Jenkins, W. T., and D'Ari, L. (1966), *J. Biol. Chem. 241,* 2845–2854.
Jenkins, W. T., and Sizer, I. W. (1960), *J. Biol. Chem. 235,* 620–624.
Kagamiyama, H., Teranishi, K., and Wada, H. (1975) *Biochem. Biophys. Res. Commun. 63,* 993–999.
Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232,* 549–558.
Martinez-Carrion, M., Turano, L., Chiancone, E., Bossa, F., Giartosio, A., Riva, F., and Fasella, P. (1967), *J. Biol. Chem. 242,* 2397–2409.
Morino, Y., and Okamoto, M. (1973), *Biochem. Biophys. Res. Commun. 50,* 1061–1067.
Morino, Y., and Watanabe, T. (1969), *Biochemistry 8,* 3412–3417.
Ovchinnikov, Yu. A., Egorov, C. A., Aldanova, N. A., Feigina, M. Yu., Lipkin, V. M., Abdulaev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyanovsky, O. L., and Nosikov, V. V. (1973), *FEBS Lett. 29,* 31–34.
Peterson, E. A., and Sober, H. A. (1954), *J. Am. Chem. Soc. 76,* 169–175.
Rando, R. R. (1974a), *Science 185,* 320–324.
Rando, R. R. (1974b), *Biochemistry 13,* 3859–3863.
Rando, R. R. (1975), *Acc. Chem. Res. 8,* 281–288.
Rando, R. R. (1977), *Methods Enzymol. 46,* 28–41.
Rando, R. R., Releyea, N., and Cheng, L. (1976), *J. Biol. Chem. 251,* 3306–3312.
Schlegel, H., and Christen, P. (1974), *Biochem. Biophys. Res. Commun. 61,* 117–123.
Simpson, R. T., Vallee, B. L., and Tait, G. H. (1968), *Biochemistry 7,* 4336–4342.
Soper, T. S., Manning, J. M., Marcotte, P. A., and Walsh, C. T. (1977), *J. Biol. Chem. 252,* 1571–1575.
Steers, E., Jr., Craven, G. R., Anfinsen, C. B., and Bethune, J. L. (1965), *J. Biol. Chem. 240,* 2478–2484.
Strausbauch, P. H., and Fischer, E. H. (1970), *Biochemistry 9,* 233–238.
Torchinsky, Yu. M., Kochkina, V. M., and Sajgo, M. (1974), *Acta Biochim. Biophys., Acad. Sci. Hung. 9,* 213–216.
Velick, S. F., and Vavra, J. (1962), *J. Biol. Chem. 237,* 2109–2122.

Effect of H^+ on the K^+ Activation of Adenosine-5′-monophosphate Aminohydrolase†

John C. W. Campbell and Clarence H. Suelter*

ABSTRACT: The activation of adenosine-5′-monophosphate aminohydrolase from rabbit skeletal muscle by H^+ has been demonstrated. Evidence is presented which indicates that the binding of H^+ and K^+ is linked, in that the dissociation constant (K_A) for K^+ activation is reduced as the pH is lowered. Concomitantly, the pK of several enzyme functional groups is changed when K^+ is added to a solution of enzyme. This change in pK results in an uptake or release of H^+, depending on the pH, and shows that K^+ interacts with the enzyme to achieve its effect. The uptake or release of H^+ provides a simple method of following conformational changes in the enzyme following interaction of K^+. The K_D for K^+ interaction monitored by following pH changes is the same within experimental error as that measured from kinetic data.

Adenosine-5′-monophosphate aminohydrolase is found in most mammalian tissues in varying levels of activity. It functions to regulate some aspects of purine nucleotide metabolism, ammonia metabolism, energy charge, and gluconeogenesis or glycolysis. The latter stems from the role that AMP[1] has on many enzyme reactions which is not shared by IMP. Most preparations of this enzyme are activated by adenine nucleotides and monovalent cations and inhibited by guanine nucleotides and inorganic phosphates (Zielke and Suelter, 1971a). Our recent studies indicated that H^+ also exerts an important effect on the enzyme activity (Suelter et al., 1968). This paper describes our attempts to determine whether H^+ and monovalent cations such as K^+ interact at the same site, or, if not, to determine the way in which H^+ affects the binding of K^+. A method for detecting conformation changes of 5′-AMP aminohydrolase not discernible by other methodologies is noted.

