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Effects of Adenine Nucleotides on Hydrogen-Ion Transport in Chloroplasts*

(spinach/coupling factor 1/photophosphorylation/electron transport/ membrane permeability)

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ABSTRACT **1–10 μM ATP stimulated H^+ uptake and slowed the release of H^+ in the dark in chloroplasts illuminated at pH values at which photophosphorylation can occur, but not at pH 6.5. This ATP stimulation of H^+ uptake was abolished by an antiserum to the chloroplast coupling factor and was reduced by the energy transfer inhibitors phlorizin and Dio-9. ATP synthesis after illumination was also enhanced by ATP. Electron flow from water to methyl viologen was inhibited by the same low concentrations of ATP.**

ADP also increased the extent of H^+ uptake in chloroplasts, even in the presence of arsenate and $MgCl_2$. In the presence of hexokinase and glucose, as well as arsenate and Mg^{++}, ADP inhibited H^+ uptake. The failure of previous investigators to observe a direct inhibition of H^+ uptake by phosphorylation was probably caused by a masking of the inhibition by the stimulation of H^+ uptake by ATP. Furthermore, the stimulation of H^+ uptake by ATP provides an explanation for its inhibition of electron flow.

Although there is little doubt that the energy stored in gradients of H^+ concentration across chloroplast membranes may be used to drive the synthesis of ATP (1), the relationship between light-dependent H^+ uptake (2) and photophosphorylation is not clear. In general, H^+ uptake in chloroplasts has properties remarkably similar to those predicted by the chemiosmotic theory of Mitchell (3). H^+ uptake is sensitive to uncouplers of photophosphorylation (2) and generates rather large transmembrane gradients in H^+ concentration (4, 5).

If the H^+ gradient were the driving force for phosphorylation, or if ATP synthesis and H^+ uptake are driven by a common intermediate, phosphorylation should decrease the extent of H^+ uptake. The accumulation of either NH_4^+ (6) or of ethylamine (5) in spinach chloroplasts, which is a reflection of H^+ uptake, is inhibited by phosphorylation. In contrast, H^+ uptake in lettuce chloroplasts was found by Karlish and Avron (7) to be stimulated by the simultaneous presence of ADP, P_i, and Mg^{++}. However, Dilley and Shavit (8) presented evidence that this stimulation might be caused by a stimulation of electron flow by ADP or $MgCl_2$ under the conditions of low-salt concentration used by Karlish and Avron (7).

In this paper, we report that ATP and ADP stimulate H^+ uptake, as well as ATP synthesis in spinach chloroplasts after illumination. We suggest that coupling-factor 1 (CF_1) alters its conformation on binding ADP or ATP. This conformational change may reduce the permeability of the membranes to H^+. These observations help to explain the results of Karlish and Avron (7) and why ADP and ATP inhibit electron transport (9).

Abbreviations: Tricine for Tris-(hydroxymethyl) methyl glycine; MES for *N*-(morpholino) ethane sulfonate; CF_1 for chloroplast coupling factor 1; PMS for *N*-methyl phenazonium methosulfate; CCP for carbonylcyanide-*m*-chlorophenylhydrazone; and STN for a solution containing 0.4 M sucrose–0.02 M Tricine·NaOH (pH 8)–0.01 M NaCl.

MATERIALS AND METHODS

Chloroplasts were prepared (10), from spinach purchased locally. Subchloroplast particles were prepared by exposure of chloroplasts to sonic oscillation (11). Chlorophyll was estimated by the method of Arnon (12).

H^+ uptake was measured at 20°C (13), except that 0.4–1.5 mM Tricine·NaOH or (*N*-(morpholino) ethane sulfonate (MES)·NaOH were present. The buffer capacity of the suspension was determined as described by Polya and Jagendorf (14). ATP formation after illumination was assayed by a modification of the method of Hind and Jagendorf (15). The dark reaction mixture contained 100 mM Tricine·NaOH (pH 8), 3 mM ADP, 2 mM potassium phosphate buffer, and carrier-free $^{32}P_i$ equivalent to 2–5 × 10^6 cpm, in a final volume of 0.5 ml. The light reaction mixture contained in a volume of 0.5 ml, either 10 mM MES·NaOH (pH 6) or 10 mM Tricine·NaOH (pH 8), 0.05 mM *N*-methylphenazonium methosulfate (PMS) or pyocyanin, 50 mM NaCl, 5 mM $MgCl_2$, and chloroplasts (equivalent to 0.1 mg of chlorophyll). After illumination for 30 sec with about 1 × 10^6 ergs/cm^2 per sec of white light at 4°C, the light reaction mixture was injected into the dark reaction mixture, which was maintained at 0–4°C. The reaction was terminated after 15 sec by the addition of 0.05 ml of 40% trichloroacetic acid and, after centrifugation, aliquots of the supernatant fluid were assayed for esterified $^{32}P_i$ (16).

