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6. Intracellular cobalt injections were made according to the technique developed by R. M. Pitman, C. D. Tweedle, and M. J. Cohen [*Science* **176**, 412 (1972)] and repeated by M. Castel, M. E. Spira, I. Parnas, and Y. Yarom [*J. Neurophysiol.* **39**, 900 (1976)].
7. The reason for this "erroneous" growth of the axons is unknown. However, it must be pointed out that during nerve cord sectioning the two cut ends are separated from each other.
8. The input resistance of the giant axons is voltage-dependent, showing delayed rectification at values positive to −75 mV. Therefore, the values were measured at membrane potential values below −90 mV [M. E. Spira *et al.* (*5*)].
9. It is expected that if the membrane properties of the sealed tip are normal then the input resistance measured at this site should be almost twice normal [J. J. B. Jack, D. Nobel, R. W. Tsein, in *Electric Current Flow in Excitable Cell* (Clarendon, Oxford, 1975), pp. 67-72]. We have computed the expected input resistance of the cut axon, taking into account the geometry of the axon 10 to 19 days after axotomy, using Rall's approximations [W. Rall, *Ann. N.Y. Acad. Sci.* **96**, 1071 (1962)]. In our computations we assumed normal membrane resistance for the sprouts. The calculations show that the input resistance is expected to be larger than that in Fig. 1G. This discrepancy indicates that at least part of the membrane of the sprouts had a lower resistance than normal.
10. Normal solution contained: NaCl, 214 n*M*; $CaCl_2$, 9 n*M*; tris, 2 m*M*; and glucose, 1 mg/ml at *p*H 7.4.
11. 4-Aminopyridine is a selective blocker of potassium channels in the giant axons of the cockroach [Y. Pichon, in *Prospective in Experimental Biology*, P. Spencer Davies, Ed. (Pergamon, Oxford, 1976), pp. 297-312].
12. We could not replace all of the Ca^{2+} as the axons deteriorated rapidly. It should be emphasized that this method only allows a comparison of the effects of removal of Ca^{2+} on membrane conductance at various times after axotomy. Extracellular Ca^{2+} is known to affect the conductance of other ions [K. Krnjevic and A. Lisiewicz, *J. Physiol. (London)* **225**, 363 (1972); R. W. Meech, *Comp. Biochem. Physiol. A* **42**, 493 (1972); C. B. Heyer and H. D. Lux, *J. Physiol. (London)* **262**, 319 (1976)].
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Gap Junctional Conductance Is a Simple and Sensitive Function of Intracellular *p*H

Abstract. *The* p*H of the cytoplasm* (pH_i) *measured with* p*H-sensitive microelectrodes in cleavage-stage blastomeres of amphibian (*Ambystoma*) and teleost (*Fundulus*) embryos is about 7.7. In electrotonically coupled cell pairs, junctional conductance is rapidly and reversibly reduced by acidification of the cytoplasm. The relation between junctional conductance and* pH_i *is the same for increasing and decreasing* p*H and is independent of the rate of change over a wide range. The relation is well fitted by a Hill curve with* K = 50 n*M* (p*K* = 7.3) *and* n = 4 *to* 5. *The closure of gap junction channels at low* pH_i *appears to be a cooperative process involving several charged sites. The absence of hysteresis and identity of effects for fast and slow* pH_i *changes implies that protons act directly on the channel macromolecules and not through an intermediate in the cytoplasm.*

Gap junctions are close appositions of cell membranes where polygonal aggregates of intramembrane particles are in one-to-one correspondence in the apposed cells (*1*). The particles are composed of proteins that form hydrophilic channels connecting the cytoplasms of the two cells. The channels permit passage of molecules whose maximum molecular weight is near 1000 and whose diameter can be somewhat greater than 1.0 nm (*1*, *2*). In excitable cells, gap junctions mediate intercellular transmission of electrical signals. In tissues that are not excitable, the junctions allow intercellular spread of nutrients and metabolites and may also transmit chemical messages (*3*). For both excitable and inexcitable cells, treatments that depress junctional conductance would be expected to disturb coordinated physiological functioning of the tissue.

