Equilibrium Kinetic Study of the Catalytic Mechanism of Oxidative Deamination of Alanine by Bovine Liver Glutamate Dehydrogenase†

Emanuel Silverstein* and Guruprasad Sulebele‡

ABSTRACT: The mechanism of reversible oxidative deamination of alanine by bovine liver glutamate dehydrogenase was studied at pH 8.0 and 25° in 13.4–20.4 mM Veronal or 106 or 193 mM Tris–acetate buffer by equilibrium kinetics utilizing isotopic exchange of substrate. In response to increase in concentration of alanine and pyruvate from below K_m to more than 10-fold greater the rates of both alanine ⇄ pyruvate (measured with [^{14}C]pyruvate) and NAD^+ ⇄ NADH (measured with [^{14}C]NADH) or $NADP^+$ ⇄ NADPH (measured with [^{14}C]NADPH) rose to plateau values. The pyruvate ⇄ alanine rate also rose to plateau values as coenzyme concentration was increased toward saturation. The NAD ⇄ NADH rate exceeded the alanine ⇄ pyruvate rate by one to two orders of magnitude. The results suggest that the mechanism of the alanine dehydrogenase reaction at pH 8.0 is alternative (random) order, and that the rate-limiting step may be either dissociation of substrate, presumably pyruvate, or a distinct hydrolysis–aminolysis step(s) if it exists in the enzymic reaction. Initial net reaction and equilibrium kinetic data also suggest that chemical transformation may be much slower in the alanine–pyruvate system as compared to glutamate–α-ketoglutarate. Minimum estimates of dissociation constants calculated from the equilibrium kinetic data are 134 μM for pyruvate and 12 μM (at 15.6 mM alanine) and 41 nM (at 218 mM alanine) for NADH.

Bovine liver glutamate dehydrogenase (L-glutamate: NAD(P)-oxidoreductase (deaminating) (EC 1.4.1.3) catalyzes the oxidative deamination of L-alanine, among other L-amino acids, at 0.3% the rate with L-glutamate at their respective pH optima (9 and 8) (Struck and Sizer, 1960). Modifiers such as ADP which increase the rate of reaction with L-glutamate tend to inhibit reaction with L-alanine, while inhibitors of L-glutamate oxidation such as GTP activate reaction with L-alanine (Frieden, 1959, 1963a,b, 1964, 1971; Tomkins *et al.*, 1961). The K_m for NADPH is independent of whether pyruvate or α-ketoglutarate is the substrate (Fisher and McGregor, 1961) and the X-ray inactivation of bovine liver glutamate dehydrogenase activity is similar with glutamate or pyruvate as substrates (Sanner and Pihl, 1972), suggesting that the same active site is involved for both substrates. It has been suggested on the basis of stopped-flow experiments that formation of the Michaelis complex is rate limiting for alanine oxidation while oxidation of glutamate is limited by the rate of dissociation of reduced coenzymes from the enzyme–coenzyme complex (Iwatsubo and Pantaloni, 1967).

In equilibrium kinetic studies of the catalytic and regulatory mechanisms of allosteric enzymes (Silverstein, 1970) we have found the catalytic mechanism of glutamate oxidative deamination by bovine liver glutamate dehydrogenase at pH 8.0 to be alternative order (random) (Silverstein and Sulebele, 1973). This report is concerned with an equilibrium kinetic investigation of the catalytic mechanism of oxidative deamination of alanine catalyzed by bovine liver glutamate dehydrogenase at pH 8.0 which suggests that the mechanism with alanine is also alternative order (random). A brief account of this work has been given (Silverstein and Sulebele, 1970).

Experimental Section

The materials and methods were previously described (Silverstein and Sulebele, 1973) except as here indicated.

Materials. L-Alanine and sodium pyruvate were purchased from the Sigma Chemical Co., St. Louis, Mo. Sodium [1-^{14}C]pyruvate (11.8 Ci/mol) was obtained from Amersham/Searle, Arlington Heights, Ill.

