Dicarboxylic Acids Link Proton Transfer Across a Liquid Membrane to the Synthesis of Acyl Phosphates. A Model for P-Type H^+-ATPases

Ian J. Colton and Romas J. Kazlauskas*

McGill University, Department of Chemistry, 801 Sherbrooke St. W., Montréal, Québec, Canada H3A 2K6

Received January 20, 1994®

H^+-ATPases are ion pumps that link proton transfer across cell membranes to the synthesis or hydrolysis of ATP. A current research goal is to understand the molecular-level mechanism of this linking. We present a chemical model that mimics some features of H^+-ATPases by linking proton transfer across a liquid membrane to the synthesis of acyl phosphates using carboxylic acid anhydride intermediates. Citraconic acid (*cis*-2-methyl-2-butenedioic acid) accelerated the transfer of protons from a pH 0.3 solution across a chloroform liquid membrane to a pH 10 solution. The mechanism involved spontaneous formation of a small amount of citraconic anhydride (0.6%) in the pH 0.3 layer. This anhydride partitioned into the chloroform layer and diffused to the pH 10 layer, where it hydrolyzed, generating two protons. When the pH 10 layer contained phosphate (1.0 M), some of the citraconic anhydride reacted with phosphate to form citraconyl phosphate, 5.0% yield. In separate experiments, we confirmed that citraconyl phosphate had high phosphoryl donor potential by reacting it with morpholine to form a phosphoramidate (11.5% yield) or with fluoride to form fluorophosphonate (32% yield). To demonstrate the link between an acyl phosphate and a proton gradient in the reverse direction, we used succinyl phosphate, whose hydrolysis occurs in two steps: formation of succinic anhydride, which consumes protons, followed by hydrolysis of succinic anhydride, which releases protons. We generated a pH gradient by carrying out these two steps in separate solutions. Hydrolysis of succinyl phosphate (3.9 mmol) at pH 6.00 started with a increase in pH to 6.16 (0.59 mmol of H^+ consumed) caused by the formation of succinic anhydride. We extracted this anhydride with dichloromethane and transferred it to a separate solution at pH 6.05. Hydrolysis of the anhydride released protons (0.36 mmol), decreasing the pH to 5.23. Our model suggests that H^+-ATPases could use acyl phosphates and carboxylic acid anhydride intermediates to link proton transfer to ATP synthesis or hydrolysis.

Introduction

Ion pumps are transmembrane proteins that catalyze the transport of ions across cell membranes.[1] These ion pumps link an energy source, light absorption or ATP hydrolysis, to the creation or dissipation of ion gradients across cell membranes. Cells use these ion gradients to drive secondary transport of metabolites, to convey nerve signals, and, in some organelles, to make ATP.

Ion pumps that link ion transport to ATP hydrolysis are also called ATPases. Researchers classify ATPases into three types: P-type ATPases, V-type ATPases, and F_oF_1-ATPases. The P-type ATPases form an aspartyl phosphate intermediate as part of their reaction cycle and are inhibited by vanadate. Vacuolar membranes, as well as a few other membranes, contain the V-type ATPases. The F_oF_1-ATPases are the most complex pumps and usually act in reverse—they synthesize ATP using a proton gradient as the driving force.

The best understood ion pumps are the P-type ATPases,[2] such as the Na^+–K^+ pump, the Ca^{2+} pump, and the H^+-K^+ pump. The Na^+–K^+ pump, or Na^+–K^+ ATPase, maintains osmotic balance in animal cells. Cardiac glycosides such as digoxin increase the force of heart contractions by inhibiting this pump.[3] The Ca^{2+} pump concentrates calcium ions into the sarcoplasmic reticulum of muscle cells. Release of calcium from this compartment signals the muscle to contract. In other cells the Ca^{2+}-ATPase concentrates Ca^{2+} into other organelles that later release the Ca^{2+} in response to specific extracellular signals. Thapsigargin, a sesquiterpene lactone from plants, promotes tumors by inhibiting Ca^{2+}-ATPases.[4] The gastric proton pump, or H^+–K^+ ATPase, pumps acid into the stomach. Inhibitors of the gastric proton pump, such as omeprazole,[5] promote the healing of gastric and duodenal ulcers. Recently, researchers found that abnormalities in a P-type ATPase may cause Menkes disease, an inborn error of copper metabolism.[6] Due to this importance of P-type ATPases in biochemistry, understanding their structure and mechanism of action is a goal of current research.

The different P-type ATPases all have similar structures. They possess catalytic subunits of similar size (approximately 100 kDa) and with similar amino acid sequence.[7] Researchers have proposed a consensus model of P-type ATPases which identifies the domains, the likely secondary structures within these domains, the mem-

® Abstract published in *Advance ACS Abstracts,* June 1, 1994.

(1) (a) Läuger, P. *Electrogenic Ion Pumps,* Sinauer Associates: Sunderland, MA, 1991. (b) Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Molecular Biology of the Cell,* 2nd ed.; Garland: New York, 1989; pp 300–323. (c) Pederson, P. L.; Carafoli, E. *Trends Biochem. Sci.* **1987**, 12, 146–150, 186–189. (d) Stein, W. D. *Transport and Diffusion across Cell Membranes*; Academic: Orlando, FL, 1986; Chapter 6.

(2) Recent reviews: Slayman, C. L.; Zucker, G. R. *Ann. N. Y. Acad. Sci.* **1989**, *574*, 233–245. Stein, W. D. *Channels, Carriers, and Pumps*; Academic: New York, 1990; Chapter 6. See also: Carafoli, E., Ed. *J. Bioenerg. Biomembr.* **1992**, *24*, 245–328.

(3) *Martindale The Extra Pharmacopeia*, 30th ed.; Reynolds, J. E. F., Ed.; Pharmaceutical: London, 1993; pp 666–670.

(4) Thastrup, O.; Cullen, P. J.; Drøbak, B.; Hanley, M. R.; Dawson, A. P. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2466–2470.

(5) Lindberg, P.; Brändström, A.; Wallmark, B. *Trends Pharmacol. Sci.* **1987**, *8*, 399–402.

(6) Vulpe, C.; Levinson, B.; Whitney, S.; Packman, S.; Gitschier, J. *Nat. Genet.* **1993**, *3*, 7–13.

0022-3263/94/1959-3626$04.50/0

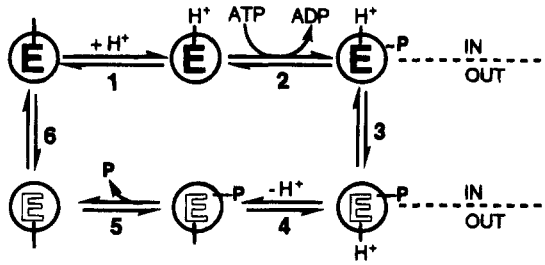


Figure 1. Schematic mechanism of action for a P-type H+-ATPase. Hydrolysis of ATP drives the transport of protons from the inside to the outside. The pumping mechanism involves a phosphoenzyme intermediate (aspartyl phosphate) and two principal conformations: one with an inward-facing binding site for the proton (solid E) and the other with an outward-facing binding site (outline E). In step 1, a proton from the inside binds to the pump; in step 2, this complex is phosphorylated by ATP to form a higher energy complex. This complex relaxes by a conformational change which reorients the proton binding site to the other side of the membrane, step 3. Release of the proton to the outside, step 4, hydrolysis of the aspartyl phosphate, step 5, and another conformational change, step 6, regenerates the original state. The molecular details of this schematic mechanism are an active topic of biochemical research.

brane-spanning regions, and the ATP-binding region. Electron microscopy and X-ray diffraction identified the basic shapes of the molecules,[8] but no one has yet reported high-resolution three-dimensional structures. Unfortunately, this structural information is still insufficient to identify the molecular mechanism of pumping.

The current hypothesis for the mechanism of action is an updated version of the Post–Albers model, which suggests that P-type ATPases link ion pumping to hydrolysis of ATP by assuming two different conformations,[9] as shown in Figure 1. Each conformation differs in its affinity for the ion and exposes the ion-binding site to opposite sides of the membrane. Hydrolysis of ATP fixes the sequence of the conformational changes and sets the direction of pumping. The current goals are to determine the structures of the two conformations, to identify what, on a molecular level, causes the change in ion affinity, and how hydrolysis of ATP causes the conformation to change. Although the answers to these questions will probably come from further studies of the structure and kinetics of the biological pumps, organic chemists can also contribute by building chemical mimics of these ion pumps.

The mechanism of action of a chemical mimic of a P-type ATPase would be much simpler to study. Mimics could suggest which functional groups are important and could test proposed mechanisms. In addition, synthetic ion pumps may be useful to concentrate ions for analysis or to remove ions from dilute waste streams. Researchers have reported chemical models of ion channels[10] and ionophores,[11] which catalyze the transport of ions driven by concentration gradients. These models helped researchers understand, among other things, the selectivity of ion transport across membranes, but did not explain how the hydrolysis of ATP could drive the transport of ions against concentration gradients. To understand this process we need models that link ion transport to the hydrolysis of ATP or to similar hydrolysis reactions.

