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ENERGY THRESHOLDS FOR ATP SYNTHESIS IN CHLOROPLASTS *

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We have investigated the ATP synthesis associated with acid-base transitions in chloroplast lamellae under conditions which allow simultaneous control of the thermodynamic variables, ΔpH, membrane potential and ΔG_{ATP}.These variables have been directly imposed rather than simply inferred. Since the initiation of labeled P_i incorporation seems to measure accurately the initiation of net ATP synthesis, the following conclusions can be drawn: (1) The proton-motive force which is just sufficient for ATP synthesis provides almost exactly the required energy for ΔG_{ATP} if the efflux of three H^+ is required for each ATP molecule formed. (2) The membrane potential and the ΔpH contribute to the proton-motive force in a precisely additive way. Thus, the threshold can be reached or exceeded by a ΔpH in the absence of a membrane potential, by a membrane potential in the absence of a ΔpH, or by any combination of membrane potential and ΔpH. With a large enough membrane potential, ATP synthesis occurs even against a small inverse ΔpH. In each instance the combined ΔpH and membrane potential necessary for initiation of ATP synthesis represent the same threshold proton-motive force.

Introduction

According to the chemiosmotic theory of ATP synthesis [1], a functional proton-motive force may be composed of either a pH gradient, an equivalent membrane potential, or any combination of the two. Under steady-state conditions, isolated chloroplast lamellar preparations probably have most of their proton-motive force in the form of a pH gradient: ion-distribution studies show little membrane potential [2] and electron transport is uncoupled by compounds that collapse the ΔpH but not by ionophores that collapse only a membrane potential [3]. However, a membrane potential can contribute to phosphorylation by chloroplasts under certain conditions. When short flashes of light are used to drive ATP synthesis, valinomycin + K^+ will inhibit phosphorylation [4]. Changes in 515 nm absorbance induced by short light flashes also indicate that a membrane potential contributes to driving phosphorylation [5]. In addition, Uribe and Li [6] and Schuldiner et al. [7] have shown that a limiting pH gradient can be supplemented by a K^+ diffusion potential to increase phosphorylation in acid-base experiments. Using chloroplasts subjected to excitation by short flashes, Gräber and Witt [8] were able to establish different values of ΔpH and $\Delta\psi$. They concluded that the two parameters were energetically equivalent. Similar conclusions were drawn from work with bacterial chromatophores in which the relative contributions of ΔpH and $\Delta\psi$ were changed by addition of various permeant and nonpermeant ions [9]. ATP synthesis in chloroplasts can also be

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* Michigan Agricultural Experiment Station Article No.10162. Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

driven by an external electric field which is thought to induce a transmembrane electric potential [10].

An additional requirement of the chemiosmotic hypothesis is that the overall proton-motive force must provide sufficient energy to permit ATP synthesis. The proton-motive force times the number of protons used to make one ATP molecule must be equal to or greater than the energy required for the formation of the phosphoryl bond of ATP (ΔG_{ATP}). As pointed out by Jagendorf [3], measurements of the ΔG_{ATP}, ΔpH and $\Delta\psi$ all in the same reaction mixture are needed for a full evaluation of the energetic aspects of the chemiosmotic model. Such measurements have been attempted under steady-state conditions with *Rhodospirillum rubrum* chromatophores using fluorescent indicators and the distribution of permeant ions for determination of ΔpH and $\Delta\psi$, and using enzymatic determination of ATP and ADP for calculation of ΔG_{ATP} [9,11]. Under a variety of conditions the indicated proton-motive force seemed thermodynamically capable of providing a sufficient driving force to account for ATP synthesis at the measured phosphate potential if at least three H^+ are assumed to transverse the membrane for each ATP molecule synthesized. A value of three was also arrived at using similar methods with chloroplasts [12].

This paper describes the results of experiments with isolated chloroplasts carried out under Jagendorf's prescribed conditions: simultaneous control of the ΔpH, $\Delta\psi$ and ΔG_{ATP}. The results should provide an unequivocal quantitative evaluation of the thermodynamic aspects of the chemiosmotic mechanism of ATP synthesis in chloroplasts. As expected, the ΔpH and $\Delta\psi$ were found to be energetically equivalent in their contribution to the proton-motive force. It appears that, under these well defined conditions, three H^+ are indeed required for each ATP molecule formed.