Radioactive Substrates. In experiments in which Veronal buffer was used [^{14}C]NADH was eluted from a DEAE-cellulose column by 0.1 M sodium acetate + 0.025 M Veronal buffer (pH 8.0). The radiochemical purity of [1-^{14}C]pyruvate solutions containing several drops of benzene at 4° was unchanged for at least 6 weeks (Silverstein and Boyer, 1964a,b). Benzene was also added to the [^{14}C]NADH solution to equalize conditions with the [^{14}C]pyruvate solution. The biological activity of bovine liver glutamate dehydrogenase has been shown to be unaffected by toluene which is structurally similar to benzene (Reisler and Eisenberg, 1972).

Experimental Design for Equilibrium Experiments. Substrate mixtures were freshly prepared in deionized water, adjusted to pH 8.0 and transferred immediately to 0°. Equilibrium reaction mixtures consisting of alanine, pyruvate, NAD, NADH, ammonium acetate, and enzyme were prepared in 106 or 193 mM Tris–acetate or 13.4–20.4 mM Veronal buffer at pH 8.0. Absorbance at 340 nM was measured before and after addition of enzyme at 25° until stable and after the slight dilution corresponding to subsequent isotope addition, and corrections in reactant concentrations and rates were made for any shift in equilibrium. Equilibrium reaction rates were measured in triplicate by addition to equilibrium mixtures (0.2 ml) of 5 or 10 μl of a tracer concentration of [^{14}C]NADH to measure NAD^+ ⇄ NADH rates or [1-^{14}C]pyruvate for pyruvate ⇄ alanine rates. For pyruvate ⇄ alanine reactions,

† From the Laboratory of Molecular Biology, Department of Medicine and the School of Graduate Studies, State University of New York, Downstate Medical Center, Brooklyn, New York 11203. *Received June 18, 1973.* This work was supported in part by grants from the National Science Foundation and the U. S. Public Health Service.

‡ Present address: Department of Chemical Technology, University of Bombay, Bombay 19, India.

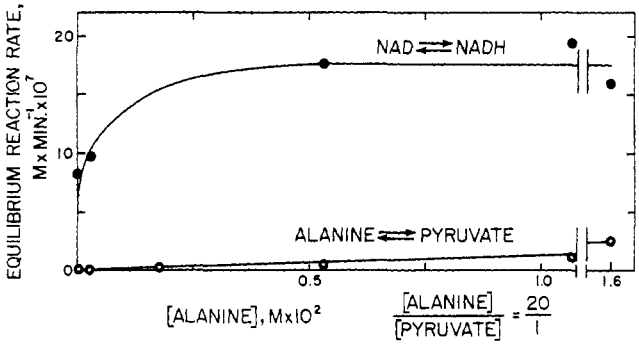

FIGURE 1: Effect of alanine and pyruvate concentration on alanine ⇀↽ pyruvate and NAD^+ ⇀↽ NADH reaction rates catalyzed by bovine liver glutamate dehydrogenase. Reaction mixtures in 0.25 ml contained 3.98 mM NAD^+, 143 μM NADH, 5 mM ammonium acetate, alanine and pyruvate as indicated, and 3.3 μM enzyme in 13.4 mM Veronal buffer at pH 8.0 and 25°: (●) NAD^+ ⇀↽ NADH; (○) alanine ⇀↽ pyruvate.

termination of reaction and substrate separation were achieved by addition of 2,4-dinitrophenylhydrazine as previously described (Silverstein and Sulebele, 1973) except that 20 μmol of sodium pyruvate was used as carrier for 2,4-dinitrophenylhydrazine precipitation and 50 μl of horse serum (any protein solution may be used) was added for compaction of the precipitate. Enzymically reactive radiosubstrate was determined by total conversion to NAD^+ or lactate in a lactate or alcohol dehydrogenase system (Silverstein and Boyer, 1964a,b, 1966; Silverstein, 1965).