In this paper we describe a chemical model that links proton transport and the synthesis of an acyl phosphate.[12] To build this model, we made two simplifications. First, we used a liquid membrane of chloroform to mimic a lipid bilayer. Second, although ATPases are membrane-spanning proteins, we used a soluble, diffusible molecule as the pump. In spite of these simplifications, this model mimics several features of biological proton pumps. It makes an acyl phosphate with high phosphoryl group transfer potential using a proton gradient as the driving force, and the reverse—it makes a proton gradient using the hydrolysis of an acyl phosphate as a driving force. Our model suggests that suitably oriented carboxyl groups in the biological pump may couple proton transport and ATP hydrolysis by way of acyl phosphates and carboxylic acid anhydride intermediates.

Results

Proton Transfer across an Organic Layer by Citraconic Anhydride. To model a proton gradient across a lipid bilayer, we separated solutions of pH 1 (0.10 M HCl) and pH 10 (50 mM K_2CO_3 buffer) with a layer of dichloromethane using a concentric ring cell (a tube within a beaker), Figure 2. Molecules from the acidic aqueous layer surrounding the tube can reach the basic aqueous layer within the tube only by passing through the dichloromethane layer at the bottom. This pH gradient was stable over several days. According to pHstat, <0.004 mmol/h of acid transferred to the basic layer.

When the acidic compartment contained a carboxylic acid, the pHstat indicated that protons transferred to the basic compartment. Analysis by ^{1}H-NMR indicated that the carboxylic acid also transferred. The rate of proton transport varied with the structure of the carboxylic acid, Figure 2. Acetic, citraconic, or maleic acids (1.0 M) in the acid compartment transferred protons rapidly, while succinic (0.40 M) or mesaconic (0.10 M) had little effect. (We used lower concentrations of succinic and mesaconic acids because they are less soluble in water than the other acids.) Substituting chloroform for dichloromethane did not change the rate of proton transport in similar experiments using citraconic acid.

This proton transfer occurred by diffusion of either (1) the carboxylic acid or (2) the carboxylic acid anhydride through the dichloromethane layer. In the case of acetic acid, protons moved by diffusion of acetic acid, Figure 3A. A two-layer experiment supported this suggestion. After equilibration of an aqueous solution of acetic acid (1.0 M, 0.1 M HCl) with a layer of $CDCl_3$, ^{1}H-NMR analysis showed

(7) Reviews: Green N. M. *Ann. N. Y. Acad. Sci.* **1992**, *671*, 104–112. Wach, A.; Schlesser, A.; Goffeau, A. *J. Bioenerg. Biomembr.* **1992**, *24*, 309–317. Nakamoto, R. K.; Rao, R.; Slayman, C. W. *Ann. N. Y. Acad. Sci.* **1989**, *574*, 165–179.

(8) Review: Stokes, D. L. *Curr. Opin. Struct. Biol.* **1991**, *1*, 555–561.

(9) Post, R. L.; Kume, S.; Tobin, T.; Orcutt, B.; Sen, A. K. *J. Gen. Physiol.* **1969**, *54*, 306S-326S. Reviews: Jencks, W. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1980**, *51*, 75–106. Hammes, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6881–6884. Tanford, C. *Ann. Rev. Biochem.* **1983**, *52*, 379–409. Jencks, W. P. *J. Biol. Chem.* **1989**, *264*, 18855–18858.

(10) Åkerfeldt, K. S.; Lear, J. D.; Wasserman, Z. R.; Chung, L. A.; DeGrado, W. F. *Acc. Chem. Res.* **1993**, *26*, 191–197. Pregel, M. J.; Jullien, L.; Lehn, J.-M. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1637–1640. Carmichael, V. E.; Dutton, P. J.; Fyles, T. M.; James, T. D.; Swan, J. A.; Zojaji, M. *J. Am. Chem. Soc.* **1989**, *111*, 767–769.

(11) Reviews: Fyles, T. M. In *Inclusion Aspects of Membrane Chemistry;* Osa, T., Atwood, J. L., Eds.; Kluwer Academic: Amsterdam, 1991; pp 59–110. Lehn, J.-M. In *Physical Chemistry of Transmembrane Ion Motions* Spach, G., Ed.; Elsevier Science: Amsterdam, 1983; pp 181–207.

(12) Preliminary communication: Colton, I. J.; Kazlauskas, R. J. *J. Org. Chem.* **1992**, *57*, 7005–7006.

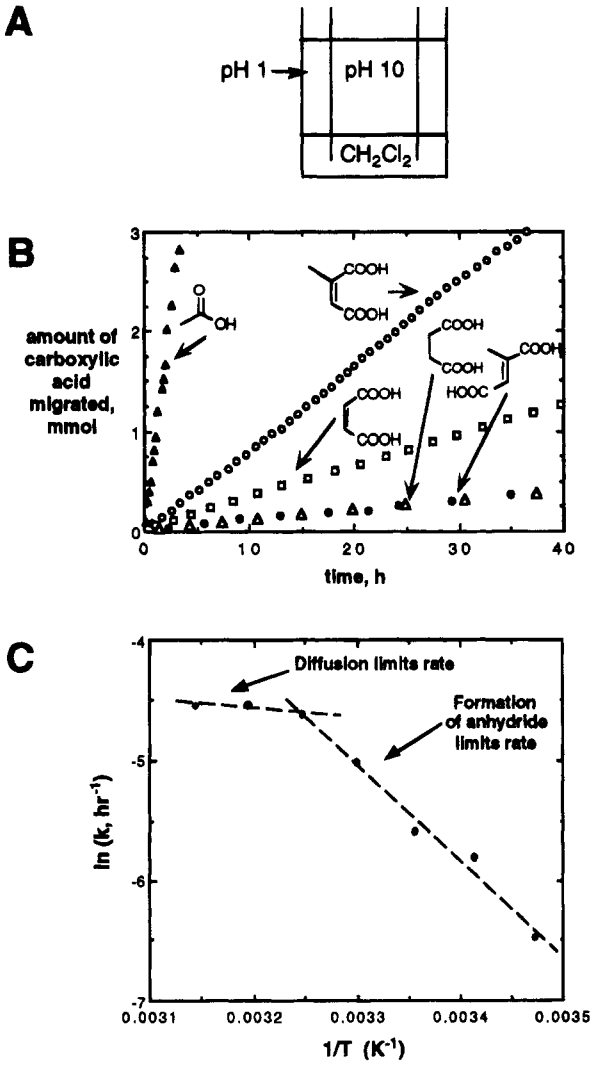


Figure 2. To mimic a proton gradient across a bilayer, we separated an aqueous layer at pH 1 from an aqueous layer at pH 10 by a layer of dichloromethane. (A) Schematic diagram of a concentric ring cell (a tube within a beaker) which separated the inner solution at pH 10 from the outer solution at pH 1 by a layer of dichloromethane at the bottom. (B) The proton gradient was stable when the acidic compartment contained only HCl (0.1 M, rate <0.004 mmol/h). When the acidic compartment contained carboxylic acids, protons were transported to the pH 10 compartment: acetic (▲ 1.0 M), citraconic (○, 1.0 M), maleic (□, 1.0 M), succinic (△ 0.40 M), mesaconic (●, 0.10 M). (C) The initial rate of proton transport mediated by citraconic acid increased with increasing temperature. In the range 15–35 °C ($1/T$ = 0.00347–0.00325 K^{-1}), the activation energy was 16 ± 2 kcal/mol, suggesting that dehydration of citraconic acid to the anhydride limits the rate in this temperature range. In the range 35–45 °C ($1/T$ = 0.00325–0.00314 K^{-1}), the activation energy was only 1.4 ± 2 kcal/mol, suggesting that diffusion of the anhydride through the chloroform layer limits the rate in this temperature range.

that the organic layer contained only acetic acid (100 ± 10 mM) and no detectable acetic anhydride (<2 mM).

For citraconic acid, protons moved by diffusion of citraconic anhydride, Figure 3B. An acidic aqueous solution of citraconic acid (1.0 M, 0.5 M HCl) contained a small equilibrium amount of citraconic anhydride (0.6 mol %) as shown by ^{1}H-NMR. The high effective molarity of the neighboring carboxylic acid in citraconic acid shifted the acid–anhydride equilibrium toward anhydride by a factor of 2 × 10^9 as compared to acetic acid, Table 1. In

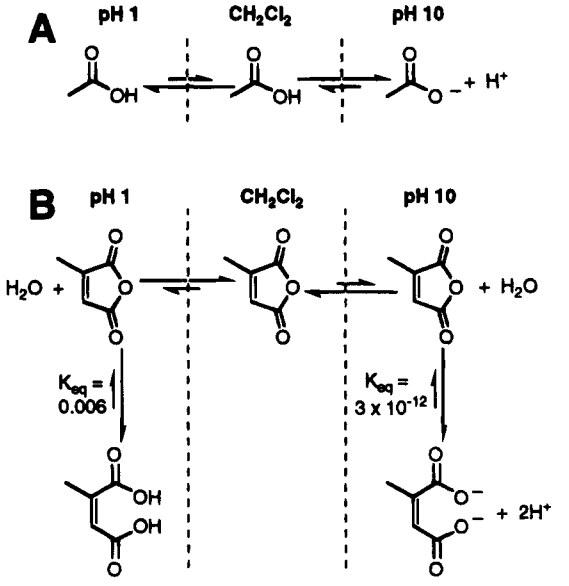


Figure 3. Mechanisms of direct and indirect proton transfer across a dichloromethane layer. (A) Direct transfer of protons across the dichloromethane layer by diffusion of acetic acid. Protons originally in the acidic compartment move to the basic compartment. (B) Indirect transfer of protons across the dichloromethane layer by diffusion of the carboxylic acid anhydride. Citraconic acid dehydrates to the anhydride in the acidic compartment. Acidic protons of citraconic acid are converted to water. In the basic compartment acidic protons are generated from water by hydrolysis of the anhydride.

other dicarboxylic acids,[13] this equilibrium shifted by as much as 10^{12}. An experiment with two layers showed that the citraconic anhydride selectively partitioned into a chloroform layer. After equilibration of an aqueous solution of citraconic acid (1.0 M, 0.5 M HCl) with a layer of $CDCl_3$, ^{1}H-NMR analysis showed that the organic layer contained only citraconic anhydride: 65±10 mM anhydride, <2 mM citraconic acid. This selective partitioning further shifted the acid-anhydride equilibrium by an additional factor of over 5000. Citraconic anhydride selectively partitioned into the chloroform because it was more soluble than citraconic acid in chloroform[14] and because the concentration of water was one thousand times lower in the chloroform layer (50 mM H_2O), thereby shifting the equilibrium toward formation of anhydride.

