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Effects of Adenosine Triphosphate and Magnesium Ions on the Fumarase Reaction*

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SUMMARY

ATP is a potent inhibitor of the activity of fumarase from yeast and pig heart (K_i = 0.005 mM and 0.03 mM, respectively). There is a sigmoid relation between the inhibition and the ATP concentration provided that Mg^{2+} is present. The sigmoid relationship is shown to be due simply to the fact that the MgATP complex does not inhibit, so that with increasing ATP concentration, the inhibition is not fully expressed until Mg^{2+} is titrated by ATP. Since several other enzymes in energy yielding pathways are known to be inhibited by free ATP but not by MgATP, this type of sigmoidal response is probably an important factor in sharpening feedback responses to ATP.

The inhibition by ATP is increased at low pH, suggesting that $ATPH^{3-}$ may be a better inhibitor than ATP^{4-}. Moreover, the ability of magnesium to diminish the inhibition is reduced at low pH, as would be expected from the fact that $ATPH^{3-}$ has a lower affinity than ATP^{4-} for Mg^{2+}. These phenomena may profoundly affect the metabolic consequences of intracellular cation movements, i.e. exchange of Mg^{2+} for H^+ between two compartments such as mitochondria and cytosol would result in the liberation of ATP from MgATP in one compartment and binding of ATP in the other, producing inverse metabolic effects in the two compartments.

During the past 10 years, a number of enzymes involved in energy production have been shown to be inhibited by ATP, an effect which is believed to be important in the feedback control of ATP formation. The best known example is phosphofructokinase, which appears to be an important control point for glycolysis (1–11). However, glycolysis of brain (5) and heart (6, 11) can be increased several-fold with only a small decrease in ATP levels. This sensitive response is believed to be due in part to the presence of multiple binding sites on phosphofructokinase, leading to a sigmoid relation between ATP concentration and inhibition (10), and in part to the fact that the inhibition of phosphofructokinase by ATP is reversed by ADP, AMP, and P_i which are formed during ATP breakdown (2, 3, 10). However, there is an additional factor that must be considered when the inhibitor is ATP, namely the fact that some of the cellular ATP exists as the MgATP complex. It can be predicted that, if an enzyme is inhibited by free ATP but not by MgATP, there will be sigmoid inhibition due simply to the fact that ATP must titrate much of the available Mg^{2+} before inhibition is expressed.

We have reported in a preliminary communication[1] that ATP is a potent inhibitor of fumarase and that the MgATP complex is not. The present paper documents this for fumarase of pig heart and yeast, and demonstrates the inhibition to be competitive with substrate. The sigmoid character of the ATP inhibition in the presence of Mg^{2+} ion is readily demonstrated, as well as a critical role of pH in the sensitivity of the magnesium effect. These observations have implications relevant to the effect of intracellular pH upon metabolism and also to studies *in vitro* of enzymes which require magnesium and which are therefore usually studied with magnesium present.

MATERIALS AND METHODS

Fumaric and malic acids were obtained from Fluka AG Chemische Fabrik, Buchs, Switzerland, and were recrystallized from deionized water. The nucleotides were obtained from Sigma and from P-L Biochemicals. The ATP was at least 99% pure while the ADP contained 1 to 3% ATP, depending on the source. The other nucleotides and chemicals were used without further purification. All solutions were prepared in deionized distilled water.

Crystalline pig heart fumarase (12) was a gift from Dr. R. A. Alberty. The enzyme was stored at 0° as a crystalline suspension in 50% ammonium sulfate. Prior to use, the crystals were washed with water by centrifugation, dissolved in 10 mM Tris-acetate and 15 mM EDTA, pH 7.0, and stored at −20° until

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[1] L. H. COHEN AND P. E. PENNER, *Fed. Proc.*, **24**, 357 (1965).

used. The enzyme is unstable in 10 mM Tris at 0° (13) but is quite stable at room temperature.