No enzyme inactivation was observed during up to 21-hr incubation at 25° in equilibrium reaction mixtures. Enzyme activity was assayed by determining the initial rate of α-ketoglutarate reduction in a reaction mixture consisting of 6.77 mM α-ketoglutarate, 140 μM NADH, 109 mM ammonium acetate, and 2.19 nM enzyme in 1.825 ml of 220 mM Tris–acetate at pH 8.0.

Results

The extent (rate of exchange at time $t \times t$) of the NAD^+ ⇀↽ NADH and alanine ⇀↽ pyruvate reactions, calculated by means of the isotopic exchange equation (McKay, 1938; Boyer, 1959), was directly proportional with time, indicating constancy of rate with time. It was thus possible to use different reaction times for the two rates which were quite disparate in order to maximize accuracy of measurement.

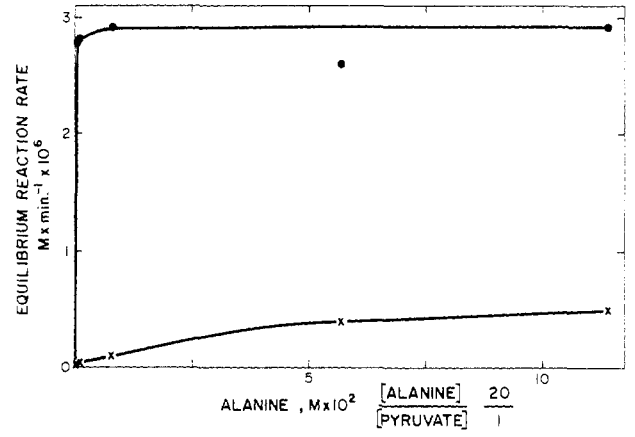

FIGURE 2: Effect of alanine and pyruvate concentration on alanine ⇀↽ pyruvate and $NADP^+$ ⇀↽ NADPH reaction rates catalyzed by bovine liver glutamate dehydrogenase. Reaction mixtures in 0.21 ml contained 9.4 mM $NADP^+$, 0.38–0.40 mM NADPH, 3.9 mM ammonium acetate, alanine and pyruvate as indicated, and 3.0 μM enzyme in 193 mM Tris–acetate at pH 8.0 and 25°: (●) $NADP^+$ ⇀↽ NADPH; (×) alanine ⇀↽ pyruvate.

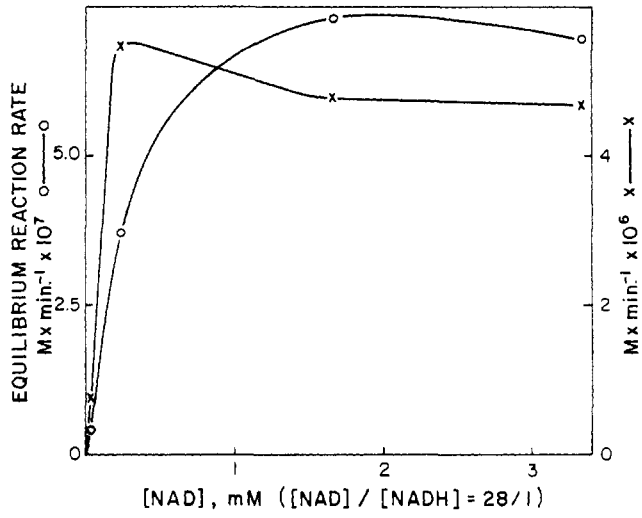

FIGURE 3: Effect of NAD^+:NADH concentration on the alanine ⇀↽ pyruvate equilibrium reaction rate catalyzed by bovine liver glutamate dehydrogenase at pH 8.0. Reaction mixtures in 0.25 ml contained 218 mM alanine and 14.7 mM pyruvate (×) or 15.6 mM alanine and 1.05 mM pyruvate (○), NAD^+ and NADH as indicated and 4.5 μM enzyme in 3.0 mM ammonium acetate and 20.4 mM Veronal buffer.

