Tomkins, G. M. (1972) *J. Steroid Biochem. 3*, 219-227.
Rousseau, G. G., Baxter, J. D., Higgins, S. J., & Tomkins, G. M. (1973) *J. Mol. Biol. 79*, 539-554.
Sherman, M. R., Barzilai, D., Pine, P. R., & Tuazon, F. B. (1979) in *Steroid Hormone Receptor Systems* (Leavitt, W. W., & Clark, J. H., Eds.) pp 357-375, Plenum Press, New York.
Siegel, L. M., & Monty, K. L. (1966) *Biochim. Biophys. Acta 112*, 346-362.
Simons, S. S., Jr., Martinez, H. M., Garcea, R. L., Baxter, J. D., & Tomkins, G. M. (1976) *J. Biol. Chem. 251*, 334-343.
Tanford, C., Nozaki, Y., Reynolds, J. A., & Makino, S. (1974) *Biochemistry 13*, 2374-2376.
Weinstein, A., Medes, G., & Litwack, G. (1967) *Anal. Biochem. 21*, 86-97.
Wira, C. R., & Munck, A. (1974) *J. Biol. Chem. 249*, 5328-5336.
Wrange, O., & Gustafsson, J.-A. (1978) *J. Biol. Chem. 253*, 856-865.

Effects of Pyrophosphate, Triphosphate, and Potassium Chloride on Adenylate Deaminase from Rat Muscle†

Thomas J. Wheeler‡ and John M. Lowenstein*

ABSTRACT: Inorganic pyrophosphate and triphosphate inhibit adenylate deaminase from rat skeletal muscle with K_I values of 10 and 1.5 μM, respectively, in the presence of 150 mM KCl at pH 7. They act by reducing the apparent affinity of the enzyme for AMP, with relatively small effects on V_{max}. The inhibitions are diminished by H^+, the K_I values increasing two- to threefold in going from pH 7.0 to 6.2, and are relieved by ADP. These properties are similar to the inhibitions produced by GTP and ATP, indicating that pyrophosphate and triphosphate act like analogues of the nucleoside triphosphates. Neither of these inhibitors shows relief of inhibition at high concentrations as do ATP and GTP. These results suggest that nucleotides interact with the inhibitor site of the enzyme primarily through their phosphate moieties and with the activator site primarily through their nucleoside moieties. As the concentration of KCl is increased from 25 to 300 mM, the apparent affinities of the enzyme for ATP, GTP, orthophosphate, pyrophosphate, and triphosphate are decreased 8-100-fold. The cooperativity of the inhibitions is increased, with the Hill coefficient rising from 1.0 to 1.3-1.8, and the maximum inhibition approaches 100%. Maximum activation by ADP is reduced from 1800% at 25 mM KCl to 80% at 200 mM KCl. Experiments with $(CH_3)_4NCl$ indicate that activation of the enzyme by KCl involves both specific K^+ effects and ionic strength effects.

Adenylate deaminase from muscle is regulated by nucleoside di- and triphosphates. ATP and GTP are inhibitors; ADP and GDP are activators (Lyubimova & Matlina, 1954; Ronca et al., 1968; Smiley & Suelter, 1967). Moreover, at high concentrations ATP and GTP reverse their own inhibition (Ashby & Frieden, 1978; Wheeler & Lowenstein, 1979b). Inorganic pyrophosphate and triphosphate also inhibit the enzyme from muscle (Nikiforuk & Colowick, 1956; Wheeler & Lowenstein, 1979a) and other tissues (Setlow & Lowenstein, 1968; Yun & Suelter, 1978). We have studied their effects on enzyme activity in order to clarify certain aspects of the nucleoside di- and triphosphate specificity of the enzyme.

The regulatory properties of adenylate deaminase are strongly dependent on the concentration and composition of added salt (Coffee & Solano, 1977; Ronca et al., 1972; Ronca-Testoni et al., 1970; Sammons et al., 1970). Previous investigators used 100-150 mM KCl as an approximation to intracellular conditions. We have examined the effects of H^+, ADP, and inhibitors on the activity of the enzyme at different KCl concentrations in order to relate such studies to conditions in vivo and to better understand the regulatory properties of the enzyme.