Yeast fumarase was partially purified from an extract of baker's yeast (*Saccharomyces cerevisiae*) which had been air-dried, suspended in 0.1 M $NaHCO_3$, and allowed to autolyse with stirring for 8 hours at 37°. After centrifugation, ammonium sulfate (36 g/100 ml) was added with stirring to the supernatant. The precipitated protein was collected by centrifugation and the supernatant fluid discarded. The pellet was dissolved in cold deionized water and an ammonium sulfate fractionation was carried out. The protein precipitating between 1.74 molal and 2.12 molal ammonium sulfate was dissolved in water. This preparation was purified 6-fold and contained a relatively small fraction of the total fumarase activity, but was used because it was very stable to freezing and contained little adenylosuccinate lyase which interferes with the assay of fumarase in the presence of AMP. The enzyme preparation was dialyzed against a solution of 15 mM EDTA and 10 mM Tris-acetate, pH 7.0, and stored frozen.

Fumarase was assayed spectrophotometrically (14, 15) with a Cary 15 recording spectrophotometer or fluorometrically with a Turner 111 fluorometer. In the spectrophotometric assay, based on fumarate disappearance, cuvettes with various light paths (2, 10, or 50 mm) and wave lengths between 220 and 305 mμ were employed in order to cover a wide range of fumarate concentrations.

The fluorometric method used in the determination of K_m for fumarate and K_i for ATP was based on the measurement of the malate formed during a 30-min incubation period. After inactivation of the fumarase, the malate was reacted with DPN in the presence of malic dehydrogenase and hydrazine, and the fluorescence of the DPNH was measured. Two concentrations of fumarase were used routinely in order to improve accuracy at the low rates obtained in the presence of inhibitor. The rate was proportional to enzyme concentration and constant for at least an hour. Details of the method are given in Fig. 4.

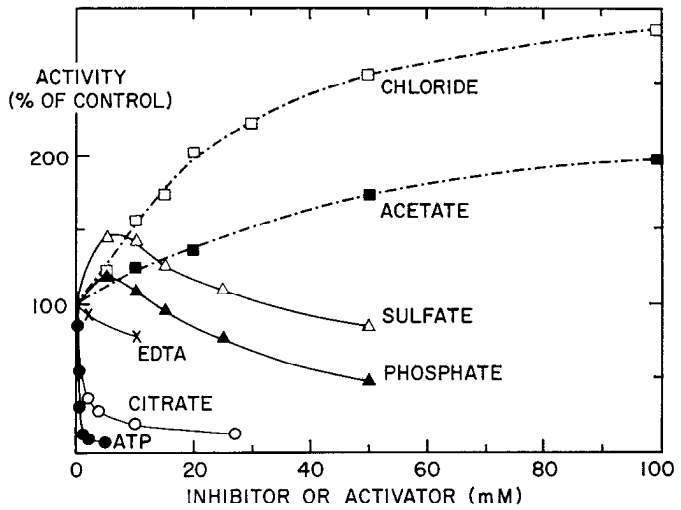

FIG. 1. The effect of anions on the activity of yeast fumarase. Enzyme activity was assayed spectrophotometrically at 25° in a medium containing 10 mM Tris-acetate, pH 7.0, 0.5 mM fumarate, and various salts in the concentrations indicated. For further details see "Materials and Methods." Potassium chloride, □-·-·-□; potassium acetate, ■-·-·-■; potassium sulfate, △——△; potassium phosphate, ▲——▲; potassium EDTA, ×——×; potassium citrate, ○——○; disodium ATP neutralized to pH 7 with potassium hydroxide, ●——●.

