Quagliariello, E., Papa, S., Saccone, C., Palmieri, F., and Francavilla, A. (1965b), *Ital. J. Biochem. XIV*, 44.

Reithel, F. J. (1963), *Advan. Protein Chem. 18*, 123.

Rogers, K. S., Hellerman, L., and Thompson, T. E. (1965), *J. Biol. Chem. 240*, 198.

Snell, E. E. (1963), *in* Comprehensive Biochemistry, Vol. 2, Florkin, M. and Stotz, E. H., Ed., New York, N. Y., Elsevier, p 52.

Tomkins, G. M., Yielding, K. L., Bitensky, M. W., and Talal, N. (1964), *Abstr. 9, 6th Intern. Congr. Biochem.*, New York, N. Y., p 694.

Glutamic–Aspartic Transaminase. XI. Reactivity toward Thiosemicarbazide*

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ABSTRACT: The second-order rate constants for the reaction of thiosemicarbazide with the phosphopyridoxal form of the pig heart extramitochondrial glutamic–aspartic transaminase were compared with those for its glutarate complex. The greater reactivity of the acidic form of the enzyme was confirmed. Glutarate prevented this increase in reactivity as the pH was lowered. Increasing the concentration of glutarate, at pH 7.9, reduces the rates of both combination and dissociation to the same extent so that the thiosemicarbazide dissociation constant is unaffected. These studies suggest the possibility that there is an obligatory sequence in the formation and dissolution of enzyme–substrate multiple ligands.

The extramitochondrial glutamic–aspartic transaminase (EC 2.6.1.1.) of pig heart is a pH indicator in the visible range due to the fact that it contains, as a chromophore, firmly bound pyridoxal phosphate (Jenkins *et al.*, 1959b). This phosphopyridoxal form may be readily shown to react not only with protons but also with both carbonyl reagents (Jenkins *et al.*, 1959a; Sizer and Jenkins, 1962) and certain dicarboxylic acids (Jenkins *et al.*, 1959b). The derivatives formed with dicarboxylic acids and carbonyl reagents are also pH indicators (Sizer and Jenkins, 1963).

The reaction with glutaric acid has recently been reinvestigated in some detail to find out how the carboxyl groups were reacting with the protein. Since the glutarate dissociation constant increased markedly with increasing pH, it was concluded that at high pH values only one carboxyl group is bound whereas both are bound at low pH values. It was found that the carboxyl group which binds at both high and low pH values does so by displacing a buffer anion from a positive site on the protein ($K_{eq} \cong 1$). The other carboxyl group binds to the acidic form of the pH-indicating chromophore.

In preliminary studies it was noted that, among carbonyl reagents, thiosemicarbazide ($NH_2CSNHNH_2$) was experimentally advantageous because it reacts slowly, absorbs only at wavelengths shorter than 310 mμ, is stable, and has pK values (1.5 and 10.3) out of the range of investigation (Sizer and Jenkins, 1963). In a series of imidazole–chloride buffers it was found that there was an increased affinity of the enzyme for the carbonyl reagent at low pH values. This was due solely to a greater reactivity of the acidic form of the enzyme, not to a faster rate of dissociation of thiosemicarbazide from the complex.

Unfortunately, these studies were made with an enzyme preparation now known to be heterogeneous by the criteria of starch gel electrophoresis and CM-Sephadex chromatography. We have, however, confirmed the earlier work with a single isozyme and compared the reactivities of the enzyme with its glutarate complexes. It was hoped that such an investigation would throw light on a possible function of one of the substrate carboxyl groups in the initial formation of a Schiff base between the substrate amino group and the bound pyridoxal phosphate.