Experimental Procedures

The purification of adenylate deaminase, assay procedures, and treatment of data were described previously (Wheeler & Lowenstein, 1979b). Sodium pyrophosphate was obtained from Fisher, sodium tripolyphosphate from Howe and French, and tetramethylammonium chloride from Eastman.

Results

Inhibition by Pyrophosphate and Triphosphate. Adenylate deaminase activity in the presence of 20 μM AMP and 150 mM KCl, pH 7.0, is inhibited 58 and 92% by 12 and 60 μM inorganic pyrophosphate, respectively. Under the same conditions it is inhibited 61 and 94% by 2 and 10 μM inorganic triphosphate, respectively. The inhibition is cooperative (n = 1.1-1.6) and becomes weaker as the pH is decreased from 7.0 to 6.2, the K_I increasing from 10 to 33 μM in the case of pyrophosphate and from 1.5 to 2.7 μM in the case of triphosphate. (K_I is defined as the concentration of inhibitor required for half-maximal inhibition.) ADP relieves the inhibition produced by both substances. For example, about 100 μM ADP completely reverses the inhibition produced by 100 μM pyrophosphate or 10 μM triphosphate. The effects of H^+ and ADP on the inhibition by pyro- and triphosphate are similar to those observed for the inhibitions caused by GTP,

† From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received January 28, 1980.* This work was supported by National Institutes of Health Grant GM-07261. Publication 1330 from the Graduate Department of Biochemistry, Brandeis University, where reprints may be obtained.

‡ Recipient of a National Science Foundation Graduate Fellowship. Supported in part by Training Grant GM-212. Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853.

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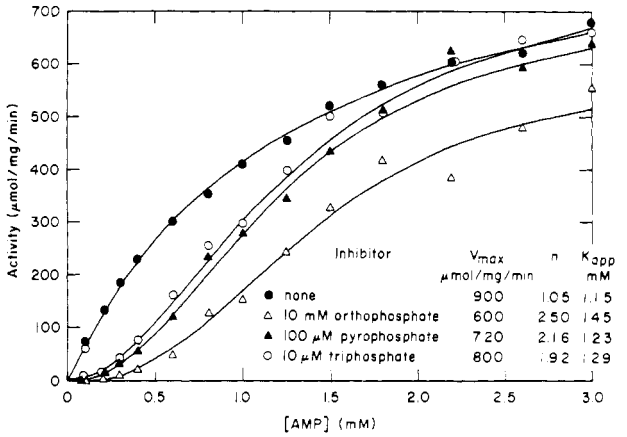

FIGURE 1: Effects of ortho-, pyro-, and triphosphate on the AMP saturation curve. Assays contained 150 mM KCl, 50 mM imidazole–HCl buffer, pH 7.0, and 0.2 μg/mL enzyme. The reaction was followed by measuring the decrease in absorbance at 262.5 nm, where the largest difference in absorbances of AMP and IMP occurs ($\Delta\epsilon_{mM}$ = 8.8) (Tornheim & Lowenstein, 1972). The curves are drawn by using the equation $v = V_{max}[[AMP]^n/([AMP]^n + K_{app}^n)]$ with the indicated values of V_{max}, n, and K_{app}. The values for V_{max} were obtained from plots of v vs. $v/[AMP]$ and those for n and K_{app} from plots of log $[v/(V_{max} - v)]$ vs. log [AMP].