In previously reported experiments on various tissues, exposure of coupled cells to saline containing high CO_2 concentrations reduced electrotonic coupling (*4*-*7*). Exposure of coupled cells to membrane-impermeant acids was without effect on coupling, suggesting that the effect of CO_2 was mediated by acidification of the cytoplasm. In some experiments, cytoplasmic acidification by CO_2 was confirmed by direct measurement of intracellular *p*H (*p*H_i) (*5*, *6*). Although uncoupling was shown to be at least partially due to decreased junctional conductance, the interpretation of most of these experiments was complicated by failure to measure junctional conductance directly (*4*, *6*).

We now report that *p*H_i and junctional conductance are strictly related during cytoplasmic acidification with weak acids. This relation suggests a direct interaction of protons with the macromolecules that comprise the junctional channels. The same treatments produce changes in nonjunctional conductance that are not simple or consistent functions of *p*H_i. The coupling coefficients (*8*), which depend on the conductances of both junctional and nonjunctional membranes, are therefore not strictly dependent on *p*H_i.

Embryos from *Ambystoma mexicanum* (late blastula stage) and *Fundulus heteroclitus* (32- to 64-cell stage) were used for these studies. Single cells or cell pairs were mechanically dissociated in saline (*9*) containing up to 0.05 percent colchicine to inhibit mitosis. Within 30 minutes after the single cells were reassociated as pairs, a steady-state level of coupling developed. Each cell of a pair was penetrated with separate current and voltage microelectrodes (3*M* KCl, 5 to 20 megohms). In addition, one cell of each pair was penetrated with a Thomas-type recessed-tip *p*H microelectrode (*10*). Current pulses were passed alternately into each cell; measurements of input and transfer resistances allowed calculation of the junctional conductance (g_j) and of the nonjunctional conductances of the two cells (g_1 and g_2) by application of the π-*t* transform (*11*). Pulses were sufficiently brief that the voltage dependence of g_j in *Ambystoma* (*12*) could be neglected.

An experiment typical of those performed on pairs of blastomeres is shown in Fig. 1. The coupling coefficients for cell pairs were generally 0.8 to 0.9 at normal *p*H_i [*Ambystoma*: *p*H_i = 7.75 ± 0.06 standard deviation (S.D.) (*13*), *N* = 9; *Fundulus*: *p*H_i = 7.67 ± 0.06 S.D., *N* = 9]. Brief application of physiological saline equilibrated with 100 percent CO_2 (arrows, Fig. 1, top) decreased the internal *p*H. The coupling decreased and then dis-

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appeared while the input conductance decreased. When the cells were rinsed with normal saline, pH_i slowly returned toward normal, and coupling was restored. A graph of pH_i and the logarithms of junctional and nonjunctional conductance each plotted as a function of time shows that the large change in g_j closely parallels the change in pH_i and that the change in g_1 and g_2 is much smaller (Fig. 1, bottom).

Figure 2A shows the relation between g_j and pH_i obtained from the data of the experiment of Fig. 1. The junctional conductance decreased along a smooth S-shaped curve. The values obtained during CO_2 washout and recovery of normal pH_i lay on the same curve as those obtained during CO_2 exposure and decreasing pH_i. The times of decrease and recovery of pH_i were 20 and 300 seconds, respectively.