Materials and Methods

Glutaric acid was prepared from Matheson glutaric anhydride by three recrystallizations from water. Thiosemicarbazide was from Eastman. Solutions which had been kept at 4° for 2 months were found to give results identical with those obtained with fresh solutions. Tris and pyrophosphate buffers were prepared from Tris base and sodium pyrophosphate by

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titration with hydrochloric acid, borate buffers by titration of sodium tetraborate with sodium hydroxide. These experiments are with only the α form of the extramitochondrial glutamic–aspartic transaminase (Martinez-Carrion *et al.*, 1965).

The reactions were followed in a Cary 15 spectrophotometer at a wavelength around 400 mμ where the products absorb substantially more than either the enzyme itself or its glutarate derivative. Both the cell holder and compartments were thermostated at 25°. Spectrophotometric titrations were performed by the addition of small aliquots of concentrated solutions with an ultraprecision micrometer pipet.[1]

Determination of Dissociation and Rate Constants

The reaction between thiosemicarbazide (T) and the phosphopyridoxal form of the transaminase (E), or its glutarate complex (EG), are slow and the final equilibria favor dissociation. A large excess of the carbonyl reagent, therefore, has to be used to measure the rate constants. With such conditions the concentration of the free thiosemicarbazide reagent does not change appreciably from that which is added so that the reaction approaches the equilibrium as if it were of pseudo first order with respect to the enzyme concentration. The apparent first-order rate constant, k', which is obtained is a measure of the rate constants in both forward and reverse directions. The reaction may be formulated

$$E \underset{k_{-1}}{\overset{k_1[T]}{\rightleftharpoons}} ET$$

The apparent first-order rate constant, $k' = k_1[T] + k_{-1}$, associated with a particular high concentration of thiosemicarbazide (T) may be obtained from the slope of a graph of the logarithm of the difference $(D_t - D_e)$ between the relevant final optical density (D_e) and that observed at time, t (D_t), with respect to the time (Frost and Pearson, 1961). The final optical density may be assumed in practice to be equivalent to the experimental value after seven reaction half-lives. The individual rate constants (k_1 and k_{-1}) may then be derived by plotting the apparent first-order rate constant, k', with respect to the associated thiosemicarbazide concentration. This yields a straight line of slope k_1, ordinate intercept k_{-1}, and abscissa intercept $(k_1/k_{-1}) = K_d$, the thiosemicarbazide dissociation constant (Sizer and Jenkins, 1963).

In the presence of glutarate but at constant buffer concentration, pH, and temperature, the reaction may be considered to involve two separate species, the enzyme itself (E) and its glutarate complex (EG). The observed rate (k_1) of the forward reaction, that is, the slope of a plot of the apparent first-order rate constant with respect to the thiosemicarbazide concentration, is dependent upon the glutarate concentration

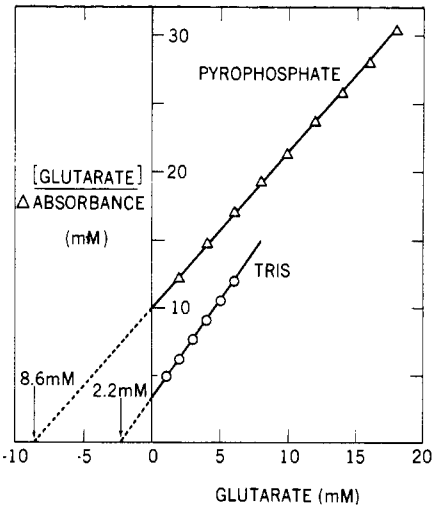

FIGURE 1: Spectrophotometric determination of the enzyme–glutarate dissociation constants at 25° in 0.05 M Tris-Cl and 0.05 M pyrophosphate buffers, pH 7.9.

if the enzyme and its glutarate complex have differing reactivities k_1' and k_1''

$$\begin{array}{ccc} & G & G \\ & + & + \\ T + E & \overset{k_1'}{\rightleftharpoons} & ET \\ \uparrow\downarrow & & \uparrow\downarrow \\ T + EG & \overset{k_1''}{\rightleftharpoons} & ETG \end{array}$$

