthesis of ATP. The stimulation of post-illumination ATP formation by a weak amine is easy to measure, but determining the increase in the storage of protons is not simple. We initially planned to use the stimulation of proton uptake as a measure of the amount of accumulated amine.

Figure 4.

Effects of aniline and pyridine on post-illumination ATP formation. The amines, when present were added to the reaction mixture prior to illumination. Chloroplast lamellae containing 300 µg of chlorophyll were illuminated for twenty seconds in a 2.0 ml reaction mixture containing the following: 0.1M sorbitol; 5mM MES-NaOH, pH 6.0; 50mM NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin and 10 µM pyocyanine. Immediately after light extinction, 1.0 ml of the following solution was added: 0.1M sorbitol; 0.2M Tricine-NaOH, pH 7.6; 50m NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin; 2mM ADP; and 20mM $Na_2H^{32}PO_4$ (7.5×10^4 cpm/µmole). The phosphorylation reaction was terminated 20 sec after light extinction by the addition of 1.0 ml of 2.0 N $HClO_4$. Temperature, 21 °C. All other conditions as described in Materials and Methods.

Figure 4.


PYRIDINE
(pK_a=5.3)
ANILINE
(pK_a=4.6)
POST-ILLUMINATION ATP SYNTHESIS
(NMOLES·MG CHLOROPHYLL^{-1})
450
300
150
0
0
10
20
30
[AMINE] × 10^3

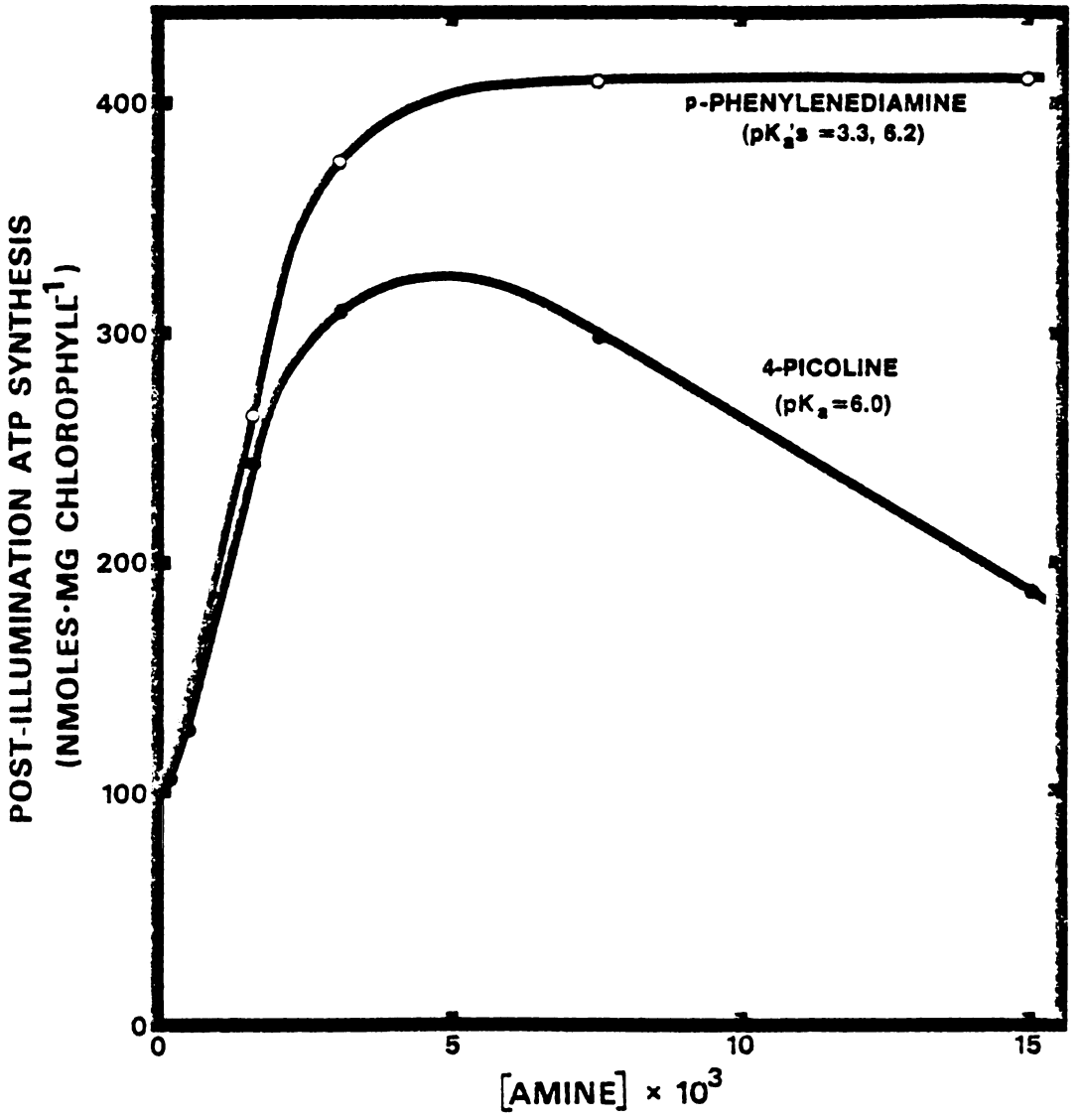


Figure 5.

Effects of p-phenylenediamine and 4-picoline on post-illumination ATP formation. Reaction conditions as described for Figure 4.

Figure 6.

Effects of N-β-hydroxyethylmorpholine (HEM) and imidazole on post-illumination ATP formation. Reaction conditions as described for Figure 4.

Figure 6.

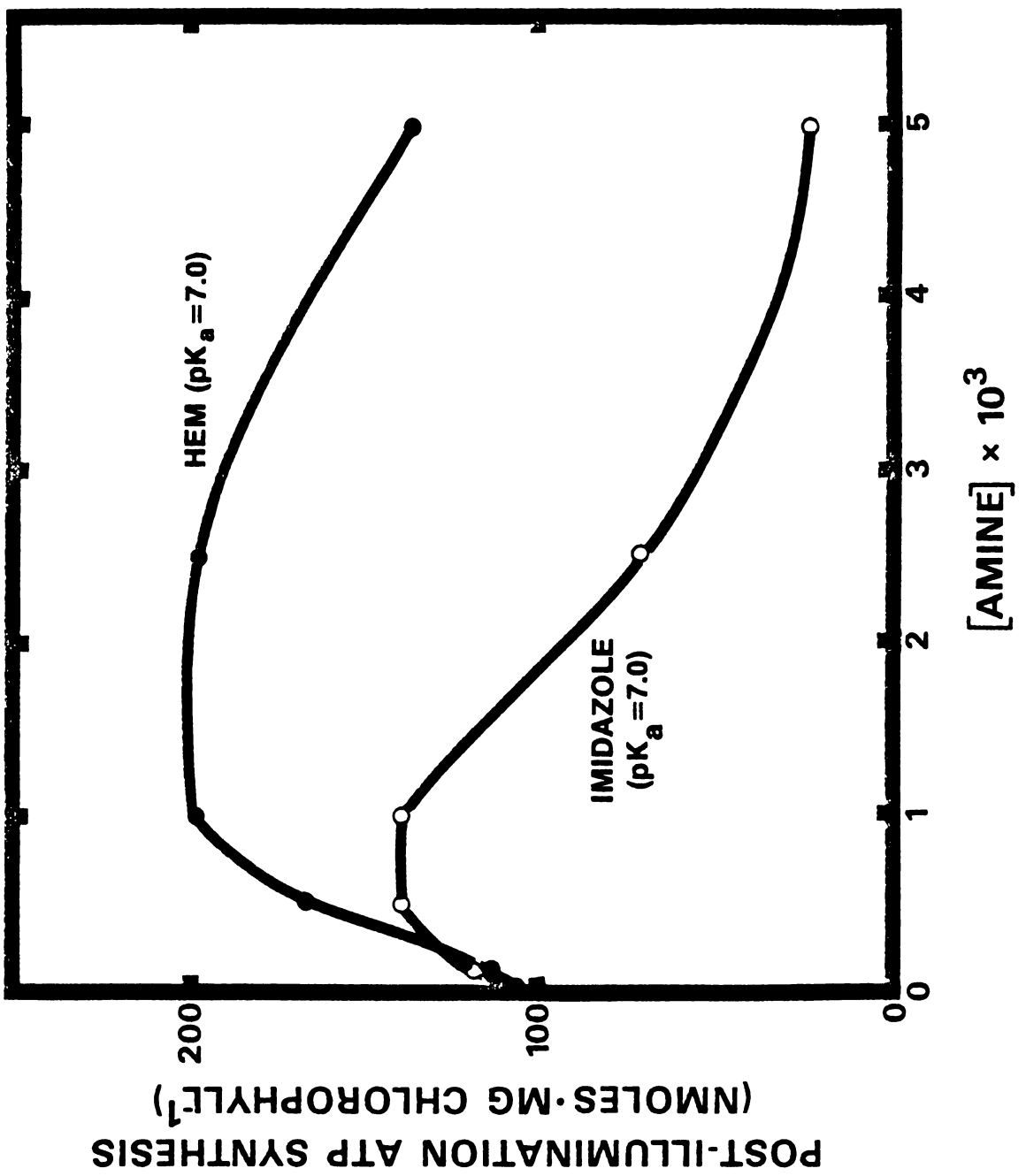
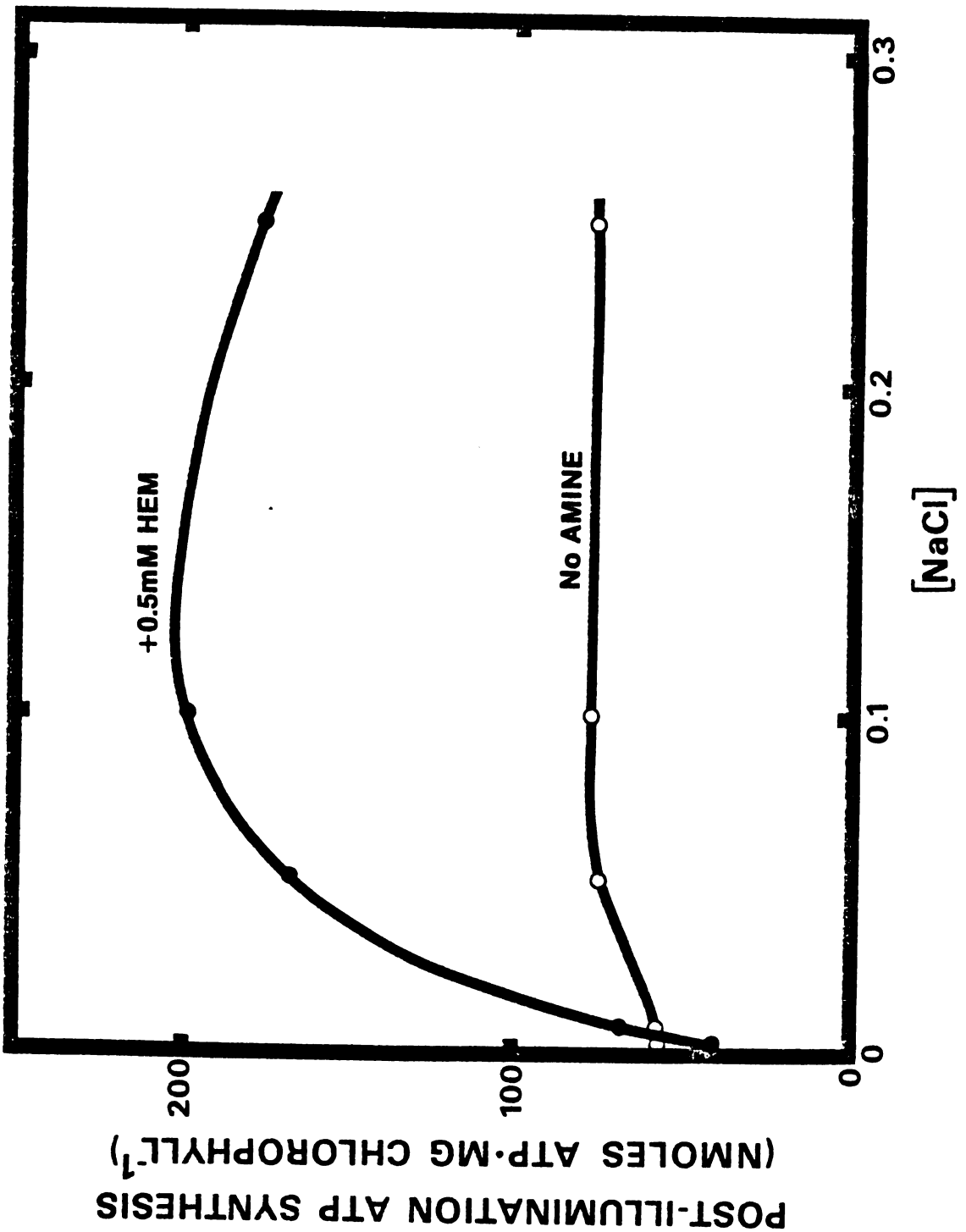

POST-ILLUMINATION ATP SYNTHESIS
(NMOLES·MG CHLOROPHYLL^{-1})
200
100
0
0
1
2
3
4
5
[AMINE] × 10^3
HEM (pK_a=7.0)
IMIDAZOLE
(pK_a=7.0)

Figure 7.

Effects of sodium chloride on post-illumination ATP formation in the presence and absence of HEM. The chloroplast lamellae were illuminated at pH 6.7 with 5mM MOPS-NaOH as buffer. All other reaction conditions as described for Figure 4.

Figure 7.


POST-ILLUMINATION ATP SYNTHESIS
(NMOLES ATP·MG CHLOROPHYLL⁻¹)
200
100
0
0
0.1
0.2
0.3
[NaCl]
+0.5mM HEM
No AMINE

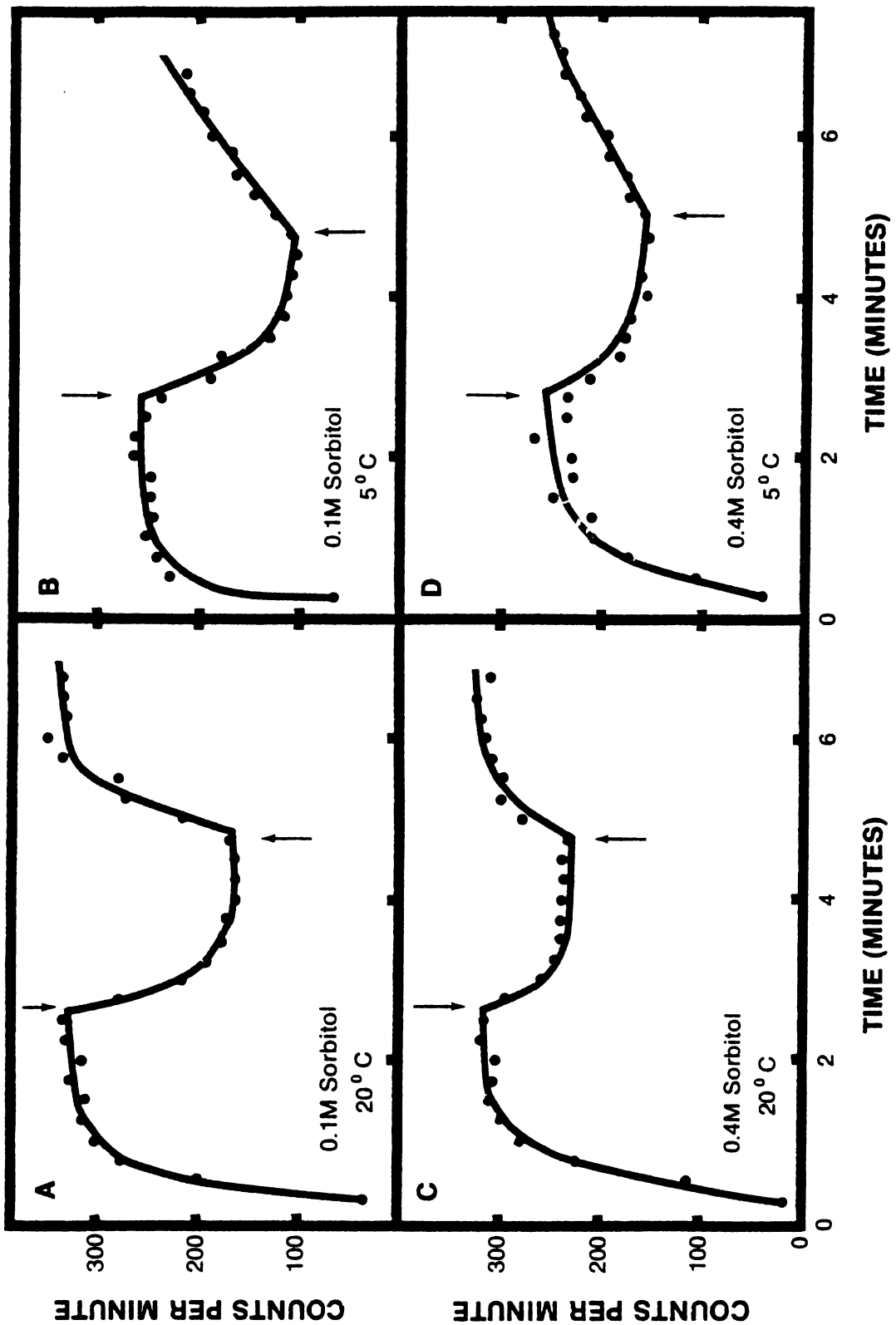
However, our earliest thinking in this area oversimplified the problem. We and other workers have concluded that a correction factor is needed to convert proton uptake to amine uptake. This correction factor is equal to the reciprocal of the fraction of amine unprotonated and can be very large below the pK_a of the amine [66]. To illustrate the necessity of the correction factor consider the following example: one pH unit below the pK_a of any amine 91% of the amine will be protonated; if the unprotonated amine is the only form which can cross the membrane then for each ten molecules of amine that are accumulated by the chloroplast 9.1 hydrogen ions will be released in the external medium. Thus for each ten molecules of protonated amine that accumulated inside the chloroplast only a single proton (0.9 proton) will seem to have been removed from the external medium if one back-titrates to the original pH with HCl.