Cytoplasmic hydrogen ions may act on junctional channels by neutralization of negatively charged groups. Protonation of n titratable sites may convert a channel from a conducting form (Ch*) to a nonconducting form (ChH_n) by the reaction:

$$\underset{\text{open}}{Ch^*} + nH^+ \rightleftharpoons \underset{\text{closed}}{ChH_n}$$

If this reaction occurs, hydrogen ion activity [H^+] should affect membrane conductance according to a form of the Hill equation (*14*)

$$G_j = K^n/(K^n + [H^+]^n) \qquad (1)$$

where G_j is the junctional conductance normalized with respect to its maximum value and K is the apparent dissociation constant. The experimental data are well approximated by this relation for K = 50 nM (apparent pK = 7.3) and for n between 4 and 5 (*15, 16*). In this formulation, n (the Hill coefficient) can be considered to represent the number of strongly cooperative titratable sites, although a larger number of sites that are less cooperative with regard to proton binding would give the same Hill coefficient (*17*).

If a channel consists of two opposed hemichannels, one in each cell membrane (*18*), and each hemichannel is closed independently by H^+, the fraction of channels open, which equals the normalized conductance, would be given by the square of the fraction of each membrane's hemichannels that was open. Hence

$$G_j = K^{2n}/(K^n + [H^+]^n)^2 \qquad (2)$$

Equation 2 fits the data about as well as Eq. 1, with pK about 7.2 and n between 3.5 and 4.0.

The nonjunctional conductances for the experiment illustrated in Fig. 1 are plotted as a function of pH_i in Fig. 2B. When CO_2-equilibrated saline is applied, both conductances drop abruptly before the cytoplasm is acidified. As pH_i decreases and then recovers during rinsing, the conductances do not have a simple relation to pH_i.

One of the two coupling coefficients (k_{21}) from the data in Fig. 1 is plotted as a function of pH_i in Fig. 2C. The smooth curve shows a plot of Eq. 1 for pK = 7.3 and n = 4.5. With regard to the effect of CO_2, the coupling coefficient is a poor index of junctional conductance. The hysteresis in the relation between cytoplasmic pH and coupling coefficient is due to the hysteresis of the effect of CO_2 on conductance of the nonjunctional membranes (Fig. 2B), even though the conductances of the nonjunctional membranes changed relatively little in the experiment illustrated. In other experiments on *Ambystoma*, and in most experiments on *Fundulus*, the nonjunctional conductances changed to a much greater extent on exposure to CO_2 (*19*).

The downward displacement of the pK of the coupling coefficient (Fig. 2C) relative to the pK of g_j (Fig. 2A) is consistent with the lower values of pK inferred from the effects of CO_2 on whole embryos (*13, 20*).

The effect of CO_2 is ascribable to acidification of the cell interior, since a similar result is obtained with external application of the weak acids lactate, propionate, and acetate, which are membrane-permeant in the undissociated form (*5*), whereas strong acids or impermeant buffers at low pH are ineffective (*5, 13*). The effects of CO_2 are not due to anoxia, since they can be obtained with the other weak acids equilibrated with air and also with gas mixtures containing 20 percent CO_2 and 80 percent O_2 (*4-7*).

Thus, conductance of gap junctions in early embryos is strongly dependent on cytoplasmic pH. The Hill equation describing the relation of conductance to pH_i suggests that closure of gap junction channels involves interaction between four to five highly cooperative sites, or more sites if there is less cooperativity.