Proton transport by maleic acid occurred by both mechanisms. After equilibration of an aqueous solution of maleic acid (1.0 M, 0.1 M HCl) with a layer of CD_2Cl_2, ^{1}H-NMR analysis showed that the organic layer contained both maleic acid (0.6 mM) and maleic anhydride (0.2 mM). The incomplete formation of anhydride may be due to the less favorable equilibrium constant for dehydration of maleic acid (<0.2 mol %) as compared to citraconic acid (0.6 mol %). Proton transport by succinic and mesaconic acid was slow, and we did not study it further.

The rate of proton transport by citraconic acid increased with increasing temperature, Figure 2C. In the range 15–35 °C, the activation energy was 16 ± 2 kcal/mol. Dehydration of succinic acid and substituted succinic acids to the anhydride showed a similar activation energy (20–22 kcal/mol);[15] therefore, we propose that the rate of dehydration of citraconic acid to the anhydride limited

(13) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183–278.
(14) Acetic anhydride is seventeen times more soluble than acetic acid in wet chloroform: Wolfenden, R.; Williams, R. *J. Am. Chem. Soc.* **1985**, *107*, 4345–4346.

Table 1. Standard Free Energies of Hydrolysis of Several Anhydrides at 25 °C

anhydride	K_{dehyd}[a]	ΔG°[b]	pK_a[c]	$\Delta G^{\circ\prime}_{pH7}$[d]	$\Delta G^{\circ\prime}_{pH10}$[d]
acetic	3.0×10^{-12}	−15.7[e]	4.76	−21.8	−30.0
succinic	1.0×10^{-6}	−8.2[f]	4.19, 5.48	−14.1	−22.3
maleic	<0.002	<−3.7[g]	1.92, 6.23	<−11.8	<−19.8
phthalic	<0.005	<−3.1[h]	2.95, 5.41	<−10.8	<−19.0
citraconic	0.006	−3.0[g]	2.29, 6.15	−10.7	−18.8
dimethylmaleic	5.0	+1.0[i]	3.1, 6.0	−4.4	−12.5
fluorophosphonate	2.0	+0.4[j]	0.55, 4.8[k]	0.04	−3.5

[a] Equilibrium constant for dehydration of the neutral acid to the anhydride. The activity of pure water is 1.0. [b] ΔG° is the standard free energy of hydrolysis in kcal/mol to the neutral carboxylic acid; see ref 18. [c] Jencks, W. P.; Regenstein, J. In *Handbook of Biochemistry*, 2nd ed.; Sober, H. A., Ed.; CRC Press: Cleveland, OH, 1970; p J-193. [d] $\Delta G^{\circ\prime}_{pH7}$ and $\Delta G^{\circ\prime}_{pH10}$ are standard free energies of hydrolysis that take into account the ionization of the carboxylic acid; see ref 18. [e] Jencks, W. P.; Barley, F.; Barnett, R.; Gilchrist, M. *J. Am. Chem. Soc.* **1966**, *88*, 4464−4467. [f] Determined by extrapolation from 60 °C to 25 °C on an Arrhenius plot: Higuchi, T.; Eberson, L.; McRae, J. D. *J. Am. Chem. Soc.* **1967**, *89*, 3001−3004. [g] Measured by ^{1}H-NMR in 1:1 H_2O/D_2O containing HCl (0.5 M). [h] Estimate from: Hawkins, M. D. *J. Chem. Soc., Perkin Trans. 2* **1975**, 282−284. [i] Vesala, A.; Jalava, J.-P. *Acta Chem. Scand. Sect. A* **1977**, *31*, 585−590. [j] Shamakhova, N. N.; Plakhotnik, V. N.; Il'in, E. G. *Koord. Khim.* **1989**, *15*, 1504−1509; *Chem. Abstr.* 1990, *112*, 85342s. [k] van Wazer, J. R. In *Phosphorus and Its Compounds*; Interscience: New York, 1958; pp 801−826.

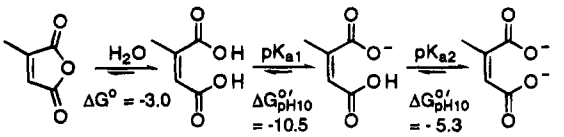
the rate of proton transport in this temperature range. The rate of proton transport using an acidic layer at pH 0.3 was 1.6 times faster than an acidic layer at pH 1.0. This increased rate supports our suggestion of a rate-limiting dehydration because the rate of dehydration of maleic acids increases slightly in more acidic solutions.[16]

In the range 35−45 °C, the rate of proton transport by citraconic acid increased only slightly with temperature; the activation energy was 1.4 ± 2 kcal/mol. Diffusion-limited processes show low activation energies of ~2 kcal/mol;[17] thus, we propose that the diffusion of the anhydride through the chloroform layer limited the rate of proton transport in this temperature range. Diffusion rates may differ in another experimental apparatus so the temperature at which the rate-determining step changes may also differ.

Proton transfer by way of a carboxylic acid anhydride intermediate was an indirect proton transfer. The protons that appeared in the basic compartment upon hydrolysis of anhydride came from water, not from the acidic compartment, Figure 3B. Nevertheless, it is a true proton transfer since a molecule of citraconic acid disappeared from the acidic compartment and the citraconate dianion and two protons appeared in the basic compartment.

This mechanism of proton transport by citraconic anhydride links proton transport across a liquid membrane to the dehydration of citraconic acid. To build a model of a proton pump, we must further link the hydrolysis of citraconic anhydride to the synthesis of molecules with high phosphoryl group transfer potential like acyl phosphates and ATP. Below, we link the hydrolysis of citraconic anhydride to the formation of citraconyl phosphate.

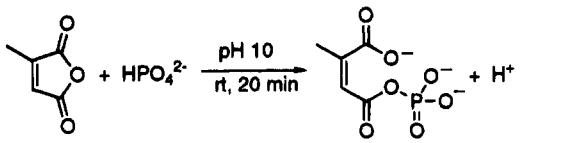
Formation of Citraconyl Phosphate Driven by Proton Transfer. The free energy of hydrolysis of citraconic anhydride at pH 10, $\Delta G^{\circ\prime}_{pH10}$, is −18.8 kcal/mol.[18] This value was calculated stepwise as shown in eq 1. Hydrolysis of the anhydride to the neutral dicarboxylic acid releases 3.0 kcal/mol, ionization of the first carboxylic acid (pK_{a1} = 2.29) releases 10.5 kcal/mol, and ionization



overall $\Delta G^{\circ\prime}_{pH10}$ = -18.8 kcal/mol (1)

of the second carboxylic acid (pK_{a2} = 6.15) releases an additional 5.3 kcal/mol, Table 1. At pH 7, the contribution due to ionization is lower; thus, the free energy of hydrolysis at pH 7 ($\Delta G^{\circ\prime}_{pH7}$) is −10.7 kcal/mol. This free energy of hydrolysis is more negative than that for biological phosphates with high phosphoryl group transfer potential (also called high-energy phosphates). For example, free energies of hydrolysis at pH 7[18] for ATP and for acetyl phosphate are −7.3 and −10.3 kcal/mol, respectively, and at pH 10 they are −11.3 and −13.8 kcal/mol, respectively.[19] Thus, citraconic anhydride is thermodynamically capable of making phosphates with high phosphoryl group transfer potential like ATP, acetyl phosphate, and other acyl phosphates.