Materials and Methods

Chloroplasts were isolated from commercial spinach (*Spinacia oleracea* L.) as described by Graan and Ort [13] except that the resuspension medium contained 5 mM Mes-bistrispropane (pH 6.7) in place of Hepes-NaOH. Phosphorylation reactions were carried out in the dark at 3°C employing the following two-stage protocol:

Stage I; 3 min incubation of chloroplast lamellar vesicles in 2 ml reaction medium at the appropriate pH with the appropriate K^+ concentration in the presence of valinomycin.

Stage II; Rapid addition of 1 ml of phosphorylation medium to bring the external pH and K^+ concentration to the appropriate levels. Nucleotides and $^{32}P_i$ were supplied in this medium. After 10 s the reactions were terminated by the additions of 1 ml 2 M perchloric acid or trichloroacetic acid containing 10 mM EDTA.

The pH of all solutions was measured at 3°C with a glass electrode. Further details of reaction conditions are given in the legends of figures and tables.

ATP synthesis was determined by measuring the incorporation of $^{32}P_i$ after absorption of the nucleotides on charcoal, deadsorption, and separation of the ATP on a Dowex AG 1x4 anion-exchange column as described by Graan and Ort [13]. In some experiments ATP synthesis was assayed by the firefly luciferin-luciferase method as described by Graan and Ort [13] except that the denatured protein was removed by centrifugation prior to the ether extraction.

The values for ΔG_{ATP} were calculated employing a value of 8.7 kcal/mol for the standard free energy of hydrolysis of ATP at pH 8.5 and 25°C [14]. The adenylate kinase inhibitor, P^1,P^5-diadenosine-5′-pentaphosphate [15], was present at 10 μM in all reactions with controlled ΔG_{ATP}. For the reactions carried out with defined ΔG_{ATP} it was necessary to purify the commercial ADP and ATP prior to use to eliminate the contamination with ATP and ADP, respectively. This was accomplished by separation of the nucleotides on a Dowex AG 1x4 anion-exchange column.

In our computations of energy levels supplied and required we used the more accessible values for 25°C rather than values for the 3°C at which our experiments were conducted. Since we are only intested in comparing thermodynamic equivalents this is of little importance; all of the values employed change in the same way with temperature.

Results

By varying the internal concentrations of K^+ through preincubation of the chloroplasts with K^+ and valinomycin, it is possible to impose a variety of transmembrane electrical potentials of known initial values when one rapidly raises the external K^+ concentration. The initial value of the K^+ diffusion potential imposed in this way is given by the Nernst equation. Similarly the initial transmembrane H^+ concentration difference can be varied by preincubation of the chloroplasts at one pH value and rapidly changing the external pH in a manner similar to that described by Jagendorf and Uribe [16].

Net ATP synthesis and the incorporation of $^{32}P_i$ into ATP

When the system of ATP synthesis is operating near a thermodynamically determined threshold, that is to say when the system is operating near equilibrium, there is no assurance that net ATP synthesis and the incorporation of $^{32}P_i$ into ATP will be the same. Indeed, if an equilibrium exists it should represent equal forward and back reactions and $^{32}P_i$ incorporation should occur in the absence of net ATP synthesis. Near equilibrium, $^{32}P_i$ incorporation might be expected to exceed net synthesis. Furthermore, the addition of unlabeled ATP in a near-equilibrium situation should diminish or eliminate net ATP synthesis (by increasing the back reaction of ATP hydrolysis) without affecting the incorporation of $^{32}P_i$. However, this seems not to be the case; $^{32}P_i$ incorporation and net ATP synthesis, as measured by the firefly assay, are essentially the same, at least under the conditions of very low ATP (Fig. 1). Moreover, the addition of unlabeled ATP (to change the ΔG_{ATP}) inhibits $^{32}P_i$ incorporation precisely as one would expect to inhibit net ATP synthesis (Fig. 6.). Unfortunately, one cannot use the firefly assay to measure small amounts of ATP formation in the presence of large amounts of ATP but, for the reason given, it seems safe to equate $^{32}P_i$ incorporation to net synthesis even in the presence of excess ATP, certainly at low ATP and probably at high ATP.