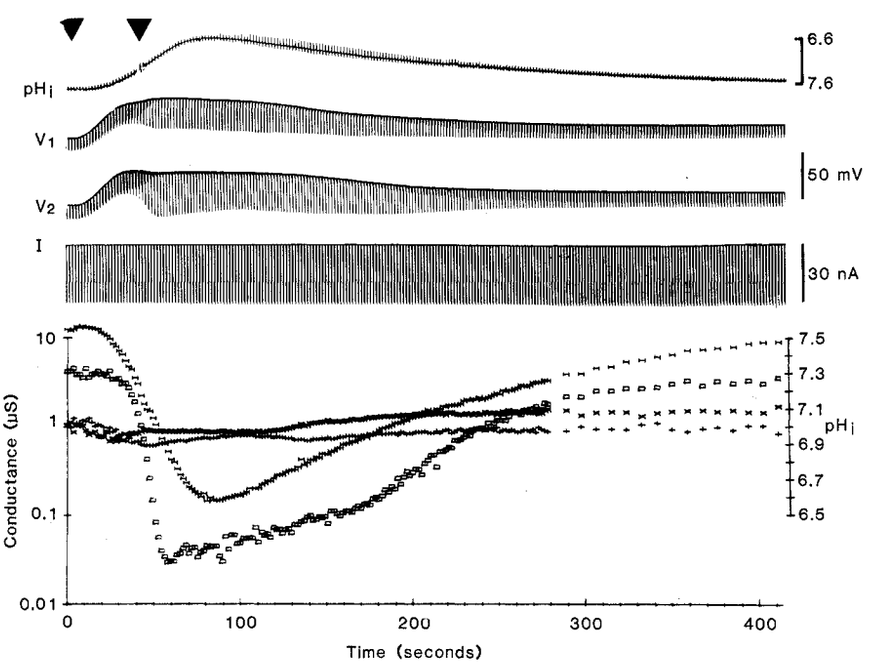

Fig. 1. Effect of CO_2 on electrotonic coupling between a pair of axolotl blastomeres. Current pulses (I) are alternately passed in cell 1 and cell 2 (V_1 and V_2), producing approximately equal-sized voltage deflections in the polarized cell and somewhat smaller potentials in the other cell. Application of saline equilibrated with 100 percent CO_2 in the superfusate (between arrowheads at the top of figure) causes cytoplasmic pH to decrease (uppermost trace, increased H^+ activity upward) and decreases the spread of current from cell to cell (seen as a decrease in the potential produced in each cell by current injected into the other). Washing the cells with CO_2-free saline at normal pH restores the pH_i and electrotonic coupling over a similar but slower time course. Junctional and nonjunctional conductances calculated from these data are plotted along the same time scale in the bottom portion of the figure. Response time for the pH electrode to measure a change of 1 pH unit was < 10 seconds; measurements were made at intervals of ~ 2 seconds. The nonjunctional conductances of the two cells (+, ×) decreased and then slowly recovered. Junctional membrane conductance (g_j, rectangular symbols) was initially about 5 microsiemens (μS) and began to decrease as pH_i (H, right ordinate) reached about 7.4. The junctional conductance reached a minimum value of about 0.02 μS at the minimum of pH_i and then recovered toward the initial value as pH_i recovered.

The observed cooperativity could arise from (i) independent binding of protons causing complete closure of the channels when sufficient sites were occupied (all-or-nothing conductance change), or (ii) cooperative binding of protons, each binding decreasing the conductance of the channel (graduated conductance change).

The apparent pK of the titratable sites in the gap junction molecule is 7.2 to 7.3 depending on the model. These are reasonable values for imidazole groups of histidine residues or the amino terminal of a polypeptide chain. These data may be relevant to structural models of the gap junction macromolecules. The lack of hysteresis in the effect of $p\mathrm{H_i}$ on junctional conductance indicates that an indirect action through change in another cytoplasmic constituent, such as calcium, would have to be rapid. Direct measurements indicate that the concentration of free calcium in the cytoplasm does not change during CO_2 uncoupling in *Fundulus* and *Xenopus* (*5*, *21*). We have recently developed a technique for perfusing one cytoplasmic face of gap junctions between pairs of *Fundulus* cells with solutions having buffered Ca^{2+} and H^+ levels. The sensitivity to H^+ is consistent with that reported here, while much higher levels of Ca^{2+} are required to decrease junctional conductance (apparent $p\mathrm{K_{Ca}}$ is 3.3 in contrast to 7.3 for $p\mathrm{K_H}$) (*22*).