Effect of Substrate Concentration on Equilibrium Reaction Rates at pH 8.0. Increasing alanine:pyruvate concentration[1] at equilibrium from highly unsaturating to saturating levels (up to 160 mM alanine and 8 mM pyruvate) in Veronal (Figure 1) or 106 mM Tris–acetate buffers resulted in increase to a plateau value in the NAD^+ ⇀↽ NADH equilibrium reaction rate and in a gradual rise in the alanine ⇀↽ pyruvate rate, ruling out a compulsory pathway in which coenzyme must bind prior to substrate. The maximum NAD^+ ⇀↽ NADH rate was 18.7 times the corresponding alanine ⇀↽ pyruvate rate in Veronal buffer and 3.4 times greater in Tris buffer under the conditions of Figure 1. The same experiment done in 193 mM Tris–acetate with $NADP^+$ yielded results similar to those with NAD^+ (Figure 2).

Increasing NAD^+:NADH concentration at equilibrium from highly unsaturating levels to above K_m resulted in a rise in the alanine ⇀↽ pyruvate rate to a plateau (Figure 3) ruling out a compulsory pathway in which substrate binds prior to coenzyme. The alanine ⇀↽ pyruvate rate rose more sharply at 218 mM than at 15.6 mM alanine in response to increasing concentration of coenzyme, suggesting that coenzyme binds more tightly in the presence of the higher substrate concentration.

Initial Rates at pH 8.0 and 8.8. The rate of oxidative deamination of glutamate was 28,200 times that for alanine at pH 8.0, while that for reductive amination of α-ketoglutarate was 1960 times that for pyruvate (Table I). Reaction of pyruvate to alanine was 22.8 times that for alanine to pyruvate (Table I). The rate of oxidative deamination of pyruvate at pH 8.8 was 29-fold higher than at pH 8.0 in Tris buffer and 18 times higher in Veronal buffer.

[1] Alanine:pyruvate concentration signifies alanine and pyruvate at constant ratio. This designation may be used for other reactants as well.

TABLE I: Initial Rates of Oxidation of Glutamate and Alanine and of Reduction of α-Ketoglutarate and Pyruvate by Bovine Liver Glutamate Dehydrogenase at 25°.

Substrate	Rate, M × min^{-1} × 10^9	
	pH 8.0	pH 8.8
Glutamate	116,000	
Alanine	4.1[a,b]	
α-Ketoglutarate	183,000[c]	
Pyruvate	93.4[c]	2690[d]
	92.2[d]	1670[e]

[a] Reaction mixtures contained 33 μmol of glutamate or alanine, 693 nmol of NAD^+, 400 μmol of Tris–acetate, 50 μg of enzyme (97 nM) in 1.605 μl for glutamate, and 300 μg of enzyme (573 nM) in 1.640 μl for alanine. [b] Rate normalized to 97 nM enzyme concentration. [c] Reaction mixtures contained 12.4 μmol of α-ketoglutarate or pyruvate, 250 nmol of NADH, 200 μmol of ammonium acetate, 50 μg (87 nM) of enzyme, and 400 μmol of Tris–acetate in 1.805 ml. [d] Reaction mixture contained 8.9 mM sodium pyruvate, 139 μM NADH, 110 mM ammonium acetate, and 97 nM enzyme in 220 mM Tris–acetate (pH 8.0) or 332 mM Tris–acetate (pH 8.8) in 1.805 ml. [e] Same conditions as in footnote *d* except for 144 mM sodium barbitone buffer instead of Tris.

Minimum estimates of dissociation constants (Boyer and Silverstein, 1963; Silverstein and Boyer, 1964b) were obtained from the $NAD^+ \leftrightharpoons$ NADH rates for pyruvate (134 μM) and from the alanine ⇀↽ pyruvate rate for NADH (12 μM at 15.6 mM alanine; 41 nM at 218 mM alanine).

Discussion

The results of this study suggest that the mechanism of alanine oxidative deamination by bovine liver glutamate dehydrogenase at pH 8.0 is alternative (random) order (noncompulsory) (Boyer and Silverstein, 1963). The pattern of suppression of $NAD^+ \leftrightharpoons$ NADH or alanine ⇀↽ pyruvate rates on increasing alanine:pyruvate or NAD^+:NADH concentrations, respectively, from highly unsaturating to saturating was not observed as anticipated for a compulsory reaction pathway with coenzyme or substrate obligatorily binding first to enzyme. The alternative (random) order mechanism is in agreement with isotope rate effect experiments (Palm *et al.*, 1972).