The Hill reaction, with methylviologen as the electron acceptor, was assayed polarographically with a Clark-type oxygen electrode. The reaction mixture was identical to that used in the H^+-uptake experiments, except that 0.1 mM methylviologen was substituted for the pyocyanin or PMS, and that 1 mM NaN_3 was present. Photophosphorylation was determined as described (10).

Hexokinase was desalted before use on a 1 × 15 cm column of Sephadex G-50 (medium), equilibrated with 25 mM sodium acetate buffer (pH 5.4) that contained 50 mM glucose. Hexokinase activity was determined enzymatically at pH 7.6 and 20°C. 1 unit is that amount of enzyme that catalyzes the formation of 1 μmol of glucose 6-phosphate per min.

Nucleotides and PMS were purchased from Sigma. The ATP analogs (α,β- and β,γ-methylene ATP) were from Miles Laboratories. Pyocyanin was prepared from PMS according to Jagendorf and Margulies (17). Dio-9 was supplied by Dr. R. J. Guillory. Hexokinase was from Boehringer-Mannheim.

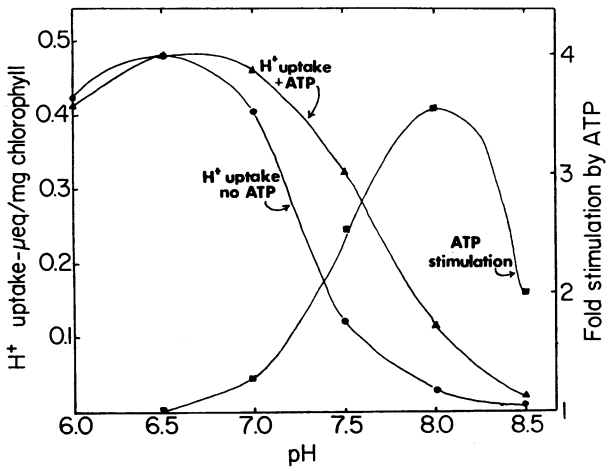

FIG. 1. Effect of pH on the stimulation of H^+ uptake by ATP. The reaction mixture (3 ml) contained 50 mM NaCl, 5 mM $MgCl_2$. 0.05 mM pyocyanin, chloroplasts equivalent to 0.3 mg of chlorophyll, and the following buffers: MES·NaOH 1.66–2.33 mM for pH 6–7 and Tricine·NaOH 1.33 mM for pH 7.5–8.5.

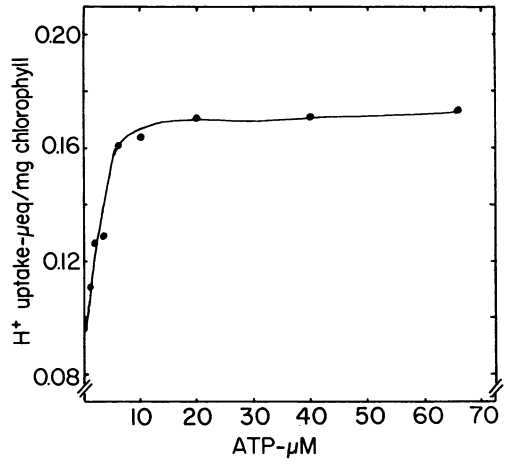

FIG. 2. Effect of ATP concentration on H^+ uptake. The reaction mixture was the same as that described in Fig. 1, except that the Tricine concentration was 1.5 mM. The pH was 7.60.

Rabbit serum containing antibodies to CF_1 was kindly provided by Dr. R. J. Berzborn.

RESULTS

Stimulation of H^+ uptake by ATP

The extent of light-dependent H^+ uptake supported by cyclic electron flow was stimulated by ATP when the assays were performed at pH values where phosphorylation takes place (Fig. 1). Maximal stimulation was observed at pH 8; no effect of ATP was detected at pH 6.5, the optimal pH for H^+ uptake. Generally, ATP stimulated the extent of H^+ uptake at pH 7.6 by 40–60%. In some experiments, this stimulation was as great as 250%, or as low as 20%.