If $[E][G]/[EG] = K$ then the observed rate of combination k_1 is given by the expression

$$k_1([E] + [EG]) = k_1'[E] + k_1''[EG]$$

or

$$k_1(1 + [G]/K) = k_1' + k_1''[G]/K$$

so that

$$(k_1' - k_1)/[G] = (k_1 - k_1'')/K$$

By carrying out the reaction with a series of glutarate concentrations, it is possible to determine a corresponding series of values for k_1, the apparent rate constant for the combination reaction. The constant k_1', for example, is thus obtained experimentally in the absence of added glutarate. It is then possible to graph $(k_1' - k_1)/[G]$ with respect to k_1. This graph is a straight line whose abscissa intercept is equal to k_1'', the reactivity of the enzyme–glutarate complex, and whose slope is equivalent to the reciprocal of the enzyme–glutarate dissociation constant, K. Between pH 6 and 9 the latter may be confirmed by direct spectrophotometric titration (Jenkins and Taylor, 1965).

[1] Roger Gilmont Instruments Inc.

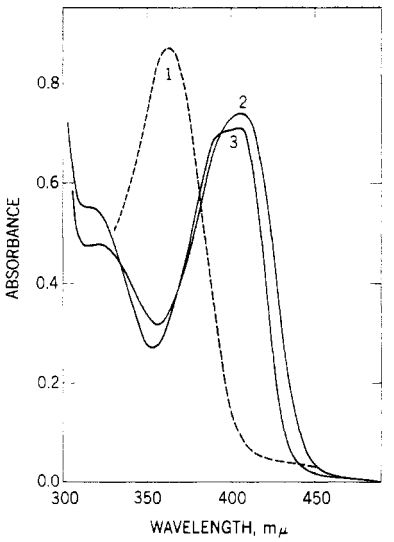

FIGURE 2: Spectra of the glutamic–aspartic transaminase (1), its binary complex with thiosemicarbazide (2), and its ternary complex with both thiosemicarbazide and glutarate (3). Tris–HCl (50 mM), pH 7.9, 23 mM glutarate, 5 mM thiosemicarbazide.

Since the buffer ions also appear to participate in the reaction, it is not easy to correlate results obtained at one pH value with those obtained at another. It is, however, valid to compare the results with and without glutarate when the other conditions are not altered.

Results

Two buffers, 0.05 M Tris–chloride and 0.05 M pyrophosphate–NaCl, were investigated at pH 7.9. Figure 1 shows how the glutarate dissociation constant was dependent upon the buffer employed. The *increase* in optical density (Δ absorbance) is proportional to the amount of complex formed. This type of plot is thus comparable to a graph of S/V with respect to substrate concentration (S) to determine the Michaelis constant from the velocity (V), in that the dissociation constant is numerically equal to the negative abscissa intercept. A more general equation was presented by Jenkins and Taylor (1965).

Figure 2 shows the spectra in 0.05 M Tris buffer, pH 7.9, of the free enzyme, enzyme–thiosemicarbazide complex, and ternary complex of enzyme–thiosemicarbazide and glutarate. Substantial spectral changes occur during complex formation.

Figure 3 shows the variation of the apparent first-order rate constant of the reaction of thiosemicarbazide as a function of thiosemicarbazide concentration and in the presence of increasing concentrations of glutarate. The interesting feature to note is that at this pH, regardless of the buffer employed, the dissociation constant of the thiosemicarbazide does not depend upon the glutarate concentration. This means that the glutarate dissociation constant also does not vary