Additive roles of ΔpH and $\Delta\psi$

Fig. 2 shows that the ΔpH and $\Delta\psi$ contribute to the proton-motive force in a precisely additive way as predicted by the chemiosmotic model. At the low ΔG_{ATP} values used, a threshold proton-motive force for ATP synthesis of approx. 165 mV was found regardless of the relative contribution of the ΔpH or $\Delta\psi$. This value for the threshold proton-motive force is in reasonable agreement with the threshold requirement observed by Jagendorf and Uribe [16] if we assume that there was no $\Delta\psi$ in their experiments.

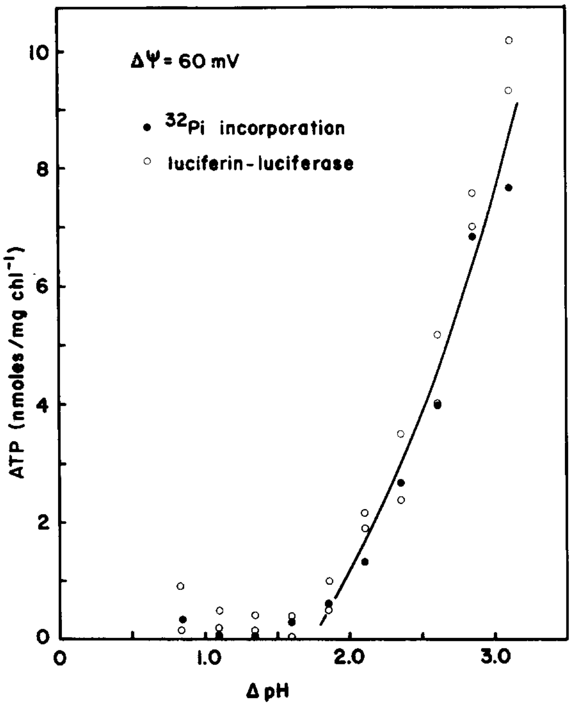

Fig. 1. ATP synthesis measured by the $^{32}P_i$ incorporation assay and the firefly luciferin-luciferase assay. Chloroplasts (50 μg Chl) were incubated for 3 min at 3°C in 2 ml of 4 mM buffer (Mes or Mops), 2 mM $MgCl_2$, 10 mM KCl, 1 μM valinomycin and 10 μM P^1,P^5-diadenosine-5′-pentaphosphate. The initial pH was adjusted by small additions of NaOH prior to the addition of the chloroplasts. The ΔpH and $\Delta\psi$ were imposed by the rapid addition of 1 ml of 200 mM Tricine-bistrispropane (pH 8.5), 2 mM $MgCl_2$, 3 mM Na_2HPO_4, 300 mM KCl and 150 μM ADP. After 10 s the reactions were terminated by the addition of 1 ml of 2 M trichloroacetic acid. The amounts of ATP synthesis as measured by the two assays are in agreement with each other, indicating that the $^{32}P_i$ incorporation method provides an accurate measure of net ATP synthesis.

We found that the yield of ATP synthesized from imposed electrochemical gradients was increased considerably when the chloroplasts were incubated at low osmotic strength prior to the