In order to avoid the problems associated with proton uptake measurements a more direct method of measuring amine uptake was devised. Using radioactive amines it was possible to monitor the concentration of amine in the external medium continuously by measuring the rate of dialysis. Typical results are shown in Figure 8. Figure 8A shows that external concentration of amine drops upon illumination of the chloroplast suspension and rises to its original concentration after the light is turned off. No uptake is seen if an uncoupler is present. As expected, the uptake is sensitive to the osmolarity of the medium which indicates that the amount of amine accumulated is related to the internal volume of the chloroplasts. See Figure 8C.

Figure 8.

Uptake and release of N-methylmorpholine (pK_a = 7.6) by chloroplast lamellae as measured by changes in the rate of dialysis. The measurements were carried out with the apparatus described in Materials and Methods. The 2.0 ml reaction mixture contained the following: 0.1M or 0.4M sorbitol; 5mM MOPS-NaOH, pH 6.7; 0.1M NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin; 10 μM pyocyanine; chloroplast lamellae containing 333 μg of chlorophyll and 0.28mM [Ring-U-^{14}C] N-methylmorpholine (0.7mCi/mmole). The flow rate of water through the lower chamber of the apparatus was 5.0 ml/min. Fractions were collected every 15 seconds and each point represents the radioactivity of the fraction just collected. The labeled amine was added to the reaction mixture at time zero, the beginning of the collection of the first fraction. The downward arrow indicates the beginning of the illumination period. The upward arrow indicates the end of the illumination period.

Figure 8.


A
0.1M Sorbitol
20°C
B
0.1M Sorbitol
5°C
C
0.4M Sorbitol
20°C
D
0.4M Sorbitol
5°C
COUNTS PER MINUTE
TIME (MINUTES)
0
100
200
300
2
4
6

It has been known for a long time that the yield of post-illumination ATP is increased if the reaction temperature is lowered from 20 °C to about 3 °C [68]. The effects of the temperature and osmoticum on amine accumulation and amine release are shown in Figures 8B and 8D respectively. The amount of amine accumulated in the light was only slightly larger at the lower temperature but the rate of release of the amine after the light was turned off was greatly reduced. The greater yield of ATP and the slower release of the accumulated amine prompted us to use the lower temperature in later experiments.

Using the flow dialysis technique for measuring amine uptake we obtained a value of 7.5 for the ratio of amine uptake to additional ATP formed. The calculation is as follows. In this experiment imidazole (0.5mM) was used at a concentration such that the amount of additional ATP was directly proportional to the concentration of amine added. See Figure 9. At this concentration of imidazole, chloroplasts containing 333 μg of chlorophyll accumulated 500 nmoles of the amine (or 1500 nmoles/mg chl). See Figure 10. Since 0.5mM imidazole gave an additional 200 nmoles ATP/mg chl the amine accumulated/additional ATP = $\frac{1500 \text{ nmoles}}{200 \text{ nmoles}}$ or 7.5 as already noted.

An amine/ATP ratio of 7.5 is an overestimate of the H^+/ATP ratio because ATP synthesis ceases before the exit of accumulated amine ceases. A truer estimate of the H^+/ATP ratio might be obtained if ATP synthesis and amine release could be measured at a point before the processes had reached completion. The kinetics of ATP formation can

Figure 9.

Stimulation of post-illumination ATP synthesis by imidazole. The amine was added to the reaction mixture prior to illumination. Chloroplast lamellae containing 341μg of chlorophyll were illuminated for 1.0 minute in a 2.0 ml reaction mixture containing the following: 0.1M sorbitol; 5mM NOPS-NaOH, pH 6.7; 100mM NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin and 10 μM pyocyanine. Immediately after light extinction, 1.0 ml of the following solution was added: 0.1M sorbitol; 0.2M Tricine-NaOH, pH 8.5; 0.1M NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin; 2mM ADP; and 20mM $Na_2H^{32}PO_4$ (1.5×10^5 cpm/μmole). The phosphorylation reaction was terminated 1.0 minute after light extinction by the addition of 1.0 ml of 2.0 N $HClO_4$. Temperature, 3 °C.

Figure 9.

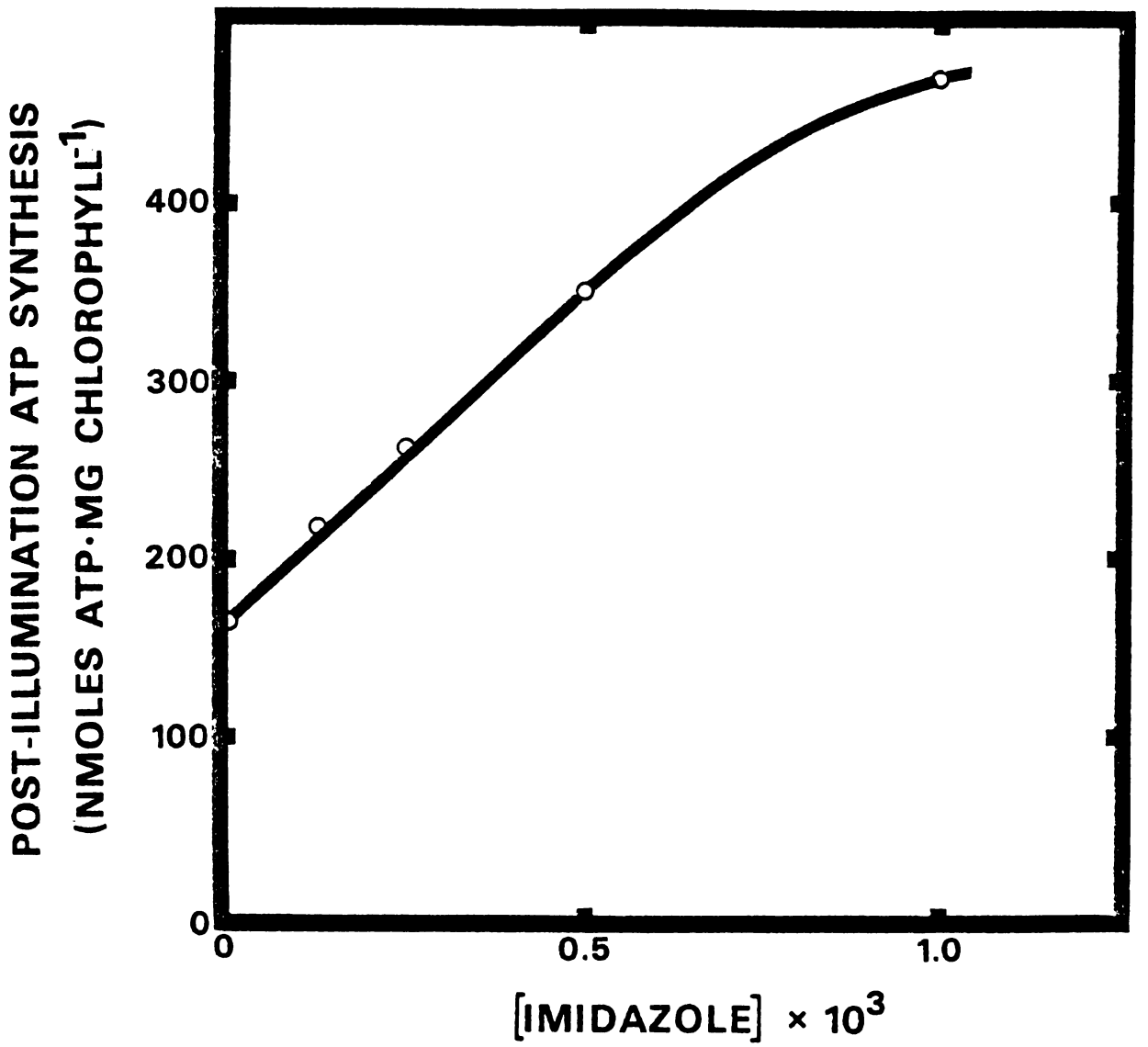
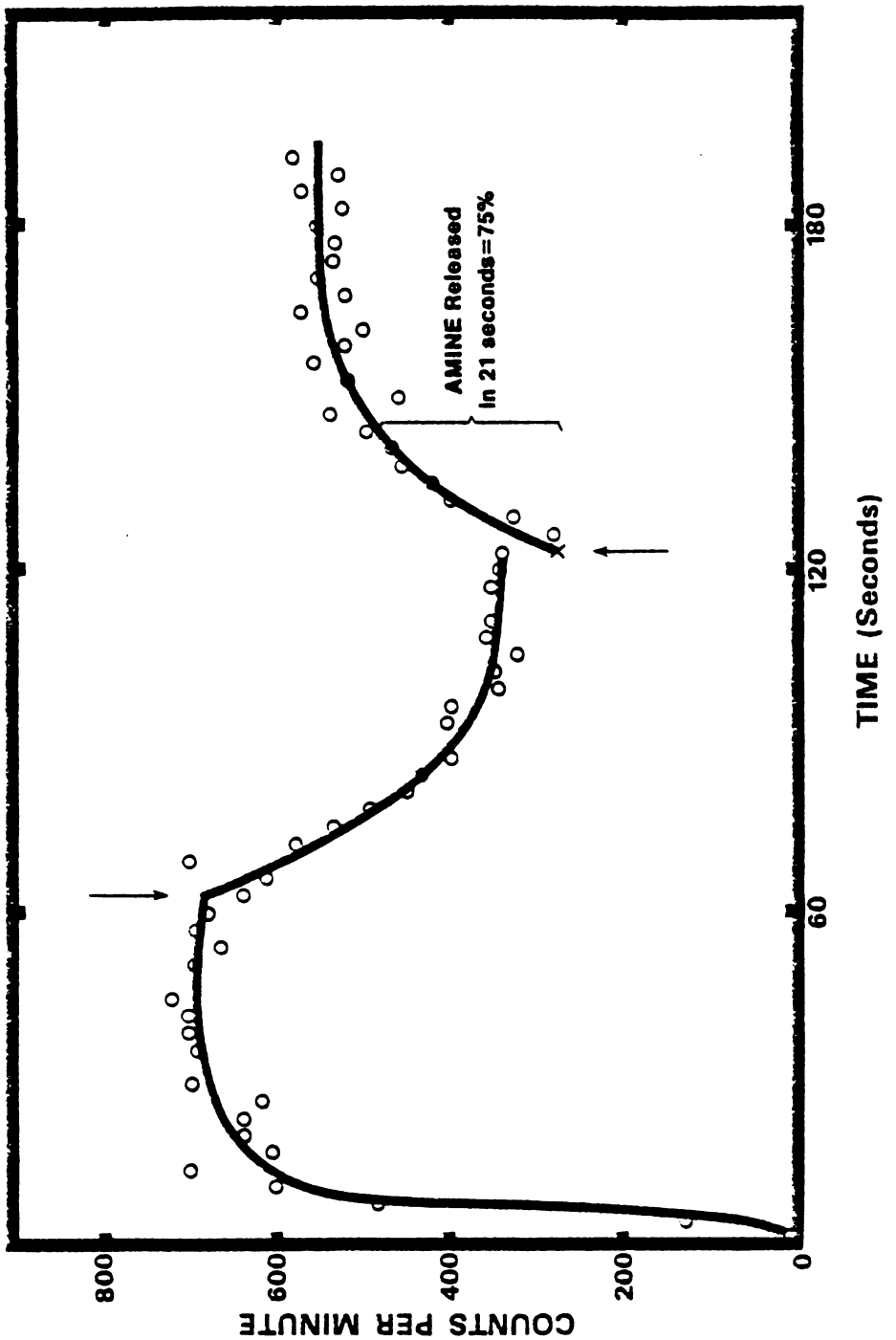

POST-ILLUMINATION ATP SYNTHESIS
(NMOLES ATP·MG CHLOROPHYLL^{-1})
400
300
200
100
0
0
0.5
1.0
[IMIDAZOLE] × 10^3

Figure 10.

Uptake and release of imidazole by chloroplast lamellae as measured by changes in the rate of dialysis. The reaction mixture was as described in Figure 8 except that 0.5mM [2-^{14}C] imidazole (3.5m Ci/mmole) was used instead of N-methylmorpholine. When the light was extinguished 0.4 ml of the following solution was added to the upper chamber: 0.1M sorbitol; 0.5M Tricine-NaOH, pH 8.5; 0.1M NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin; 5mM ADP; and 50mM Na_2HPO_4. The dilution of the reaction and change in pH resulted in a new lower steady-state level of radioactivity in the effluent when all of the amine had been released by the chloroplasts. The (X) at fraction number 41 (123 sec) shows the amount of radioactivity in fraction number 41 corrected for this change in the final steady-state value of radioactivity. The flow rate of water through the lower chamber was 12 ml/minute. Fractions were collected every three seconds. Temperature, 3 °C.

Figure 10.