As little as 1 μM ATP stimulated H^+ uptake (Fig. 2). Half-maximal stimulation was given by 3 μM ATP; saturation was achieved at 10 μM. H^+ uptake in subchloroplast particles prepared by exposure of chloroplasts to sonic oscillation (11) was also enhanced by ATP.

Although ATP stimulated H^+ uptake in the absence of added Mg^{++}, 1 mM EDTA abolished this effect of ATP. If, however, 1 mM $MgCl_2$ was present, EDTA did not prevent the ATP stimulation of H^+ uptake. Thus, Mg^{++}–ATP, rather than ATP itself, is probably the stimulator.

Neither arsenate nor P_i, at concentrations up to 1 mM affected the ATP stimulation of H^+ uptake. Since the endogenous P_i concentrations in our chloroplast preparations are low (about 0.07 μmol/mg of chlorophyll), it is unlikely that the small amount of P_i contributed by the chloroplasts to the reaction mixture (about 7 μM) was enough to satisfy a P_i requirement for the ATP stimulation. Avron *et al.* (9) reported that the ATP inhibition of electron flow was unaffected by P_i.

At pH 7.6, the decay of the light-induced pH rise in the dark was slowed by ATP. No effects of ATP on the kinetics of H^+ uptake were detected at pH 6.5. The decay of the pH rise in chloroplasts illuminated in the presence of pyocyanin was, in many experiments, complex. Whereas the plots of log (Δ pH) versus time for the pH decay in the absence of ATP were generally monophasic, these plots in the presence of ATP were frequently biphasic (Fig. 3). During the first 8–10 sec in the dark, the decay in the presence of ATP was fast, although not as fast as that observed in the absence of ATP. The remainder of the decay was usually considerably slower. In the presence of methylviologen, the dark decay in the presence and absence of ATP was monophasic, and pronounced inhibitions of the rate of the dark decay were observed. Although it is clear that ATP reduced the rate of the pH decay in the dark, it is not readily possible to correlate this rate reduction by ATP to the increased extent of H^+ uptake because of the complex kinetics.

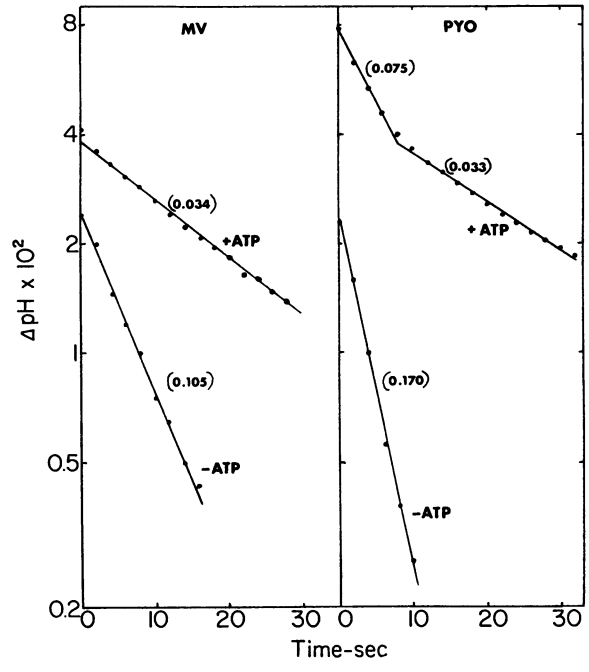

FIG. 3. Effect of ATP on the decay of the pH rise after illumination. The reaction mixture for H^+ uptake with pyocyanin was the same as that described in Fig. 1. For H^+ uptake in the presence of methylviologen, 0.1 mM methylviologen and 1 mM NaN_3 were added in place of pyocyanin. *MV* stands for methylviologen and *PYO* for pyocyanin. The numbers in brackets are the apparent first-order rate constants derived from these graphs in sec^{-1}. The ATP concentration was 20 μM.

TABLE 1. *Effect of several nucleotides on H^+ uptake and electron flow*

Nucleotide	H^+ uptake (μeq/mg chlorophyll)	Methylviologen reduction (μeq/mg chlorophyll per hr)	K_d (sec^{-1})
None	0.098	261	0.127
ATP	0.154	162	0.036
GTP	0.102	240	0.118
ITP	0.105	240	0.102
UTP	0.091	266	0.098

The nucleotide concentrations were 20 μM. H^+ uptake was assayed at pH 7.60 with methylviologen as the electron acceptor. Tricine was present at 0.67 mM. K_d stands for the apparent first-order rate constants for the pH decay in the dark.