The mechanism for alanine dehydrogenation is thus similar to that for glutamate dehydrogenation at pH 8.0 (Silverstein and Sulebele, 1973) despite the reciprocal changes in their activity in relation to each other with regard to such parameters as pH and allosteric effectors (Tomkins *et al.*, 1961; Frieden, 1964; Bitensky *et al.*, 1965). The similarity in mechanism is also compatible with evidence that both substrates utilize the same catalytic center (Fisher and McGregor, 1961; Sanner and Pihl, 1972).

The basic similarity in mechanism, on the one hand, and the great disparity in rate and reciprocal activity changes, on the other hand, suggest that the rates of rate-limiting and possibly other steps may differ quantitatively and probably qualitatively. The alanine ⇀↽ pyruvate rate was markedly lower than the $NAD^+ \leftrightharpoons$ NADH rate in these experiments. If the reaction mechanism is a concerted one (including possible iminopyruvate formation) (Figure 4, Reaction Scheme 1) this disparity suggests that the rate-limiting step is substrate dissociation. On the other hand, if the reaction proceeds through an iminopyruvate intermediate whose reaction to pyruvate and NH_4^+ is much slower than the coenzyme requiring reaction of the imino acid intermediate to alanine (Figure 4, Scheme 2), as well as all enzyme dissociation steps, the exchange rate disparity could be due to the slow imino intermediate–pyruvate step. In this case the coenzyme exchange could proceed at the more rapid oxidation–reduction rate or coenzyme dissociation rates, whichever is slower, while the alanine ⇀↽ pyruvate rate is limited by the rate of the subsequent hydrolysis–aminolysis steps. If a discrete imino acid intermediate–pyruvate step exists, but it as well as the oxidation–reduction step are rapid in comparison to dissociation steps, then substrate dissociation is still likely to be rate limiting and explain the low substrate interchange rate in comparison to coenzyme rate.

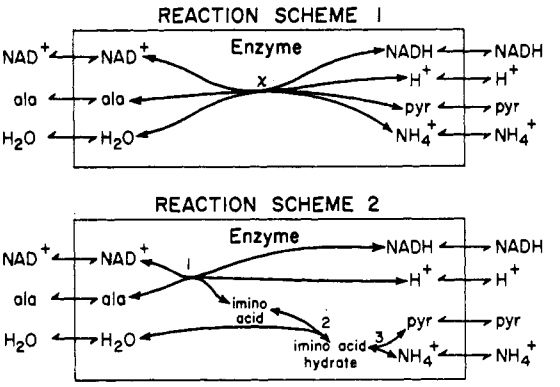

FIGURE 4: Two possible reaction pathways for the reversible alanine dehydrogenase reaction catalyzed by bovine liver glutamate dehydrogenase. Each rectangle represents an enzyme molecule. Pyr and ala signify pyruvate and alanine, respectively. Binding and dissociation steps are depicted by arrows to the left and the right. In Reaction Scheme 1 a concerted reaction is depicted, which could include imino acid formation in complex χ. The rates of chemical transformation of the various reactants are essentially equal. Inequality of $NAD^+ \leftrightharpoons$ NADH and alanine ⇀↽ pyruvate rates could occur if chemical transformation is not rate limiting and substrate dissociation is much slower than coenzyme dissociation. In Reaction Scheme 2 three separate chemical transformation steps are depicted (1, 2, and 3). If step 1 and dissociation steps are rapid compared to 2 and/or 3 it is possible for the $NAD^+ \leftrightharpoons$ NADH rate to be much faster than the alanine ⇀↽ pyruvate rate.