AMINE Released
In 21 seconds=75%
COUNTS PER MINUTE
0
200
400
600
800
TIME (Seconds)
60
120
180

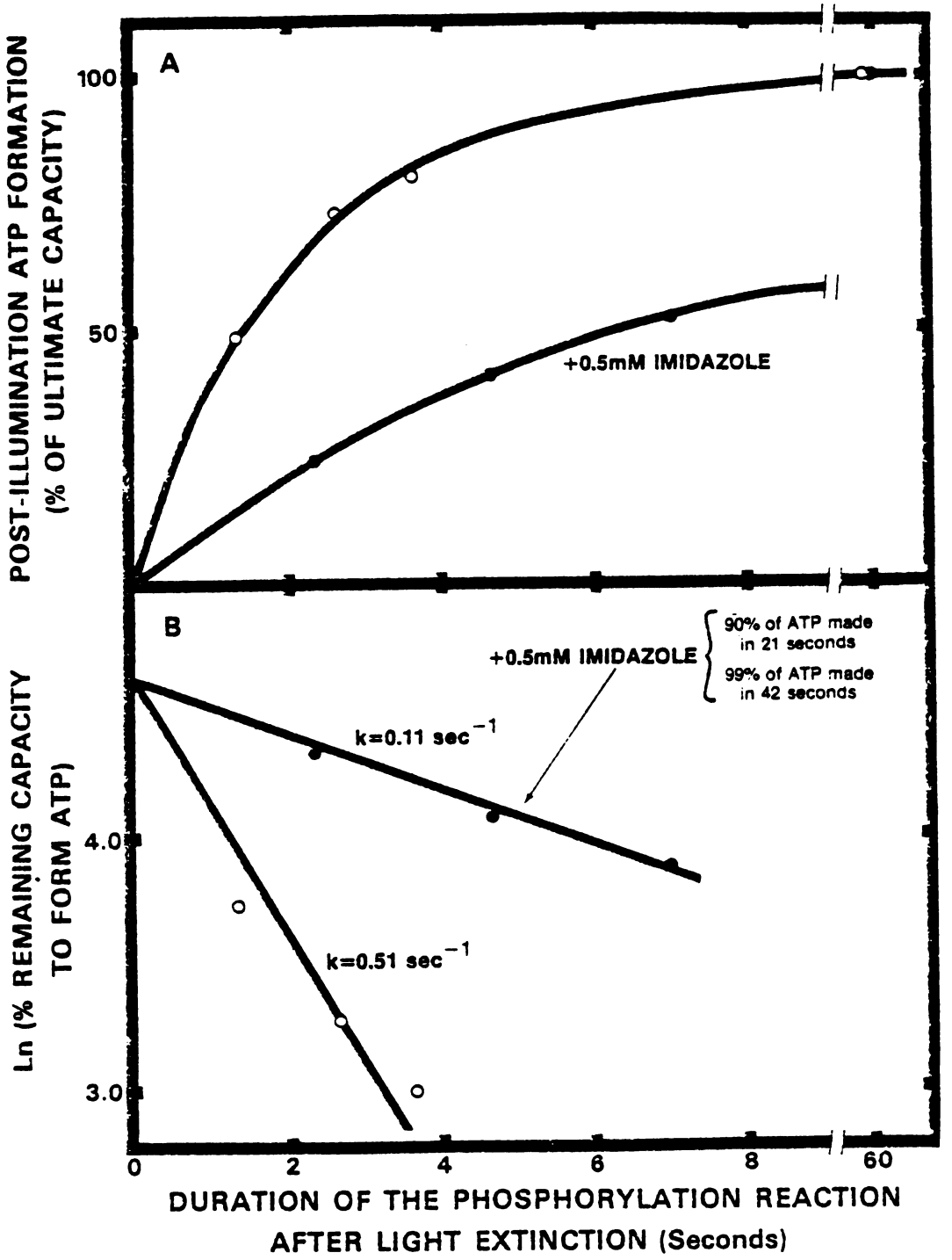
be determined by terminating the dark reaction after different periods of time. Data from an experiment of this type are shown in Figure 11A. The capacity of the chloroplasts to make ATP after the light was turned off decayed almost exponentially with time and the decay was much slower if imidazole was present during the illumination. If one assumes that the decay of the high energy state is a first order reaction then one would expect a straight line if the logarithm of the remaining ability to make ATP is plotted against time. The apparent first order decay of post-illumination ATP formation are illustrated in Figure 11B. Using the rate constant obtained from Figure 11 one can calculate that in the presence of 0.5 mM imidazole 90% of the ATP will be made in 21 seconds. In 21 seconds 75% of the accumulated amine was released (see Figure 10). The ratio of amine released to ATP formed then becomes $\frac{1125 \text{ nmoles}}{180 \text{ nmoles}}$ or 6.25.

Despite our original hopes the use of weak amines would not allow us to obtain a reliable measurement of the H^+/ATP ratio. The response time of our flow dialysis device along with its inherent noise level prevented us from accurately measuring the amount of amine released in the first few seconds after the light was turned off. Even if we could accurately measure the initial release of this amine, we would be unable to distinguish the source of protons used in ATP formation (endogenous buffering vs. exogenous amine). However, this research did uncover several interesting observations in addition to those already described.

Figure 11.

Time course of post-illumination phosphorylation in the presence and absence of 0.5mM imidazole. Reaction conditions as described for Figure 9 except that the phosphorylation was terminated with an injection of $HClO_4$ after various lengths of time using the digital timer and injection device described in Materials and Methods. The amount of ATP formed in a 60 second dark stage was 396 nmoles·mg chlorophyll^{-1} in the presence of 0.5mM imidazole and 180 nmoles·mg chlorophyll^{-1} without the amine. In A the ATP formed is plotted (as a fraction of the total which could be formed) against the delay in the injection of $HClO_4$. In B, using the same data, the logarithm of the remaining capacity to form ATP is plotted against the delay in the injection of $HClO_4$ to give a measure of the decay of the phosphorylation capacity during phosphorylation at pH 8.5. Although the data obtained in the absence of imidazole in this experiment do not fit a straight line very well, the straight line shown is probably justified on the basis of the results shown in Figure 12 and by Izawa's results [32]. Temperature, 3°C.

Figure 11.


A
100
50
POST-ILLUMINATION ATP FORMATION
(% OF ULTIMATE CAPACITY)
+0.5mM IMIDAZOLE
B
+0.5mM IMIDAZOLE
90% of ATP made in 21 seconds
99% of ATP made in 42 seconds
k=0.11 sec^{-1}
k=0.51 sec^{-1}
4.0
3.0
Ln (% REMAINING CAPACITY TO FORM ATP)
0
2
4
6
8
60
DURATION OF THE PHOSPHORYLATION REACTION
AFTER LIGHT EXTINCTION (Seconds)

The decay of the capacity for ATP formation appeared to be much slower in the presence of imidazole. See Figure 11. In this experiment the rate constants reflected both the rate at which ATP was being formed and the rate at which the high energy state decayed along paths which did not lead to ATP synthesis. The rate of the dissipative leak of the high energy state can be measured by itself if one delays the addition of ADP and $^{32}P_i$ after the light is turned off. The results of this type of experiment are shown in Figure 12. The decay of the high energy state is much slower in the presence of HEM than in its absence. However, the presence of aniline had little effect on the decay of the high energy state even though the yield of ATP was increased. This suggests that the pK_a of the internal buffering plays a role in determining the rate of decay of the high energy state which is not surprising in view of the fact that the pK_a of the amine must determine the concentration of hydrogen ions when they are released from storage in the protonated amine.

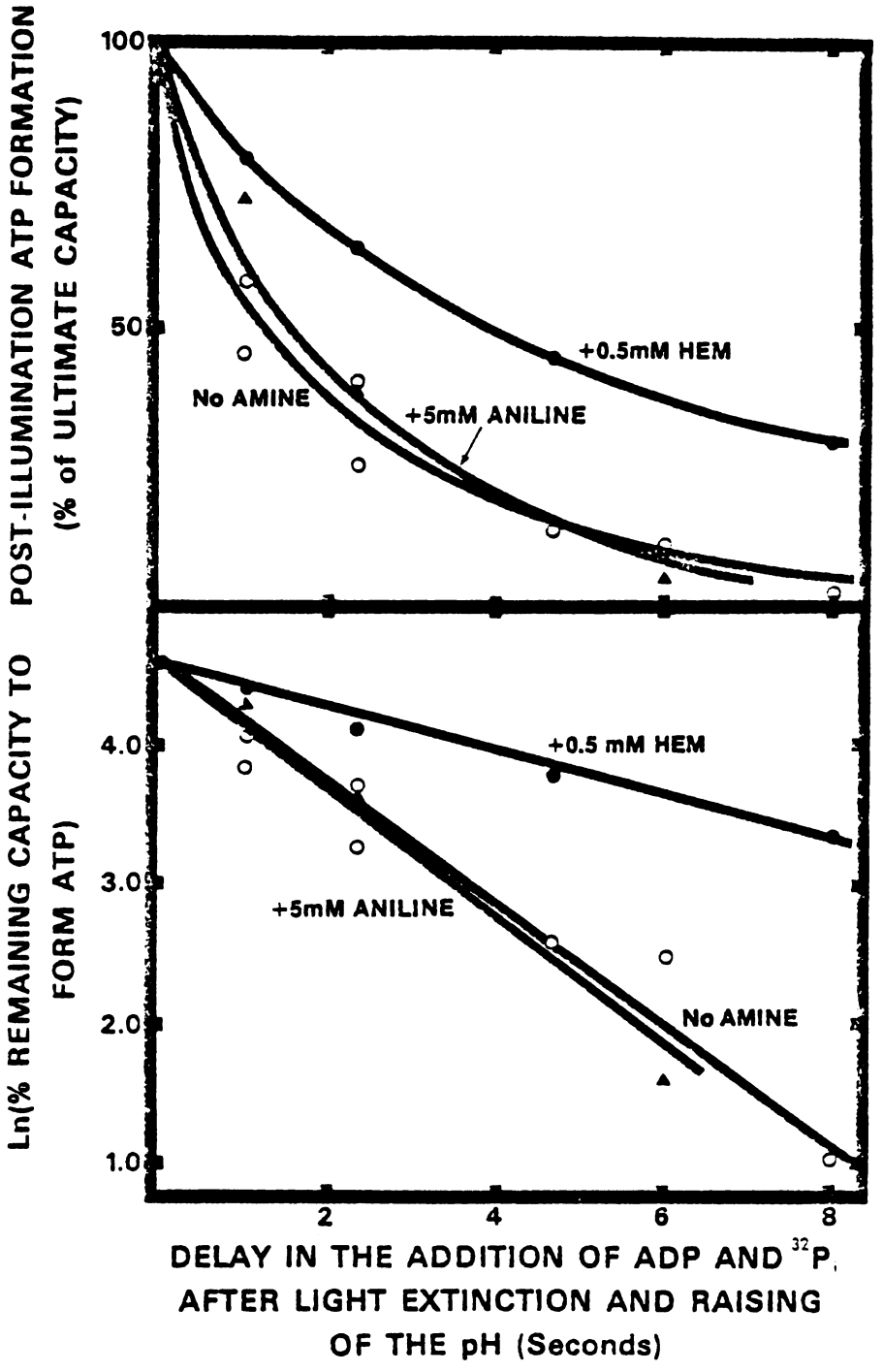
The kinetics of the decay of the high energy state and the question of the availability of the hydrogen ion stored in the internal protonated amine will be considered in the discussion section.

Regardless of the nature of the high energy state, one would expect the yield of post-illumination ATP to be reduced if the ratio of product (ATP) to reactants (ADP + Pi) became too high. In general, one would expect the yield of ATP to be sensitive to the concentrations of reactants and product if the magnitude of the driving force for ATP synthesis was equal or nearly equal to the thermodynamically

Figure 12.

Decay of the phosphorylation capacity in the absence of phosphorylation at pH 7.6. The amines, when present, were added prior to illumination. Chloroplast lamellae containing 340 μg of chlorophyll were illuminated for 20 seconds in a 2.0 ml reaction mixture containing the following: 0.1M sorbitol; 5mM MOPS-NaOH, pH 6.7; 0.1M NaCl; 2mM $MgCl_2$; 2 mg/ml bovine serum albumin and 10 μM pyocyanine. After the light was shut off, 0.67 ml of the following solution was injected: 0.1M sorbitol; 0.3M tricine-NaOH, pH 7.6; 0.1M NaCl; 2mM $MgCl_2$ and 2 mg/ml bovine serum albumin. A second injection (0.33 ml containing 2 μmoles of ADP and 20 μmoles of $NaH^{32}PO_4$) was made after a variable length of time using the digital timer and injection system described in Materials and Methods. Sixty seconds after the second injection the reaction was terminated with $HClO_4$. The amount of ATP formed with no time delay in the addition of ADP + $^{32}P_i$ was 65 nmoles/mg chlorophyll in the absence of amine, 199 nmoles/mg chlorophyll in the presence of 0.5mM HEM, and 96 nmoles/mg chlorophyll in the presence of 5mM aniline. Temperature, 22 °C.

Figure 12.


POST-ILLUMINATION ATP FORMATION
(% of ULTIMATE CAPACITY)
100
50
+0.5mM HEM
No AMINE
+5mM ANILINE
Ln(% REMAINING CAPACITY TO FORM ATP)
4.0
3.0
2.0
1.0
+0.5 mM HEM
+5mM ANILINE
No AMINE
2
4
6
8
DELAY IN THE ADDITION OF ADP AND $^{32}P_i$
AFTER LIGHT EXTINCTION AND RAISING
OF THE pH (Seconds)

imposed energy requirement for the phosphorylation of ADP. For instance a larger pH gradient should be required when the ATP concentration is high than is required when it is very low. Table 1 shows that the yield of ATP was insensitive to the concentrations of ADP and ATP when the ratio of product to reactants was changed over three orders of magnitude. This insensitivity implies that ATP synthesis stops before the driving force drops to the thermodynamic threshold. In other words, the phosphorylation reaction as carried out in these experiments must involve some irreversible and therefore energetically wasteful steps.

Table 1.

Effects of ADP and ATP Concentrations on Post-Illumination ATP Synthesis. Chloroplasts containing 346 μg of chlorophyll were added to each reaction. The concentration of N-methylmorpholine during illumination was 0.25mM. The nucleotides (ADP and ATP) were added to the chloroplasts with the dark stage buffer and the concentrations listed indicated the concentration in the reaction mixture after the addition of the dark stage buffer. All other conditions as described in Figure 9.

		Post-illumination ATP synthesis (nmoles ATP·mg chlorophyll^{-1})	
[ADP] X 10^3	[ATP] X 10^3	-Amine	+NMM
1.8	--	154	278
0.6	--	154	285
0.6	0.077	159	284
0.6	0.25	159	294
0.6	0.77	156	282
0.6	2.5	154	292

Section II The Role of Membrane Potentials and pH Gradients in ATP Synthesis by Isolated Chloroplasts

A potentially interesting phenomenon was observed in the dark controls for the post-illumination ATP synthesis experiments. In these experiments the illumination was performed at pH 6.7 while the subsequent dark phosphorylation occurred at pH 8.5. Table 2 shows that chloroplasts which were not illuminated apparently formed 27 nmoles ATP·mg chl^{-1} in response to the upward pH change and that the synthesis was insensitive to DCMU, an electron transport inhibitor. However, there was a net synthesis of ATP by the chloroplasts of only about 9 nmoles ATP·mg chl^{-1}. The other 18 nmoles ATP·mg chl^{-1} was non-biological in origin since it could be "formed" when the chloroplasts were denatured with perchloric acid before the addition of ADP and $^{32}P_i$ or even when chloroplasts were not added. This apparent non-biological ATP synthesis was, in fact, not ATP synthesis at all. It is a measure of the polyphosphate contamination in the commercial $H_3{}^{32}PO_4$. Any organic ^{32}P (including ATP) and polyphosphate remain in the aqueous phase after the phosphomolybdate extraction with organic solvent which removes the unreacted $^{32}P_i$.

Since the ATP formed in the dark by a small pH increase was only a fraction of the background pyrophosphate level it was deemed necessary to abandon the organic extraction of the $^{32}P_i$-molybdate complex in favor of a technique which would eliminate the pyrophosphate problem. Such a technique had been developed by Boyer's laboratory [61]. It Involved the adsorbtion of all nucleotides onto charcoal followed by

Table 2.

Apparent ATP synthesis in the dark resulting from a small pH increase. All conditions are described in Figure 9 except that the chloroplasts were not illuminated and pyocyanine was not present. In Experiment 1 the chloroplasts were denatured with perchloric acid before the addition of the pH 8.5 solution containing ADP and $^{32}P_i$. In Experiment 2 the chloroplasts were not added to the reaction. In Experiment 3 the chloroplasts were subjected to a pH increase of 1.8 units in the dark and the same pH increase was imposed in the presence of 5 μM DCMU in Experiment 4. The temperature in this experiment and all subsequent experiments was 3 °C. Note that the fraction designated "ATP" refers to all radioactive phosphate not removed as phosphomolybdate and that the amount formed in the absence of chloroplasts represents background counts not actually in ATP.

	pH change			nmoles "ATP"/mg chl
(1)	6.7 →	$HClO_4$ →	ADP + $^{32}P_i$	18.4
(2)	6.7 →	8.5	(-chl)	(18.1)
(3)	6.7 →	8.5		27.4
(4)	6.7 →	8.5	(+DCMU)	26.7

elution from the charcoal and separation of the nucleotides using a quaternary amine anion-exchange column.