Effects of ATP and other nucleotides

Even though ATP enhanced H^+ uptake coupled to electron flow from water to methylviologen, methylviologen reduction was inhibited by ATP (Table 1). Methylviologen reduction and H^+ uptake were assayed in the same reaction mixture. Similar low concentrations of ATP were required to inhibit methylviologen reduction as in the inhibition of ferricyanide reduction (9) or the stimulation of H^+ uptake. GTP, ITP, and UTP at 20 μM had little effect on either H^+ uptake or methylviologen reduction, whereas 20 μM ATP stimulated the extent of H^+ uptake by 57% and inhibited electron flow by about 30%. Furthermore, only ATP markedly slowed the rate of H^+ efflux in the dark. Even at 100 μM, UTP, AMP, and the ATP analogs, α,β- and β,γ-methylene-ATP, had no effect on H^+ uptake.

Effects of inhibitors and uncouplers

To attempt to localize the site that interacts with ATP to cause the stimulation of H^+ uptake, the effects of several inhibitors of photophosphorylation were investigated. A rabbit antiserum to CF_1 abolished the stimulation of H^+ uptake by ATP (Table 2). In this experiment, cyclic photophosphorylation was inhibited 80% by the antiserum, whereas

TABLE 2. *Inhibition of the ATP stimulation of H^+ uptake by an antiserum to CF_1*

Chloroplast treatment	H^+ uptake −ATP (μeq/mg chlorophyll)	H^+ uptake +ATP (μeq/mg chlorophyll)	PMS-dependent phosphorylation (μmol P_i esterified/hr per mg chlorophyll)
Control serum	0.128	0.234	531
Antiserum to CF_1	0.073	0.076	105

Aliquots of about 1 ml of a chloroplast suspension (2.5 mg of chlorophyll per ml) were incubated for 15 min at 0°C with either 1.5 ml of control serum or 1.5 ml of the antiserum to CF_1. The chloroplasts were then sedimented by centrifugation for 10 min at 10,000 × *g*, resuspended in 5 ml of STN, and sedimented by centrifugation. The pellets were finally resuspended in about 0.5 ml of STN. H^+ uptake was determined with pyocyanin as the mediator of cyclic electron flow at pH 7.6. The Tricine concentration was 0.67 mM.

H^+ uptake in the absence of ATP was inhibited 40%. Mouse anti-CF_1 serum has no effect on H^+ uptake, even when phosphorylation was inhibited in excess of 80% (18). This difference between the mouse and rabbit sera is not understood, and is currently under investigation. The rabbit anti-CF_1 serum also prevented the inhibition of electron flow by ATP.

Phlorizin (19) and Dio-9 (20), energy-transfer inhibitors that interact with CF_1, attenuated the stimulation of H^+ uptake by ATP, but did not abolish it, even at concentrations that severely inhibit phosphorylation (Table 3). Furthermore, these reagents enhanced H^+ uptake at pH 7.6. Energy-transfer inhibitors were shown to enhance H^+ uptake (7, 21); very recently, it was reported (22) that this stimulation is pH dependent. Little stimulation of H^+ uptake at pH 6–6.5 was observed, whereas large stimulations were detected at pH 8.

In contrast, neither 2 mM ethylamine nor 10 μM carbonylcyanide-*m*-chlorophenylhydrazone (CCP) markedly affected the degree of stimulation by ATP, even though they decreased the extent of H^+ uptake by 50–60% (Table 4). Thus, partial uncoupling of phosphorylation from electron flow is not sufficient to prevent the stimulation of H^+ uptake by ATP.

Effect of ATP on ATP synthesis after illumination

Since H^+ uptake and the formation of X_E (defined as the high-energy state generated in the light that can drive ATP synthesis in a subsequent dark-reaction) are closely correlated (23), it was expected that ATP should also enhance X_E formation. As may be seen in Table 4, this was the case. Whereas 0.1 mM ATP added to the light stage at pH 6.0 had little effect on X_E formation, at pH 8 ATP stimulated X_E formation nearly 3-fold. At 0.1 mM, GTP, ITP, UTP, AMP, and β,γ-methylene-ATP had little or no effect on X_E formation at pH 8.0.