It should be noted that there is considerable evidence (Olson and Anfinsen, 1953; Strecker, 1953; Frieden, 1963a) against a mechanism originally proposed by von Euler (von Euler *et al.*, 1938) in which only the oxidation–reduction part of the reaction is enzymatic

$$\text{amino acid} + NAD^+ \overset{\text{enzymic}}{\rightleftharpoons} \text{imino acid} + \text{NADH} + H^+ \quad (1)$$

$$\text{imino acid} + H_2O \overset{\text{nonenzymic}}{\rightleftharpoons} \text{hydrated imino acid} \overset{\text{nonenzymic}}{\rightleftharpoons} \text{keto acid} + NH_4^+ \quad (2)$$

Since there is evidence against a significant reaction pathway involving imino acid formation and dissociation from enzyme, imino acid dissociation has not been included in Figure 4 (Reaction Scheme 2). However, bovine liver glutamate dehydrogenase enhances the rate of formation of α-iminoglutarate from NH_4^+ and α-ketoglutarate about fourfold, but this enhanced rate is one-tenth of the α-ketoglutarate → glutamate rate in the presence of NADH (Hochreiter *et al.*, 1972). It is thus not clear whether the complete pyridine nucleotide linked enzymatic reaction, α-ketoglutarate ⇀↽ L-glutamate,

shares part of its reaction mechanism in common with the α-ketoglutarate ⟷ α-iminoglutarate reaction, or whether the reaction proceeds differently in the presence of all reactants wherein enzyme conformation and reactant positioning might be different. It would certainly seem reasonable that some acceleration by enzyme of the α-ketoglutarate ⟷ α-iminoglutarate rate should be present in either case since NH_4^+ and α-ketoglutarate can bind at the catalytic center and the proximity of the two should certainly enhance the possibility of their reaction to enzyme bound α-iminoglutarate.

Equilibrium kinetic data would seem to rule out an enzyme-bound α-ketoglutarate ⟷ α-iminoglutarate reaction as overall rate limiting for the glutamate dehydrogenase reaction since the α-ketoglutarate ⟷ glutamate equilibrium rate was higher rather than lower than the NAD ⟷ NADH rate (Silverstein and Sulebele, 1973). However, the possibility does exist that such a step might be rate limiting for alanine dehydrogenase as already explained. The similarity of allosteric modifier action on the rate of α-iminoglutarate formation from α-ketoglutarate and on the rate of the alanine dehydrogenase reaction suggests the possibility that a similar rate-limiting step may be affected in the two cases. Thus the imino ⟷ keto chemical transformation step or reactant dissociation steps (alanine:pyruvate; α-iminoglutarate as suggested by Hochreiter *et al.* (1972)) could be rate limiting and similarly affected by allosteric modifiers in the two systems.

Since the rate of pyruvate reductive amination was considerably more rapid than alanine oxidative deamination (Table I), pyruvate dissociation may be rate limiting in the overall reaction, if substrate dissociation is rate limiting. On the other hand, the initial rate with pyruvate was still far less than the rate with α-ketoglutarate (Table I), suggesting that alanine dissociation or chemical transformation in pyruvate reductive amination may be slower than glutamate dissociation or chemical transformation in α-ketoglutarate reductive amination.

In equilibrium kinetics with isotopic exchange one may simultaneously observe more than one substrate interchange. Thus, if the rates of chemical transformation and coenzyme dissociation were similar in the alanine and glutamate systems, but only substrate dissociation differed, one would expect the NAD^+ ⟷ NADH rate to be similar in the two systems. Instead one finds the NAD^+ ⟷ NADH rate in the alanine system to be about two to three orders of magnitude less than that observed in the glutamate system when enzyme concentration differences are taken into account. It would appear reasonable that such a large difference may be accounted for by difference in the rate of chemical transformation, and that differences in coenzyme dissociation rate, if any, would not account for the large difference observed. Perhaps the alignment of the monocarboxylic acid interconvertible pair, alanine–pyruvate, with the catalytic groups at the catalytic center is not as favorable for catalysis as is the case with glutamate–α-ketoglutarate. The experimental results thus suggest that while substrate dissociation may be rate limiting in reversible alanine oxidative deamination, chemical transformation (including oxidation–reduction) may also be much slower than in the glutamate reaction.