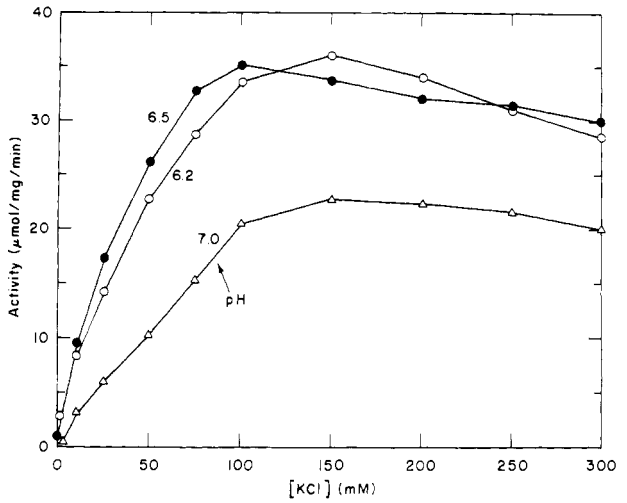

FIGURE 2: Effect of KCl concentration on adenylate deaminase activity in the presence of 20 μM AMP. The experimental conditions were as described for Figure 1 except that [KCl] was varied as indicated.

ATP, and orthophosphate (Wheeler & Lowenstein, 1979b).

Effects of Inorganic Phosphates on the Substrate Saturation Curve. The effects of 10 mM orthophosphate, 100 μM pyrophosphate, and 10 μM triphosphate on the AMP saturation curve of adenylate deaminase in the presence of 150 mM KCl and at pH 7.0 are shown in Figure 1. The effect of each inhibitor is to convert the curve from a hyperbolic to a sigmoidal form and to reduce the reaction velocity at low [AMP]. The Hill coefficient, n, increases from 1.05 to 1.9–2.5. There is an apparent decrease of V_{max} by 10–30%; however, the extrapolation to V_{max} is relatively inaccurate. ATP and GTP have similar effects (Coffee & Solano, 1977).

Effects of KCl and Tetramethylammonium Chloride. We established previously that in the presence of 20 μM AMP, 150 mM KCl, and various effectors adenylate deaminase activity is proportional to enzyme concentration in the range 0.02–1.0 μg/mL (Wheeler & Lowenstein, 1979b). For the purposes of the present paper we also measured the specific activity at low [KCl]. In the presence of 20 μM AMP and 25 mM KCl at pH 7.0, the specific activity did not decrease between 1.0 and 0.1 μg of enzyme/mL. In all experiments