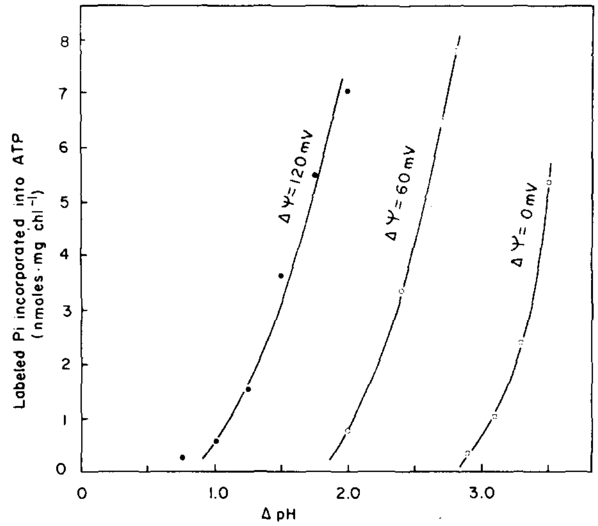

Fig. 2. Incorporation of labeled P_i into ATP by various combinations of ΔpH and membrane potentials. Chloroplasts (100 μg Chl) were incubated for 3 min at 3°C in 2ml of 4 mM buffer (Mes, bistrispropane, or glutamate), 2 mM $MgCl_2$, 100 mM sorbitol, 1 μM valinomycin and 20 mM KCl for $\Delta\psi$ of 0 mV, 10 mM KCl for $\Delta\psi$ of 60 mV or 1 mM KCl for $\Delta\psi$ of 120 mV. The initial pH was adjusted by small additions of HCl or bistropropane prior to the addition of the chloroplasts. The ΔpH and $\Delta\psi$ were imposed by the rapid addition of 1 ml of 200 mM Tricine-bistrispropane (pH 8.5), 2 mM $MgCl_2$, 3 mM $Na_2H^{32}PO_4$(2.5 μCi/μmol), 1.5 mM ADP,and 20 mM KCl for $\Delta\psi$ of 0 mV or 300 mM KCl for $\Delta\psi$ of 60 and 120 mV. Initial pH = 8.5 − indicated pH change. *Note*: As predicted from thermodynamic considerations, each increase of 60 mV in the membrane potential decreased the H^+ concentration gradient required for ATP synthesis by 1 pH unit; either a membrane potential of 60 mV or a H^+ concentration gradient of 1 pH unit increases the work potential of the charged H^+ by the same amount. It follows that at each membrane potential the threshold proton-motive force is the same, approx. 165 mV or its equivalent, an H^+ concentration gradient of 2.7 pH units. Note that in all of the figures and tables, the membrane potentials listed are the nominal, initial potentials calculated for the imposed K^+ concentration gradient.

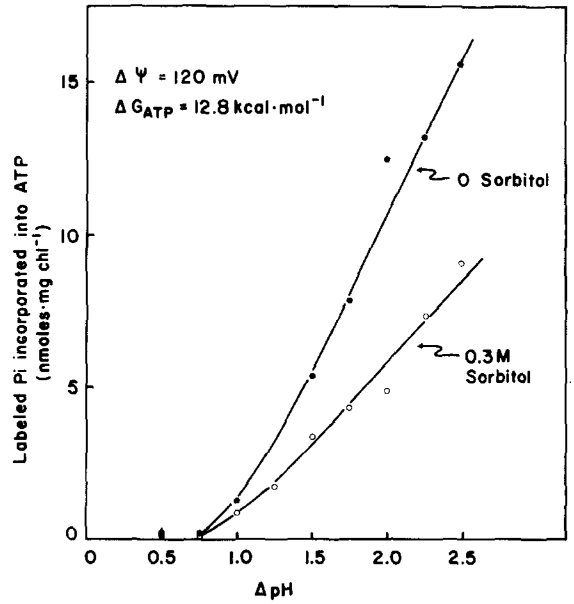

Fig. 3. Effect of osmolarity on that incorporation of labeled P_i into ATP which results from imposed electrochemical gradients. Conditions as in Fig. 2 except for the variations in the concentration of sorbitol in the stage I incubating medium and the presence of 0.5 mM ATP in the final stage (in order to define ΔG_{ATP}). Note that the low osmolarity increased the incorporation of P_i into ATP without changing the threshold proton-motive force required for incorporation of labeled P_i into ATP.

addition of the phosphorylation stage (Fig. 3). The reason for this increase is not yet clear to us. It is well known that as the osmotic strength decreases the internal volume of the chloroplasts increases and the amount of buffer which can be stored therein should increase correspondingly. However, in our system, the increased yield of ATP at low osmotic strength does not appear to be due to the presence of exogenous buffer inside, since the yield is not affected by the concentration of buffer in the incubation medium (Fig. 4). It is possible that the internal volume of the chloroplasts affects the lifetime of the K^+ diffusion potential. Thus, the larger chloroplast vesicles may have a longer-lived membrane potential (since salts entering at the same rate would then raise the internal K^+ concentration more slowly). Therefore, ATP synthesis might continue longer, resulting in increased ATP yields. Note that the magnitude of the K^+ diffusion potential seems not to be influenced by the osmotic strength, since the threshold for ATP synthesis is not changed by different osmolarities (Fig. 3).