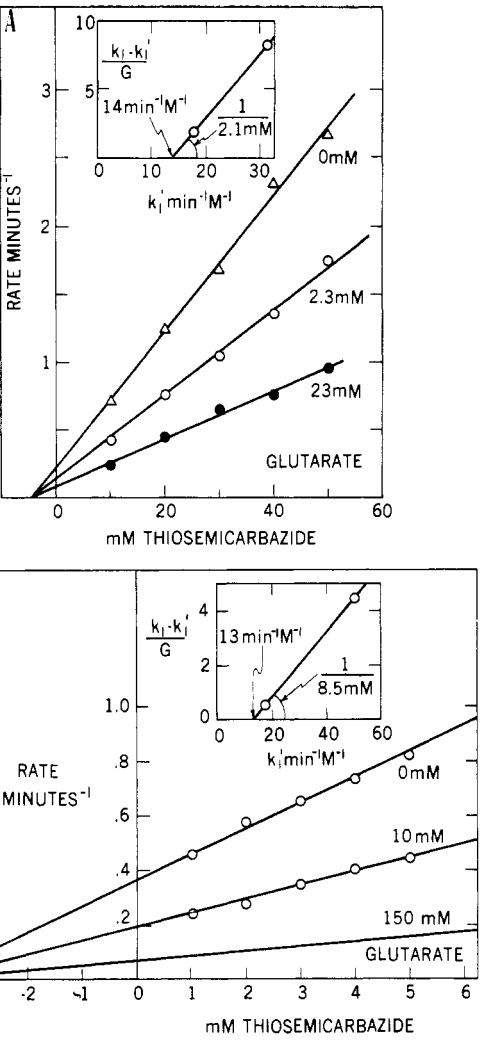

FIGURE 3: Effect of glutarate on the reactivity of the enzyme with thiosemicarbazide. (A) 0.05 M Tris–Cl. (B) 0.05 M pyrophosphate. No points are shown for the highest glutarate concentration since this line was determined with more thiosemicarbazide.

with increasing concentrations of thiosemicarbazide. This may have meant that there was no interaction between the two binding sites. There is, however, a marked effect of glutarate on the enzymatic *reactivity*. The insets in Figure 3 shows that there was a good agreement between the glutarate dissociation constants derived from their effect on the thiosemicarbazide reactivity (the reciprocals of the slopes of the graphs shown in the insets) with those determined directly under comparable conditions (Figure 1) regardless of the buffer employed. The insets also show that the rates of combination with an excess of glutarate, that is, the abscissa intercepts in the insets, became independent of the buffer employed. The effect of the buffer appears to be an inherent property of the buffer type, for the rate of the reaction, unlike the glutarate dissociation constant, was not dependent upon the *concentration*

TABLE I: Reactions of Glutamic–Aspartic Transaminase and Pyridoxal Phosphate with Thiosemicarbazide.

Reaction	0.05 M KPO_4, pH 6.3, 25°			0.05 M Pyrophosphate, pH 7.9, 25°			0.05 M Tris–Cl, pH 7.9, 25°			0.1 M Borate, pH 10, 25°		
	K_d[a]	k_{+1}	k_{-1}	K_d	k_{+1}	k_{-1}	K_d	k_{+1}	k_{-1}	K_d	k_{+1}	k_{-1}
PLP[b] + T $\rightleftharpoons$ PLP–T	<1	57	<0.05	<1	6.4	<0.01	<1	1.8	<0.01	—	*c*	—
E + T $\rightleftharpoons$ E–T	0.35	840	0.3	3.8	95	0.36	4.5	50	0.22	6	29	0.18
EG + T $\rightleftharpoons$ EG–T	18	16	0.3	3.8	13	0.05	4.5	14	0.06	2.3	7.3	0.02
E + G $\rightleftharpoons$ EG	0.06[d]	—	—	8.6[d]	—	—	2.2[d]	—	—	29[d]	—	—
				8.5			2.1					

[a] Units: K_d, mM; k_{+1}, $min^{-1}\ M^{-1}$; k_{-1}, min^{-1}. [b] Abbreviations: T, thiosemicarbazide; G, glutarate; PLP, pyridoxal phosphate; E, EG, and ET, free phosphopyridoxal enzyme and its glutarate and thiosemicarbazide complex. [c] Reaction not second order. [d] Values obtained by glutarate titration.

of Tris buffer. A marked catalytic effect of pyrophosphate buffer was previously noted by Jenkins *et al.* (1959a) in the reaction with isonicotinic acid hydrazide.