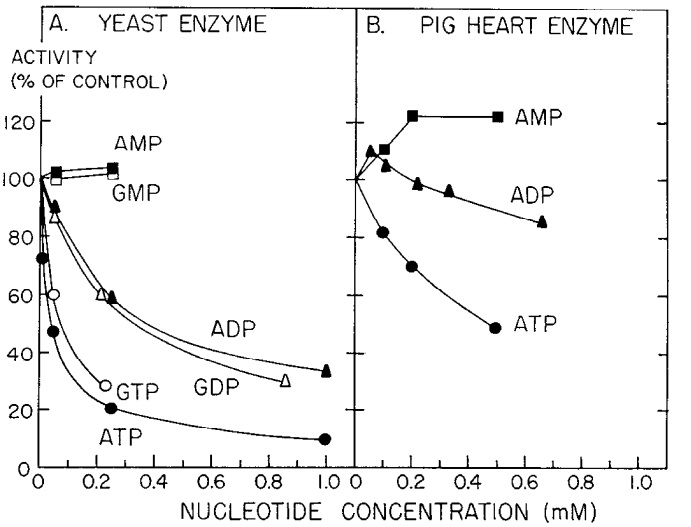

FIG. 2. The effect of nucleoside mono-, di-, and triphosphates on the activity of fumarase. Enzyme activity was assayed under the conditions described for Fig. 1 except for the fumarate concentration. *A*, yeast enzyme, 0.2 mM fumarate. In the experiments with AMP present, interference by the adenylosuccinase reaction was eliminated by assaying fumarase activity at 259.5 mμ, the isoabsorptive point of the adenylosuccinase reaction. *B*, pig heart enzyme, 0.1 mM fumarate.

Tris-acetate buffer was used throughout this study so that pH could be altered without affecting anion concentration, as recommended by Frieden and Alberty (16). Acetate was found to be the least potent stimulant anion tested, approximately 100 mM being required for maximal effect. For this reason, 10 mM Tris-acetate buffer did not prevent observation of stimulatory effects of other anions.

RESULTS

Effect of ATP and Other Anions—Various anions have been reported to stimulate or inhibit pig heart fumarase, depending on the particular anion and on the pH (15, 17). The effects of several anions, including compounds believed to have regulatory significance, upon the initial rate of malate formation were therefore studied. In the first experiment with the yeast enzyme a high substrate concentration was used (0.5 mM or approximately 100 × K_m of fumarate) since the presence of ATP interfered with the spectrophotometric enzyme assay at the low wave lengths needed when low substrate concentrations were used. As seen in Fig. 1, the effects of anions depend upon their charges. Singly charged anions (chloride, acetate) stimulate the enzyme; doubly charged anions (sulfate, phosphate) stimulate at low concentration but inhibit at high concentration; and ions of higher charge only inhibit. However, it is clear that ionic charge is not the only factor; phosphate is a better inhibitor than sulfate even though at pH 7 the concentration of divalent phosphate is lowered; citrate is a much more potent inhibitor than EDTA although both are triply charged at pH 7. Also, fructose 1,6-diphosphate is a much weaker inhibitor than inorganic pyrophosphate (not shown). The best inhibitor tested in this experiment is ATP which is predominantly triply charged.[2]

[2] ATP is approximately 70% protonated at an ionic strength of 0.01 and pH 7 (18).

The effect of ATP is compared with that of other nucleotides in Fig. 2. At a fumarate concentration of 0.2 mM (approximately 40 × K_m), the nucleotide concentrations required for 50% inhibition of the yeast enzyme were 0.04 mM ATP, 0.08 mM GTP, 0.33 mM GDP, or 0.4 mM ADP. Thus ATP was about twice as potent as GTP and 10 times as potent as ADP. In a separate experiment, CTP and UTP were found to inhibit to about the same extent as GTP. Neither AMP nor GMP at 0.25 mM was inhibitory. The results obtained with the pig heart enzyme were similar (Fig. 2*B*), except that all of the nucleotides were weaker inhibitors than in the case of the yeast enzyme, and some stimulation by low concentrations of ADP and by AMP was observed. Indeed, at sufficiently high fumarate concentrations, even ATP stimulates the pig heart enzyme, but not the yeast enzyme. This is seen in Fig. 3 (*upper*) by the fact that the *lines* for inhibited and uninhibited rates of the heart enzyme cross at about 0.7 mM fumarate. When the effect of ATP on the reaction in the reverse direction is examined (Fig. 3, *lower*) the *lines* meet at about 1.6 mM malate, but do not cross. Thus, in either direction, the inhibition of the pig heart enzyme is completely abolished by moderate substrate concentrations.