Pyridine greatly enhances both X_E formation and H^+ uptake in chloroplasts at pH values of 7 or greater (24). Pyridine is probably accumulated by the chloroplasts in response to H^+ uptake and acts as an internal buffer. ATP stimulated H^+ uptake to the same extent in the absence or presence of 10 mM pyridine, which enhanced H^+ uptake at pH 7.6 by nearly 10-fold (Table 5). ATP decreased the rate of H^+ efflux in the presence of pyridine to a similar extent as it did in its absence. ATP also stimulated X_E formation in the presence of 10 mM pyridine. These results make it rather unlikely that ATP stimulates H^+ uptake by acting as an internal buffering agent.

TABLE 3. *Effects of energy transfer inhibitors and uncouplers on the ATP stimulation of H^+ uptake*

Experiment	Inhibitor or uncoupler	H^+ uptake −ATP (μeq/mg chlorophyll)	H^+ uptake +ATP (μeq/mg chlorophyll)	Stimulation by ATP (%)
I	None	0.090	0.239	166
	Phlorizin (1 mM)	0.167	0.277	66
	Dio-9 (5 μg/ml)	0.194	0.291	50
II	None	0.194	0.310	60
	Ethylamine (2 mM)	0.079	0.122	54
	CCP (10 μM)	0.122	0.187	53

ATP was present at 0.1 mM. The reaction mixture contained pyocyanin. The initial pH was 7.60, and the Tricine concentration was 1.25 mM in Expt. I and 0.67 mM in Expt. II.

TABLE 4. *Effect of ATP on after-illumination ATP synthesis*

pH of light stage	Addition to light stage	P_i esterified (nmol/mg chlorophyll)
6.0	None	36.9
6.0	0.1 mM ATP	32.1
8.0	None	2.6 ± 0.2
8.0	ATP	7.1 ± 0.2

The light- and dark-stage reaction mixtures are described in *Methods*. Chloroplasts, equivalent to 0.1 mg of chlorophyll, were used in each assay. Standard deviations were obtained from triplicate determinations.

Effects of ADP on H^+ uptake

ADP stimulated H^+ uptake and X_E formation to the same extent as did ATP at similar concentrations. The rate of the pH decay in the dark was also slowed by ADP. Furthermore, rabbit anti-CF_1 serum abolished the ADP effect, as well as that of ATP. High concentrations of Dio-9 only partially prevented the ADP stimulation of H^+ uptake, as was the case for ATP.

The stimulation of H^+ uptake by ADP may be caused by ATP, rather than ADP itself. Commercial ADP preparations are contaminated with ATP. The ATP content of our ADP, assayed enzymatically, was about 2–2.5%. Thus, a reaction mixture containing 100 μM ADP would also contain 2–2.5 μM ATP, enough to elicit detectable stimulations of H^+ uptake. Furthermore, some ATP could be formed by the action of adenylate kinase or by photophosphorylation, since a small amount of P_i was present in the chloroplasts.

The effect of phosphorylation on H^+ uptake was, therefore, reexamined; we took precautions to exclude ATP from the reaction mixture. As shown in Table 6, 0.1 mM ADP stimulated pyocyanin dependent H^+ uptake in this experiment by 42%. Neither 1 mM arsenate nor 15 units of hexokinase markedly affected H^+ uptake in the presence or absence of ADP. In the presence of both hexokinase and arsenate, ADP inhibited H^+ uptake by 32%. With hexokinase and arsenate present, there should have been little ATP in the reaction mixture. The ATP in the ADP would be removed by the hexokinase, and arsenate would prevent the formation of ATP by photophosphorylation. Similar results were obtained when H^+ uptake was assayed in the presence of 10 mM pyridine or in the presence of methylviologen, rather than pyocyanin. Thus, when ATP is excluded, photophosphorylation decreases the extent of H^+ uptake.

DISCUSSION

Mechanism of the ATP stimulation

Since very low ATP concentrations stimulate H^+ uptake, it is unlikely that this stimulation is caused by either an internal buffering action of the ATP or by passive transport of a negatively charged Mg^{++}–ATP complex (7). ATP hydrolysis can support H^+ uptake in the dark (25) in chloroplasts previously exposed to dithiothreitol in the light to induce the Mg^{++}-dependent ATPase (26, 27). However, no sulfhydryl compound was added in our experiments, and the apparent K_m for ATP in the stimulation of H^+ uptake (3 μM) is much lower than that of chloroplast ATPase reactions. Thus, ATP-dependent H^+ uptake was probably negligible.