Results at pH 6.3 and 10.0. pH 7.9 was selected because at that pH the enzyme itself is wholly in the basic form (pK = 6.3), the derivatives formed with glutarate alone, and glutarate together with thiosemicarbazide, having pK values around pH 9, are mostly in their acidic forms. The derivative with thiosemicarbazide alone has a pK of about 7.9. At pH 6.3, therefore, one may derive some idea of the reactivity of the acidic forms of the enzyme and its glutarate complex and at pH 10 some idea of the reactivity of the basic forms of the complexes formed in the reactions.

The results which were obtained are summarized in Table I. The noteworthy features of this table are as follows. (1) There is a marked increase in the rate of the combination of thiosemicarbazide (k_1) with the enzyme–buffer anion complex (E) as the pH is lowered with little change in the rate of the back reactions (k_{-1}). The over-all effect is to reduce the dissociation constant (K_d). (2) There is no comparable increase in the rate of the forward reaction of the enzyme–glutarate complex (EG). The rate of thiosemicarbazide dissociation is little affected. (3) The presence of bound glutarate causes a substantial increase in the thiosemicarbazide dissociation constant at low pH values from 0.35 to 18 mM. From this it follows that thiosemicarbazide prevents the great *reduction* in glutarate dissociation constant as the pH is reduced. Since this increase in enzyme affinity has been ascribed to the formation of an ionic bond with the second glutarate carboxyl group, the formation of this bond is apparently hindered. The reactivity of the enzyme–glutarate complex at pH 7.9 was essentially the same for 0.05 M pyrophosphate buffer (13 $min^{-1}\ M^{-1}$) and 0.05 M Tris–Cl buffer (14 $min^{-1}\ M^{-1}$); the reactivities of the enzyme–anion complexes differ by a fraction of two.

Discussion

When the glutamic–aspartic transaminase was first isolated (Jenkins and Sizer, 1957), its spectra were found to differ from those of pyridoxal phosphate but appeared to be similar to those of the pyridoxal phosphate imines (Metzler, 1957; Heinert and Martell, 1963). Jenkins and Sizer (1957) therefore suggested that the pyridoxal phosphate aldehyde group was bound to the protein through an amino group. The latter was subsequently shown to be an ϵ-amino group of a lysine residue (Hughes *et al.*, 1962; Polyanovsky and Keil, 1963).[2] Since this implied that the aldehyde group might be blocked, the reactivity of the chromophore toward a variety of carbonyl reagents was immediately tested. When it was found that these reagents reacted extremely rapidly, it was suggested (Jenkins *et al.*, 1959a) that the aldimine linkage was inherently more reactive than the free aldehyde, and that the reaction of the substrate amino group was a displacement, that is to say an aminolysis, rather than condensation–dehydration. Cordes and Jencks (1962) obtained much better evidence for this mechanism. They noted that the formation of the free pyridoxal phosphate semicarbazone was specifically catalyzed by aniline and morpholine. The reaction studied by Cordes and Jencks was also susceptible to general base catalysis. The *catalytic effect of carboxylate ions* in removing a proton from the attaching amino group has since been investigated (Koehler *et al.*, 1964). The reaction mechanism proposed by Cordes and Jencks is essentially the same as that which is believed to occur between the enzyme and thiosemicarbazide (Figure 4) where B is the catalytic base. It was earlier suggested that when a carboxyl group of glutarate reacted with the aldimine nitrogen atom in the enzymic reaction, it should have the effect, at pH values of about 8, of stabilizing a positive charge on the nitrogen atom of this group and thereby increase the reactivity of the carbon atom to nucleophilic attack. Protonation of the aldimine nitrogen by lowering the pH causes

[2] It now appears that this type of aldimine linkage is a characteristic feature of all phosphopyridoxal enzymes.