Materials and Methods

A. *Enzyme Purification.* 5′-AMP aminohydrolase (EC 3.5.4.6) was purified from mature rabbit back muscle in the manner of Smiley et al. (1967). All buffers and reagents were of the same composition as those used by Smiley et al. (1967). The purification was achieved by direct elution of the enzyme from cellulose phosphate with 1.0 M KCl, 1 mM 2-mercaptoethanol, pH 7.0, rather than a 0.45 to 1.0 M KCl gradient as in the original procedure. Specific activities of between 80 and 130 units per mg of protein were obtained at 50 μM 5′-AMP, pH 6.3. The enzyme was routinely stored at 4 °C, in the presence of 1.0 M KCl, 1 mM 2-mercaptoethanol, pH 7.0, under a nitrogen atmosphere. There was negligible loss of activity after 3 weeks. In all experiments, enzyme was used within 2 weeks of preparation.

B. *Enzyme Assays.* 5′-AMP aminohydrolase was assayed using the spectrophotometric assay of Kalckar (1947). For

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received May 10, 1977.* Supported by a grant from the National Science Foundation. Michigan State Agricultural Experiment Station Journal Article No. 8097.

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonate; 5′-AMP, adenosine 5′-monophosphate; IMP, inosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; ORD, optical rotatory dispersion; CD, circular dichroism.

5′-AMP concentrations above 0.1 mM, the modifications of Smiley and Suelter (1967) were used. The changes in optical density at 265 nm or at 285 nm were measured and converted to μmol per min using the conversion factors of 8.86 μmol mL^{-1} cm^{-1} at 265 nm and 0.30 μmol mL^{-1} cm^{-1} at 285 nm. All assays were started by addition of enzyme.

For routine assays, the following buffer was used: 50 mM Tris–Mes, 150 mM KCl, 50 μM 5′-AMP, pH 6.3. For determinations of K_A, the buffer used was 90 mM Tris–Mes, 50 μM 5′-AMP, 300 mM $(CH_3)_4NCl$, at the appropriate pH. One unit of enzyme activity is defined as μmol of 5′-AMP deaminated per min at 30 °C.

The Michaelis–Menten parameters K_m, K_A, and V_{max} were calculated from initial velocity data as described by Wilkinson (1961). Hill slopes were calculated from plots of $\log[v/(V - v)]$ vs. $\log(s)$ drawn by eye.

C. *Removal of Activating Cations.* Activating cations were removed in either of the following two ways. For determination of K_A values, where protein concentrations below 0.5 mg mL^{-1} were suitable, a small volume of purified enzyme was passed over a column of Sephadex G-25, previously equilibrated with the desired buffer. The second procedure involved extensive dialysis. Dialysis tubing (Union Carbide Corporation) was boiled in 2 mM NaEDTA for 1 h, rinsed twice with deionized water, and stored in 2 mM NaEDTA at 4 °C. Before use, all tubing was washed exhaustively with deionized water. Since it was found that ionic strength was critical for enzyme stability, the following dialysis procedure was adopted. One milliliter of purified enzyme in 1.0 M KCl was dialyzed successively against two 500 mL volumes of 0.5 M KCl and 0.3 M KCl, each for 12 h. Each solution also contained 1.0 mM Tris–Mes, at the appropriate pH, and 1.0 mM 2-mercaptoethanol. Next, the enzyme was dialyzed against two 500 mL volumes of 0.5 M $(CH_3)_4NCl$, 1 mM Tris–Mes, each for 12 h. The 0.5 M $(CH_3)_4NCl$ concentration was used, instead of lower concentrations, because of the increased stability noted in this solvent.

D. *Protein Determination.* Protein concentrations were determined by the tannic acid method of Katzenellenbogen and Dobryszycka (1959), by the method of Lowry et al. (1951), or by use of an extinction coefficient of 0.92 mg cm^{-1} mL^{-1} as determined by Zielke and Suelter (1971b). For the turbidometric method, all reagents were filtered before use. Bovine serum albumin in 1% NaCl solution was used as a standard.

E. *Proton Release and Uptake Experiments.* Protons released or absorbed by enzyme upon addition of K^+ were detected in either of two ways. The first method involved the use of the dye, resazurin. The second method involved the use of a hydrogen ion sensitive electrode (Sargent-Welch S-30070 combination electrode), in conjunction with a sensitive differential amplifier (Heath/Schlumberger EU-200-30), coupled to a recorder (Heath/Schlumberger EU 205-1). For either method, activating cations were removed from 5′-AMP aminohydrolase by extensive dialysis as described above.