It is conceivable that $p\mathrm{H_i}$ changes sufficiently under physiological conditions to alter junctional conductance. Large changes in extracellular pH occur in various pathological conditions such as ischemia, hypoxia, and seizures (*23*). The changes in $p\mathrm{H_i}$ in cells producing the extracellular acidity are presumably greater than the extracellular changes, although they have not been well characterized. It remains to be determined whether concentrations of CO_2, lactate, or other weak acids are large enough under these conditions to shift $p\mathrm{H_i}$ in otherwise unaffected cells to the point where communication through gap junctions is interrupted (*24*).

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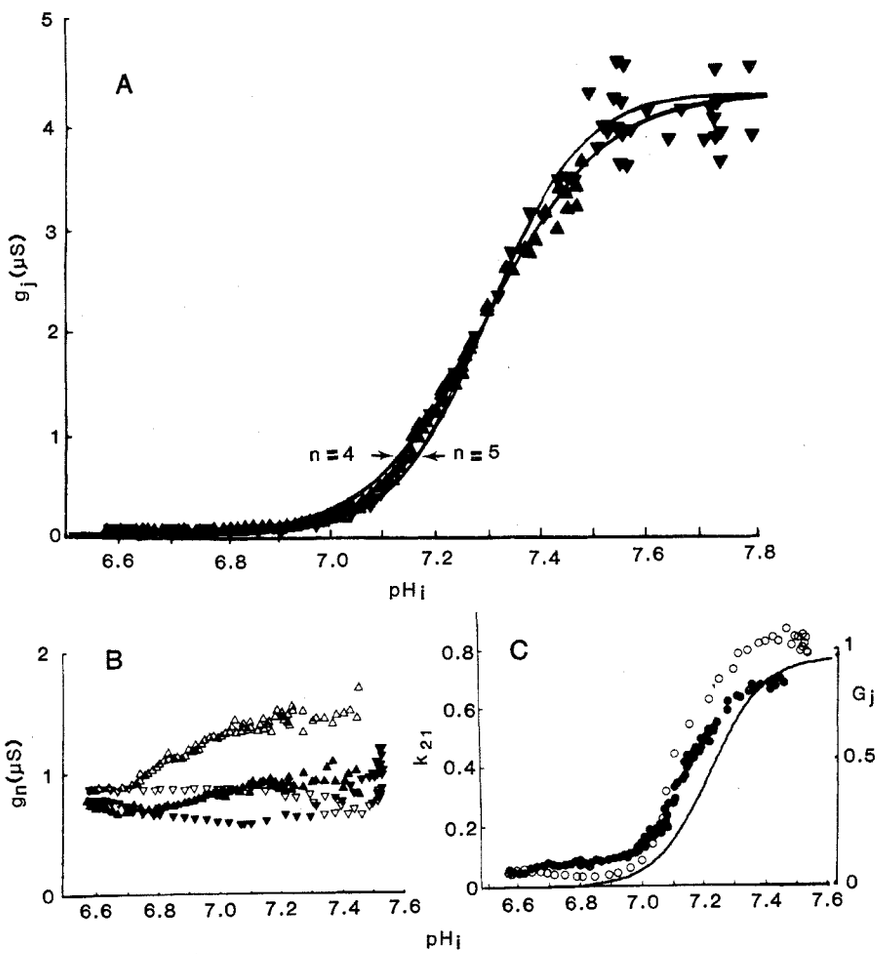

Fig. 2. Analysis of junctional and nonjunctional conductances and the coupling coefficient as functions of $p\mathrm{H_i}$ from the experiment shown in Fig. 1. (A) The relation between g_j and $p\mathrm{H_i}$ is sigmoid, and the points obtained during decreasing pH (triangle with one vertex pointing down) and for increasing $p\mathrm{H_i}$ (triangles pointing up) fall close together. The data are well fitted by the equation $G_j = K^n/(K^n + [\mathrm{H^+}]^n)$, where G_j is normalized g_j; K, the apparent dissociation constant, is about 50 nM (pK = 7.3); and n, the effective number of strongly cooperative titratable sites, is 4 to 5. Curves for $n = 4$ and $n = 5$ are plotted. The g_j values for higher $p\mathrm{H_i}$ (7.6 to 7.8) were obtained before the record of Fig. 1 began. (B) Relations between nonjunctional conductances (g_n) and $p\mathrm{H_i}$ of the two cells [cell 1, open symbols; cell 2, filled symbols; rising and falling $p\mathrm{H_i}$ indicated as in (A)]. The relations are not monotonic and exhibit marked hysteresis. (C) Relation between coupling coefficient (k_{21}) and pH from the data of Fig. 1. The relation shows hysteresis for decreasing (open circles) and increasing (filled circles) $p\mathrm{H_i}$. The solid line is G_j calculated for $n = 4.5$ and $K = 50$ nM, according to the equation above. The apparent pK for k_{21} is less than that for G_j.