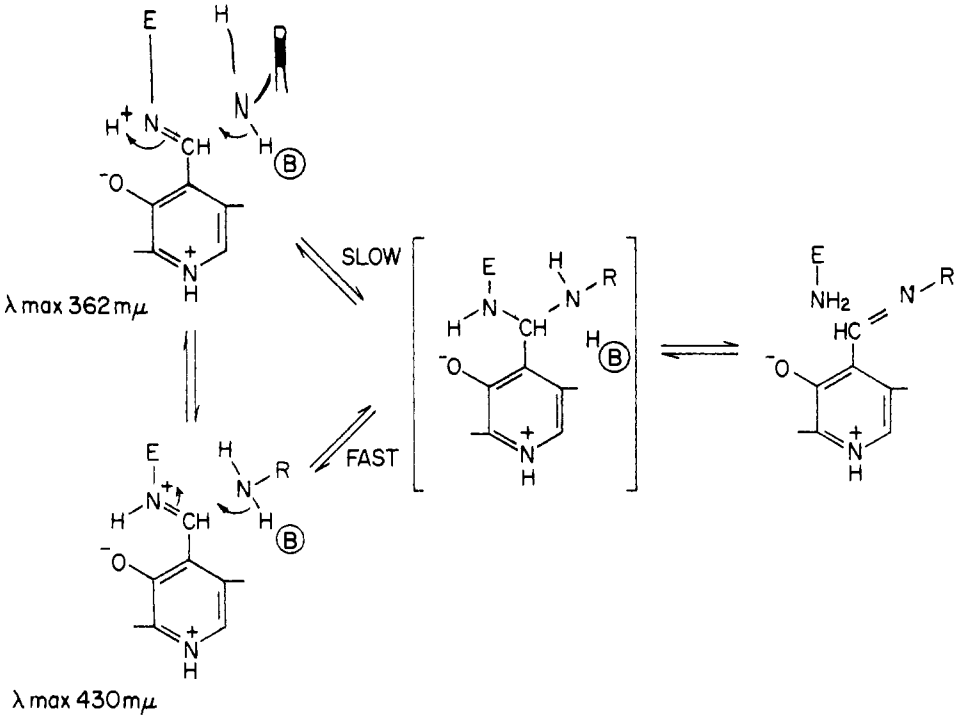

FIGURE 4: Proposed aminolytic reaction mechanism for Schiff base formation.

a comparable spectral change and does have a catalytic effect. This catalytic effect presumably is greater, however, than would be obtained by binding a *carboxyl* group to this same nitrogen atom (Sizer and Jenkins, 1963). There were thus two ways indicated by which combination with glutarate would be expected to catalyze the combination with thiosemicarbazide.

In two respects, therefore, the results reported in this paper are a disappointment for although an interaction between glutarate binding and aldimine reactivity was found, the glutarate binding decreased the rate of formation of the thiosemicarbazide derivative. That this arises through steric hindrance or other artifacts of this model system is very likely for the enzyme reacts with thiosemicarbazide even more slowly at pH 6.3 than with free pyridoxal phosphate.

On the basis of optical rotatory dispersion (ORD) measurements, it has been concluded that when carbonyl reagents react with the phosphopyridoxal form of the enzyme, the aldimine linkage is broken and that the lysine ϵ-amino group involved in this linkage is thus liberated (Torchinsky and Koreneva, 1964; Braunstein, 1965). Although glutarate will bind to the thiosemicarbazide complex and this complex is a pH indicator, the affinity for glutarate of the enzyme is no longer markedly pH dependent. It therefore appears that the second carboxyl group cannot be bound in the enzyme–thiosemicarbazide complex. This is in agreement with the hypothesis that it is the aldimine nitrogen atom or the aldimine grouping which binds the second glutarate carboxyl at low pH.