Although it is not yet understood how incubation of the chloroplasts at low osmolarity increases the yield of ATP from imposed electrochemical gradients, we used the phenomenon to good effect. Swollen chloroplasts were able to synthesize easily measured amounts of ATP using a membrane potential in the absence of a ΔpH (Fig. 5). In fact, with a large enough membrane potential, some ATP was made even against a small inverse ΔpH.

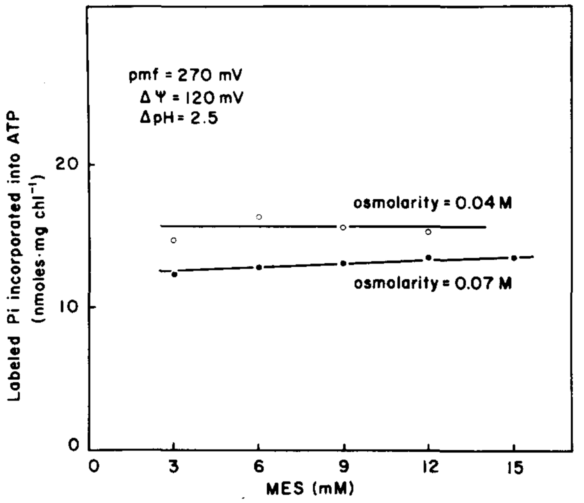

Fig. 4. Lack of effect of exogenous buffer on the incorporation of P_i into ATP. Conditions as in Fig. 2 except the concentration of Mes buffer in the incubation stage was varied as indicated and the osmolarity was kept constant at two different levels by the addition of sorbitol. Note that as in Fig. 2 osmolarity affected the incorporation of P_i, even though the concentration of buffer did not. Therefore, it is difficult to ascribe the larger yield of ATP at lower osmolarity to an increased reservoir of internal H^+ unless somehow the swelling exposes new sites of endogenous buffering.

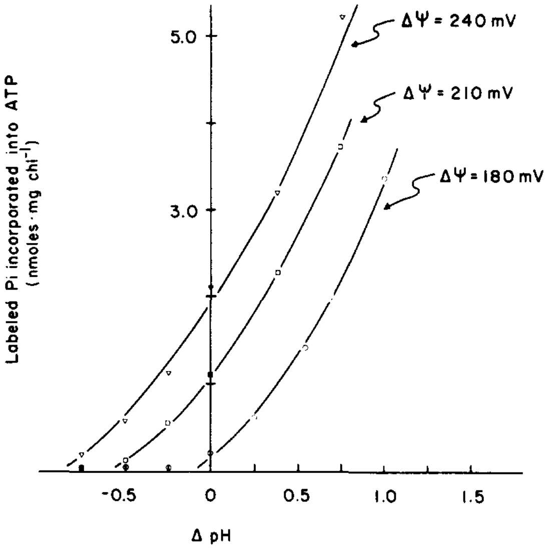

Fig. 5. Incorporation of labeled P_i into ATP in the absence of an H^+ concentration gradient. Conditions as Fig. 2 except that the concentrations of K^+ in the incubation stage were: 0.01 mM for an initial, nominal $\Delta\psi$ of 240 mV; 0.03 mM for 210 mV; and 0.1 mM for 180 mV. Concentration of K^+ in the final reaction mixture was raised to 100 mM. As in Fig. 1, the membrane potential and ΔpH are additive. Hence, with a large enough membrane potential, P_i can be incorporated into ATP even against a ΔpH in the wrong direction.

Of course, similar results were seen with chloroplasts incubated at high osmolarity but the yields were lower (data not shown).