The kinetics of the inhibition by ATP in the millimolar range of substrate concentration was found to be quite complex for both enzyme preparations, particularly because of the well known substrate activation of fumarase at high fumarate levels (15). However, at the low substrate concentrations, the inhibition of both enzymes was found to be competitive, with

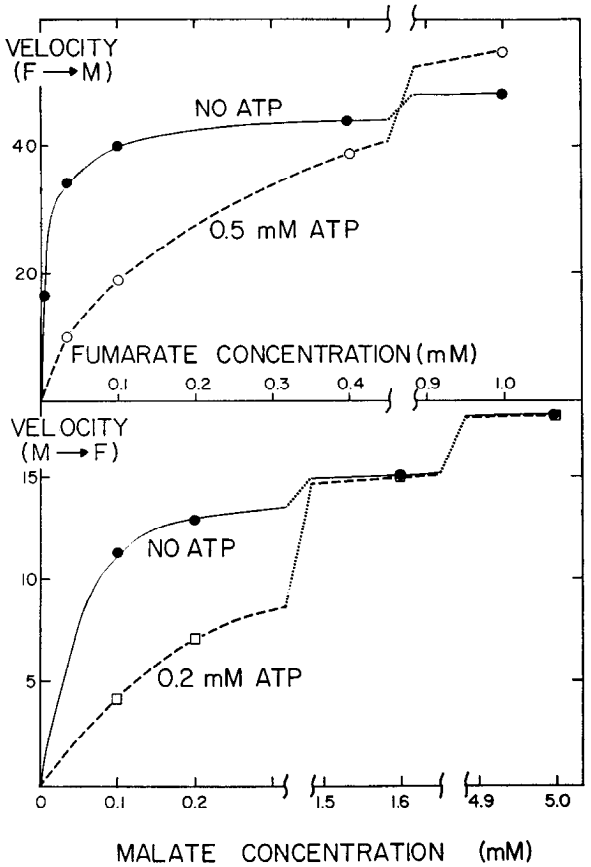

FIG. 3. The effects of ATP on the activity of pig heart fumarase as a function of substrate concentration. Enzyme activity was assayed as described for Fig. 1 with fumarate (*F*) or malate (*M*) and ATP concentrations as indicated.