The results of an experiment using Boyer's procedure is shown in Table 3. In this experiment the background level of radioactive contamination had been reduced to 0.04 nmoles·mg chl^{-1}. Although a pH increase of two units in this case produced only 3 nmoles ATP·mg chl^{-1}, $^{32}P_i$ incorporation into ATP was markedly reduced when ATP was substituted fro ADP. Therefore, under the conditions employed, $^{32}P_i$ incorporation into ATP was not a result of the ATP-P_i exchange reaction which is known to be stimulated by a large pH change [69].

The ATP synthesis with small pH changes which we were observing appeared inconsistent with the early work of Jagendorf and Uribe [23]. In their pioneering work on acid-base ATP synthesis, a pH increase of about three units was found to be necessary before ATP formation resulted. This discovery of a Δ pH threshold requirement for ATP synthesis could be interpreted quite easily by Mitchell's chemiosmotic theory of ATP synthesis. Mitchell's theory states that the driving force for ATP synthesis resides in the transmembrane electrochemical potential gradient of protons and that the energy from a small number of protons is pooled to phosphorylate one molecule of ADP. If the energy made available when this small number of protons traverse the transmembrane pH gradient does not provide the required minimum for ATP synthesis, then phosphorylation should not occur. Thus one would predict that if a pH gradient were being used to drive ATP synthesis there should be a minimum pH difference of several units required before any ATP would be formed.

Table 3.

Comparison of the amount of radioactive ATP formed in the dark in the presence of ADP or ATP. Chloroplasts containing 791 µg of chlorophyll were added to a 2.0 ml reaction mixture which contained the following: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 0.1M KCl; 2mM $MgCl_2$; the concentration of ADP or ATP listed above and 6.67 mM NaH_2PO_4. The pH of the initial stage was varied with small additions of KOH made before the addition of the chloroplasts. One minute after the chloroplasts were added to the initial reaction mixture, 1.0 ml of the following solution was added: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 0.1M KCl; 2mM $MgCl_2$; the concentration of ADP or ATP present in the initial stage; 6.67mM $Na_2H^{32}PO_4$ (8×10^5 cpm/µmole) and 0.2M tricine-KOH, pH 8.5. The incorporation of $^{32}P_i$ into ATP was measured using Boyer's procedure in this experiment and all subsequent experiments. Details of the procedure are recorded in Materials and Methods.

Nucleotide	pH Change	nmoles ATP/mg chl
0.75mM ADP	6.7 → $HClO_4$ → ADP + $^{32}P_i$	0.04
0.75mM ADP	6.4 → 8.5	2.94
0.75mM ADP	8.6 → 8.5	0.36
0.33mM ATP	6.3 → 8.5	0.65
0.33mM ATP	8.4 → 8.5	0.44

Since our experiments showed that a pH increase of less than two units was making ATP we decided to see if any pH threshold could be observed. Our experiments showed that a plot of ATP synthesis against the pH difference extrapolated through zero pH change, i.e. no threshold ΔpH was found. See Figure 13. These results posed serious mechanistic or thermodynamic problems for the chemiosmotic theory since a pH difference of 0.5 units could scarcely provide the requisite energy for the phosphorylation of ADP unless the energy from a large number of protons could be pooled.

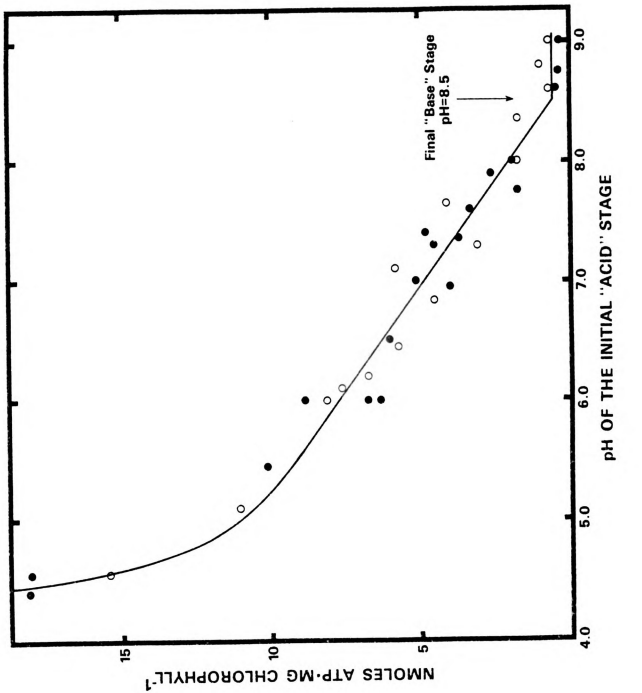
Our results could be accomodated in the chemiosmotic theory if in addition to a pH change we were unknowingly creating a membrane potential (positive inside) which would be expected to add to the proton electrochemical potential gradient. Several workers have shown that a pH increase which is too small to allow any ATP synthesis can support phosphorylation if a membrane potential is imposed along with the pH change [27,28]. In these experiments the membrane potential was formed by the introduction of potassium along with valinomycin, a potassium-specific ionophore. It therefore became very important to establish whether or not any membrane potentials were inadvertently being created in our experiments.

Since a membrane potential might develop from a sudden change in the ionic composition of the medium the chloroplasts were suspended in, our first objective was to make the initial "acid" stage as much like the "final" basic stage as possible. The open circles in Figure 13 show that addition of ADP and P_i to the acid stage had no effect on the yield of ATP. The only remaining differences between the initial

Figure 13.

ATP synthesis by chloroplast lamellae as a result of small pH increases in the dark. The final pH was 8.5 in all of the reactions. The 2.0 ml stirred "acid" stage contained the following: 0.1M sorbitol; 0.1M KCl; 1 mg/ml bovine serum albumin, 2mM $MgCl_2$ and chloroplasts containing 400-650 μg chlorophyll. The pH of the initial stage was varied with small additions of HCl or KOH made before the addition of the chloroplasts. One minute after the chloroplasts were added to the "acid" stage, 1.0 ml of the following "base" stage solution was added: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 2mM $MgCl_2$; 20mM $NaH^{32}PO_4$ (2×10^5 cpm/μmole); 2.25mM ADP; 0.2M tricine-KOH, pH 8.5. Experiments recorded with open circles differed only in that ADP and P_i were added to the "acid" stage.

Figure 13.


Final "Base" Stage
pH=8.5
NMOLES ATP·MG CHLOROPHYLL-1
15
10
5
4.0
5.0
6.0
7.0
8.0
9.0
pH OF THE INITIAL "ACID" STAGE

and final stages were the obvious pH differences and the ionic composition of the buffer used in the final stage to establish the final pH. Table 4 shows that the addition of KCl to the initial stage had very little effect on the yield of ATP resulting from a small pH increase when tricine-KOH was used as the buffer in the final stage. Similar results were obtained when sodium or lithium chlorides were added to reactions buffered by tricine-NaOH or tricine-LiOH respectively. Therefore it seemed to us that no diffusion potentials could be contributing to the observed ATP synthesis.

However, in an attempt to reduce even further the possibility of ion gradients, the chloroplasts were incubated in the initial stage medium (including ADP, P_i and KCl) for 60 minutes before raising the pH and assaying for ATP synthesis. The results of this incubation experiment are shown in Figure 14. These results are remarkably similar to those of Jagendorf and Uribe [23]. The difference in the results shown in Figure 13 and Figure 14 is due solely to the long pre-incubation in KCl. Table 5 shows that a similar long incubation in any of the alkali chlorides tested eliminated ATP synthesis when tricine-KOH was used as the buffer in the final stage.

It appeared from this that alkali cations were playing an important role in the phenomenon we were observing. An experiment was done to determine what function that the final base stage buffer was playing in the ATP synthesis. See Table 6. The buffers that were tested fell into two groups. The buffers which contained alkali cations allowed the formation of modest amounts of ATP while the

Table 4.

Effect of one minute incubations with salts on the yield of ATP from a small pH increase. The concentration of the alkali chlorides, when present in the pH 6.5 acid stage was 0.14M. All other conditions as described in Table 3 except that the buffer used in the base stage was varied as indicated above.

Salt In Acid Stage	Buffer In Base Stage	pH 6.5 → pH 8.5 nmoles ATP/mg chl
——	Tricine-LiOH	7.14
LiCl	Tricine-LiOH	6.63
——	Tricine-NaOH	8.63
NaCl	Tricine-NaOH	7.32
——	Tricine-KOH	7.42
KCl	Tricine-KOH	7.28

Figure 14.

The effect of prolonged (one hour) incubation of chloroplast lamellae in KCl on the yield of ATP from an acid-base transition. The chloroplast lamellae (690 μg chlorophyll per reaction) were incubated in the following solution: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 2mM $MgCl_2$; 0.14M KCl; 0.75mM ADP and 6.67mM KH_2PO_4, pH 6.5. The composition of the acid stage was identical to the pre-incubation medium except for the pH which was varied with small additions of HCl or KOH made before the addition of the chloroplasts. One minute after the pH of the initial acid stage had been adjusted, 1.0 ml of the following solution was added: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 2mM $MgCl_2$; 0.75mM ADP; 6.67mM K_2HPO_4 (6.3×10^5 cpm/μmole); 0.2M tricine-KOH, pH 8.5.

Figure 14.

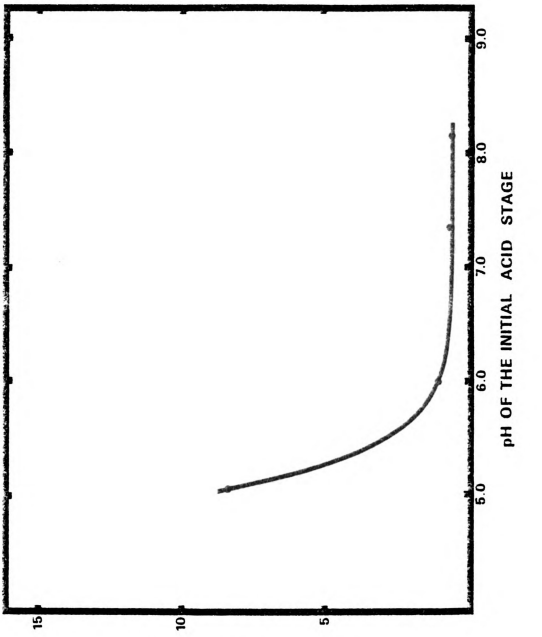

NMOLES ATP·MG CHLOROPHYLL⁻¹
15
10
5
5.0
6.0
7.0
8.0
9.0
pH OF THE INITIAL ACID STAGE

Table 5.

Effect of one hour incubation of chloroplast lamellae in alkali chlorides on the yield of ATP resulting from a small pH increase. Chloroplast lamellae containing 410 μg chlorophyll were incubated for 60 minutes in 2.0 ml of the following solution: 0.1M sorbitol; 1 mg/ml bovine serum albumin, pH 6.5; 2mM $MgCl_2$; ± 0.14M alkali chloride as indicated above. After the incubation period was over 1.0 ml of the base stage solution described in Figure 13 was added.

Salt In Incubation Medium	pH 6.5 → pH 8.5 nmoles ATP/mg chl
——	4.40
LiCl	1.21
NaCl	1.11
KCl	1.37
RbCl	0.55

Table 6.

Yield of ATP in the dark as a function of the cations present in the buffer used to raise the pH. The 2.0 ml stirred pH 6.5 solution consisted of: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 2mM $MgCl_2$; 6.67mM NaH_2PO_4; 0.75mM ADP; and chloroplast lamellae containing 490 µg chlorophyll. One minute after the chloroplasts were added to the pH 6.5 stage, 1.0 ml of the following solution was injected: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 2mM $MgCl_2$; 6.67 mM $Na_2H^{32}PO_4$ (6 X 10^5 cpm/µmole); 0.76mM ADP; and the indicated buffer (200mM, pH 8.5).

Buffer In The Base Stage	ATP formed as a result of a change in pH from 6.5 to 8.5 nmoles ATP/mg chlorophyll
Tricine-LiOH	7.14
Tricine-NaOH	8.63
Tricine-KOH	7.42
Glycylglycine-KOH	7.73
Tricine-Bistrispropane	1.17
Tricine-Arginine*	1.55
Glycylglycine-Bistrispropane	1.27
Bistrispropane·HCl	0.77
Tris·HCl	1.48

*Arginine added as the free base

buffers which did not contain alkali cations hardly supported any ATP synthesis at all. Table 7 also shows that ATP synthesis failed when no alkali ions were present in the final base stage but that ATP synthesis was restored when K^+ ions were added to the otherwise alkali ion-deficient medium. Figure 15 shows that the yield of ATP from a small pH change is proportional to the concentration of alkali salt in the final stage.

In summary, ATP synthesis by small pH increases required a sudden concomitant increase in alkali salt concentration. Prolonged incubation with an alkali salt eliminated the ATP formation. Although a membrane potential (positive inside) is a possible consequence of a sudden increase in alkali salt concentrations, we had not yet ruled out the possibility that a sudden increase in salt inside the chloroplast rather than a membrane potential was responsible for the stimulation of ATP synthesis. If a membrane potential is responsible for the stimulation of ATP synthesis one would expect to be able to manipulate the yield of ATP by varying the relative permeation rates of the alkali cation and its accompanying anion. Table 8 shows that the stimulation of ATP formation seen when KCl is added to the basic stage is greatly increased when valinomycin is present. Valinomycin greatly increases the permeability of the membranes to potassium and therefore should increase the membrane potential. However, if potassium is present at the same concentration with the permeant anion thiocyanate no stimulation of ATP synthesis is observed. Much of the effectiveness of potassium can be restored, in the presence of thiocyanate, if valinomycin is also present. These data strongly

Table 7.

Dependence of ATP synthesis on the presence of an alkali ion in the base stage. The indicated buffer (200mM, pH 8.5) and salt (0.14M KCl) were added to the base stage solution. All other conditions as described in Table 6.

Buffer in Base Stage	KCl in Base Stage	ATP Formed As A Result of a Change in pH from 6.5 to 8.5 (nmole/mg chl)
Tricine-KOH	-	4.77
Bistrispropane·HCl	-	0.77
Bistrispropane·HCl	+	4.62
Tricine-Bistrispropane	-	1.17
Tricine-Bistrispropane	+	3.51
Tricine-Arginine	-	1.55
Tricine-Arginine	+	3.90
Tris·HCl	-	1.48
Tris·HCl	+	2.47

Figure 15.

Yield of ATP resulting from a small pH increase as a function of the concentration of potassium in the basic stage. The pH of the initial acid stage was 6.0. The buffer in the base stage was tricine-bistris-propane, pH 8.0. Dipotassium succinate was added to the base stage solution to give the final concentrations indicated above. Chloroplast lamellae containing 576 μg chlorophyll were present in each reaction. Reaction conditions otherwise as in Table 6.

Figure 15.