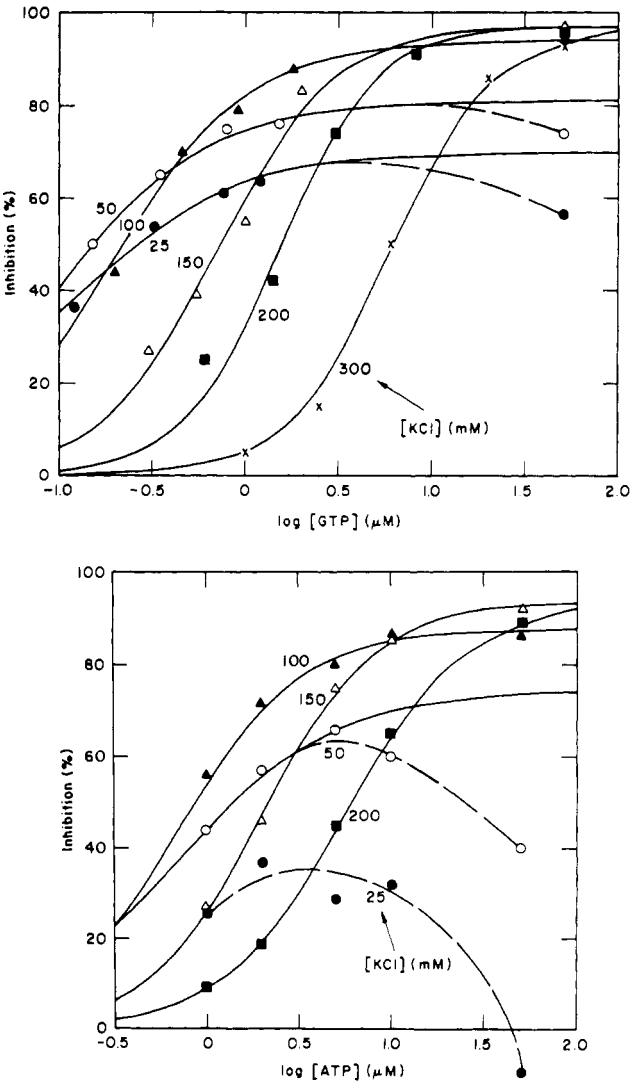

FIGURE 3: Effects of KCl concentration on inhibition of adenylate deaminase by GTP and ATP. Assays contained 10 μM AMP, [KCl] as indicated, 50 mM imidazole–HCl buffer, pH 7.0, and 0.2 μg of enzyme/mL. The temperature was 20 °C. The solid lines are drawn according to the equation $I = I_{max}[[L]^n/([L]_n + K_I^n)]$, where [L] is the inhibitor concentration, using the parameters listed in Table I. The values for I_{max} were obtained from plots of I vs. $I/[L]$ and those for n and K_I from plots of log $[I/(I_{max} - I)]$ vs. log [L]. The broken lines were not drawn by using the equation. The point drawn below the x axis in the ATP graph corresponds to 10% activation.

reported here the enzyme was used at a concentration of 0.2 μg/mL.

Adenylate deaminase activity in the presence of 20 μM AMP is maximum at KCl concentrations between 100 and 150 mM (Figure 2). Inhibition of activity at higher KCl concentrations is probably due to chloride ion (Ronca et al., 1972). Potassium acetate and sulfate were tested in the same concentration range at pH 7.0, but the activity was less than that observed with potassium chloride for all concentrations of potassium ions.

The chloride inhibition complicates the KCl curve, making it difficult to assess the degree to which the enzyme is activated by a given concentration of potassium ions. However, the enzyme appears more readily saturated by K^+ at pH 6.2–6.5 than at pH 7.0, in agreement with the results of Campbell & Suelter (1977).

The effect of KCl on the inhibition of the enzyme by GTP and ATP is shown in Figure 3. Values for maximum inhibition (I_{max}), K_I, and n obtained in these experiments, as well

Table I: Effect of [KCl] on Inhibition Parameters at 10–20 μM AMP and pH 7.0

inhibitor[a]	parameter[b]	[KCl] (mM) 25	50	100	150	200	300
GTP	I_{max}	0.70	0.81	0.94	0.97	0.97	0.97
	n	1.0	1.1	1.25	1.4	1.7	1.8
	K_I (μM)	0.1	0.1	0.2	0.7	1.5	6
ATP	I_{max}	c	0.75	0.88	0.94	0.94	
	n	c	1.0	1.3	1.4	1.3	
	K_I (μM)	c	0.7	0.7	2.0	5.6	
orthophosphate	I_{max}	0.96	0.99		1.00		1.00
	n	1.0	1.0		1.5		1.7
	K_I (mM)	0.4	0.4		1.2		3.4
pyrophosphate	I_{max}	0.92			0.98		0.99
	n	1.0			1.5		1.6
	K_I (μM)	1.1			8		65
triphosphate	I_{max}	0.91			0.99		0.99
	n	1.3			1.5		1.6
	K_I (μM)	0.12			1.4		13

[a] [AMP] was 10 μM for the experiments with GTP and ATP and 20 μM for the others. Orthophosphate was added as potassium phosphate, pH 7, and KCl was added to give the indicated concentration of K^+. [b] I_{max} is the extrapolated maximum inhibition, n the Hill coefficient, and K_I the inhibitor concentration producing half-maximal inhibition. [c] Not determined because of biphasic curve.