TABLE 5. *Effects of pyridine on H^+ uptake and ATP synthesis after illumination*

Additions	H^+ uptake (μeq/mg chlorophyll)	ATP formation after illumination (nmol P_i esterified/ mg chlorophyll)
None	0.090	—
0.1 mM ATP	0.181	—
10 mM pyridine	1.81	29.6
Pyridine + ATP	3.84	50.4

H^+ uptake was assayed at pH 7.60 with pyocyanin. The pH of both the light and the dark reaction mixtures in the assay of ATP synthesis was 8.0. Pyocyanin was used as the mediator of cyclic electron flow. The Tricine concentration was 0.67 mM.

ATP probably reduces the permeability of chloroplast membranes to H^+, since the rate of H^+ efflux from chloroplasts in the dark is slowed by ATP. The sensitivity of the ATP stimulation of H^+ uptake to the CF_1 antiserum indicates that CF_1 is involved in this stimulation. Since the rabbit antiserum to CF_1 and removal of CF_1 from chloroplast membranes (19) both inhibit H^+ uptake, it is probable that CF_1 is required in some way for H^+ uptake. It is possible that the conformation of CF_1 may affect the properties of the membrane. On binding ATP, CF_1 may alter its conformation; this change may, in turn, reduce the permeability of the membrane to H^+.

Relation of the ATP stimulation of H^+ uptake to its inhibition of electron flow

Quite similar concentrations of ATP were required to stimulate H^+ uptake and inhibit electron flow. Moreover, energy-transfer inhibitors (28) and CF_1 antibody attenuated both effects of ATP. The ATP inhibition of electron flow may be explained in terms of its stimulation of H^+ uptake. In the chemical hypothesis, electron flow in the absence of ADP and P_i results in the accumulation of a high-energy intermediate ($X \sim I$). I is postulated to be required for electron flow through a coupling site. Therefore, an increase in $X \sim I$ by ATP would decrease the concentration of I and inhibit electron flow.

In the chemiosmotic theory (3), electron flow directly generates an electrochemical gradient (in H^+) across the membranes. The back pressure of this gradient reduces the rate

TABLE 6. *Effect of arsenate and hexokinase on the stimulation of H^+ uptake by ADP*

Additions	H^+ uptake −ADP (μeq/mg of	H^+ uptake +ADP chlorophyll)	Effect of ADP (%)
None	0.122	0.173	+42
1 mM arsenate	0.114	0.168	+46
15 units hexokinase	0.120	0.180	+50
Arsenate + hexokinase	0.132	0.090	−32

H^+ uptake was assayed at pH 7.6 with pyocyanin. The reaction mixture contained 50 mM glucose, in addition to 50 mM NaCl, 5 mM $MgCl_2$, 1 mM Tricine, and chloroplasts equivalent to 0.15 mg of chlorophyll (in 1.5 ml). The ADP concentration was 0.1 mM.

of electron flow until the rate of H^+ uptake is equal to that of H^+ loss. Since ATP reduces the rate of H^+ loss, electron flow is inhibited.

It has been suggested (4) that the internal pH may control the rate of electron flow in chloroplasts, a low internal pH resulting in a low flow rate. By increasing H^+ uptake, ATP should decrease the internal pH and inhibit electron flow.

Effects of ADP on H^+ uptake

ADP stimulates H^+ uptake, even in the presence of hexokinase and glucose, but this stimulation may be caused by ATP. Some ATP may be formed by phosphorylation and this ATP, still bound to CF_1, may exert its effects before it can be trapped by the hexokinase.

Dilley and Shavit (8) and Schwartz (29) found that phosphorylation decreases the extent of H^+ uptake. These authors, as well as Karlish and Avron (7), also observed a stimulation of H^+ uptake by ADP, Mg^{++}, and arsenate, which they attributed to a stimulation of electron flow by these reagents. Since Dio-9, at 4 μg/ml, fully blocks the stimulation of the rate of electron flow by the phosphorylation reagents, the enhancement of electron flow cannot be the only cause of the stimulation of H^+ uptake. It is more likely that this stimulation is caused by ATP. As we show, the ATP (or ADP) stimulation of H^+ uptake was only partially inhibited by Dio-9.

Although the physiological significance of this effect of ATP is not clear, it is apparent that the interaction between small molecules and a membrane protein can regulate the properties of the membrane.

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