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8. The coupling coefficient, k_{12}, is defined as the voltage recorded in cell 2 divided by the voltage in cell 1, with current injected into cell 1. In a two-cell system, $k_{12} = g_j/(g_2 + g_j)$ where g_2 is the nonjunctional conductance of cell 2 and g_j is the junctional conductance. In systems with multiple connectivity, the coupling coefficient can be calculated from input and transfer conductances, but it is a function of both junctional and nonjunctional conductances and evaluation of individual conductances can be difficult [see (*11*)].
9. Saline used with *Ambystoma* embryos contained, in millimoles per liter: NaCl, 58.2; KCl, 0.7; $Ca(NO_3)_2$, 0.3; $MgSO_4$, 0.8; Na_2HPO_4, 0.8; KH_2PO_4, 0.1; and $NaHCO_3$, 0.4 [M. C. Nui and V. C. Twitty, *Proc. Natl. Acad. Sci. U.S.A.* **39**, 985 (1953)]. Saline used with *Fundulus* contained, in millimoles per liter: NaCl, 120; KCl, 1.3; $CaCl_2$, 1.8; $NaHCO_3$, 4.8 [double-strength Holtfreter solution, from G. M. Cavenaugh, *Formulae and Methods of the Marine Biological Laboratory Chemical Room* (Marine Biological Laboratory, ed. 6, Woods Hole, Mass., 1975), p. 71]. Both solutions were buffered to pH 7.6 with 4 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid].
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16. Equations of this form with $n = 4.5$ and $pK = 7.28$ to 7.31 fit data obtained with CO_2 exposure equally well in four other pairs of *Ambystoma* embryos and five pairs of *Fundulus* blastomeres.
17. For example, the hemoglobin molecule has four oxygen binding sites, but the Hill coefficient is 2.7 to 3 [see M. F. Perutz, *Nature (London)* **228**, 726 (1970)].
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19. Nonjunctional conductance decreases before pH_i changes when cell pairs are treated with any low pH solution, including membrane-impermeant strong acids. The pH_i at which the late rise in nonjunctional conductance occurs depends upon the rate of change of pH_i and might be mediated by a slower secondary process, such as an increase in cytoplasmic calcium.
20. In considering the significance of uncoupling assayed electrically, it should be noted that a coefficient for coupling of an inert tracer molecule (expressed as a concentration ratio) is determined by the ratio of permeabilities through junctional and nonjunctional membranes, rather than by the ratio of the electrical conductances of those membranes. If, as we believe, gap junction channels are closed by decreased pH_i in an all-or-none fashion, changes in junctional conductance are a good measure of changes in permeability to any (permeable) molecule. Electrical conductance of nonjunctional membranes may be due to a variety of channels that are specifically permeable to simple ions, and therefore changes in nonjunctional conductance or in electrical coupling coefficients are probably poor measures of changes in nonjunctional permeability or coupling coefficients for small molecules.
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Concentration Oscillations and Efficiency: Glycolysis

Abstract. *The oscillations observed in glycolysis are analyzed from the point of view of efficiency of free energy conversion. It is suggested that the mechanisms generating these oscillations may have evolved in order to reduce the dissipation of free energy.*