Presumably the rate-limiting steps in glutamate and alanine catalysis are altered in opposite directions by such factors as small molecular modifiers and pH because they are different. It appears possible from the present results that substrate dissociation or a discrete aminolysis–hydrolysis step, if it exists, limits the alanine oxidative deamination rate, and chemical transformation which includes the oxidation–reduction step may limit the rate of reaction with glutamate (Silverstein and Sulebele, 1973; Malcolm, 1972). Thus, it is possible that the rate of chemical transformation or coenzyme dissociation, for example, may be similarly affected for the two substrates with a change in pH or in the presence of an allosteric effector, but a simultaneous and opposite effect on substrate dissociation (or perhaps a distinct aminolysis–hydrolysis step if present) would result in opposite effects on observed rates with the two substrates.

It is not clear why there was a tendency for substrate interchange to rise with increasing substrate concentration after coenzyme interchange reached constancy (Figures 1 and 2). Perhaps substrate binding at noncatalytic center binding sites of low affinity results in acceleration of the step rate limiting for substrate interchange.

Acknowledgment

We thank Mr. Harold Geller for capable technical assistance in some of these experiments.

References

Bitensky, M. W., Yielding, K. L., and Tomkins, G. M. (1965), *J. Biol. Chem. 240*, 663.
Boyer, P. D. (1959), *Arch. Biochem. Biophys. 82*, 387.
Boyer, P. D., and Silverstein, E. (1963), *Acta Chem. Scand. 17*, S195.
Fisher, H. F., and McGregor, L. L. (1961), *J. Biol. Chem. 236*, 791.
Frieden, C. (1959), *J. Biol. Chem. 234*, 815.
Frieden, C. (1963a), *Enzymes, 3rd Ed., 7*, 3.
Frieden, C. (1963b), *J. Biol. Chem. 238*, 3286.
Frieden, C. (1964), *Brookhaven Symp. Biol. 17*, 98.
Frieden, C. (1971), *Annu. Rev. Biochem. 40*, 653.
Hochreiter, M. C., Patek, D. R., and Schellenberg, K. A. (1972), *J. Biol. Chem. 247*, 6271.
Iwatsubo, M., and Pantaloni, D. (1967), *Bull. Soc. Chim. Biol. 49*, 1563.
Malcolm, A. D. B. (1972), *Eur. J. Biochem. 27*, 453.
McKay, H. A. C. (1938), *Nature (London) 142*, 997.
Olson, J. A., and Anfinsen, C. B. (1953), *J. Biol. Chem.202*, 841.
Palm, D., Limberg, B., Thanner, F., and Berger, W. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353*, 742.
Reisler, E., and Eisenberg, H. (1972), *Biochim. Biophys. Acta 258*, 351.
Sanner, T., and Pihl, A. (1972), *Radiat. Res. 51*, 155.
Silverstein, E. (1965), *Anal. Biochem. 12*, 199.
Silverstein, E. (1970), *Biochim. Biophys. Acta 198*, 148.
Silverstein, E., and Boyer, P. D. (1964a), *Anal. Biochem. 8*, 470.
Silverstein, E., and Boyer, P. D. (1964b), *J. Biol. Chem. 239*, 3901.
Silverstein, E., and Boyer, P. D. (1966), *Biochem. Prep. 11*, 89.
Silverstein, E., and Sulebele, G. (1970), *Biophys. J. 10*, 123a.
Silverstein, E., and Sulebele, G. (1973), *Biochemistry 12*, 2164.
Strecker, H. J. (1953), *Arch. Biochem. Biophys. 46*, 128.
Struck, J., Jr., and Sizer, I. W. (1960), *Arch. Biochem. Biophys. 86*, 260.
Tomkins, G. M., Yielding, K. L., and Curran, J. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 270.
von Euler, H., Adler, E., Günther, G., and Das, N. B. (1938), *Hoppe-Seyler's Z. Physiol. Chem. 254*, 61.