In accord with this expectation, citraconic anhydride reacted with aqueous phosphate at pH 10 to give citraconyl phosphate (18−23% yield based on citraconic anhydride) and release one proton, eq 2. Acetic, succinic, and maleic



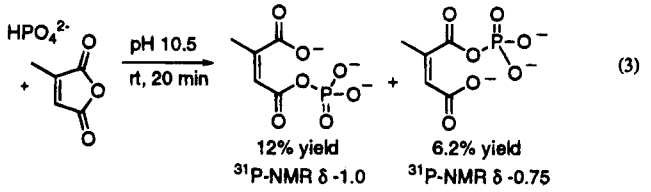
18-23%, ^{31}P-NMR δ -1.0 (2)

anhydrides also react with aqueous phosphate to give the corresponding acyl phosphates.[20,21] All four acyl phosphates showed similar ^{31}P-NMR chemical shifts (citraconyl, δ −1.0; acetyl, δ −1.4; succinyl, δ −1.2; maleyl, δ −1.0) and similar coupling between the carbonyl carbon and phosphorus (citraconyl, $^2J_{C-P}$ = 7.2 Hz; acetyl, 8.1 Hz; succinyl, 8.6 Hz; maleyl, 7.2 Hz). The ^{13}C-NMR of citraconyl phosphate also showed a coupling between the

(15) Higuchi, T.; Eberson, L.; McRae, J. D. *J. Am. Chem. Soc* **1967**, *89*, 3001−3004.
(16) Eberson, L. *Acta Chem. Scand.* **1964**, *18*, 1276−1282.
(17) Gillen, K. T.; Noggle, J. H. *J. Phys. Chem.* **1970**, *53*, 801−809.
(18) Free energies of hydrolysis refer to 1.0 M as the standard state, use 1.0 for the activity of pure water and take into account the ionization of the starting materials and products at the pH indicated. These pH-dependent free energies of hydrolysis refer most directly to the experimental results. Jencks, W. P. In *Handbook of Biochemistry*, 2nd ed.; Sober, H. A., Ed.; CRC Press: Cleveland, OH, 1970; pp J-181−J-186.
(19) Hydrolysis of ATP to ADP and phosphate above pH 7 releases one proton. For this reason hydrolysis becomes more favorable by 1.4 kcal/mol/pH unit at 25 °C as the pH increases (Alberty, R. A. *J. Biol. Chem.* **1969**, *244*, 3290−3902). Similarly, we extrapolated $\Delta G^{\circ\prime}_{pH7}$ for acetyl phosphate to pH 10 using 4.95 for the pK_a of acetyl phosphate (DiSabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4400−4405).
(20) Avison, A. W. D. *J. Chem. Soc.* **1955**, 732−738. Kazlauskas, R. J.; Whitesides, G. M. *J. Org. Chem.* **1985**, *50*, 1069−1076.
(21) Hildebrand, J. G.; Spector, L. B. *J. Biol. Chem.* **1969**, *244*, 2606−2613.

methine carbon (identified by an APT experiment) and phosphorus, $^3J_{C-P}$ = 7.0 Hz, establishing that the phosphoryl group was linked to the carboxylate shown.

At higher pH, 10.5, citraconic anhydride reacted with aqueous phosphate to give two acyl phosphates: ^{31}P-NMR δ −1.0 (12% yield) and −0.75 (6.2% yield), eq 3. These



(3)

two acyl phosphates also formed at pH 11, but in lower yield, 6.4% and 2.1%, respectively. The new acyl phosphate at δ −0.75 was tentatively assigned as the other regioisomer of citraconyl phosphate. To support this suggestion, we examined the reactions of other unsymmetric cyclic anhydrides with aqueous phosphate. All gave two acyl phosphates according to ^{31}P-NMR: methyl succinic anhydride (δ −1.1 and −1.3, 1:1), 2,2-dimethylsuccinic anhydride (δ −1.1 and −1.4, 3:2) and itaconic anhydride (δ -0.8 and −1.2, 4:1). The major regioisomers in the last two cases were those with the phosphoryl group linked to the least sterically-hindered carboxylate according to ^{13}C-NMR.

For citraconyl phosphate, rapid hydrolysis of the δ −0.75 regioisomer may also contribute to the predominance of δ −1.0 regioisomer. The half-life of the δ −0.75 regioisomer at pH 10.5 was only 12 min; at pH 10 and below, we never detected this regioisomer. Due to this instability, all other experiments in this paper involve only the more stable, major regioisomer of citraconyl phosphate.

The stability of citraconyl phosphate (major regioisomer) varied with pH in a manner similar to succinyl and maleyl phosphate and different from that for acetyl phosphate, Figure 4A. A change in the mechansim of hydrolysis accounts for the sharp increase in the rate of hydrolysis of succinyl phosphate below pH 6.[22-24] In basic solution, nucleophilic attack at phosphorus predominates (P—O bond breaking), while below pH 6, intramolecular attack of a carboxylate gives succinic anhydride (C—O bond breaking), Figure 4B. Since citraconyl phosphate also shows a similar sharp increase in the rate of hydrolysis below pH 8.5, we propose that hydrolysis of citraconyl phosphate below pH 8.5 proceeds by way of a citraconic anhydride intermediate.

To demonstrate synthesis of citraconyl phosphate using proton transfer as the driving force, we added potassium phosphate (1.0 M) to the basic compartment of the experiment described in Figure 2A. Some of the citraconic anhydride from the chloroform layer reacted with phosphate, instead of water, to form citraconyl phosphate, Figure 5. The ^{31}P-NMR showed the formation of citraconyl phosphate: 5.7 mM, 5.0% yield based on the amount of citraconic acid transferred. In five similar experiments the yield ranged from 0.6% to 7.0%.

Citraconyl phosphate does not form spontaneously from citraconate and phosphate in aqueous solution. This formation of the citraconyl phosphate trapped some of the energy available in the hydrolysis of citraconic

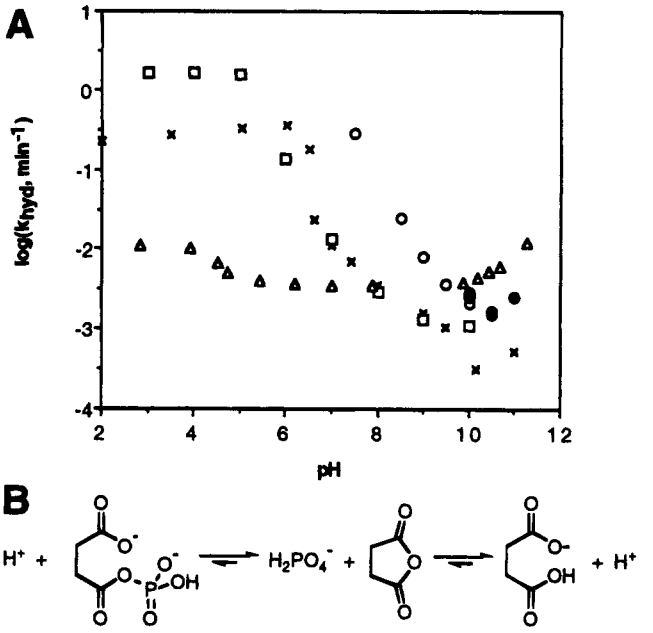


Figure 4. Hydrolysis of acyl phosphates. (A) The rate of hydrolysis of several acyl phosphates as a function of pH. Rates of hydrolysis of citraconyl phosphate (major isomer, ○) and maleyl phosphate (□) were measured at 25 °C by pHstat (open symbols) or by ^{31}P-NMR (filled symbols). The data for acetyl phosphate (△) were taken from the work of Koshland (Koshland, D. E., Jr. *J. Am. Chem. Soc.* **1952**, *74*, 2286–2292) and succinyl phosphate (×) from ref 24. Acyl phosphates of dicarboxylic acids hydrolyze at least 10 times more rapidly in acidic solution than in basic solution, while the hydrolysis of acetyl phosphate varies only slightly in this pH range. (B) The fast hydrolysis of succinyl phosphate below pH 6 proceeds via an anhydride intermediate (ref 22–24). Since pH-dependence of the rate of citraconyl phosphate hydrolysis is similar to that for succinyl phosphate, we propose that citraconyl phosphate also hydrolyzes by way of an anhydride below pH 8.5.

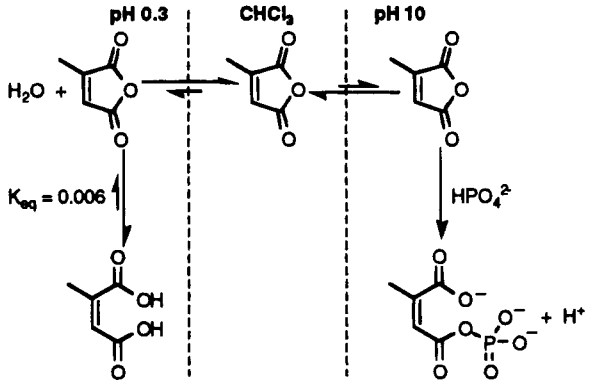


Figure 5. Mechanism for formation of citraconyl phosphate driven by indirect proton transfer from pH 0.3 to pH 10.

anhydride in the form of an acyl phosphate. Acyl phosphates such as acetyl phosphate have high phosphoryl group transfer potential and can make ATP from ADP in enzyme catalyzed reactions. However, we do not know of any kinases that accept citraconyl phosphate, so we demonstrated that citraconyl phosphate has high phosphoryl group transfer potential using nonenzymic phosphoryl transfers.