Table II: Effect of Added Salts on GTP Inhibition[a]

[KCl] (mM)	[Me_4NCl] (mM)	activity (μmol mg^{-1} min^{-1}) 0 μM GTP	0.6 μM GTP	6 μM GTP
0	50	0.6	0.4	
0	150	0.3	0.3	0.2
0	300	0.3	0.2	0.2
50	0	11.9	1.7 (85)	
50	100	20.0	9.0 (45)	
50	250	20.8	22.3 (0)	
150	0	24.7	18.3 (36)	1.7 (93)
150	150	20.7	20.1 (3)	7.7 (61)
300	0	22.5	22.2 (2)	15.3 (32)

[a] Assays contained 20 μM AMP, 50 mM imidazole-HCl buffer, pH 7.0, and 0.2 μg/mL enzyme. Percent inhibition is indicated in parentheses.

as in similar experiments with ortho-, pyro-, and triphosphate, are listed in Table I. For each inhibitor, K_I increases with increasing [KCl]. This effect can be very large; for example, the K_I for triphosphate increases 100-fold between 25 and 300 mM KCl. Moreover, the inhibition shows little cooperativity at 25–50 mM KCl but becomes progressively more cooperative as the KCl concentration increases. I_{max} falls between 70 and 95% at low [KCl], increasing to nearly 100% at high [KCl]. The enzyme is inhibited by low concentrations of ATP and GTP, but reversal of this inhibition occurs at high ATP and GTP concentrations (Ashby & Frieden, 1978; Wheeler & Lowenstein, 1979b). Ronca et al. (1972) reported that the biphasic effects of ATP seen at low [KCl] disappear at higher [KCl]. Comparison of the results shown in Figure 3 with our previous results (Wheeler & Lowenstein, 1979b) shows that the reversal of the inhibition does not disappear with added KCl but is shifted to higher concentrations of ATP and GTP.

We tested whether the effect of KCl on the GTP inhibition of adenylate deaminase is due to K^+, Cl^-, or ionic strength. The activity of the enzyme was measured in the presence of various combinations of KCl and $(CH_3)_4NCl$ (Table II). $(CH_3)_4NCl$ reduces the inhibition produced by GTP in the presence of 50 or 150 mM KCl, indicating that the reversal

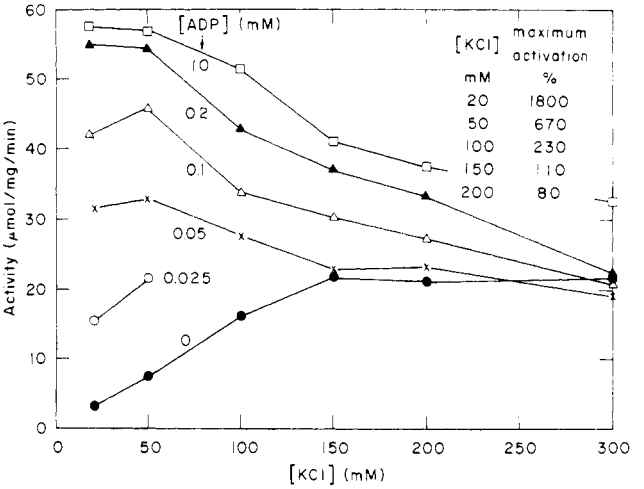

FIGURE 4: Effect of KCl concentration on the activation of adenylate deaminase by ADP in the presence of 20 μM AMP. The experimental conditions were as described for Figure 1 except that the pH was 7.0 and [KCl] was varied as indicated. Extrapolated values for maximum activation at various KCl concentrations are listed.

of the inhibition by higher amounts of KCl is not due solely to K^+. Note that in the presence of $(CH_3)_4NCl$ the enzyme is activated at lower levels of KCl, even though $(CH_3)_4NCl$ itself is not an activator. Thus, $(CH_3)_4NCl$ increases the effectiveness of K^+ as an activator, presumably by providing an increase in ionic strength. The inhibition produced by 6 μM GTP is much weaker in the presence of 300 mM KCl than in the presence of 150 mM KCl plus 150 mM $(CH_3)_4NCl$, indicating effects specifically due to K^+.