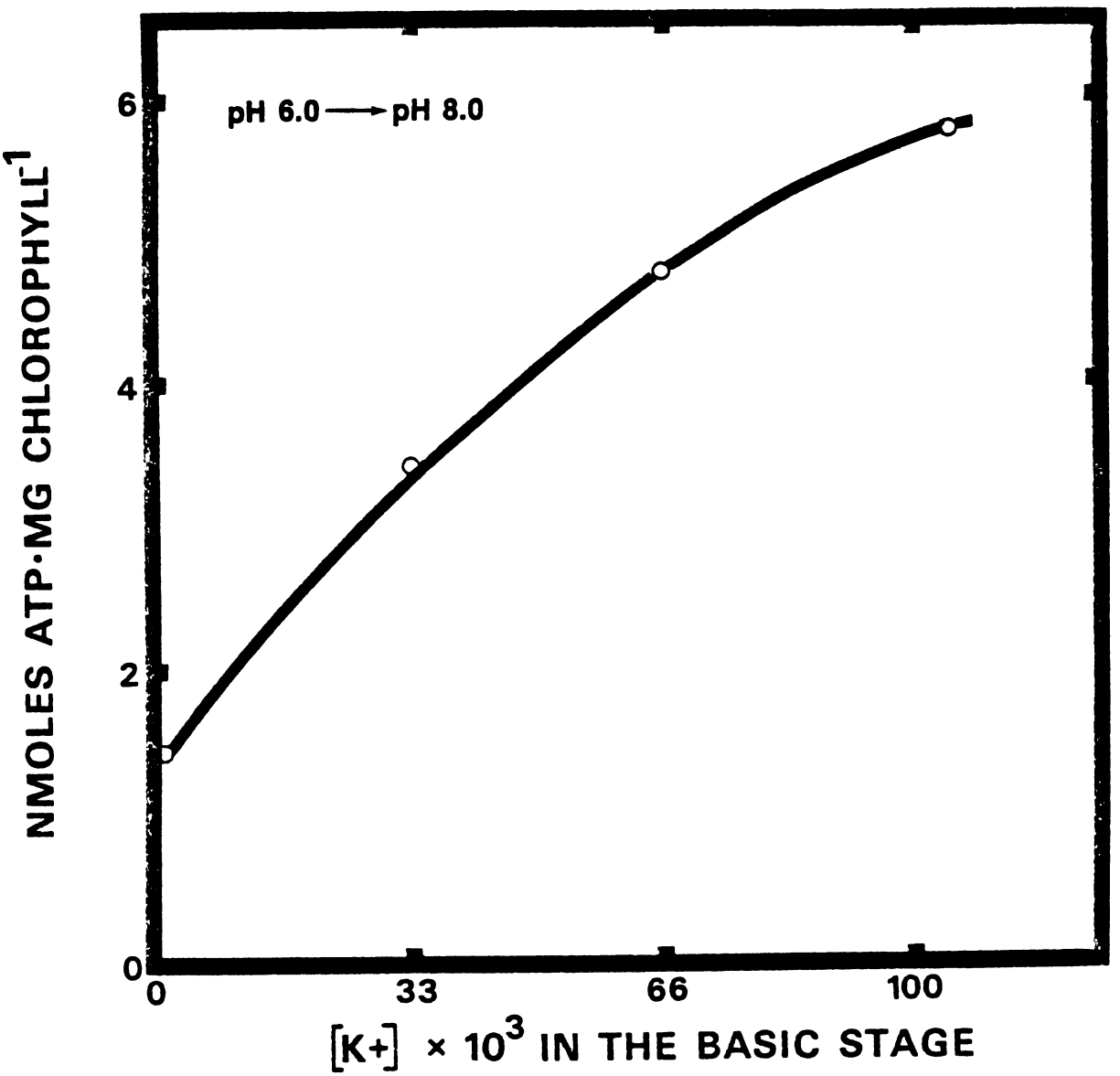

pH 6.0 → pH 8.0
NMOLES ATP·MG CHLOROPHYLL^{-1}
0
2
4
6
0
33
66
100
[K+] × 10^3 IN THE BASIC STAGE

Table 8.

The effect of permeant ions on the amount of ATP synthesized. Tricine-bistrispropane was used to raise the pH in all of the reactions. Valinomycin (1 μM), when present, was added to the acid stage. The potassium salts (150mM) along with ADP and P_i were present only in the 1.0 ml which was added to provide the basic stage. All other conditions as in Table 6.

Potassium Salt Added in Basic Stage	ATP Formed as a Result of a Change in pH from 6.0 to 8.0 (nmoles/mg chlorophyll)	
	-Valinomycin	+Valinomycin
None	2.69	2.10
KCl	5.31	20.45
KSCN	2.14	11.28

suggest that the potassium salt is creating a membrane potential (positive inside) which is stimulating ATP synthesis since increasing the permeation of cations increases the yield of ATP while increasing the permeation of anions decreases the yield. It is tempting to suppose that this diffusion potential combines with a sub-threshold ΔpH to provide a sufficient driving force for ATP synthesis.

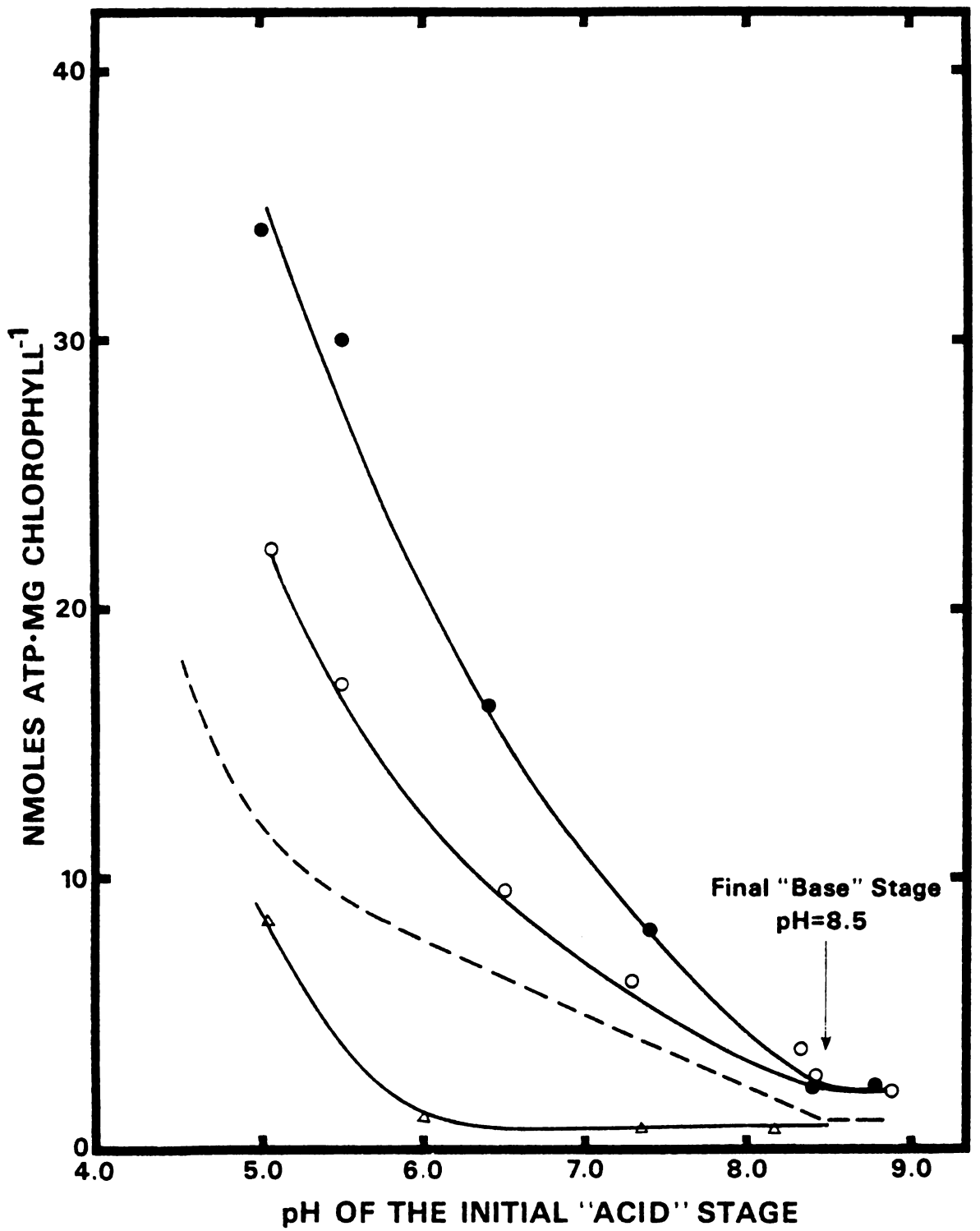
The fact that ATP synthesis extrapolates through zero pH change in Figure 13 indicates that under the conditions employed the membrane potential alone must have sufficient energy to drive the phosphorylation reaction. If this were not so one would expect to find some ΔpH threshold, that is to say if the membrane potential were providing only some fraction of the requisite energy, the ΔpH would have to provide the rest.

In light of the results shown in Figure 14 and Table 8 we were able to increase the yield of ATP from a given pH change above that shown in Figure 13. The conditions which increased the yield of ATP are those conditions which would be expected to increase the membrane potential generated. By the argument given above the increased membrane potential should now certainly EXCEED the thermodynamic requirement for ATP synthesis. We found, surprisingly, that regardless of the apparent magnitude of the membrane potential (inferred from cation gradients and estimated by increased ATP synthesis), the yield of ATP still extrapolated through zero pH change. See Figure 16. Thus, a membrane potential appears to increase the efficiency with which a small pH change is used to make ATP, but it is without effect unless it is accompanied by a pH difference across the thylakoid membrane.

Figure 16.

The effect of small pH increases on the yield of ATP under conditions which should alter the membrane potentials generated. The pH of the initial stage was adjusted with small additions of HCl or bistris-propane. The experimental conditions were as in Figure 13 except as noted below. In all cases the second "basic" stage contained potassium salts. Bottom curve (Δ —— Δ) 60 minute preincubations with 0.1M KCl, hence little diffusion generated transmembrane potential (curve taken from Figure 14). Lower middle curve (- - - -), only one minute incubation with 0.1M KCl, hence some diffusion potential left (curve taken from Figure 13). Upper middle curve, (O —— O) K^+ present only in the base stage, hence greater diffusion potential. Top curve (● —— ●) valinomycin (1 μM) present in both stages but K^+ present only in the "base" stage, hence probably the largest diffusion potential.

Figure 16.


40
30
20
10
0
NMOLES ATP·MG CHLOROPHYLL^{-1}
4.0
5.0
6.0
7.0
8.0
9.0
pH OF THE INITIAL "ACID" STAGE
Final "Base" Stage
pH=8.5

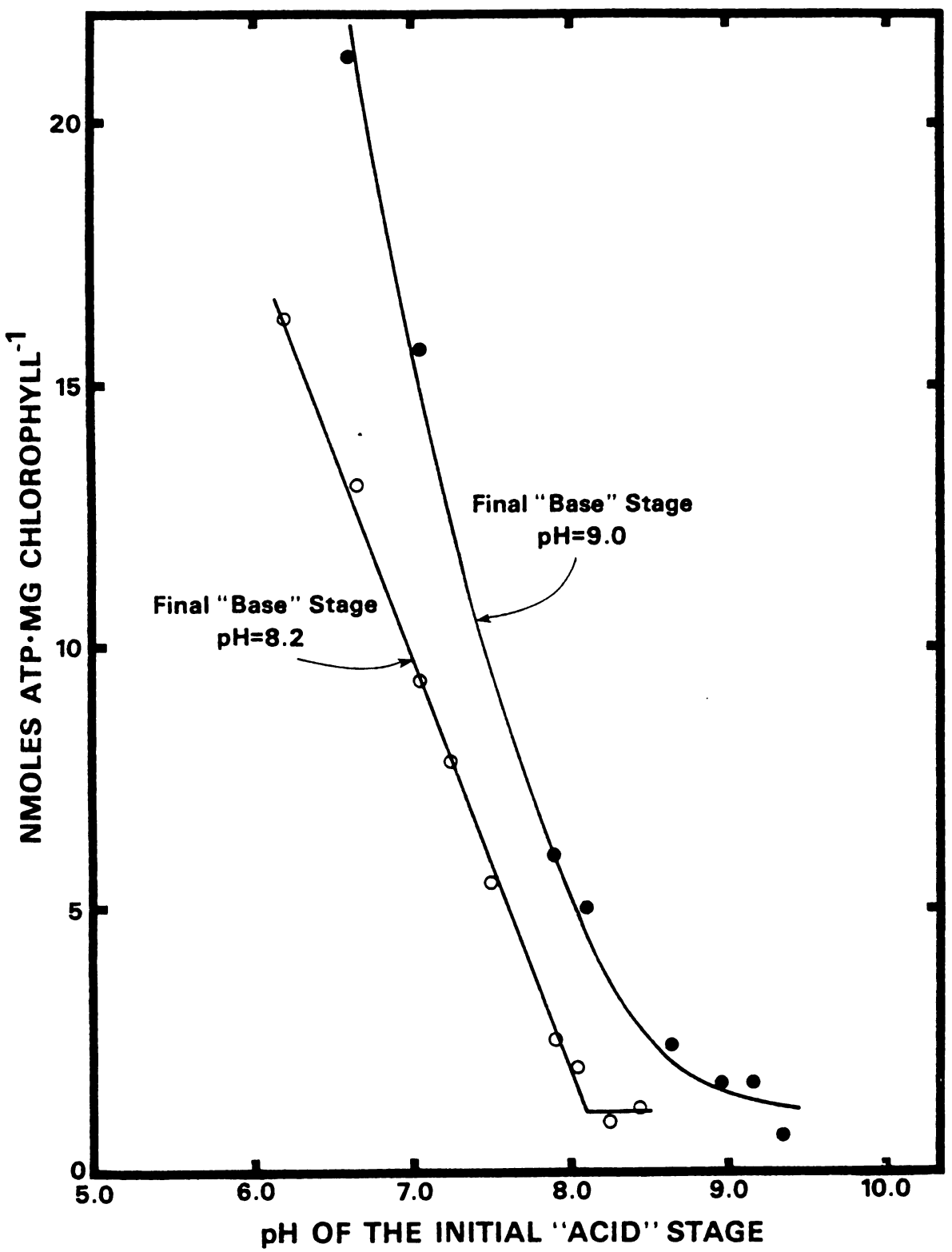
It seemed possible that the convergence of the curves in Figure 16 at pH 8.5 reflected some inherent property of the chloroplast membrane. Some factor needed for ATP synthesis might have decreased with increasing pH and have been exhausted at pH 8.5. In such a situation the magnitude of the membrane potentials generated would have been irrelevant. However, the extrapolation of ATP synthesis through zero pH change is only a property of the chloroplast membranes at pH 8.5 when the final pH is 8.5. The extrapolation of ATP synthesis through zero pH change was also seen when the base stage pH was changed to 8.2 or 9.0 as shown in Figure 17. Regardless of the pH of the basic stage a membrane potential sufficient to drive ATP synthesis was without effect if the pH inside the chloroplasts was not more acid than the pH outside. Figure 17 also shows that ATP synthesis is proportional to the pH difference, as has already been shown for ATP synthesis driven by large pH changes [23]. Thus more ATP is made if the pH is raised from 7.0 to 9.0 than if the pH is raised from 7.0 to 8.2. We have also noted that very small pH changes are used somewhat less efficiently at pH 9.0 than at pH 8.2 whereas larger pH changes are used somewhat more efficiently at pH 9.0 than at pH 8.2.

If the chemiosmotic hypothesis is correct in asserting that the synthesis of ATP is coupled to the outward movement of protons, the yield of ATP from an acid-base transition might be increased if the supply of protons was increased. Remarkable increases in the yield of ATP from a large pH change have been demonstrated when appropriate buffers have been added to the acid stage [23]. A buffer which increases ATP synthesis must be able to enter the chloroplast at the acid pH and

Figure 17.

The effect of small pH increases on the yield of ATP at two different final pH's. Conditions as in Figure 13 except as follows: all potassium salts were removed from the "acid" stage; dipotassium succinate (75mM) was used in the "base" stage; the base stage buffer was bistrispropane succinate (200mM); valinomycin (1 μM) was present in all reactions.

Figure 17.


NMOLES ATP·MG CHLOROPHYLL^{-1}
20
15
10
5
0
5.0
6.0
7.0
8.0
9.0
10.0
pH OF THE INITIAL "ACID" STAGE
Final "Base" Stage
pH=9.0
Final "Base" Stage
pH=8.2

release its protons inside the chloroplast when the external pH is raised. If the ATP synthesis resulting from small pH changes is also using an internal pool of protons one might expect that appropriate exogenous buffers might be found which would stimulate ATP synthesis. Indeed, several buffers have been found which do increase the yield of ATP from small pH changes. The buffer giving the greatest stimulation of ATP synthesis from a 2 pH unit increase is N-β-hydroxyethylmorpholine (HEM). Therefore the stimulation by HEM (pK_a 7.0) has been studied in the greatest detail.

The stimulation of ATP synthesis by HEM was difficult to detect at first because HEM, like other aliphatic amines, is an uncoupler of chloroplast phosphorylation. The synthesis of ATP by small pH changes is much more sensitive to uncoupling by HEM than is steady-state photophosphorylation. Figure 18 shows that 2.2 mM HEM will give a 50% inhibition of the ATP formation resulting from a small pH change whereas the same level of uncoupling of steady-state photophosphorylation requires 20 mM HEM at pH 8.0 (Bell, D., personal communication). However the inhibition of small pH change ATP synthesis by HEM was eliminated when the chloroplasts were incubated for 60 minutes with the amine. In fact, a small stimulation of ATP synthesis was observed after a long pre-incubation of the chloroplasts at the appropriate concentration of HEM (Figure 18, 3 mM and 10 mM HEM).