F. *K_A Determination.* Purified 5′-AMP aminohydrolase was freed of activating cations according to the procedure described in Materials and Methods. Initial velocities were determined as a function of K^+ concentration using the assay procedure described previously. An initial estimate of K_A was determined by plotting the data as suggested by Hofstee (1952). Here the residual activity of the enzyme in the absence of cation was subtracted from the initial velocity values at each cation concentration. More precise values of K_A were then determined, by using this initial estimate of K_A to design kinetic experiments as suggested by Cleland (1967). Initial velocities in triplicate were determined at five concentrations of cation between 20 and 80% saturation. Protein concentrations in the assay were maintained as high as technically feasible to reduce enzyme inactivation during assay (Hemphill and Suelter, 1971).

TABLE I: Kinetic Constants for K^+ Activation of 5′-AMP Aminohydrolase at pHs 6.2, 6.5, and 6.8.[a]

pH	K_A (mM)	V^b	n^c
6.2	1.96 ± 0.16	96.8 ± 13.9	0.98 ± 0.03
6.5	3.46 ± 0.25	109.0 ± 20.0	1.01 ± 0.08
6.8	6.10 ± 0.26	113.5 ± 12.0	1.02 ± 0.01

[a] Reaction mixtures contained 90 mM Tris–Mes, 300 mM $(CH_3)_4NCl$, and 50 μM 5′-AMP. [b] Velocity (μmol min^{-1} mg^{-1}) at saturating concentrations of KCl. [c] Hill slope as determined from plots of $\log(v/V - v)$ vs. $\log(s)$.

G. *Reagents.* The Tris-base, Mes, 2-mercaptoethanol, and 5′-AMP (Na^+) were obtained from Sigma Chemical Co. (St. Louis, Mo.). $(CH_3)_4NCl$ and resazurin, certified type, were obtained from Eastman (Rochester, N.Y.). The $(CH_3)_4NCl$ was recrystallized twice from 2-propanol and stored at 110 °C until use. The cellulose phosphate used in the purification was obtained from Brown Corp. (Berlin, New Hampshire). All other reagents used were of reagent grade or better.

Results

A. *Kinetic Constants for K^+ Activation of 5′-AMP Aminohydrolase as a Function of pH.* The K^+ activation constant, along with V_{max} and the Hill slope, n, were determined as a function of pH in order to establish the nature of the interaction of H^+ and K^+ with enzyme. Activating cations were removed from purified enzyme by gel filtration, as described in Materials and Methods. Initial velocities were then measured at 30 °C vs. K^+ ion concentration at 50 μM 5′-AMP. Values for n_H were determined from Hill plots. The values of K_A at various pH values, calculated as described in Materials and Methods, are given in Figure 3 and Table I. These experiments demonstrate that the K_A for K^+ activation decreases with increasing H^+ concentration, which suggests that K^+ and H^+ are not competing for the same site. Maximum velocity and Hill slope ($\eta_H = 1$) under these conditions were independent of pH. These last observations are consistent with previous data (Hemphill et al., 1971).

B. *Release or Uptake of H^+ upon K^+ Binding to 5′-AMP Aminohydrolase.* Resazurin, a proton sensitive dye, was used to examine uptake or release of H^+ by enzyme upon addition of K^+ to a solution of enzyme. Figure 1 presents the visible absorption spectrum of resazurin at a concentration of 50 μM in 45 mM Tris–Mes at various pH values. A maximum absorption at 600 nm was observed for the ionized form and 530 nm for the protonated form of dye. Titration of resazurin with H^+ produced data which fitted a theoretical curve, calculated from the Henderson–Hasselbalch equation, using a pK of 5.7 (see insert of Figure 1).

Uptake or release of H^+ upon K^+ binding to enzyme, in the presence of resazurin, was then examined by monitoring absorbance changes at 600 nm. Potassium was added to solutions containing 1.5 mg of enzyme dissolved in 1 mL of buffer composed of 1.0 mM Tris–Mes, 300 mM $(CH_3)_4NCl$, and between 5.0 and 15 μg of resazurin. The amount of dye added depended on the pH of the experiment so as to ensure a significant change in absorbance at the higher pH values. Absorbance experiments performed below approximately pH 6.15 showed an increase in absorbance indicating an uptake of H^+