These data clearly demonstrate that the threshold proton-motive force can be reached or exceeded by a ΔpH, a $\Delta\psi$, or any combination of ΔpH and $\Delta\psi$.

Effect of ΔG_{ATP} on the threshold proton-motive force

In principle, the proton-motive force represents the thermodynamic force driving the flow of protons through the membrane-bound H^+-ATPase. As we have already pointed out, the proton-motive force times the number of protons used to make one ATP molecule must be equal to or greater than the free energy change required for the synthesis of ATP if there is to be any net ATP synthesis. Therefore, the threshold proton-motive forces observed must vary with the ΔG_{ATP} if they represent true thermodynamic thresholds. The rather precise additivity of the ΔpH and $\Delta\psi$ in determining the threshold proton-motive force for ATP synthesis (Fig. 2 and 5) suggests that these observed threshold proton-motive forces give a reasonably accurate picture of the maximum energy initially available per H^+. Furthermore, the fact that the threshold proton-motive force for $^{32}P_i$ incorporation into ATP varies in the predicted way with ΔG_{ATP} (Fig. 6) argues strongly that the observed threshold does indeed represent the thermodynamic threshold.

From the observed minimum proton-motive force required to initiate ATP synthesis at known values of ΔG_{ATP}, the minimum number of H^+ required can be deduced. The results summarized in Table I imply that the proton-motive force which is just sufficient for ATP synthesis provides almost exactly the energy for ΔG_{ATP}, if the efflux of 3 H^+ is required for each ATP molecule formed. Although there is some dispute about the absolute value of the standard ΔG_{ATP}, there is no dispute at all about the effect of ATP/ADP ratios on ΔG_{ATP}. Fig. 6B shows the effect of a 10-fold change in the ATP/ADP ratio on the threshold ΔpH required for the incorporation of $^{32}P_i$ into ATP. If only one H^+ were required, a change of 10-fold in the