If the stimulation of ATP synthesis by HEM is a result of an additional supply of available internal hydrogen ions, stimulation by HEM should be a function of the amount of HEM inside the chloroplasts.

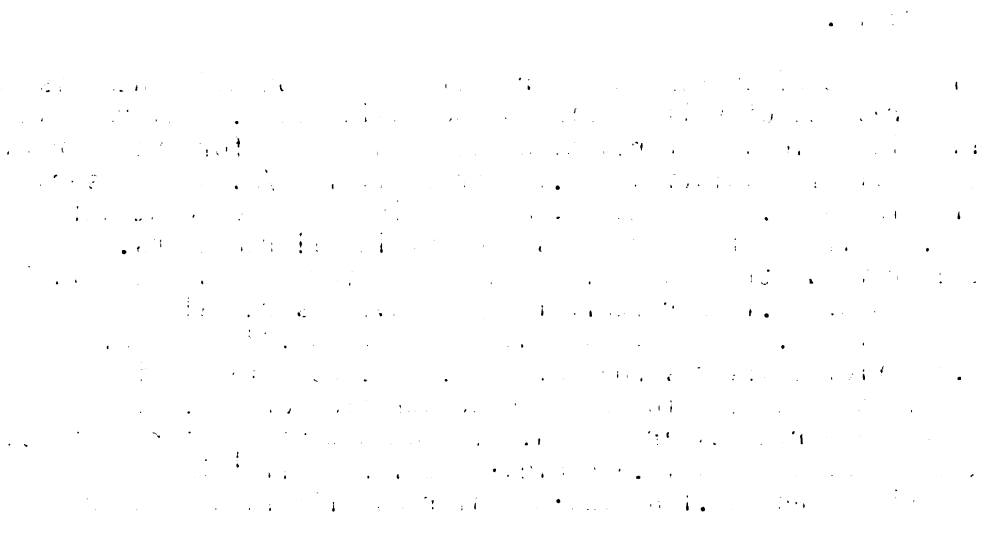


Figure 18.

The effect of hydroxyethylmorpholine (HEM) on ATP synthesis in the presence of valinomycin and potassium ions. Chloroplast lamellae (616 μg per reaction) were incubated for one hour in the following solution: 0.1M sorbitol; 1 mg/ml bovine serum albumin, pH 6.0; 2mM $MgCl_2$; and the indicated concentration of HEM. Valinomycin (1 μM) was present in all reactions. After the preincubation, one ml of the following base stage solution was added: 0.1M sorbitol; 1 mg/ml bovine serum albumin; 2mM $MgCl_2$; 2.25mM ADP; 20mM $Na_2H^{32}PO_4$ (2 X 10^5 cpm/μmole); 0.2M tricine-bistrispropane, pH 8.4; 0.225M dipotassium succinate; and the indicated concentration of HEM. The results of two experiments are shown. The amount of ATP formed in the absence of HEM was 19.5 nmoles·mg chlorophyll^{-1} in one experiment and 21.1 nmoles·mg chlorophyll^{-1} in the other experiment.

Figure 18.

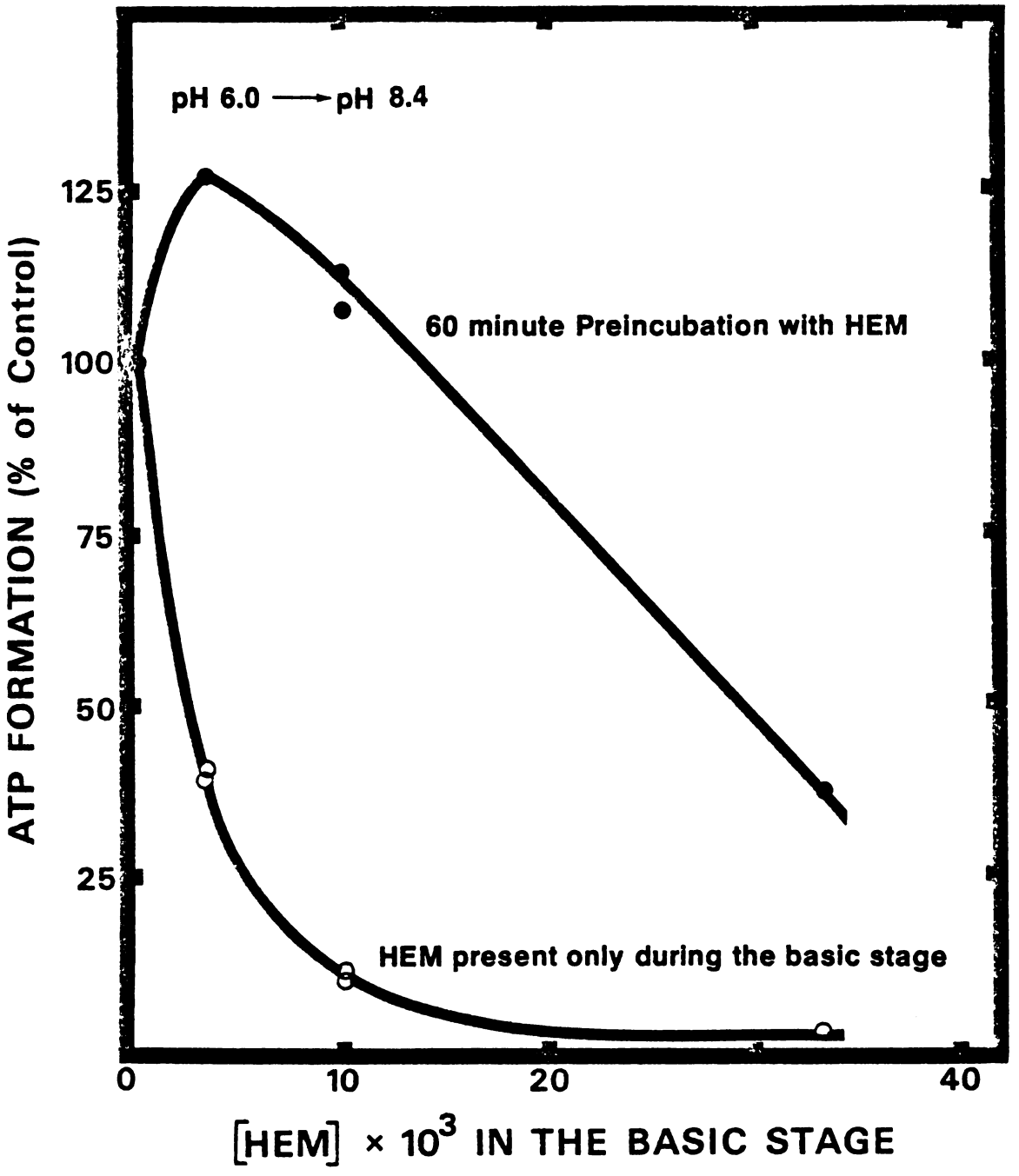

pH 6.0 ⟶ pH 8.4
60 minute Preincubation with HEM
HEM present only during the basic stage
ATP FORMATION (% of Control)
125
100
75
50
25
0
10
20
40
[HEM] × 10³ IN THE BASIC STAGE

Figure 19 shows that there is a range of HEM concentrations in which the increase in the yield of ATP is a linear function of the amount of amine added. Note that the same was true of 3-(dimethylamino)-propionitrile (pK_a 7.0). More importantly, the stimulation of ATP synthesis by a fixed concentration of HEM was strongly affected by the osmotic strength of the reaction mixture whereas, the formation of ATP in the absence of HEM was only weakly affected by osmotic strength (Figure 20). It is well known that the internal volume of chloroplast lamellar fragments is a function of the osmotic strength of the suspending solution [58,70]. Therefore as the osmotic strength decreases the internal volume of the chloroplasts increases and the amount of buffer which can be stored also increases. Buffer-stimulated ATP synthesis by large pH changes shows a similar sensitivity to increases in osmotic strength [70].

It should be pointed out that the stimulation of small pH change ATP synthesis by HEM remains entirely dependent on the presence of a membrane potential. Figure 20 also shows that the presence of HEM is without effect unless valinomycin and K^+ are also added to the reaction.

The slow change from uncoupling to stimulation of ATP formation by HEM with increased periods of pre-incubation was examined in further detail. The uncoupling and stimulating effects of HEM cancelled each other after a 3 minute incubation of the chloroplasts with HEM at pH 6.0 (See Figure 21). The half-time for the stimulation of ATP synthesis was about ten minutes (Figure 21). The increase in the yield of ATP seen with increased incubation time presumably

Figure 19.

Stimulations of ATP synthesis by two different pK_a 7.0 amines. Chloroplast lamellae containing 413 µg chlorophyll were incubated with the indicated amine for one hour at pH 6.0. All experimental conditions as described in Figure 18 except that the sorbitol concentration was lowered to 0.05M.

Figure 19.

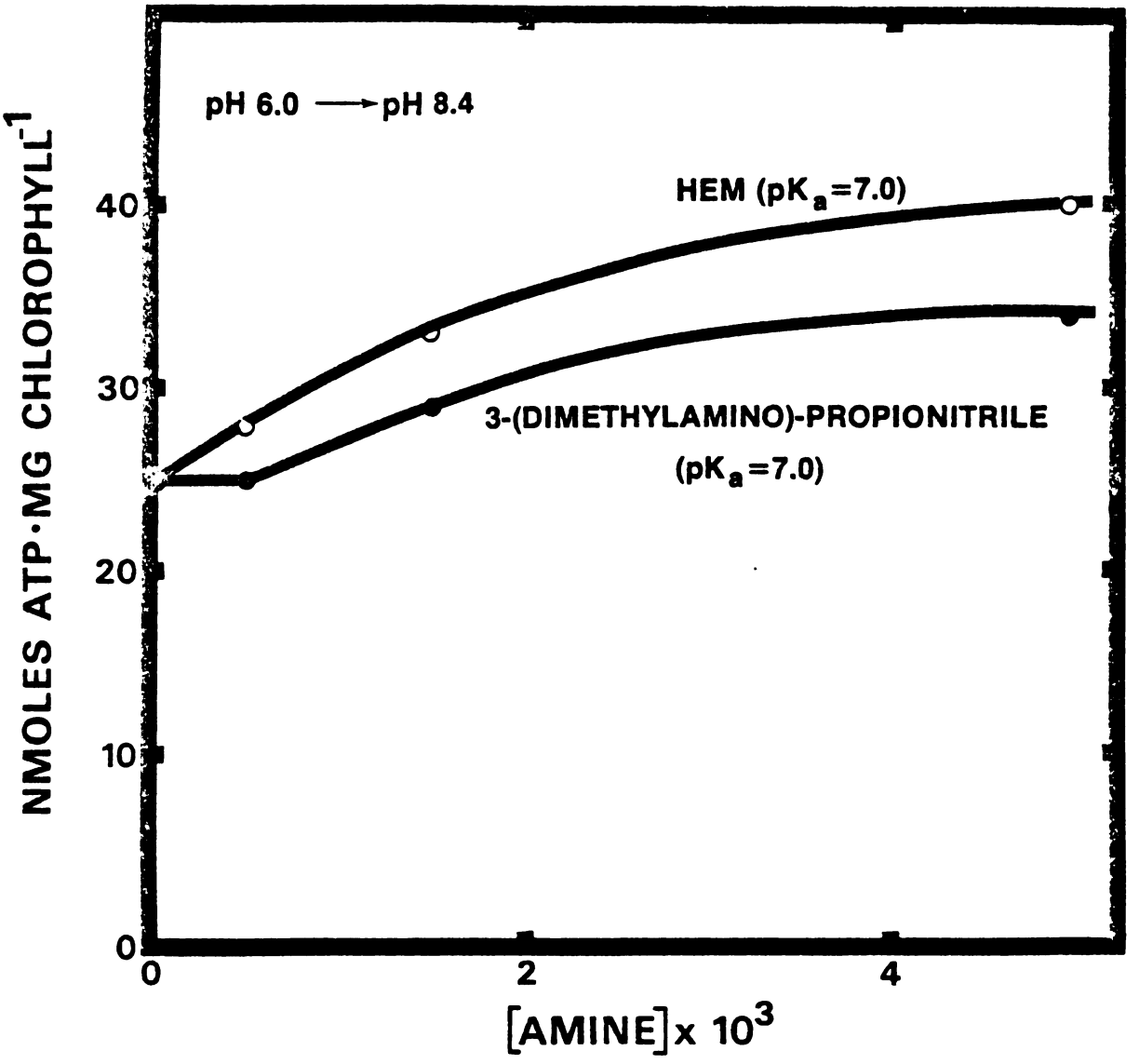

pH 6.0 ⟶ pH 8.4
HEM (pK$_a$=7.0)
3-(DIMETHYLAMINO)-PROPIONITRILE
(pK$_a$=7.0)
NMOLES ATP·MG CHLOROPHYLL^{-1}
40
30
20
10
0
0
2
4
[AMINE]x 10^3

Figure 20.

The effect of osmoticum on the yield of ATP in the presence and absence of HEM. Chloroplast lamellae containing 390 μg of chlorophyll were incubated for one hour with or without 5mM HEM. All reaction conditions as described in Figure 18 except that the sorbitol concentration in the acid and base stages was varied as indicated. Note that ATP synthesis is entirely dependent on the potassium ion gradient.

Figure 20.