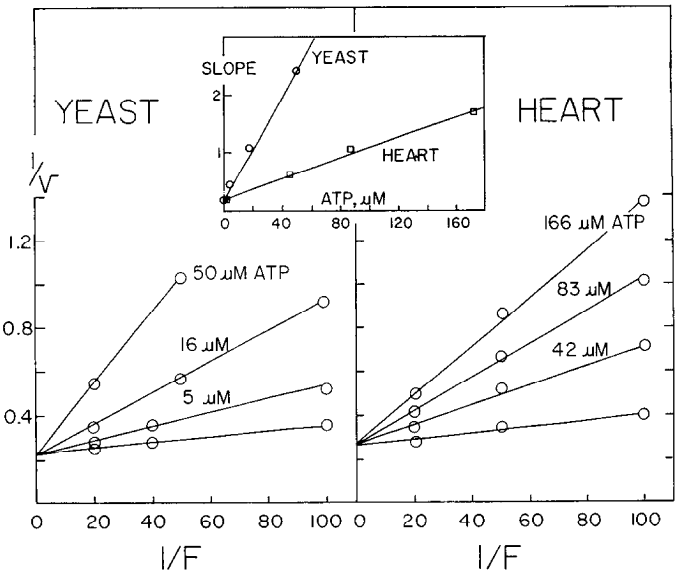

FIG. 4. Inhibition of yeast and pig heart fumarases by ATP. Fumarate concentration (*F*) is expressed as millimolar, and velocity as micromoles per hour. The *inset* shows replots of slope against ATP for the two preparations. Enzyme activity was assayed fluorometrically, each point being the average of three determinations. About 10^{-4} or 3 × 10^{-5} units of fumarase was added to each of a series of tubes containing 3 ml of 17 mM Tris-acetate buffer pH 7, substrate, and inhibitor. After 30 min of incubation at 23° the reaction was stopped by addition of 50 μl of 2 N NaOH and 10 min at 90°. The samples were cooled and 1.5 ml of buffer (0.2 M Tris, 1 M hydrazine base, 1 mM EDTA, 0.515 mM DPN, pH 9.1). To each tube was added 0.3 units of malate dehydrogenase which had been freed of traces of fumarase by passage through Sephadex G-75. After 1½ hours of incubation at room temperature the fluorescence was read (Corning 7-60 primary filter and Corning 4-94 plus Kodak Wratten 8 secondary filters) and compared with a standard malate curve.

TABLE I

Effect of pH on inhibition of fumarase by ATP

Enzyme activity was assayed by the spectrophotometric method in a medium containing 16 mM Tris-acetate, 0.15 mM fumarate, and ATP at the concentrations indicated.

pH	Inhibition	
	Heart fumarase, 0.25 mM ATP	Yeast fumarase, 0.025 mM ATP
	%	
6.0	60	62
6.5	41	53
7.0	44	49
7.5	15	24
8.0	8	11

K_i values of ATP of 0.005 mM and 0.03 mM, for the yeast enzyme and pig heart enzyme, respectively, at pH 7 (Fig. 4). The inhibition is inversely related to pH, as shown in Table I. For the heart enzyme the K_i at pH 8 was determined and found to be 0.1 mM, about 3 times higher than that at pH 7. The results suggest that $ATPH^{3-}$ is more inhibitory than ATP^{4-}, although the possibility that the effect of pH is largely upon the enzyme cannot be ruled out.

Effect of MgATP—A preliminary experiment showed that, in the absence of ATP, neither Mg^{2+} nor Ca^{2+} at concentrations

of up to 15 mM had any effect upon the activity of fumarase from yeast other than the slight stimulation due to the counter anion, acetate. In order to determine whether MgATP inhibited the enzyme, an experiment was carried out in which the total ATP was varied over a 12-fold range, and Mg^{2+} concentrations arranged such that the free ATP concentration was held constant at a level that causes about 60% inhibition. From the results of this experiment, shown in Table II, it is clear that the presence of a large excess of Mg^{2+} abolishes the inhibition completely (*cf.* the top two rows), and that the inclusion of MgATP at concentrations of up to about 12 times the free ATP level had no effect upon the inhibition by free ATP.

In a separate experiment, Ca^{2+} was also found to antagonize the inhibition, but less effectively than did Mg^{2+}, as expected from the fact that it binds nucleoside triphosphates less strongly than does Mg^{2+} (19). Thus, in an experiment in which inhibition of the yeast enzyme by 0.25 mM GTP alone was 72%, the inhibition by GTP in the presence of 0.5 mM Ca^{2+} was 39%, and inhibition in the presence of 0.5 mM Mg^{2+} was 18%.

TABLE II

Correlation of inhibition of yeast fumarase with concentration of free ATP

Enzyme activity was assayed in a medium containing 10 mM Tris-acetate, pH 7, 0.3 mM fumarate, ATP, and magnesium at the concentrations indicated. Magnesium was added as the ATP salt at pH 7, thus obviating a pH drop which occurs when magnesium forms a complex with ATP.