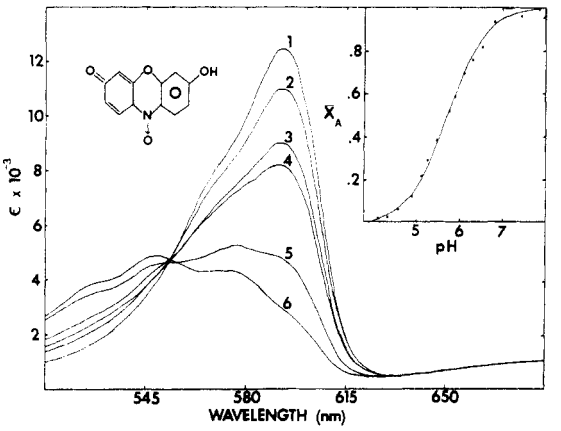

FIGURE 1: Visible absorption spectrum and titration curve. Absorption spectra of 50 μM resazurin were obtained in 45 mM Tris-Mes at the following pHs: 6.50 (1), 6.10 (2), 5.80 (3), 5.70 (4), 5.30 (5), and 4.80 (6). Extinction coefficient (ϵ, L mol^{-1} cm^{-1}) is plotted vs. wavelength (nm). The fraction of change in absorbance, $\bar{X}_A$, at 600 nm was determined for a solution of 2.5 μg mL^{-1}. Resazurin dissolved in 100 mM Tris-Mes as a function of pH. The solid line is a theoretical curve calculated using the Henderson-Hasselbalch equation assuming a pK of 5.7.

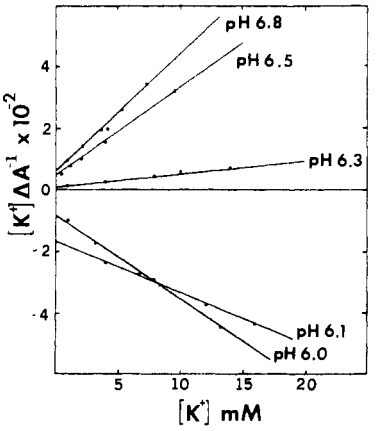

FIGURE 2: Change in absorbance of resazurin following K^+ binding to 5'-AMP aminohydrolase plotted according to Eadie-Hofstee (1952) as a function of pH, as indicated on the figure.

by enzyme. At higher pH values, a decrease in absorbance was observed indicating a release of H^+ by enzyme. Additional experiments at pH values below pH 6.0 were attempted, but due to the precipitation of enzyme, accurate absorbance readings were not possible. Experiments above pH 7.0 were not performed because earlier work demonstrated that enzyme at these pH values showed anomalous kinetics (Hemphill et al., 1971). As a control, K^+ was added to buffer minus enzyme. Sufficiently concentrated solutions of K^+ were added so as to eliminate a dilution effect. In addition, levels of enzymatic activity were measured before and after each experiment and were found to change less than 5%.

Data from these titration experiments were analyzed as follows. At zero cation concentrations, the absorbance at 600 nm was noted and was then subtracted from absorbance at 600 nm obtained after each addition of cation to give a net change in absorbance. Net changes in absorbances showed hyperbolic saturation with increasing K^+ concentration and were found to fit eq 1:

$$\Delta A = \frac{\Delta A_{max}[K^+]}{K_D + [K^+]} \quad (1)$$

where ΔA is change in absorbance, and ΔA_{max} change in absorbance at saturating concentrations of K^+. The data were

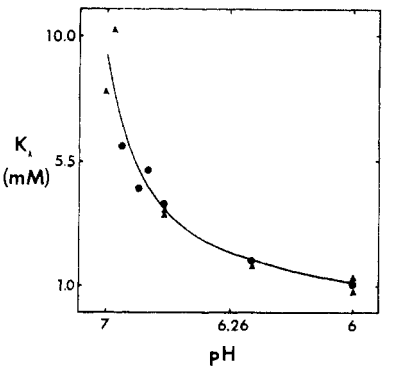

FIGURE 3: The pH dependence of K_A for K^+ activation of 5'-AMP aminohydrolase. Activation constants (K_A's) were determined from equilibrium (▲) or kinetic (●) experiments (experimental conditions and data treatment are given in Results).

analyzed statistically in terms of the Lineweaver-Burk transformation, as described by Wilkinson (1961), and then plotted as indicated in Figure 2. The K_D values determined from such experiments are plotted as a function of H^+ in Figure 3.