The activating effect of ADP was reported to be greatest at low concentrations of KCl; little or no activation was seen at 100 mM KCl (Coffee & Solano, 1977; Ronca et al., 1972; Ronca-Testoni et al., 1970). The dependence of the activation on the concentration of KCl is shown in Figure 4. Although the maximum activation decreases greatly with increasing [KCl], significant activation is still seen at [KCl] as high as 200 mM. The decrease in activity with increasing [KCl] is most apparent at high concentrations of ADP.

Discussion

Inorganic pyrophosphate is a powerful inhibitor of adenylate deaminase, with a K_I of about 10 μM in the presence of 20 μM AMP and 150 mM KCl, pH 7.0 (Table I). Pyrophosphate levels of several micromolar have been detected in liver (Guynn et al., 1974). Thus, if the liver enzyme is equally sensitive to pyrophosphate, or if pyrophosphate occurs at similar levels in muscle, pyrophosphate could contribute to the inhibition of adenylate deaminase.

The inhibition of adenylate deaminase by inorganic pyrophosphate and triphosphate is similar to that produced by low concentrations of ATP and GTP in the following respects. The inhibitions have similar pH dependence and are relieved by ADP and Mg^{2+} (not shown), have similar KCl dependence (Table I), and produce similar effects on the AMP saturation curve (Figure 1). Thus, these two compounds appear to act as analogues of GTP and ATP and can be used as tools for studying the regulatory properties of the enzyme.

The K_I for inorganic triphosphate is 1.4 μM in the presence of 150 mM KCl, which is similar to the K_I for ATP and only twice that for GTP (Table I). This suggests that most of the binding specificity of the inhibitory site is for the triphosphate moiety of the nucleoside triphosphate molecule. The observation that UTP, CTP, and ITP inhibit adenylate deaminase (Ronca et al., 1968; Ronca-Testoni et al., 1970) supports this

interpretation. In the presence of 20 μM AMP and 150 mM KCl, adenylate deaminase shows biphasic ATP and GTP inhibition curves (Wheeler & Lowenstein, 1979b). It is maximally inhibited by 50 μM ATP, but the inhibition decreases above 100 μM ATP. Reversal of the inhibition by ATP is presumably due to ATP binding to an activator site. The inhibition by GTP shows similar behavior, but reversal of the inhibition begins to occur only above 300 μM GTP. Moreover, ADP is a better activator than GDP. Thus, adenine nucleotides bind more tightly to the activator site than guanine nucleotides (Wheeler & Lowenstein, 1979b). Hypoxanthine nucleotides can also function as activators (Ashby & Frieden, 1978). No relief of inhibition by pyrophosphate and triphosphate is observed upon raising their concentrations as high as 1 mM (not shown). Taken together these observations indicate that the activator site interacts primarily with the nucleoside moieties of nucleoside di- and triphosphates.

The K_I for all inhibitors listed in Table I increases when the KCl concentration is raised from 25–50 to 250–300 mM.[1] At the same time the Hill coefficient increases significantly, and the extrapolated maximum inhibition approaches 100%. The change in I_{max} with KCl concentration occurs because at low KCl concentrations the residual activity is a greater fraction of the uninhibited activity. Previous studies with a small number of inhibitor concentrations showed that the inhibitory effects of GTP, ATP, and orthophosphate are reduced at high KCl concentrations (Coffee & Solano, 1977; Ronca et al., 1972; Smiley & Suelter, 1967). The results in Table II show that an increase in $[K^+]$ at constant $[Cl^-]$ reduces the inhibition produced by GTP, indicating a specific role of K^+. In contrast, Ronca et al. (1972) concluded that the reduced inhibition observed at high salt concentrations was primarily due to the anion.

Potassium ions decrease the extent of the activation of the enzyme by ADP, but significant activation is still observed at 200 mM KCl (Figure 4). This is in contrast to Coffee & Solano (1977), who observed no activating effect of ADP at KCl concentrations above 20 mM and concluded that it seems likely that at in vivo levels of K^+, ADP plays an insignificant role in the regulation of AMP deaminase. Our previous results indicate that the primary role of ADP may be to relieve the inhibition produced by GTP and orthophosphate (Wheeler & Lowenstein, 1979b).