Total ATP	Total magnesium	MgATP=[a]	Free ATP[a]	Inhibition
mM	*mM*	*mM*	*mM*	%
0.25	7.5	0.25	0.001	4
0.07	0	0	0.07	61
0.09	0.04	0.03	0.06	61
0.10	0.06	0.04	0.06	63
0.44	0.48	0.36	0.07	60
0.65	0.74	0.57	0.075	63
0.81	1.03	0.75	0.06	62
0.85	0.98	0.77	0.08	68
0.86	1.15	0.81	0.051	61

[a] Calculated values for pH 7 and ionic strength of 0.01 assuming dissociation constants determined by Phillips, George, and Rutman (18).

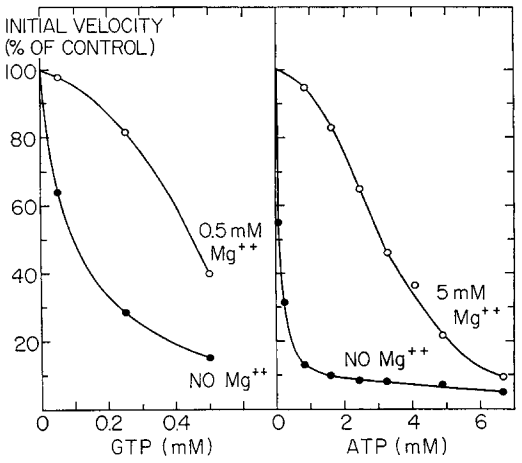

FIG. 5. Antagonism of the GTP and ATP inhibition of yeast fumarase by Mg^{2+}. Enzyme activity was assayed as described for Fig. 1 in the presence of 0.2 mM fumarate (*left*) or 0.3 mM fumarate (*right*) and the concentrations of GTP, ATP, and Mg^{2+} ion indicated. Mg^{2+} was added as magnesium acetate. When omitted, potassium acetate was added in its place. Thus the concentration of acetate ion was the same throughout.

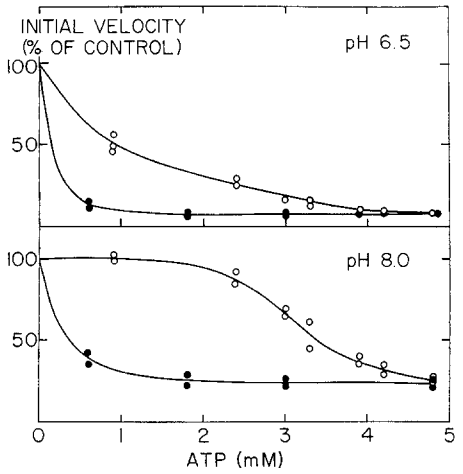

FIG. 6. Effect of Mg^{2+} on the ATP inhibition at low and high pH. Enzyme activity was assayed spectrophotometrically in a medium containing 15 mM Tris-acetate, 0.2 mM fumarate, 3 mM Mg^{2+}, and ATP as indicated at pH 6.5 (*upper*) and pH 8.0 (*lower*). Other conditions were as in Fig. 5.

As in the case of the yeast enzyme, the inhibition of the pig heart enzyme by ATP was abolished by Mg^{2+}, but an extensive study of this effect was not carried out because Mg^{2+} had a small stimulatory effect upon the activity in the absence of ATP.

As stated earlier, it would be predicted from the failure of MgATP to inhibit that the relation between inhibition and ATP concentration should be sigmoid in the presence of Mg^{2+} but not in the absence of Mg^{2+}. This is shown to be the case in the data of Fig. 5 at two Mg^{2+} concentrations. It can also be seen that in both experiments the nucleotide approaches full effectiveness as it titrates the Mg^{2+}, despite a 10-fold difference in magnesium concentration in the two experiments. This further supports the idea that the sigmoid curve results from the fact that MgATP does not inhibit.