Transfer of the Phosphoryl Group from Citraconyl Phosphate to Morpholine and to Fluoride. To support the notion that citraconyl phosphate has high phosphoryl group transfer potential, we transferred this

(22) Higuchi, T.; Flynn, G. L.; Shah, A. C. *J. Am. Chem. Soc.* **1967**, *89*, 616–622.
(23) Moore, S. A.; Jencks, W. P. *J. Biol. Chem.* **1982**, *257*, 10874–10881.
(24) Walsh, C. T.; Hildebrand, J. G.; Spector, L. B. *J. Biol. Chem.* **1970**, *245*, 5699–5708.

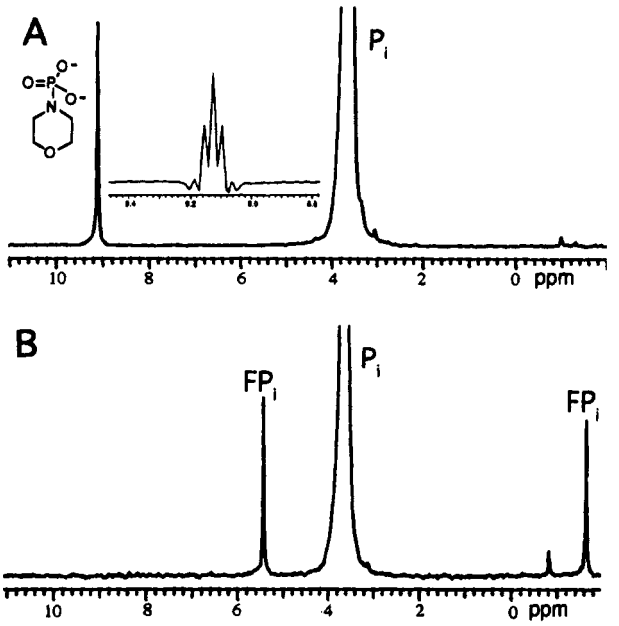
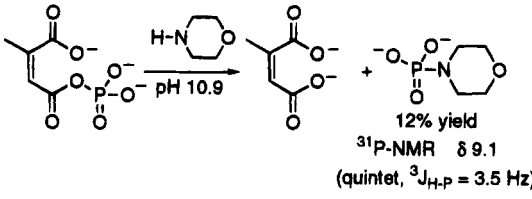


Figure 6. ^{31}P-NMR spectra of the reaction mixtures where the phosphoryl group from citraconyl phosphate transferred to morpholine and to fluoride. (A) Morpholinophosphonate, δ 9.1, formed in 12% yield from the reaction of citraconyl phosphate (170 mM) with morpholine (1.0 M). The inset shows the proton-coupled ^{31}P-NMR spectrum (resolution-enhanced) of the δ 9.1 resonance. (B) Fluorophosphonate, δ 1.9 (d, $^1J_{P-F}$ = 860 Hz), formed in 32% yield from reaction of citraconyl phosphate (160 mM) with 1,4-diazabicyclo[2.2.2]octane (0.5 M) and KF (1.6 M). The resonance at δ −0.8 is a trace of remaining citraconyl phosphate.

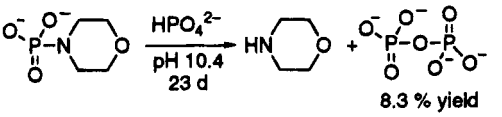
phosphoryl group to morpholine to form a phosphoramidate and to fluoride to form fluorophosphonate. Reaction of citraconyl phosphate (170 mM) and morpholine (1.0 M) gave morpholinophosphonate in 12% yield, based on citraconyl phosphate, eq 4. We identified morpholino-



(4)

phosphonate by its ^{31}P-NMR spectrum: δ 9.1 (quintet, $^3J_{H-P}$ = 3.5 Hz), Figure 6A. This chemical shift ($\delta \sim 10$) is characteristic of phosphoramidates[25,26] and the multiplicity of the hydrogen−phosphorus coupling is consistent with the assigned structure. Succinyl and itaconyl phosphates also reacted with morpholine to form morpholinophosphonate in 8.8% and 37% yield, respectively. Morpholinophosphonate was stable at pH ~10.4 ($t_{1/2}$ = 12.3 d) but hydrolyzed completely at pH 7 overnight, consistent with the known stability of phosphoramidates in basic solution and their lability in acidic solution.[27] Acetyl phosphate also reacts with macrocylic amines to form phosphoramidates;[25,28] however, reaction with morpholine gave only a 2.4% yield of morpholinophosphonate.[29] The major product was 4-acetylmorpholine resulting from attack at the carbonyl.

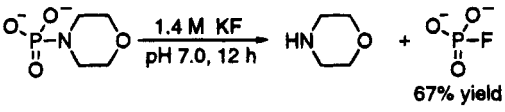
Phosphoramidates have high phosphoryl group transfer potential and have been proposed as high-energy intermediates in enzyme-catalyzed phosphoryl transfer reactions. Herschlag and Jencks estimated the free energy of hydrolysis of $^+H_3NPO_3^{2-}$ to be 12 kcal/mol.[30] Hosseini and Lehn showed that phosphoramidates react with phosphate to form pyrophosphate[25] and with ADP to form ATP.[28] We confirmed that morpholinophosphonate also had high phosphoryl group transfer potential by reacting it with phosphate to form pyrophosphate in 8.3% yield, eq 5.



(5)

In concentrated salt solutions, acetyl phosphate reacted directly with phosphate to give pyrophosphate[31] in up to 10% yield; under similar conditions using citraconyl phosphate we did not detect any pyrophosphate. Our two-step formation of pyrophosphate from citraconyl phosphate (eqs 4 and 5) yielded pyrophosphate in only 1% yield overall; nevertheless, it demonstates that citraconyl phosphate has high phosphoryl group transfer potential.

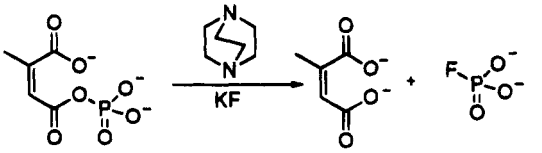
The phosphoryl group from morpholinophosphonate also transferred to fluoride in 67% yield to form fluorophosphonate, which we identified by its ^{31}P-NMR spectrum: δ 1.9 (d, J_{P-F} = 860 Hz), Figure 6B. The reaction of fluoride



(6)

was faster and gave a higher yield than the reaction with phosphate. Herschlag and Jencks[32] also noted an increased reactivity of fluoride in phosphoryl transfer from phosphorylated pyridines and attributed this increase to the high affinity of fluoride for phosphorus.

We could also form fluorophosphonate from citraconyl phosphate in a single reaction, eq 7. Reaction of citraconyl



(7)

phosphate (160 mM) with potassium fluoride (1.6 M) in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO, 0.5 M) at pH 10.6, 25 °C, gave fluorophosphonate in 32% yield, based on the starting amount of citraconyl phosphate. Jencks previously reported that acetyl phosphate transferred its phosphoryl group to fluoride in 34% yield under similar conditions.[29]

(25) Hosseini, M. W.; Lehn, J.-M. *J. Chem. Soc., Chem. Commun.* **1985**, 1155–1157; *J. Am. Chem. Soc.* **1987**, *109*, 7047–7058.
(26) *N*-Phosphoethylenediamine, δ 8.9: Lightcap, E. S.; Frey, P. A. *J. Am. Chem. Soc.* **1992**, *114*, 9750–9755.
(27) Bruice, T. C.; Benkovic, S. J. *Bioorganic Mechanisms*; Benjamin: New York, 1966; Vol. 2, pp 71–85.
(28) Hosseini, M. W.; Lehn, J.-M. *J. Chem. Soc., Chem. Commun.* **1988**, 397–399; **1991**, 451–453.
(29) DiSabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4393–4400.
(30) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1990**, *112*, 1942–1950. For the reaction $H_2O + {}^+H_3NPO_3^{2-} \leftrightarrow HPO_4^{2-} + NH_4^+$ where the activity of pure water is 1.0 and the reactants and products are ionized exactly as shown.
(31) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108*, 7938–7946.
(32) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1990**, *112*, 1951–1956.

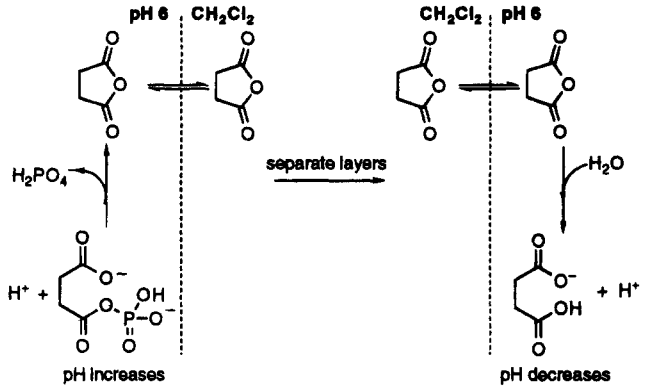


Figure 7. Mechanism for formation of a pH gradient during hydrolysis of succinyl phosphate. At pH 6, succinyl phosphate collapsed to succinic anhydride and phosphate. This reaction consumed a proton so the pH of the solution increased. We extracted the succinic anhydride with dichloromethane and transferred it to another pH 6 solution. Hydrolysis of succinic anhydride released a proton so the pH of this second aqueous solution decreased. This experiment generated differences in pH of up to 1.5 pH units.