Adenylate deaminase from muscle is activated by K^+ and Na^+ ions at low concentrations of AMP but not by $(CH_3)_4N^+$ and $Tris^+$ ions (Smiley & Suelter, 1967). However, because the enzyme is inhibited by all anions that have been tested, the precise contribution made by K^+ ions cannot easily be evaluated. Although $(CH_3)_4N^+$ ions do not activate the enzyme, they enhance the activation by K^+ and reduce the inhibition by GTP in the presence of K^+ (Table II). These findings indicate that activation and inhibition of the enzyme are sensitive to ionic strength.

[1] ATP forms complexes with K^+, and this could contribute to reduced inhibition by nucleoside triphosphates at increased [KCl]. Using the equations $[KATP^{3-}]/([K^+][ATP^{4-}]) = 10\ M^{-1}$ (Melchior, 1954) and $[HATP^{3-}]/([H^+][ATP^{4-}]) = 8.9 \times 10^6\ M^{-1}$ (Smith & Alberty, 1956), it can be calculated that at pH 7.0 and 25, 150, and 300 mM KCl the amount of ATP not complexed with K^+ is 88, 56, and 39% of the total, respectively. We assume GTP is complexed to a similar extent. Thus, formation of KATP and KGTP may be involved in reducing the inhibition by these nucleotides. However, the change in K_I values with [KCl] is much greater than the change in [nucleoside triphosphate] not complexed with K^+; thus, additional factors must be involved in reducing the inhibition by raising [KCl]. Moreover, if a decrease in the amount of free nucleotide were solely responsible for the reduced inhibition, then increasing K^+ would simply shift the inhibition curves to higher nucleotide concentrations, without affecting the cooperativity of inhibition. However, Table I indicates that the cooperativity is increased at higher [KCl].

References

Ashby, B., & Frieden, C. (1978) *J. Biol. Chem. 253*, 8728–8735.

Campbell, J. C. W., & Suelter, C. H. (1977) *Biochemistry 16*, 4836–4839.

Coffee, C. J., & Solano, C. (1977) *J. Biol. Chem. 252*, 1606–1612.

Guynn, R. W., Veloso, D., Lawson, J. W. R., & Veech, R. L. (1974) *Biochem. J. 140*, 369–375.

Lyubimova, M. N., & Matlina, E. S. (1954) *Dokl. Akad. Nauk SSSR 94*, 927–930.

Melchior, N. C. (1954) *J. Biol. Chem. 208*, 615–627.

Nikiforuk, G., & Colowick, S. P. (1956) *J. Biol. Chem. 219*, 119–129.

Ronca, G., Raggi, A., & Ronca-Testoni, S. (1968) *Biochim. Biophys. Acta 167*, 626–629.

Ronca, G., Raggi, A., & Ronca-Testoni, S. (1972) *Ital. J. Biochem. 21*, 305–321.

Ronca-Testoni, S., Raggi, A., & Ronca, G. (1970) *Biochim. Biophys. Acta 198*, 101–112.

Sammons, D. W., Henry, H., & Chilson, O. P. (1970) *J. Biol. Chem. 245*, 2109–2113.

Setlow, B., & Lowenstein, J. M. (1968) *J. Biol. Chem. 243*, 3409–3415.

Smiley, K. L., Jr., & Suelter, C. H. (1967) *J. Biol. Chem. 242*, 1980–1981.

Smith, R. M., & Alberty, R. A. (1956) *J. Am. Chem. Soc. 78*, 2376–2380.

Tornheim, K., & Lowenstein, J. M. (1972) *J. Biol. Chem. 247*, 162–169.

Wheeler, T. J., & Lowenstein, J. M. (1979a) *J. Biol. Chem. 254*, 1484–1486.

Wheeler, T. J., & Lowenstein, J. M. (1979b) *J. Biol. Chem. 254*, 8994–8999.

Yun, S.-L., & Suelter, C. H. (1978) *J. Biol. Chem. 253*, 404–408.