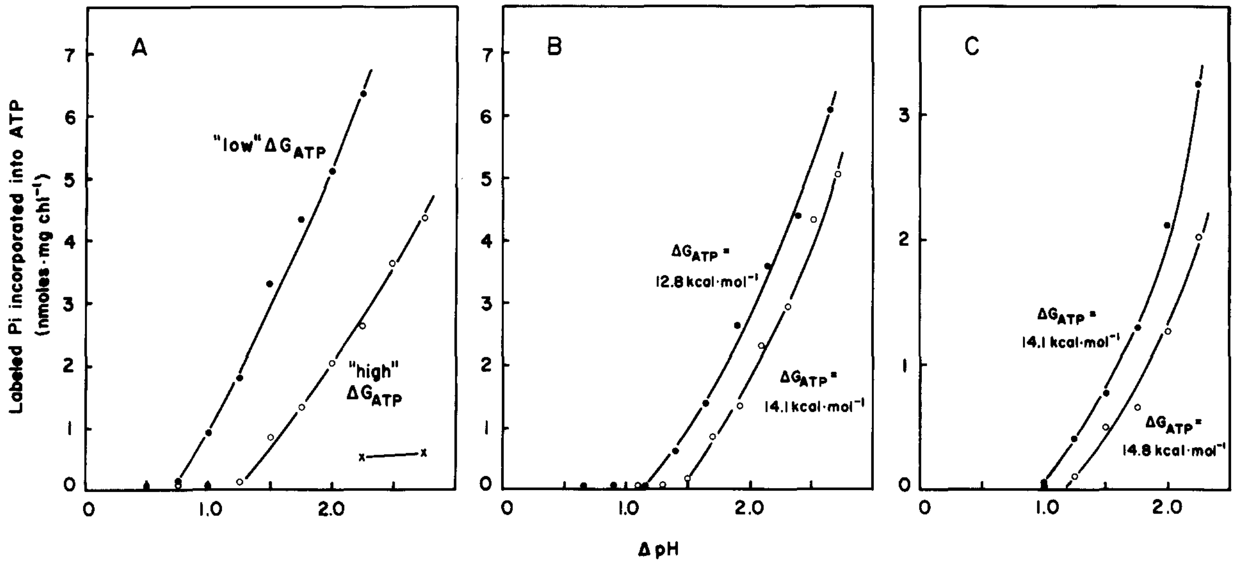

Fig. 6. Effect of the energy requirement for ATP synthesis (ΔG_{ATP}) on the threshold proton-motive force for incoporation of P_i into ATP. Reaction conditions as in Fig. 2 for a membrane potential of 120 mV except that ADP and ATP were added in the indicated amounts and all reaction mixtures contained P^1,P^5-diadenosine-5′-pentaphosphate. (A) Curve designated low ΔG_{ATP} shows values obtained with 0.5 mM commercial ADP (containing approx. 3% ATP) while curve designated high ΔG_{ATP} shows values obtained with 0.5 mM commercial ATP (containing approx. 4% ADP). Removal of the ADP from the ATP resulted in almost no incorporation of labeled P_i into ATP (x———x). (B) Both curves show values obtained with 2.5 mM ATP. Data in upper curve (●———●) were obtained when 2.5 mM ADP was present with the ATP, while data in lower curve (○———○) were obtained when 0.25 mM ADP was present with the ATP. (C) Both curves show values obtained with 0.05 mM ADP. Data in upper curve (●———●) were obtained when 0.5 mM ATP was present with the ADP while the lower curve (○———○) was obtained when 1.5 mM ATP was present with the ADP.

ATP/ADP ratio would necessitate a change in the threshold ΔpH of 1.0 unit, if two H^+ were required a change in the threshold ΔpH of 0.5 units would result, and if three H^+ were required there should be a change in the threshold ΔpH of 0.33 units. The observed change in Fig. 6B is close to 0.3 units. Similarly, Fig. 6C shows the effect of a 3-fold change in the ATP/ADP ratio and a roughly commensurate change in the threshold. Thus, data from Fig. 6B and C are also reasonably consistent with a requirement of three H^+ per ATP molecule formed and the argument is in this case independent of the absolute value of ΔG_{ATP}. Of course with such small changes in the predicted thresholds, the data of Fig. 6 alone would not provide a very conclusive value for the H^+/ATP ratio.

Melandri et al. [17] and Graan and Ort [13] have also observed changes caused by the phosphate potential on the threshold for ATP synthesis driven by single turnover flashes, but their observations are largely qualitative.

Discussion

The magnitude of the yield of ATP in response to the imposition of a transmembrane H^+ activity gradient is difficult to interpret. In the experiments described here, there is no way of predicting the lifetime of the ΔpH or $\Delta\psi$ and no way of knowing the relative importance of reaction duration versus reaction velocity. Fortunately, by looking primarily at the threshold proton-motive force for ATP synthesis, we beg these questions; we ask only what initial ΔpH and initial $\Delta\psi$ are required before any net ATP synthesis can begin. Nevertheless, the yield data do provide some significant surprises. The fact that the slopes of the plots of ATP yield against ΔpH are essentially parallel at different values of $\Delta\psi$ is surprising because at different pH ranges the supply of internal H^+ must be very different. This, by itself, would imply that the limiting factor in ATP synthesis is the duration of the $\Delta\psi$, not the duration of the ΔpH.

TABLE I

OBSERVED THRESHOLD PROTON-MOTIVE FORCE FOR THE INCORPORATION OF LABELED P_i INTO ATP AT DIFFERENT VALUES OF ΔG_{ATP}

Conditions as in Fig. 2 except that the amounts of ADP and ATP were varied to give the calculated ΔG_{ATP} values indicated, and all reaction mixtures contained 10 μM P^1,P^5-diadenosine-5′-pentaphosphate. Each threshold proton-motive force was determined by extrapolation.

Nominal initial ΔG_{ATP} (kcal/mol ATP)	$\Delta\psi$ (mV)	Observed proton-motive force (kcal/mol H^+)	Apparent H^+/ATP
10.0	120	4.2	2.4
11.3	120	4.2	2.7
11.3	0	3.9	2.9
12.8	90	4.2	3.0
12.8	120	4.4	2.9
12.8	120	4.5	2.8
12.8	120	4.5	2.8
12.8	120	4.5	2.8
12.8[a]	120	4.4	2.9
12.8	150	4.1	3.1
14.1	150	4.5	3.0
14.1	90	4.4	3.2
14.1	0	4.8	2.9
14.1[a]	120	4.8	2.9
14.1[b]	120	4.2	3.3
14.3	120	4.9	2.9
14.3	120	4.9	2.9
14.8[b]	120	4.5	3.3
			Average = 2.9 ± 0.2

[a] From Fig. 6B.