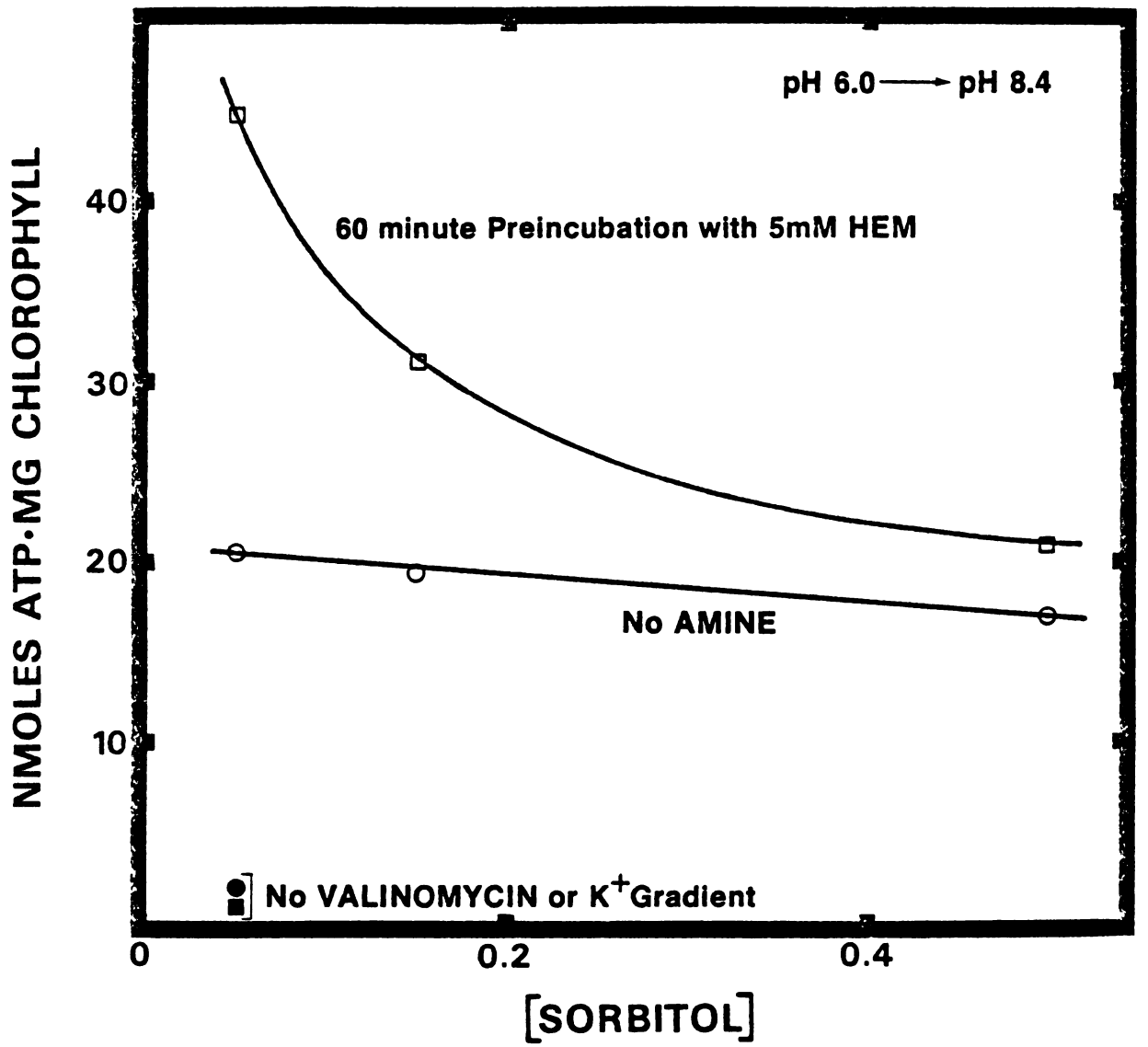
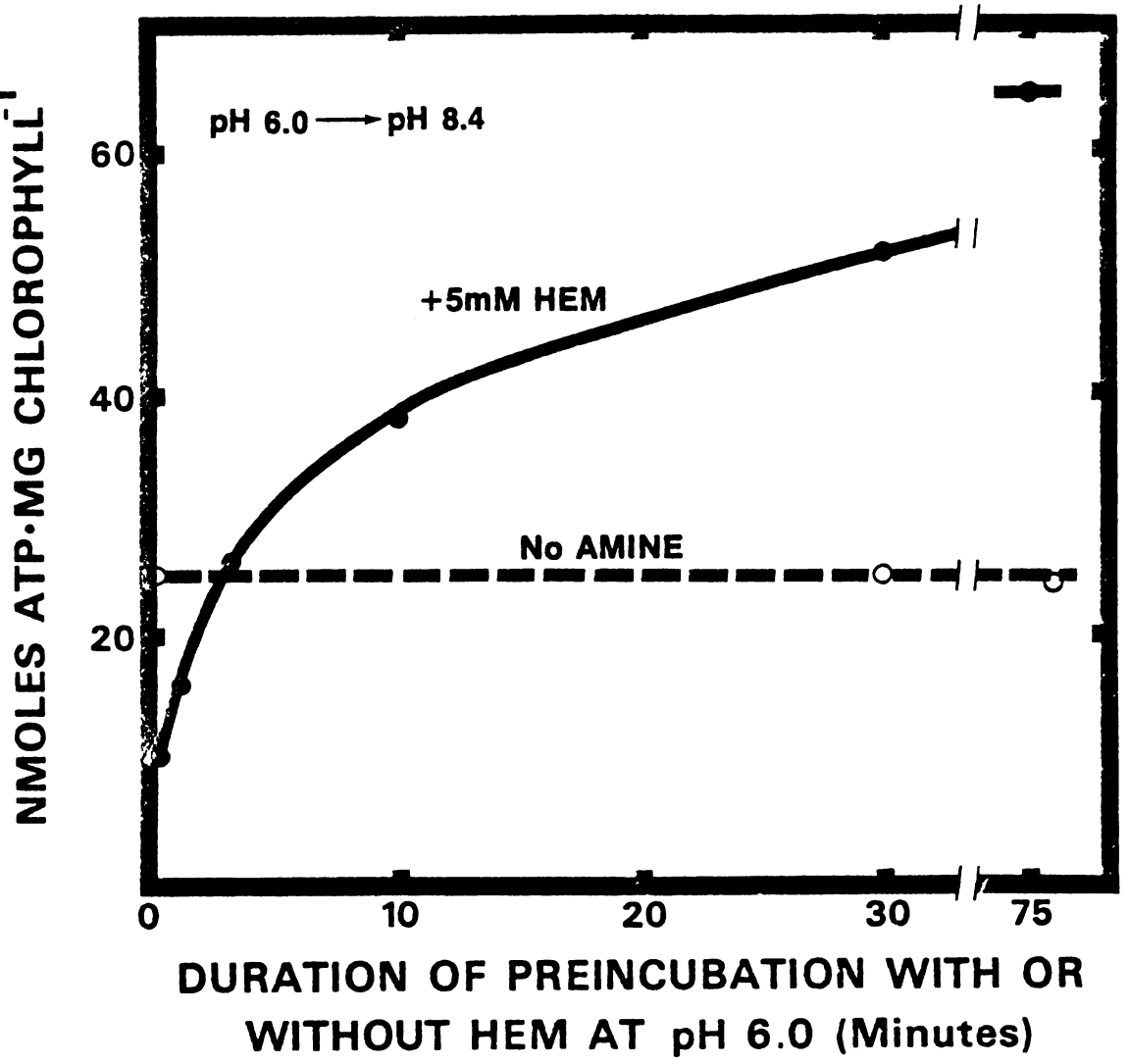

pH 6.0 ⟶ pH 8.4
60 minute Preincubation with 5mM HEM
No AMINE
No VALINOMYCIN or K+Gradient
NMOLES ATP·MG CHLOROPHYLL
40
30
20
10
0
0.2
0.4
[SORBITOL]

Figure 21.

The effect of HEM on acid-base ATP synthesis as a function of the time of preincubation with the amine. All reaction conditions as described in Figure 18 except that the concentration of sorbitol was lowered to 0.01M.

Figure 21.


pH 6.0 → pH 8.4
+5mM HEM
No AMINE
60
40
20
0
10
20
30
75
NMOLES ATP·MG CHLOROPHYLL^{-1}
DURATION OF PREINCUBATION WITH OR WITHOUT HEM AT pH 6.0 (Minutes)

reflects the entry of the protonated amine with a counter ion into the chloroplast. Similar rates of movement across chloroplast membranes have been observed with some inorganic ions [71].

The ability to stimulate the ATP synthesis associated with small pH changes is not common to all buffers which have pK_a near 7.0. Imidazole (pK_a 7.0), phosphate (pK_a 6.8), and MOPS (pK_a 7.1) gave no stimulation of ATP synthesis even though the pH increase passed through their buffering range. See Table 9. This experiment was done under conditions which should have maximized the effect of an added buffer (long incubation, low osmotic strength). Under identical conditions HEM increased ATP synthesis by 150%! The slight inhibition of ATP formation which was found with sodium phosphate can probably be attributed to an influx of sodium which lowered the subsequent diffusion potential in the basic stage. The same degree of inhibition was seen following a pre-incubation with an equivalent concentration of sodium chloride (Table 9).

We believe that all of the buffers tested in Table 8 should have entered the internal aqueous space of the chloroplasts although we have no rigorous proof that they did so in our experiments. Ort has measured the entry of phosphate into the chloroplast at pH 8.0 and found that the half-time for entry was less than 30 seconds at 4 °C [58]. Table 10 shows the results of an experiment similar to the one in Table 9 except that the pH of the pre-incubation with the buffer was raised from 6.0 to 8.0. Phosphate, imidazole, and MOPS were still found to be unable to stimulate ATP synthesis. With a similar high pH pre-incubation the stimulation by HEM, although reduced, was still evident.

Table 9.

The effect of various buffers on the yield of ATP. I. Chloroplast lamellae contained 516 µg of chlorophyll were incubated with the indicated addenda (5mM) for 90 minutes at pH 6.0. The concentration of sorbitol was 0.01M. Reaction conditions otherwise as in Figure 18.

Addendum	ATP Formed as a Result of a Change in pH from 6.0 to 8.4 (nmoles/mg chlorophyll)
None	25.73
HEM·HCl	64.59
MES-NaOH	33.41
MOPS-NaOH	25.25
NaH_2PO_4	20.89
NaCl	19.74
Imidazole·HCl	24.08

Table 10.

The effect of various buffers on the yield of ATP. II. Reaction conditions: the same as described in Table 9 except that the chloroplast lamellae (460 μg per reaction) were incubated at pH 8.0 for sixty minutes in the "acid" stage solution. The pH was then lowered to 6.1 with small aliquots of HCl and held there for two minutes before the addition of the "base" stage solution.

Addendum	ATP Formed as a Result of a Change in pH from 6.1 to 8.4 (nmoles/mg chlorophyll)
None	11.24
HEM·HCl	17.70
MES-NaOH	6.58
MOPS-NaOH	6.48
Na_2HPO_4	7.39
NaCl	9.20
Imidazole·HCl	8.00

The ability of an internal buffer to increase the yield of ATP resulting from a small pH change seems to depend on some undetermined property of the buffer.

The high light intensity, steady-state driving force for ATP synthesis in chloroplasts is believed to consist almost entirely of a ΔpH. Membrane potentials measured under these steady-state conditions are generally very small [20,72,73]. There are, however, two conditions reported in chloroplasts where the membrane potential seems to assume a much larger proportion of the driving force for ATP synthesis. These conditions are: 1. steady-state photophosphorylation at low light intensities [74]; 2. short illuminations ($<$50 msec) at high light intensity [58,75]. In both of these situations, ATP synthesis is sensitive to valinomycin + K^+ whereas steady-state photophosphorylation is hardly affected at all. The similarity of the two conditions is also shown by an enhanced sensitivity to certain uncouplers, especially those weak acids which are thought to act by allowing hydrogen ions to leak across the membrane (e.g. CCCP or FCCP). Moreover, the uncoupler octylamine is an exceptionally potent inhibitor of ATP synthesis with either brief illumination periods [76] or small pH changes. Table 11 documents the fact that uncouplers such as SF-6847, 2,4-dinitrophenol, FCCP and octylamine are more effective in inhibiting the ATP synthesis driven by small pH changes than they are at inhibiting steady-state photophosphorylation. In contrast, methylamine shows identical effectiveness in the two phosphorylation systems. This suggests that the same energized conditions of the membrane may exist under all three

Table 11.

Relative sensitivities of steady-state photophosphorylation and small-pH-change phosphorylation to uncouplers. Conditions for induction of ATP synthesis by small pH changes as in Figure 13. The indicated uncouplers were added at the beginning of the 1.0 minute acid incubation period.

	Concentration of the uncoupler giving 50% inhibition of ATP synthesis		Ratio of sensitivities
Uncoupler	Phosphorylation with small pH changes (6.5 → 8.5)	Steady-state photophosphorylation at pH 8.5*	Δ pH/steady-state
methylamine	200μM	200μM	1
neutral red	20μM	30μM	1.5
atebrin	2μM	15μM	7.5
FCCP	0.2μM	1.5μM	7.5
2,4-dinitrophenol	60μM	500μM	8.3
imidazole	0.75mM	7.0mM	9.3
2,4-lutidine	0.4mM	4.0mM	10
HEM	2.5mM	2.5mM	10
SF-6847	0.02μM	0.3μM	15
octylamine	2μM	70μM	35

*Steady-state photophosphorylation was assayed by measuring $^{32}P_i$ incorporated into ATP resulting from a 2.0 minute illumination at a saturating light intensity in 2.0 ml of the following solution: 200mM sorbitol; 50mM TAPS, pH 8.5; 2mM $MgCl_2$; 100mM $Na_2H^{32}PO_4$; 0.75mM ADP; 10mM KCl; 0.5mM $K_3Fe(CN)_6$ and chloroplasts containing 30 μg chlorophyll. Temperature 19 °C. Assays were performed by D. Bell.

conditions, small pH change, brief illumination, or low light intensity, and that the same little-understood mechanism of ATP synthesis operates in all three cases.

In addition, ATP synthesis driven by small pH changes is very sensitive to uncoupling by weak amines if the amines have not been pre-incubated with the chloroplasts. See Table 11. The three amines with pK_a's near 7.0 (imidazole, 2,4-lutidine, N-β-hydroxyethylmorpholine) all show about a 10-fold greater effectiveness in inhibiting the ATP formation accompanying small pH changes than in inhibiting steady-state phosphorylation. Since the phosphorylation occurs with essentially all of the amine unprotonated (pH 8.5) and therefore probably able to penetrate the chloroplast membrane, one can view the amine as a permeant base capable of neutralizing the internal aqueous space of the chloroplast. The movement of the unprotonated, uncharged amine across the chloroplast membrane should have no effect on any membrane potential which may exist. It would therefore be surprising to find that compounds which collapse only the pH gradient are such potent uncouplers of membrane potential-dependent ATP synthesis were it not for the already demonstrated absolute dependence of the ATP synthesis on the pH gradient.

DISCUSSION

Section I

One of the most important areas which should be examined to test the validity of the chemiosmotic hypothesis is the stoichiometry between proton translocation and ATP formation. Equally important is the magnitude of the transmembrane proton electrochemical activity gradient available during phosphorylation. These factors combine to determine the maximum free energy available to meet the free energy requirement for the phosphorylation of ADP.

Many workers have attempted to measure the H^+/ATP ratio in chloroplasts and the reported values vary from 2 to 4. Using a glass pH electrode, Carmeli measured the initial rates of proton uptake during the hydrolysis of radioactive ATP and found that the H^+/ATP ratio approached 2 [77]. Schwartz also found an H^+/ATP ratio of 2 when he compared the steady-state rate of phosphorylation to the initial rate of proton efflux when the light was turned off [78]. However, when the experiment was redone in the presence of valinomycin and K^+ by Schroder an H^+/ATP ratio of 3 was obtained [73]. Valinomycin and K^+ were added to eliminate any membrane potential which might accelerate or decrease the rate of proton efflux. Witt has shown that ATP synthesis increases the decay of an absorbance change at 515 nm. His calculations based on ion movements led to H^+/ATP ratios of 3 and

4 [79,80]. Portis and McCarty claim that the logarithm of the phosphorylation rate was linearly related to the pH gradient with the slope (about 3) being equal to the H^+/ATP ratio [21]. Finally, Izawa measured the relationship between light-driven proton accumulation and post-illumination ATP synthesis [32]. He found an H^+/ATP ratio of 5 but after making a correction for the dissipative leak of protons he arrived at an H^+/ATP ratio of about 2.5.

As already pointed out, the original goal of the research reported in this thesis was to attempt to measure the H^+/ATP ratio by measuring the additional ATP formed in post-illumination ATP formation reactions due to the additional accumulation of H^+ due to buffering. However, the approach proved inadequate to the task. While we were able to measure the accumulation of the buffering amine and the increase of ATP formation, we were not able to determine with sufficient accuracy the efficiency with which protons stored in the accumulated amines were used to make ATP; we did not know how many of the stored protons could be released before the internal pH rose to a level which precluded phosphorylation.

We did observe the exponential decay of the capacity to make ATP after light extinction, which has been reported previously [32,34]. This phenomenon was observed both in the presence and absence of amines which increase the yield of post-illumination ATP synthesis (Figure 12). The decay of the ability to make ATP presumably reflects the raising of the internal pH until the transmembrane proton electrochemical activity gradient is no longer capable of driving the phosphorylation reaction. Thus as the pH rises fewer protons are stored in the

protonated amine and at some pH phosphorylation ceases. An exponential decay would be expected if the rate of decay depended on the concentration gradient of some limiting extruded ion. If the decay of the ability to make ATP after light extinction is limited by the movement of hydrogen ions then:

1. rate of efflux of $H^+ = k ([H^+]_{in} - [H^+]_{out})$.

If the process is to be exponential we must also assume that the change in concentration of H^+ is proportional to the amount of H^+ extruded:

2. rate of efflux of $H^+ = k' \, d[H^+]_{in}/dt$

Combining equation 1 and equation 2 gives:

3. $k'd[H^+]_{in}/dt = k([H^+]_{in} - [H^+]_{out})$

When $[H^+]_{out}$ is much smaller than $[H^+]_{in}$ equation 3 becomes:

4. $d[H^+]_{in}/dt = k'' \, [H^+]_{in}$

which is the description of an exponential process. Clearly the conditions are met if there is no internal buffering and the rate of H^+ efflux is dependent only on the concentration of H^+. The conditions should also be met when the internal buffering is provided by an amine which can permeate the membranes freely in the unprotonated for [N] but cannot permeate the membrane in the protonated form [NH^+]. In this case, the concentration of unprotonated amine inside is at all times in equilibrium with the concentration of unprotonated amine outside and therefore essentially constant. The tendency of an amine to

lose a proton is given by the dissociation constant:

5. $K=[H^+][N]/[NH^+]$

If [N] is held constant, equation 5 yields:

6. $[H^+] = K'[NH^+]$

Since any efflux of H^+ represents a depletion of NH^+ (in effect NH^+ is being extruded in the form of H^+ and N), the efflux of any particular amount of H^+ causes a proportional change in $[H^+]$ in according to equation 6. That is to say, the conditions of equation 4 are met and exponential decay in the store of protons is to be expected. It should be noted, however, that the presumed threshold concentration of stored H^+ required for phosphorylation should make for a marked difference between the decay of the stored protons and the decay in the ability to make ATP. Only the store of protons should decline exponentially, not the ability to make ATP.