C. *Stoichiometry of H^+ Release or Absorption upon K^+ Binding to 5'-AMP Aminohydrolase as a Function of pH.* A H^+-sensitive glass electrode coupled to a differential amplifier (see Experimental Procedures for a description of equipment used) was used to determine the stoichiometry of H^+ release or absorption following addition of K^+ to an enzyme solution. In these experiments, the enzyme was freed of activating cations by dialysis, according to the procedure outlined in the experimental procedure. The pH of solutions containing 1.5 mg/mL of enzyme in a buffer composed of 1.0 mM Tris-Mes, and 300 mM $(CH_3)_4NCl$ at the appropriate pH, was then monitored upon addition of aliquots of stock 1 M KCl. A control experiment was performed in which the pH of the above buffer solution, minus enzyme, was monitored upon addition of K^+. No detectable change in pH was observed. All experiments were performed under a nitrogen atmosphere to prevent absorption of CO_2 by the buffer. Data generated in this manner showed hyperbolic saturation with increasing K^+ concentration and were found to fit eq 1, where $\Delta[H^+]$ is substituted for ΔA, and where $(\Delta H)_{max}$ is the change in $[H^+]$ at saturating concentrations of K^+. Stoichiometry of H^+ release or absorption was then calculated by expressing $(\Delta pH)_{max}$ as an amount of OH^- or H^+ required to back titrate the pH changes. After saturating levels of K^+ had been reached ($100 \times K_A$), several aliquots of $(CH_3)_4NOH$ or HCl were added with pH change being noted after each addition. From such data, standard curves of ΔpH vs. OH^- or H^+ concentrations were constructed. The slopes, $\Delta pH/[OH^-]$, calculated from a least-squares fit of data to a straight line were then used, knowing the volume of an enzyme solution, to express $(\Delta pH)_{max}$ as an amount of OH^- or H^+ taken up or liberated. These experiments were repeated over the pH range of 6.0 to 7.0. The data are summarized in Figure 4.

Discussion

The data presented earlier are consistent with the following conclusions. First, as indicated by the experiments with the dye, resazurin, and by direct pH measurements, K^+ interacts with 5'-AMP aminohydrolase in the absence of substrate AMP. Furthermore, the K_D for K^+ interaction measured with the pH electrode or dye was not significantly different from those determined kinetically. Thus, as is the case with most monovalent cation activated enzymes, the cation interacts with the

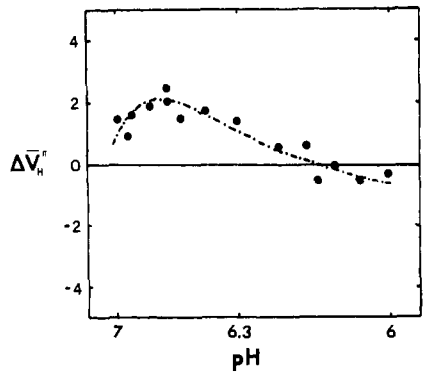

FIGURE 4: Difference titration data and a theoretical curve calculated with eq 2. Changes in pH, using equipment as described in Materials and Methods, of solutions containing 1.5 mg mL^{-1} 5′-AMP aminohydrolase dissolved in 1.0 mM Tris-Mes, and 300 mM $(CH_3)_4NCl$ upon addition of KCl. All experiments were performed under an atmosphere of N_2. A line calculated using eq 2 is shown. The constants used in the calculation were: 6 sites with pK shifting from 5.78 to 6.22, 6 sites with pK shifting from 6.75 to 6.20, and 6 sites shifting from 7.70 to 8.20 when K^+ is added.

enzyme to achieve its effect rather than with the substrate (Suelter, 1974). Secondly, since the K_A for K^+ activation decreased with decreasing pH, K^+ and H^+ do not interact at the same site on the enzyme surface. Likewise, the interaction of H^+ with the enzyme results in a decrease in the K_A for K^+.

The stoichiometry of H^+ binding can be estimated by fitting difference titration data to eq 2 (Aune and Tanford, 1969)

$$\Delta V_{H^+}{}^n = \sum_i^l \left(\frac{n_i a_H}{a_H + K_i} - \frac{n_i a_H}{a_H + K_i^{(A)}} \right) \qquad (2)$$