A number of biochemical reaction systems show periodic changes (oscillations) in the concentrations of their intermediates (*1*, *2*). The most extensively studied case of such metabolic oscillations is that of glycolysis, particularly in yeast cells and in cell-free extracts of yeast. Much effort has been devoted to determining the detailed mechanisms that lead to oscillatory behavior (*3*–*6*). Very little is known, however, about the reasons for the emergence of such mechanisms in the course of evolution. There is an argument that they are accidental by-products of regulatory features that are built into the system for the purpose of control. We suggest here another reason: oscillations may enhance the efficiency of free energy conversion from sugar to ATP (*7*). In an anaerobic environment, where glycolysis provides most of a cell's free energy supply, this may be a factor that favors an evolutionary development toward oscillatory operation.

Glycolysis is the degradation of sugars to pyruvate, which is further metabolized to alcohol (in yeast fermentation), lactic acid (in muscle), or acetyl coenzyme A (under aerobic conditions). Depending on the available source of free energy, which may be any of a variety of sugars or glycogen, there are different entries into the glycolytic pathway (*8*). Their point of convergence is the level of F6P, after which there are eight more reaction steps in the overall reaction

$$F6P + 2P_i + 3ADP + NAD^+ \rightarrow 2PYR + 3ATP + 2NADH + H^+$$

The overall drop in Gibbs free energy (ΔG) is about 14 kcal/mole. This does not mean, however, that in each of the eight steps the free energy decreases by ~ 1.8 kcal/mole, or $3RT$ (R is the gas constant and T is absolute temperature). Rather, the evidence points to a free energy profile with three distinct steps (Fig. 1). (The free energy profile takes full account of all reactants in each reaction step; however, we show only one identifying reactant on each level.)

The first reaction in the sequence, catalyzed by PFK, is highly irreversible (*9*, *10*). This is the primary oscillophor (generator of species oscillations) in the system, so that by necessity the reaction must proceed far from equilibrium. The relatively large Gibbs free energy change is attained in this step by conversion of ATP to ADP. Several models have been proposed that explain the occurrence of spontaneous oscillations on the basis of known regulatory features of PFK such as product activation, substrate inhibition, and allosteric properties (*3*–*5*).

The last reaction, which is catalyzed by PK, also takes place far from equilibrium; in fact, about half the total free energy loss occurs at this point. The enzymatic activity pattern of PK contains a number of features that make this reaction another likely candidate for being an oscillophor: there is activation by FDP, which alone could give rise to oscillations (*11*); furthermore, there is cooperativity with respect to the substrate PEP and inhibition by the product ATP (*12*, *13*). However, no experimental observations have been reported of independent oscillations in the lower part of the glycolytic pathway. These facts suggest the hypothesis, as yet unconfirmed, that the system is below marginal stability with respect to the onset of oscillations, but is in a regime where perturbations of the steady state decay in an oscillatory way. This provides the system with a resonance potentiality when driven by external oscillations—that is, those generated in the PFK reaction. We will see what effect this has on the efficiency of free energy throughput.

There is almost equilibrium among the intermediates FDP through PEP ($\Delta G \lesssim RT$) except for a drop in ΔG that probably occurs between GAP and 3PG. This step involves the allosteric enzyme GAPDH and couples glycolysis to the oxidation reaction ($NAD^+/NADH$).

Evidence for the free energy profile of Fig. 1 comes from concentration measurements (*14*, *15*) and from an analysis of phase relations in the oscillatory mode (*16*). Appreciable phase lags have been found only across the three steps mentioned—the PFK, PK, and GAPDH reactions. Hence these are slow steps compared to the others, which indicates that they are the farthest from equilibrium (*17*). The phase lag at the center (GAPDH) is very sensitive to changes in the steady-state ratios ATP/ADP and $NAD^+/NADH$, whereas the shifts across PFK and PK appear to be independent of such details.

From this partitioning of the total free energy decrease, we postulate the fol-

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