We estimated the free energy of hydrolysis of fluorophosphonate using the same stepwise approach that we used for citraconic acid above. From the equilibium constant for hydrolysis (2.0 in acidic solution[33]) and the pK_a's (0.55, 4.8[34]), we estimated the free energy of hydrolysis at pH 7 and pH 10 to be 0 and −3.5 kcal/mol, respectively. Although hydrolysis of fluorophosphonate is favorable at pH 10, it is not sufficiently favorable to drive the synthesis of ATP. Fluorophosphonate possesses only moderate phosphoryl transfer potential.

This ability to form two species with high phosphoryl group transfer potential, morpholinophosphonate and pyrophosphate, as well as a molecule with moderate phosphoryl group transfer potential, fluorophosphonate, confirms that citraconyl phosphate contains an activated phosphoryl group.

Formation of a pH Gradient Driven by Hydrolysis of Succinyl Phosphate. Hydrolysis of succinyl and phthalyl phosphate below pH 6 proceeds via the carboxylic acid anhydride, Figure 4B.[22-24] If the starting succinyl phosphate exists as the dianion as shown, then the first step, formation of the carboxylic acid anhydride, consumes 1 equiv of protons. The second step, hydrolysis of the carboxylic acid anhydride, releases 1 equiv of protons. No net consumption or release of protons occurs, but if the two steps occur in sequence then the pH should first increase then decrease back to the original. In reality, succinyl phosphate at pH 6.0 probably exists as a mixture of dianion and monoanion[35] so that less than 1 equiv of protons is consumed. Hydrolysis of succinic anhydride to the acid (pK_a = 4.19, 5.48) releases 1.5 equiv of protons at pH 6; thus, the net reaction releases protons.

To model the formation of a pH gradient we used extraction to separate the formation and hydrolysis of succinic acid. We added 1 N HCl to a solution of succinyl phosphate (0.39 M, 3.9 mmol) containing succinate (0.39 M) and K_2HPO_4 (0.38 M) to decrease the pH to 6.00 and immediately extracted this solution with dichloromethane (3 × 20 mL). After extraction, the pH of the original solution had increased to 6.16. A back-titration indicated that 0.59 mmol of protons had been consumed. The combined dichloromethane extracts were equilibrated with succinate buffer (20 mM, pH 6.05) for 1 h. The pH of this second aqueous layer decreased to 5.23. A back-titration indicated that 0.36 mmol of protons had been released. This 0.88 unit difference in pH of the two solutions came from the hydrolysis of succinyl phosphate. In another experiment, the pH increased to 6.23 in the original solution and dropped to 5.49 in the second solution.

Five experiments using an initial pH of 5.00 yielded similar results. The observed pH changes for the first aqueous layer after extraction ranged from a decrease in pH to 4.97 to an increase in pH to 5.23. The decrease corresponded to a net release of 0.2 mmol protons, indicating an inefficient extraction of succinic anhydride, while the increase corresponded to a net consumption of 1.26 mmol of protons. The pH of the second layer decreased in all five experiments to between 4.37 and 3.64, corresponding to a release of 0.51−1.72 mmol of protons. The number varied depending on the efficiency and speed of the extraction procedure. Thus, the extraction procedure separated the two steps of the hydrolysis of succinyl phosphate, thereby allowing the pH to increase in one solution and decrease in the other. These experiments demonstrated that the hydrolysis of an acyl phosphate can also generate a pH gradient.

Discussion

This work describes the first synthesis of an acyl phosphate from a carboxylic acid and phosphate where the energy for the dehydration reaction comes from a proton gradient. These reactions help define the thermodynamic requirements of a proton pump. The pH of the two layers differed by nine units, which corresponded to an available energy of 12.3 kcal/mol at 25 °C for each proton transferred. Citraconic acid anhydride transferred two protons; thus, 24.5 kcal/mol was available. Hydrolysis of citraconic anhydride in the basic layer released this energy as heat. However, when the basic layer contained phosphate, the formation of citraconyl phosphate captured some of this energy. We estimate that the cost of making citraconyl phosphate from citraconate and phosphate at pH 10 is ~14 kcal/mol, similar to that for acetyl phosphate (13.8 kcal/mol). The sum of these two reactions, Figure 8, shows that thermodynamics favors the overall reaction by ~11 kcal/mol.[36]

We demonstrated that the phosphoryl group in citraconyl phosphate is indeed an activated phosphoryl group by transferring it to morpholine and to fluoride. The phosphoryl group of morpholinophosphonate further transferred to phosphate to form pyrophosphate. This ability to make phosphates known to have high phosphoryl transfer potential from citraconyl phosphate confirms that it is a high-energy intermediate. Some proton pumps in bacteria and plants, known as H^+-PPases,[37] use pyro-

(33) Shamakhova, N. N.; Plakhotnik, V. N.; Il'in, E. G. *Koord. Khim.* **1989**, *15*, 1504−1509; *Chem. Abstr.* **1990**, *112*, 85342s.

(34) van Wazer, J. R. In *Phosphorus and Its Compounds*; Interscience Publishers: New York, 1958; pp 801−26

(35) We esimate pK_{a3} to be 6.6 from the sharp rise in the rate of hydrolysis of succinyl phosphate at this pH. The value of pK_{a2} is not known; however, since the structure of succinyl phosphate is similar to glutaric acid (pKa = 4.34, 5.42), we estimate that pK_{a2} is near 5, suggesting significant amounts of both monoanion and dianion.

(36) Our reaction also contained a small (~24×) gradient of citraconic acid: 980 mM in the acid phase, 40 mM in the basic phase after 10 h. We do not believe this gradient contributes significantly to the overall energy balance compared to the proton gradient of 10^9 and did not include the citraconic acid gradient in the energy analysis.

(37) Reviews: Baltscheffsky, M.; Nyren, P. *Methods Enzymol.* **1986**, *126*, 538−545. Rea, P. A.; Poole, R. J. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44*, 157−180.

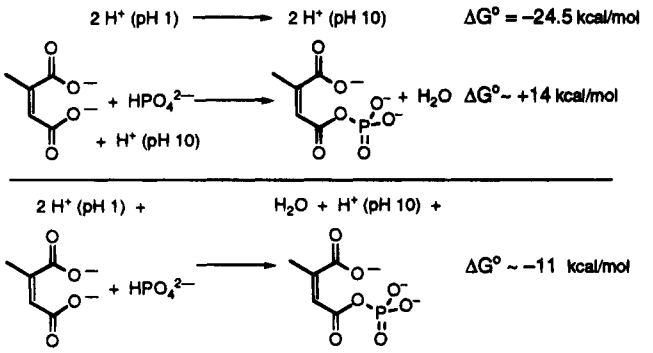


Figure 8. Energy balance for formation of citraconyl phosphate driven by proton transport. Transport of two protons from pH 1 to pH 10 releases 24.5 kcal/mol. We estimate that dehydration of citraconate and phosphate to citraconyl phosphate costs ~14 kcal/mol because the synthesis of acetyl phosphate at pH 10 costs 13.8 kcal/mol. Note that the formation of an acyl phosphate at pH 10 consumes a proton. Thermodynamics favors the net reaction, transport of two protons from pH 1 to 10 coupled to the formation of citraconyl phosphate at pH 10, by ~11 kcal/mol. Note that the net reaction releases only one proton at pH 10.

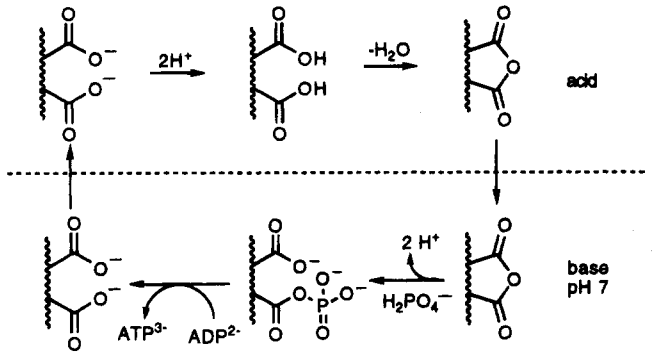


Figure 9. Proposed mechanism for a proton pump involving carboxylic acid anhydride and acyl phosphate intermediates. On the acid side of the membrane, protons add to two carboxylates. If the carboxyl moieties are held close to one another by the protein, then dehydration to the anhydride may be facile. Next, a conformational change transfers, or exposes, the anhydride group to the basic side of the membrane. Phosphate reacts with the anhydride to form an acyl phosphate, which then transfers the phosphate to ADP forming ATP. Finally, a second conformational change returns the carboxylates to the original state. A reverse reaction, where ATP is hydrolyzed in order to pump protons, could occur by following the reactions in the opposite direction.

phosphate as the energy source, so the formation of pyrophosphate directly mimics a biologically occurring pump.

We have also demonstrated the reverse process, the formation of a proton gradient during the hydrolysis of another acyl phosphate, succinyl phosphate. We used succinyl phosphate for this experiment because it is more stable than citraconyl phosphate and therefore easier to handle.