where $\Delta V_{H^+}{}^n$ is the net difference in numbers of H^+ released or absorbed by enzyme upon addition of saturating levels of K^+; K_i and $K_i^{(A)}$ are H^+ dissociation constants in absence and presence of K^+, respectively. l is the number of different types of functional binding groups undergoing a pK change and n, the number of groups (H^+ binding sites) within each type. Figure 4 shows a plot of $\Delta V_{H^+}{}^n$ as a function of the H^+ concentration. In attempts to fit the date with eq 2, we varied the total number of H^+ binding sites, H^+ dissociation constants, and direction of pK shifts. The theoretical line obtained with eq 2 drawn through the data of Figure 4 assumes that a total of 18 H^+ binding sites per subunit were perturbed by the K^+ binding, as indicated in the legend of Figure 4. Attempts to fit the data with a smaller number of H^+ binding sites undergoing a pK change were not successful. On the other hand, it will not be possible to obtain a unique fit of the data if the pK of 18 H^+ binding sites is allowed to vary; the simplest interpretation is that the interaction of K^+ with the enzyme results in a change in the pK of several H^+ binding sites, reflecting a change in conformation of the enzyme.

The suggestion of a reviewer that subunit association-dissociation may be responsible for the change in pK of H^+ binding sites cannot be unambiguously eliminated. The most convincing evidence negating this argument is that given by Ellis et al. (1971) showing that enzyme at 0.75 mg mL^{-1} in 0.1 M $(CH_3)_4NCl$ has the same specific activity as that at 1.7 mg mL^{-1}. Concentrations of enzyme lower than 0.75 mg mL^{-1} show lower specific activities indicative of a concentration dependent inactivation such as subunit dissociation or loss of the required divalent cation, Zn^{2+} (Zielke and Suelter, 1971b). Since the experiments of Figure 4 were completed at 1.5 mg mL^{-1}, it seems reasonable to argue that the $\Delta V_{H^+}{}^n$ data do not reflect this concentration-dependent inactivation. We believe that the increased stabilization of the enzyme in K^+ (Hemphill et al., 1971) reflects the same K^+ induced conformation as that noted by the $\Delta V_{H^+}{}^n$ data. It is important to note that this change in conformation was not discernible by ORD, CD, and fluorescent techniques (unpublished observations). If the release or uptake of H^+ results from a general conformational change, monitoring pH changes either with a dye or by an electrode represents a convenient way to follow such changes in this enzyme.

The physiological significance of the activation of 5′-AMP aminohydrolase by H^+ is not appreciated. We are aware of the observations that the conformations of proteins, including their allosteric properties, are controlled by H^+ (Wieker and Hess, 1971; Garel and Labousse, 1970; Antonini and Brunori, 1970). However, the way in which H^+ affects or regulates the activity of various enzymes which catalyze reactions that are important in the metabolism of AMP in muscle has not as yet been assessed.

Acknowledgments

We acknowledge the assistance and loan of equipment from Professor Norman Good, Department of Botany and Plant Pathology, Michigan State University.

References

Antonini, E., and Brunori, M. (1970), *Annu. Rev. Biochem. 39,* 977-1042.

Aune, K. C., and Tanford, C. (1969), *Biochemistry 8,* 4579-4585.

Cleland, W. W. (1967), *Adv. Enzymol. 29,* 1-32.

Ellis, K. J., Kuntz, K., and Sturtevant, J. M. (1971), *J. Biol. Chem. 246,* 6631-6637.

Garel, J. R., and Labousse, B. (1970), *J. Mol. Biol. 47,* 41-56.

Hemphill, R. M., Zielke, C. L., and Suelter, C. H. (1971), *J. Biol. Chem. 246,* 7237-7240.

Hofstee, H. J. (1952), *Science 116,* 329-331.

Kalckar, H. M. (1947), *J. Biol. Chem. 167,* 461-475.

Katzenellenbogen, W., and Dobryszycka, W. M. (1959), *Clin. Chim. Acta 4,* 515.

Lowry, D. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265-275.

Smiley, K. L., Jr., Berry, A. J., and Suelter, C. H. (1967), *J. Biol. Chem. 242,* 2502-2506.

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

Suelter, C. H. (1974), in Metal Ions in Biological Systems, Vol. 3, Segel, H., Ed., New York, N.Y., Marcel Dekker, (1968), pp 201-251.

Suelter, C. H., Kovacs, A. L., and Antonini, E. (1968), *FEBS Lett. 2,* 65-68.

Wieker, H. J., and Hess, B. (1971), *Biochemistry 10,* 1243-1248.

Wilkinson, G. N. (1961), *Biochem. J. 80,* 324-332.

Zielke, C. L. (1970), Ph.D. Thesis, Michigan State University.

Zielke, C. L., and Suelter, C. H. (1971), *Enzymes, 3rd Ed. 4,* 47-77.

Zielke, C. L., and Suelter, C. H. (1971b), *J. Biol. Chem. 246,* 2179-2186.