[b] From Fig. 6C.

On the other hand, if one does the reciprocal experiment, varying the $\Delta\psi$ at various levels of ΔpH a similar family of parallel curves is obtained (data not shown). Since the duration of $\Delta\psi$ cannot fail to be a function of the magnitude of $\Delta\psi$ (the K^+ ratio inside and outside), we are left with a mystery.

The use of $^{32}P_i$ incorporation into ATP as a measure of net ATP synthesis needs to be defended. The method has been shown to be valid for high rates of ATP synthesis [19], i.e., when the system is operating far from equilibrium. However, the situation could be very different near the thermodynamic threshold where equilibrium is being approached. Indeed, in conventional solution thermodynamics $^{32}P_i$ incorporation should go on at equilibrium:

$$X_E + ADP + {}^{32}P_i \underset{v_2}{\overset{v_1}{\rightleftharpoons}} X + ATP$$

where X_E is the energized state, X is the nonenergized state, v_1 is the rate of the forward reaction and v_2 that of the reverse reaction hydrolyzing ATP. In fact, it is very difficult to imagine the meaning of a threshold energy requirement except in terms of such an equilibrium where v_1 and v_2 just balance. Nevertheless, our experiments tell us that $^{32}P_i$ incorporation and net ATP synthesis are identical at low ATP and the effect of high ATP on changing the threshold intercept without changing the slope of the ATP synthesis line would be difficult to explain if $^{32}P_i$ incorporation and net ATP synthesis were not also identical even in the presence of high ATP. This suggestion of no ATP-P_i exchanges during ATP synthesis has important implications regarding the mechanism of photophosphorylation and raises questions as to the relevance of exchange reactions observed in the presence of dithiothreitol.

In any event, this distinction between ATP-P_i exchange and net ATP synthesis is important to the interpretation of our experiments. If the exchange reaction occurred freely, the $^{32}P_i$ incorporation we have observed would reflect the energy requirements for activation of the ATPase rather than the energy requirement for the formation of ATP. Fortunately, the firefly assay of net ATP synthesis precludes this interpretaion.

The effect of osmolarity (Figs. 3 and 4) on the yield of ATP in these experiments is a phenomenon in need of further investigation. It is well known that the ATP yield in acid-base transition experiments is greatly affected by some buffers. However, we failed to observe any effect of the relatively impermeant buffers we used. Nevertheless, swollen vesicles produced more ATP than shrunken vesicles. This is difficult to understand, since the supply of H^+ inside should not be affected by the internal volume unless external buffers can penetrate, in which case the increase in ATP with swollen vesicles should be a function of the concentration of buffer. But it is not. Of course, it is possible that the duration of $\Delta\psi$ is prolonged by a larger internal volume, since the entering K^+ should then raise the internal K^+ concentration more slowly.

The synthesis of a molecule of ATP must involve the transport of at least three H^+. No other interpretation of the data is possible unless we are completely misinterpreting the role of H^+ in photophosphorylation. It does not necessarily follow, however, that the theoretical ATP-to-electron pair ratio in noncyclic phosphorylation is 1.33 as has been claimed [20]. There is as yet no reliable information on the H^+-to-electron transport ratio. We do known that Photosystem II electron transport supports ATP synthesis, as does Photosystem I electron transport, and therefore there must be at least two sites of noncyclic photophosphorylation [21,22], two reactions generating H^+. But Photosystem I is about twice as efficient as Photosystem II in generating ATP [23] and the possibility remains that the transport of electrons from Photosystem II to Photosystem I generates two H^+ not one H^+. If so, the theoretical P/e_2 ratio of the overall electron transport may be 2.0 rather than 1.33 [24].

Acknowledgements

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