Still further evidence on this point comes from a study of the effect of pH upon the ATP inhibition in the presence of magnesium. If $MgATP^{2-}$ does not inhibit, then displacement of Mg^{2+} by H^+ should diminish the effect of magnesium, provided that $ATPH^{3-}$ is a good inhibitor of the enzyme. As shown in Fig. 6, the effectiveness of Mg^{2+} in producing sigmoidicity is practically abolished at pH 6.5, and markedly increased at pH 8.0 as compared with pH 7. This result supports the view that $ATPH^{3-}$ is an inhibitory form of the nucleotide.

DISCUSSION

Although it has been known for several years that anions have pronounced effects, both stimulatory and inhibitory, on the fumarase-catalyzed reaction, the possibility that these effects might reflect regulatory properties of the enzyme had not been explored, probably because fumarase does not conform to the classical picture of a "regulatory" protein, *i.e.* it does not catalyze an obvious first step of a pathway, does not catalyze an irreversible step, and does not display a sigmoid relation between rate and substrate concentration. The sensitivity to ATP of the

yeast and heart enzymes (K_i = 0.005 mM and 0.03 mM, respectively), however, appears to be greater than that of several other glycolytic and citric acid cycle enzymes (10, 20–25), so that significant control of other enzymes by ATP without simultaneous control of fumarase would seem improbable.

Although the most obvious function of the ATP inhibition of fumarase might seem to be feedback control of the citric acid cycle, it is unlikely that any single step controls this cycle. The reactions of the cycle serve not only to generate ATP, but also to direct products of catabolism into synthetic paths such as gluconeogenesis, lipogenesis, and the synthesis of porphyrins and amino acids. In view of this multiplicity of functions, a complex feedback network affecting many enzymes may be needed to regulate the direction of traffic through the cycle, and one might expect the evolution of mechanisms of control of the rates of several reactions of the cycle and of the concentrations of most of the intermediates as well. The kinetics of the ATP inhibition of pig heart fumarase suggests that ATP inhibition is a means for the control of substrate level rather than the control of throughput, since the substrates at moderately high concentrations can completely overcome the ATP inhibition, as shown in Fig. 3.

A specific role for the control of fumarate or malate concentration can only be a matter of speculation at present. However, there is suggestive evidence that variations in fumarase activity may be of biological significance. First of all, Williamson[3] has observed that the malate to fumarate ratio in perfused rat liver engaged in gluconeogenesis from lactate or pyruvate is 7.3 to 9.5, significantly higher than the equilibrium value of 3.2 at 37° (26). Since this value is probably the average for the cytosol and mitochondrial compartments (both of which contain fumarase (27–30)), it is quite possible that the displacement from equilibrium in one compartment may be still greater. Thus the enzyme level in liver is low enough that variations in its activity could affect the levels of malate and fumarate. In brain, however, the malate to fumarate ratio is reported to be 6 (31), closer to the equilibrium value. Secondly, the fumarase activity of liver supernatant is known to be increased 4-fold in alloxan diabetes (32), suggesting that the rate of the reaction catalyzed by this enzyme has some metabolic significance.

Although the biological function of the effect of ATP remains uncertain, the enzyme serves as a useful model system to investigate phenomena that may be of general importance. The results obtained herein illustrate that a sigmoid relation between inhibition of an enzyme and inhibitor concentration in the presence of divalent cations need not be interpreted only in terms of multiple binding of inhibitor to the enzyme. More important, they bring into focus the importance of the relative concentrations of divalent cation, nucleotides, and other ligands in determining the kinetics of enzymes regulated by ATP. In the presence of Mg^{2+} a small decrease in the amount of ATP results in a large decrease in the free ATP concentrations; thus enzymes regulated by free ATP would be particularly sensitive to ATP breakdown. A number of enzymes besides fumarase are known to be inhibited more strongly by free ATP than by $MgATP^{2-}$ (phosphofructokinase (10); aldolase (20); citrate synthetase (23, 24)), suggesting that this property may be a factor in the ability of cells to respond metabolically to the breakdown of only a small fraction of the ATP.