Our model also permits us to propose a chemically and thermodynamically reasonable path for a P-type H+-ATPase in Figure 9. In the clockwise direction, downhill proton transport drives ATP synthesis; in the counterclockwise direction hydrolysis of ATP drives uphill proton transport. The first step in the clockwise direction is formation of a carboxylic acid anhydride. The folding of the protein may place the carboxylic acid moieties of aspartate or glutamate residues close to one another so that an anhydride would form easily. This formation of the anhydride is more thermodynamically favorable in acidic solution where the carboxylates are protonated. A conformational change could either move this anhydride to the basic side of the membrane or rearrange the protein to expose the anhydride to the basic side. Reaction with phosphate to form an acyl phosphate, followed by transfer of the phosphoryl group to ADP, makes ATP and releases two protons. Another conformational change returns the carboxylates to the starting point. This mechanism agrees with currently proposed mechanism for P-type ATPases because it involves two conformations and an aspartyl phosphate intermediate.

Our model has demonstrated the feasibility of several of these steps. First, we showed the formation of the anhydride, and its reaction with phosphate to form an acyl phosphate. Next, we transferred the phosphoryl group of citraconyl phosphate to several nucleophiles, but did not show transfer to ADP to form ATP. Others have demonstrated enzyme-catalyzed phosphorylation of ADP by acetyl phosphate and other acyl phosphates so this step is reasonable. Our model emphasizes that the chemical potential of a carboxylic acid anhydride depends on the pH of the solution.

An unusual feature of our model is the indirect mechanism of proton transport by way of a carboxylic acid anhydride intermediate. Although some biological proton transfers[38] are direct, for example, by transfer of a fatty acid (COOH form) across a bilayer,[39] others may be indirect. For example, CO_2, the anhydride of carbonic acid, crosses cell membranes rapidly and releases acid upon hydration. No one knows whether the P-type H+-ATPases catalyze direct or indirect proton transport, but since the P-type ATPases transport metal ions like Na^+, K^+, and Ca^{2+} across the cell membranes directly, most researchers assume that H+-ATPases also catalyze direct transport. Currently, there is no evidence for or against an anhydride intermediate in the mechanism of action of P-type H+-ATPases.

Although our model helps chemists understand the thermodynamic requirements for a H+-ATPase, it does not attempt to mimic any of the kinetic features of a P-type ATPase. The kinetic mechanism of our model differs significantly from the kinetic mechanism of a biological pump. Our model relies on diffusion of a soluble molecule across an organic layer, while the biological pump uses a conformational change in a transmembrane protein. Our model uses an indirect proton transport, while at least some biological pumps use direct transport. Our model relies on uncatalyzed phosphoryl transfers and suffers from competing hydrolysis, while the biological pump accelerates and directs phosphoryl transfer and limits the competing hydrolysis. Answers to these kinetic questions will probably come from direct study of the biological pumps.

Experimental Section

General. Citraconic acid and anhydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetyl,[20] maleyl, and succinyl phosphates and methyl succinyl phosphates[21,24] were prepared as described previously. These acyl phosphates were characterized in solution by ^1H-, ^{13}C-, and ^{31}P-NMR, Table 2. The supplementary material contains copies of several of

(38) *Proton Passage across Cell Membranes*; Bock, G., Marsh, J., Eds.; Ciba Foundation Symposium 139; John Wiley: Chichester, 1988.
(39) Kamp, F.; Hamilton, J. A. *Biochemistry* **1993**, *32*, 11074−11086.

Table 2. NMR Data for Carboxylates and Acyl Phosphates[a]

carboxylate/acyl phosphate	^{31}P-NMR, δ	^{1}H-NMR, δ	^{13}C-NMR, δ
acetate		1.92 (s, 3H)	23.3 (s), 181.4 (s)
acetyl phosphate	−1.4 (s)	2.11 (s, 3H)	22.1 (d, $^{3}J_{C-P}$ = 4.5 Hz). 172.2 (d, $^{2}J_{C-P}$ = 8.1 Hz)
succinate		2.41 (s, 4H)	34.1 (s), 182.3 (s)
succinyl phosphate	−1.2 (s)	2.46 (br t, 2H), 2.62 (br t, 2H, ^{3}J = 6.9 Hz)	31.8 (d, $^{3}J_{C-P}$ = 4.9 Hz), 32.0 (s), 173.3 (d, $^{2}J_{C-P}$ = 8.6 Hz), 181.5 (s)
maleate		6.04 (s, 2H)	130.4 (s), 175.3 (s)
maleyl phosphate	−1.0 (s)	5.86 (d, 1H, ^{3}J = 12.0 Hz), 6.65 (d, 1H)	119.3 (d, $^{3}J_{C-P}$ = 6.7 Hz), 142.1 (s), 164.4 (d, $^{2}J_{C-P}$ = 7.2 Hz), 175.5 (s)
citraconate[b]		1.92 (d, 3H), 5.5 (q, 1H, ^{4}J = 1.6 Hz)	20.3 (s), 120.1 (s), 148.3 (s), 174.1 (s), 179.9 (s)
citraconyl phosphate[b]	−1.0 (s)	2.03 (d, 3H), 5.6 (q, 1H, ^{4}J = 1.6 Hz)	20.7 (s), 113.9 (d, $^{3}J_{C-P}$ = 7.0 Hz), 156.8 (s), 164.4 (d, $^{2}J_{C-P}$ = 7.2 Hz), 178.9 (s)
methyl succinate[c]			17.2 (s), 40.1 (s), 42.3 (s), 181.8 (s), 185.5 (s)
methyl succinyl phosphate[c]	−1.1 (s), −1.3 (s)		16.2 (s), 17.4 (s), 38.2 (d, $^{3}J_{C-P}$ = 4.6 Hz), 38.8 (s), 39.8 (d, $^{3}J_{C-P}$ = 4.4 Hz), 40.9 (s), 172.8 (d, $^{2}J_{C-P}$ = 8.4 Hz), 176.1 (d, $^{2}J_{C-P}$ = 9.2 Hz), 181.0 (s), 184.9 (s)
2,2-dimethyl succinate[c]		1.15 (s, 6H), 2.38 (s, 2H)	25.4 (s), 41.9 (s), 48.4 (s), 181.2 (s), 187.1 (s)
2,2-dimethyl succinyl phosphate[c]	−1.1 (s), −1.4 (s)	1.18 (s, 6H), 1.23 (s, 6H), 2.48 (s, 2H), 2.59 (s, 2H)	24.7 (s), 25.6 (s), 41.5 (d, $^{3}J_{C-P}$ = 4.8 Hz), 41.6 (s), 46.1 (d, $^{3}J_{C-P}$ = 4.2 Hz), 47.5 (s), 172.1 (d, $^{2}J_{C-P}$ = 9.0 Hz), 177.8 (d, $^{2}J_{C-P}$ = 9.9 Hz), 180.3 (s), 186.3 (s)
itaconate[d]		3.16 (s, 2H), 5.38 (m, 1H), 5.85 (m, 1H)	42.0 (s), 121.9 (s), 142.0 (s), 175.7 (s), 180.5 (s)
itaconyl phosphate[d]	−0.8 (s), −1.2 (s)	3.20 (s, 2H), 3.38 (s, 2H), 5.52 (m, 1H), 5.77 (m, 1H), 5.94 (m, 1H), 6.33 (m, 1H)	40.0 (d, $^{3}J_{C-P}$ = 4.9 Hz), 40.7 (s), 124.0 (s), 128.9 (s), 136.9 (d, $^{3}J_{C-P}$ = 4.5 Hz), 139.8 (s), 166.2 (d, $^{2}J_{C-P}$ = 8.0 Hz), 172.1 (d, $^{2}J_{C-P}$ = 8.8 Hz), 175.0 (s), 179.7 (s)

[a] ^{1}H-NMR spectra were run at 200 MHz in 1:1 H_2O/D_2O at pH 10.0. ^{13}C-NMR spectra were run at 50 MHz in 1:1 D_2O/H_2O at pH 9.0. ^{31}P-NMR spectra were run at 121 MHz in H_2O at pH 10.0. ^{31}P-NMR and ^{13}C-NMR spectra were ^{1}H-decoupled. [b] ^{13}C NMR (75 MHz), 3.8:1 H_2O/D_2O, pH 9.0. [c] ^{13}C NMR (75 MHz), 1:1 D_2O/H_2O, pH 10.0. [d] ^{13}C NMR (75 MHz) 4.2:1 H_2O/D_2O, pH 10.0.

these spectra. Chemical shifts for ^{1}H-NMR were referenced to internal dioxane at 3.76 ppm or to 3-(trimethylsilyl)propanesulfonic acid, DSS, at 0 ppm. Internal dioxane at 66.5 ppm was used as the reference for ^{13}C-NMR. Chemical shifts for ^{31}P-NMR spectra were referenced to external 85% phosphoric acid at 0 ppm. ^{31}P-NMR spectra were acquired without a relaxation delay unless otherwise indicated; we estimate an integration error ±10% based on comparison with spectra acquired with a 30-s relaxation delay.

Citraconyl Phosphate. Citraconic anhydride (0.90 g, 8.0 mmol) was added in four portions over 10 min to dipotassium phosphate buffer (8.0 mL, 1.0 M, pH 10.0) at room temperature. The pH of the reaction mixure was maintained at 10.0 using a pHstat, which controlled the addition of KOH solution (5.0 M). Reaction was complete in ~20 min as measured by the slowing in the release of acid. Citraconyl phosphate was characterized in solution by NMR, Table 2. The actual ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra are included in the supplementary material.