Moreover, it is not at all clear why the decay in the store of protons should be exponential when this store of internal protons resides in endogenous buffers, since such buffers cannot have diffusible unprotonated forms and their release of protons is <u>not</u> linearly related to the change in $[H^+]_{in}$. In other words, the change in the internal concentration of H^+ is not proportional to the amount of H^+ extruded. The buffering curves described by the Henderson-Hasselbalch equation upset this proportionality. Rather the amount of H^+ extruded is almost linearly related to the change in pH (or log of H^+) over much of the buffering range [81]. Nevertheless, there is

ample evidence that the decay of the store of accumulated protons in chloroplast lamellar vesicles is exponential or nearly so even in the absence of exogenous buffering substances. It is important to note that the concentration of hydrogen ions in an aqueous system, and therefore the pH, is determined by the difference in the concentrations of cations other than H^+ from the concentration of anions other than OH^-. Perhaps the decay in the capacity of chloroplast lamellae to make ATP after light extinction is limited by the movement of some ion other than the proton and it is the abundance of this other ion (e.g. chloride?) which is declining exponentially.

There is another aspect of the decay of the ability to make ATP which should be discussed at this time. Post-illumination ATP synthesis must stop at some point because the driving force for ATP formation drops to a level which is smaller than the free energy requirement for the phosphorylation of ADP. Since increasing the ATP/ADP ratio would be expected to increase the free energy requirement for ATP synthesis, it would also be expected to increase the pH gradient required to drive the reaction assuming, of course, no wasteful irreversible steps. An increase in the required pH gradient should reduce the yield of post-illumination ATP, since this would allow fewer protons to exit from the thylakoid before the pH gradient dropped to an inadequate level. The fact that changing the ATP/ADP ratio over three orders of magnitude had no effect on the yield of ATP suggests that, as we have already pointed out, ATP synthesis stops before the driving force drops to the thermodynamic threshold. See Table 1. While it is clear that the coupling factor is reversible in the sense that ATP formation is

coupled to proton movement in one direction and ATP hydrolysis is coupled to proton movement in the opposite direction, it appears that the synthesis of ATP does indeed involve energetically wasteful reactions. Alternatively, it may be that there are unsuspected energy sources such as other ion gradients or membrane potentials and that the requirement for a threshold pH gradient is illusory (see below).

Section II

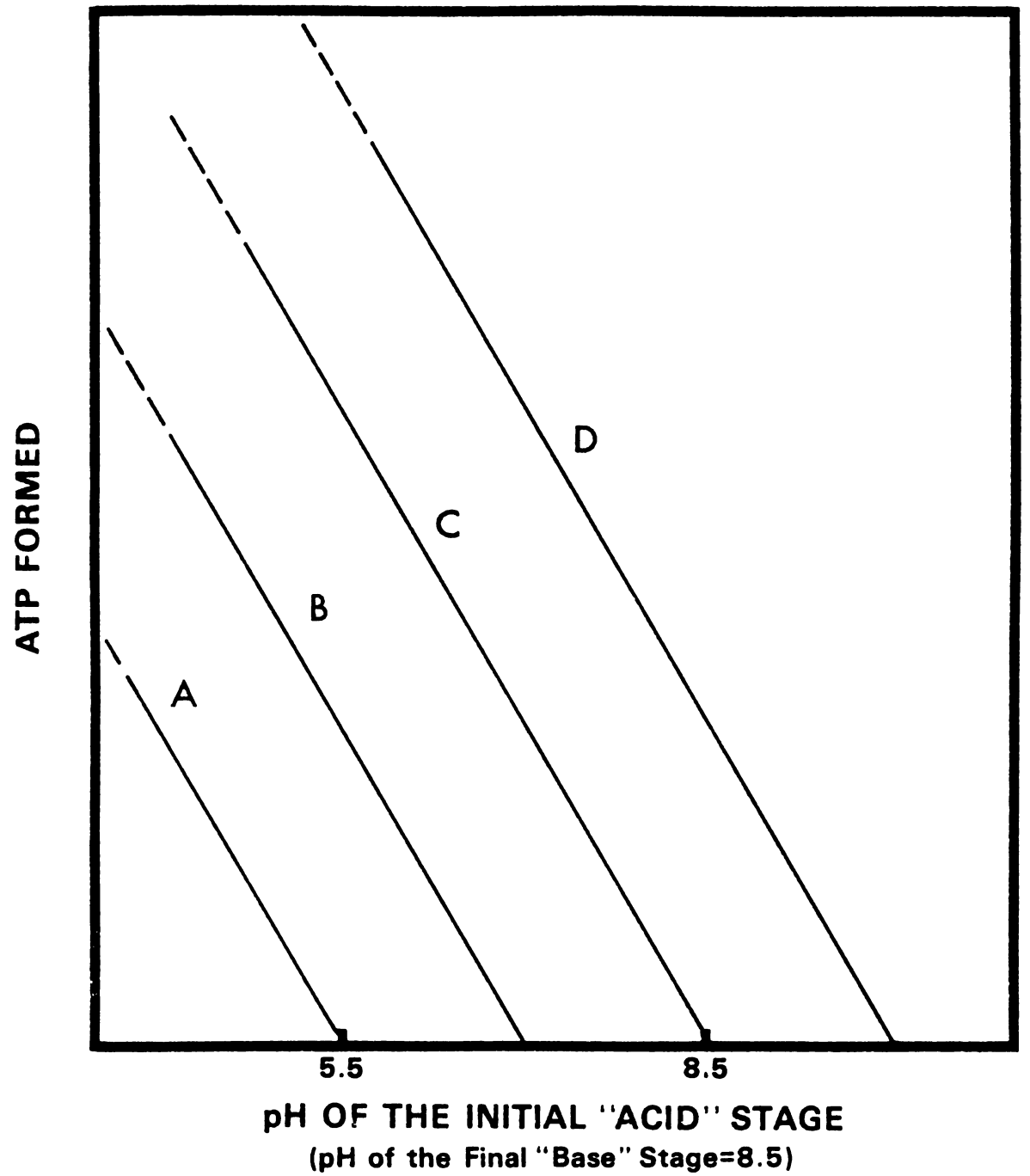
Any proposed mechanism for ATP synthesis must necessarily describe the source of energy which is capable of driving the reaction. Mitchell's chemiosmotic hypothesis [6] proposes that ATP formation is linked to the movement of a small number of protons and that the energy needed to drive this phosphorylation resides in a transmembrane electrochemical activity gradient of protons, or "protonmotive force". The protonmotive force, or leaving tendency of the hydrogen ions, consists of the transmembrane hydrogen ion concentration gradient and the transmembrane charge imbalance. The two components of the protonmotive force are thermodynamically indistinguishable and, one would think, mechanistically equivalent.

One of the strongest arguments in favor of the chemiosmotic model of phosphorylation is the fact that an imposed hydrogen ion gradient across chloroplast lamellar vesicles can make ATP [23]. If the pH difference is large enough ATP can be made without any membrane potential, but with smaller pH differences no ATP is made unless there is also a membrane potential, positive inside [27,28,29]. Figure 22 shows the interrelationship of pH difference, membrane potential and ATP synthesis which one would expect if a composite protonmotive force were driving hydrogen ions out through the coupling factor to make ATP in a pH jump experiment. This figure is based on the assumption that the pH of the inside of the lamellar vesicle rises in a more or less linear fashion as hydrogen ions are extruded, which is probably true at least over a fairly narrow range of pH values. It is also assumed

Figure 22.

Predicted relationship of pH increases to ATP formation at different membrane potentials (positive inside). Line A, no membrane potential. Line B, a membrane potential applied which, by itself, provides less than the threshold protonmotive force. Line C, membrane potential equals the threshold. Line D, membrane potential exceeds the threshold. It is assumed that the amount of ATP formed is proportional to the H^+ extruded, that the extrusion of H^+ causes a linear increase in internal pH, and that the membrane potential does not change much during the very few seconds that ATP is being made.

Figure 22.


ATP FORMED
A
B
C
D
5.5
8.5
pH OF THE INITIAL "ACID" STAGE
(pH of the Final "Base" Stage=8.5)

that a pH difference of 3.0 units is capable of providing sufficient protonmotive force for phosphorylation in the absence of any membrane potential. A final assumption is that the membrane potential is stable and long-lived when compared to the pH gradient. Line A shows the expected relation of ATP formation to the internal pH when there is no membrane potential. Phosphorylation should proceed until the internal pH has risen to 5.5 and the amount of ATP formed should reflect the number of hydrogen ions available before their extrusion raises the pH to this threshold level. Line B shows the ATP synthesis expected if a sub-threshold membrane potential is applied, that is to say a membrane potential which is not itself sufficient to provide a protonmotive force capable of driving phosphorylation. Line C shows the expected relation of ATP synthesis to the initial pH if the membrane potential alone provides a threshold protonmotive force. In this case, no ATP will be formed unless there is a pH gradient too, since the extrusion of any hydrogen ions would otherwise raise the inside pH above the outside pH and the resulting inverse pH gradient would decrease the already marginal protonmotive force. However, if there is a pH gradient in the right direction, hydrogen ions can be extruded through the coupling factor until the inside pH rises to the level of the outside pH. Line D shows the phosphorylation to be expected if a membrane potential is imposed which by itself provides a protonmotive force in excess of that thermodynamically necessary for ATP synthesis. Phosphorylation should proceed freely until the extrusion of hydrogen ions provides an inverse pH gradient which brings the composite protonmotive force below the threshold value.

The discrepancies between our observations and the predictions shown in Figure 22 are very great. Our observations do not seem to bear out the contention that the membrane potential and the pH difference are, in fact, equivalent driving forces. There is an absolute requirement for a pH difference, the inside being more acid than the outside, if significant amounts of ATP are to be formed. This is true no matter how large or small the membrane potential. On the other hand, the membrane potential allows small pH increases to be used to make ATP. Thus ATP synthesis is both a function of a membrane potential and a function of the pH difference, but it is not a function of the sum of the membrane potential and the pH difference as one would expect if the onset of ATP synthesis depended on some threshold protonmotive force.

Since the membrane potential and the pH difference clearly ARE equivalent thermodynamically, it must be that they are not equivalent mechanistically; the pH difference must be doing something which is essential for phosphorylation, something which a membrane potential cannot do by itself. Furthermore the thing provided by the pH difference is quantitatively related to the magnitude of the pH change since ATP synthesis at any membrane potential is a nearly linear function of the pH change. It is not easy to visualize the mechanism for this absolute and quantitative dependence on small pH changes. If the main driving force causing hydrogen ions to leave is the membrane potential (as must be the case with small pH changes), the dependence of phosphorylation on a pH rise cannot involve the supply of internal hydrogen ions. The supply depends on the internal buffering at the

internal pH and does not depend on the small pH change. Nevertheless, in the presence of a membrane potential, goodly amounts of ATP are formed if the pH is raised from 8.2 to 9.0 whereas almost no ATP is formed with the same potential if the pH remains at 8.2. Yet the hydrogen ions available to be pushed out must be almost the same in each case. We can only reiterate the lesson of Figure 22. According to the chemiosmotic picture of phosphorylation, a sufficient membrane potential should make ATP and the small pH change from 8.2 to 9.0 should be almost irrelevant.

The relationship between the change in pH and the availability of hydrogen ions for ATP synthesis remains obscure but it is nevertheless a very real relationship. While it is clear that certain permeant amines can increase the yield of ATP resulting from a small pH change in the presence of a membrane potential, it is also clear that other buffers with similar pK's cannot function in this manner. See Table 9. The stimulation of ATP synthesis by permeant amines suggests that the reservoir of hydrogen ions is a determining factor in the yield. The fact that the amount of ATP formed is proportional to the magnitude of the pH change can be taken as evidence for the same thing. On the other hand, the inefficiency of buffers like MOPS and phosphate in stimulating ATP synthesis when they are almost certainly inside the chloroplast lamellae is difficult to understand. It is conceivable that the greater lipid solubility of HEM allows it to equilibrate into regions of the membrane which are not accessible to MOPS or phosphate. This might allow HEM to donate protons to the phosphorylation machinery with much greater efficiency than is possible in the case of hydrophilic buffers. However, this does not explain the inability of

imidazole to stimulate ATP synthesis since its solubility properties and pK_a are very similar to those of HEM.

It is well to remember, however, that we have no certain knowledge of what we are measuring when we measure the ATP made. Are we measuring rates of ATP synthesis or duration of the phosphorylation reaction or some combination of these? Or is it possible that the membrane potential is limiting in some stage of the reaction while the Δ pH is limiting at another stage? Again the consistent quantitative relationship between Δ pH and ATP formed argues that the Δ pH is usually limiting. Moreover, there is reason to believe that the membrane potential is much longer lived than the Δ pH. Certainly a very long preincubation with inorganic monovalent cation is required to eliminate the diffusion potential-dependent phosphorylation and during all of this long preincubation the diffusion potential probably remains.

The mechanism of ATP synthesis with small pH increases is different from the mechanism of ATP synthesis with large pH increases. With large pH increases the hydrogen ion concentration gradient across the membrane is thermodynamically sufficient to drive phosphorylation and no requirement for a membrane potential is observed. The converse is not the case; even though the membrane potential must be providing almost all of the energy for the phosphorylation when the pH change is small, none of the membrane potentials we have been able to impose produces much ATP without a concomitant pH increase. It is true that in experiments where the chloroplasts were subjected to no pH increase, we sometimes observed the formation of tiny amounts of ATP comparable to those reported

by Uribe as resulting from the imposition of a membrane potential alone [29]. In our hands, this small amount of ATP varied from experiment to experiment in the range of 0.5 to 2 nmoles·mg chl^{-1} and did not seem to be determined by the membrane potential imposed. The same very low level of ATP synthesis was seen with a pH decrease as well as with no pH increase. As we have already pointed out, the phosphorylation which occurs with small pH changes resembles the phosphorylation which occurs at very low light intensities [74] or with very brief periods of illumination [75,76]. All three phenomena seem to be dependent on a membrane potential and all three are exceptionally sensitive to hydrogen ion-carrying uncouplers of the CCCP and dinitrophenol type. Probably the phosphorylation which occurs as a result of short periods of illumination takes place with very small pH difference across the membrane, a conclusion which is not surprising in view of the fact that internal buffers do not need to be acidified before phosphorylation can start [58].

In addition, the phosphorylation which occurs with small pH changes is much more sensitive to uncoupling by weak amines than steady-state photophosphorylation is. The lipid solubility of the unprotonated amine and the lipid insolubility of the protonated amine would suggest that a weak amine could only neutralize a pH gradient and not affect any charge distribution. The fact that weak amines are indeed good uncouplers of membrane potential-dependent ATP synthesis resulting from small pH increases would be perplexing were it not for the already demonstrated requirement of a pH difference for ATP synthesis.

The author wishes to stress that the problems posed by his observations have to do with mechanisms, not with energetics. The requisite energy for ATP synthesis can be provided entirely by a sufficient pH difference across the membrane, or entirely by a sufficient membrane potential as when the pH differences are very small. But, regardless of the source of the energy, a pH difference is mechanistically required and the amount of ATP formed depends on the magnitude of the pH difference. It is not easy to picture any property of the pH difference other than its contribution to the hydrogen ion leaving tendency. We must somehow imagine how this pH gradient-generated driving force can have consequences different from the consequences of a driving force contributed by a membrane potential. Perhaps in this unexplained observation lie clues as to the nature of the phosphorylation reaction. Apparently the hydrogen ion concentration difference exerts an effect without the trans-membrane charge difference contributing to the hydrogen ion activity in some unknown reaction. That is to say, the essential hydrogen ion reactions must be occurring without hydrogen ions being pushed by the electric field. The hydrogen ion reactions having been completed, the membrane potential must then act on the product to provide most of the energy for ATP synthesis.

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