[3] J. R. Williamson and E. T. Browning, personal communication.

It would seem to be important that the energy-producing paths of metabolism should be especially responsive to small changes in ATP. ATP is consumed in so many cell functions that depletion of the ATP reserves is an ever-present threat to cell economy. This is particularly so because of the fact that ATP is needed to prime the major energy-producing paths; if the ATP level were to drop below that required for maximal activity of these paths, the ATP depletion would accelerate. It is therefore important that the enzymes that control the energy-producing paths be responsive to very small changes in ATP. A number of mechanisms to assure sharpness of response of enzymes to ATP breakdown have been proposed in the past, such as regulation by ADP, AMP, and P_i, as well as by ATP (2, 3, 33); competitive balance between ATP and ADP or AMP (3, 10); and the existence of multiple regulator-binding sites on the enzymes. All of these phenomena would be markedly enhanced in the presence of Mg^{2+} or Ca^{2+} ions, as a result of the type of sigmoidal effect described here.

Because of the effects of divalent cations on the concentration of free ATP, intracellular movements of cations would be expected to have important effects upon the metabolic rates in the various cellular compartments. As previously pointed out[1] Mg^{2+} by complexing ATP, converts an inhibitor of certain regulatory enzymes into substrates for the same or other enzymes (10, 20, 23, 24), so that a change in Mg^{2+} concentration at a given site would have a profound metabolic effect. Thus, transport of Mg^{2+} into the mitochondria from the cytosol would have the effect of decreasing free ATP in the mitochondria and increasing it in the cytosol. Moreover, this effect would be enhanced by the reverse movement of protons known to occur during Mg^{2+} transport (34), since protons compete with Mg^{2+} for ATP. The ability of protons to enhance the ATP inhibition of fumarase in the presence of Mg^{2+} is in fact shown by the data of Fig. 6. This kind of pH effect might in part explain the extreme pH dependence of the phosphofructokinase reaction observed by Trivedi and Danforth (35). These workers concluded that the effect was related in some way to ATP inhibition. Examination of their data shows that the effect was observed when the ATP level was slightly less than that of magnesium, *i.e.* under conditions where the level of free ATP would be highly pH-dependent. If it is indeed the case that the pH effect on this enzyme is partly the result of the competition between protons and Mg^{2+} for ATP, then the replacement of Mg^{2+} of the cytosol by protons would have a very large effect upon the enzyme and therefore upon glycolysis. Shifts of Mg^{2+} between mitochondria and cytosol might thus be an important factor in the inverse relationships known to exist between respiration and glycolysis, such as the Pasteur and Crabtree effects.

Of paramount importance to the thesis that the partition of nucleotides between complexed and uncomplexed forms plays a role in metabolic control is the question of whether enough uncomplexed ATP is present at the site of the enzyme to have a substantial inhibitory effect, since in some tissues the total magnesium concentration considerably exceeds the ATP concentration. Unfortunately, for most tissues there is insufficient firm evidence about the intracellular concentrations of magnesium, the fraction present as Mg^{2+}, or the extent of compartmentation to permit an answer to this question. Recently, however, a method for estimating the Mg^{2+} from the measured K_{eq} of adenylate kinase has been devised (36). It was concluded that in human erythrocytes, in which compartmentation is

minimal, the concentration of uncomplexed ATP is approximately 285 μmoles per liter of cells, 30% of the total ATP. If other tissues are similar in the portion of ATP uncomplexed, then the effect of ATP is a significant factor affecting the rate of the fumarase reaction.[4]

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[4] *Note Added in Proof*—Prof. H. A. Krebs has brought to our attention an early publication mentioning that under certain conditions the malate to fumarate ratio in minced sheep heart is as low as 1.1 (37). This deviation from equilibrium is further support for the view that fumarase is rate-limiting in some part of the cell.

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