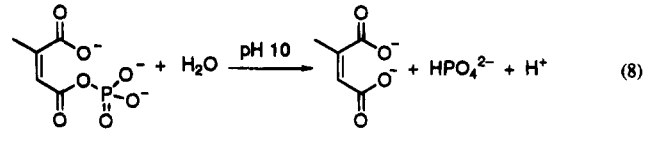
Itaconyl phosphates were prepared as a 4:1 mixture of regioisomers by a literature method[21] and characterized in solution by NMR, Table 2. The actual ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra are included in the supplementary material. The major regioisomer came from attack of phosphate at the least hindered carbonyl as shown by the $^{3}J_{C-P}$ coupling to the methylene carbon in the ^{13}C-NMR.

2,2-Dimethylsuccinyl phosphates were prepared as a 3:2 mixture of regioisomers by a literature method[21] and characterized in solution by NMR, Table 2. The actual ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra are included in the supplementary material. The major regioisomer came from attack of phosphate at the least hindered carbonyl as shown by the $^{3}J_{C-P}$ coupling to the methylene carbon in the ^{13}C-NMR. The resonance for the methylene carbon was identified by a DEPT experiment.

Rate of Proton Transfer through an Organic Layer. A glass tube was clamped ~2 mm above the bottom of a 100-mL beaker, containing dichloromethane (25 mL) and a stirring bar. Acidic solution (0.1 M HCl, 20 mL) was added to the outside compartment, and then buffer (50 mM K_2CO_3, pH 10.0, 25 mL) was added to the inside compartment, and finally additional acidic solution (20 mL) was added to the outside compartment to equalize the level of the dichloromethane at both aqueous interfaces. The top of the apparatus was completely covered with parafilm. The dichloromethane layer was stirred with a magnetic stirrer (110 ± 10 rpm). The initial rate of proton transport (<0.004 mmol H^+/h) was measured by the volume of 5.0 M KOH required to maintain the pH at 10.0 using a pHstat. When the HCl solution was replaced by a solution of carboxylic acid (0.1−1.0 M) and HCl (0.1 M), protons transferred more rapidly as shown in Figure 2B. A detailed diagram of this apparatus is contained in the supplementary material of ref 12.

Activation Energy for the Citraconic Acid-Mediated Proton Transfer. The initial rate of proton transfer was measured as above (1.0 M citraconic acid, pH 0.3/$CHCl_3$/1.0 M K_2HPO_4, pH 10) using a separate experiment for each temperature. The first-order rate constants at each temperature were derived from the slope of a plot of $\ln(A/A_o)$ as a function of time where A_o represents initial amount of citraconic acid in the acidic aqueous phase (40 mmol) and A represents the amount remaining. The error limits for activation energy are twice the standard error.

Decomposition of Citraconyl Phosphate. (a) pHstat. The rate of release of one proton upon hydrolysis of citraconyl phosphate, eq 8, was monitored at pH 10 for >5 half-lives by



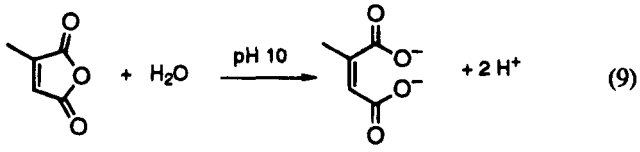
(8)

pHstat which controlled the addition of KOH solution (5.0 M). **(b) ^{31}P-NMR.** Aliquots (500 μL) were taken every 30 min from a solution of citraconyl phosphate maintained at pH 10.0 by pHstat. The decrease in the area of the citraconyl phosphate was monitored by ^{31}P-NMR for 1 half-life. The decomposition followed first-order kinetics in both cases. Data are summarized in Figure 4A.

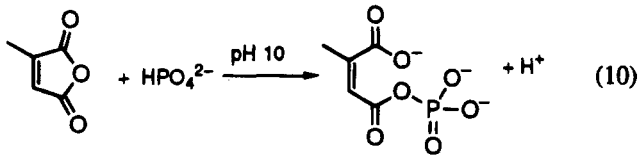
Formation of Citraconyl Phosphate Driven by a Proton Gradient. The tube in a beaker apparatus described above was prepared using phosphate buffer (1.0 M K_2HPO_4, pH 10.0) as the basic layer, aqueous citraconic acid (1.0 M) containing HCl (0.5 M) as the acidic layer, and chloroform as the organic layer. The initial rate of proton transfer was 0.29 mmol H^+/h. After 20 h, ^{31}P-NMR (40° pulse (5.3 ms), ~1100 transients, 0.6-s acquisition time, no relaxation delay) of an aliquot from

the inner compartment showed citraconyl phosphate, δ −1.0, 5.7 mM, 0.15 mmol. The signal to noise ratio was 22:1 for citraconyl phosphate. These NMR spectrum acquisition parameters may have underestimated the amount of citraconyl phosphate by 10−20% because spectra of more concentrated solutions of citraconyl phosphate acquired with a 60-s delay (10 transients) showed 10−20% more citraconyl phosphate than those acquired without a delay. Nevertheless, we did not use a delay with dilute samples of citraconyl phosphate because hydrolysis of citraconyl phosphate during acquisition would offset any increase due to more complete relaxation. No correction was made for this possible underestimation.

The total amount of citraconic anhydride transferred from the acidic layer was determined from the pHstat. Since hydrolysis of citraconic anhydride liberates two protons, eq 9,



(9)



(10)

while formation of citraconyl phosphate liberates one proton, eq 10, the total amount of protons released is $2CA_B + CP$, where CA_B is the amount of citraconic acid in the basic compartment and CP is the amount of acyl phosphate in the basic compartment.

Since ^{31}P-NMR gave 0.15 mmol for CP and the total amount of base titrated was 5.77 mmol, CA_B was 2.81 mmol. The total amount of citraconic acid transferred from the acidic compartment was 3.0 mmol (7.4%). The yield of citraconyl phosphate was therefore 5.0%. In five similar experiments the yield ranged from 0.6% to 7.0%.

Transfer of Phosphoryl Group from Citraconyl Phosphate to Morpholine. Morpholine (0.88 mL, 10.0 mmol) was added to a freshly-prepared solution of citraconyl phosphate (170 mM, 10.0 mL) and allowed to react overnight at room temperature. The initial concentration of citraconyl phosphate was determined by ^{31}P-NMR using a relaxation delay of 60 s. The initial pH of the reaction mixture, 10.87, decreased to 10.63 the next day. The ^{31}P-NMR spectrum revealed a new peak at δ 9.1 (quintet, $^3J_{P-H}$ = 3.5 Hz), in 11.5% yield, based on the starting amount of citraconyl phosphate.

Transfer of the Phosphoryl Group from Morpholinophosphonate to Fluoride. Potassium fluoride (1.4 M) was added to a solution of morpholinophosphonate, prepared as above, and the pH was lowered to 7.0. After reaction overnight at room temperature, ^{31}P-NMR revealed the formation of fluorophosphonate (δ 1.9 (d, $^1J_{P-F}$ = 860 Hz)) in 67% yield based on the starting amount of citraconyl phosphate.

Transfer of Phosphoryl Group from Citraconyl Phosphate to Fluoride. 1,4-Diazabicyclo[2.2.2]octane (DABCO, 0.56 g., 5.0 mmol) and anhydrous potassium fluoride (0.96g, 16.5 mmol) were added to a freshly prepared solution of citraconyl phosphate (160 mM, 10.0 mL) at pH 10.0 and allowed to react overnight at room temperature. The initial concentration of citraconyl phosphate was determined by ^{31}P-NMR using a relaxation delay of 60 s. The initial pH of the reaction mixture, 10.84, had decreased to 10.60 the next day. A ^{31}P-NMR spectrum acquired using a relaxation delay of 60 s revealed the formation of fluorophosphonate in 32% yield, based on the starting amount of citraconyl phosphate.

Making the pH Gradient. A solution of succinyl phosphate (0.38 M, 10 mL, 3.8 mmol) containing succinate (0.39 M) and K_2HPO_4 (0.39 M) was brought down from pH 9.8 to pH 4.99 with 1 N HCl and rapidly extracted (3 × 20 mL) with CH_2Cl_2. The initial concentration of succinyl phosphate was determined by ^{31}P-NMR using a relaxation delay of 30 s. Immediate analysis of this aqueous solution revealed that that the pH had gone up slightly to 5.04 (back-titration indicated that 0.26 mmol protons had been consumed). The combined CH_2Cl_2 extracts (60 mL) were then equilibrated with 20 mM NaOAc buffer, pH 5.00 (30 mL) for approximately 1 h. Following the separation of the CH_2Cl_2 layer, the pH of the sodium acetate buffer was 3.86 (1.04 mmol protons had been released).

Acknowledgment. We thank NSERC Canada for financial support, Dr. Françoise Sauriol for help with the NMR analysis, and Prof. Tom Fyles (University of Victoria) for critically reading the manuscript.

Supplementary Material Available: Selected spectra (^{1}H, ^{13}C, and ^{31}P NMR) of acyl phosphates (18 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.