The affinity of the enzyme for glutarate is comparable to that for maleate so that the carboxyl binding sites must be close together. Since there is an interaction between one of these carboxyl binding sites and the aldimine grouping, some influence of the buffer on the reactivity of this group, independent of its concentration, would be anticipated if the (adjacent) carboxyl binding site is normally masked by a buffer anion. The elimination of this effect when glutarate displaces the various buffer anions, as was observed, would also be expected.

One of the continuing problems in enzymology has been to determine whether there is a preferred or obligatory order of addition when several substrates are bound simultaneously to a single enzyme. Since each substrate is, presumably, bound by multiple enzyme–substrate ligands, an obligatory sequence of formation of these is also a possibility. When an amino acid reacts with a transaminase, for example, it is of interest to determine whether the α-carboxyl group must bind to the enzyme before the α-amino group reacts with the pyridoxal phosphate prosthetic group. If it does react first, it is still of interest to find out whether it remains bound prior to the reaction; in other words, to determine through how many succeeding interconversions of enzyme–substrate complexes a ligand, once formed, remains intact. In our recent study of glutarate binding to the glutamic–aspartic transaminase, we found that the first enzyme–substrate ligand to be formed at high pH values, where glutarate is ultimately bound by only a single carboxyl group to an enzymatic site previously masked by a buffer anion, was an ionic linkage between the *other* carboxyl group and the aldimine chromophore. The derivative with both carboxyl groups bound to the enzyme is an intermediate in the reaction. The probability thus arises that binding the carboxyl group to the aldimine

is not necessary for Schiff base formation and might even be inhibitory. Since several compounds are known which undergo a transamination with bound pyridoxal phosphate and yet have no α-carboxyl group, there is no theoretical objection to the hypothesis that this group is unnecessary in one of the component steps in the transamination reaction, namely, the aminolysis of the pyridoxal aldimine double bond by the substrate amino group.

References

Braunstein, A. E. (1965), *Vitamins Hormones 22*, 451.

Cordes, E. H., and Jencks, W. P. (1962), *Biochemistry 1*, 773.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley, p 47.

Heinert, D., and Martell, A. E. (1963), *J. Am. Chem. Soc. 85*, 183.

Hughes, R. C., Jenkins, W. T., and Fischer, E. H. (1962), *Proc. Natl. Acad. Sci. U. S. 43*, 1615.

Jenkins, W. T., Orlowski, S. A., and Sizer, I. W. (1959a), *J. Biol. Chem. 234*, 2657.

Jenkins, W. T., and Sizer, I. W. (1957), *J. Am. Chem. Soc. 79*, 2655.

Jenkins, W. T., and Taylor, R. T. (1965), *J. Biol. Chem. 240*, 2907.

Jenkins, W. T., Yphantis, D. A., and Sizer, I. W. (1959b), *J. Biol. Chem. 234*, 51.

Koehler, K., Sandstrom, W., and Cordes, E. H. (1964), *J. Am. Chem. Soc. 86*, 2413.

Martinez-Carrion, M., Riva, F., Turano, C., and Fasella, P. M. (1965), *Biochem. Biophys. Res. Commun. 20*, 206.

Metzler, D. E. (1957), *J. Am. Chem. Soc. 79*, 485.

Polyanovsky, O. L., and Keil, B. A. (1963), *Biokhimya 28*, 372.

Sizer, I. W., and Jenkins, W. T. (1962), *Methods Enzymol. 5*, 677.

Sizer, I. W., and Jenkins, W. T. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., Braunstein, A. E., Fasella, P. M., and Rossi-Fanelli, A., Ed., New York, N. Y., Pergamon, p 123.

Torchinsky, Y. M., and Koreneva, L. G. (1964), *Biochim. Biophys. Acta 79*, 426.