Mechanism of Inactivation of γ-Aminobutyrate Aminotransferase by 4-Amino-5-fluoropentanoic Acid. First Example of an Enamine Mechanism for a γ-Amino Acid with a Partition Ratio of 0†

Richard B. Silverman* and Benedict J. Invergo

Departments of Chemistry and of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60201

Received May 30, 1986; Revised Manuscript Received July 25, 1986

ABSTRACT: The mechanism of inactivation of pig brain γ-aminobutyric acid aminotransferase (GABA-T) by (*S*)-4-amino-5-fluoropentanoic acid (**1**, R = CH_2CH_2COOH, X = F) previously proposed [Silverman, R. B., & Levy, M. A. (1981) *Biochemistry 20*, 1197–1203] is revised. *apo*-GABA-T is reconstituted with [4-^{3}H]pyridoxal 5′-phosphate and inactivated with **1** (R = CH_2CH_2COOH, X = F). Treatment of inactivated enzyme with base followed by acid denaturation leads to the complete release of radioactivity as 6-[2-hydroxy-3-methyl-6-(phosphonoxymethyl)-4-pyridinyl]-4-oxo-5-hexenoic acid (**4**, R = CH_2CH_2COOH). Alkaline phosphatase treatment of this compound produces dephosphorylated **4** (R = CH_2CH_2COOH). These results support a mechanism that was suggested by Metzler and co-workers [Likos, J. J., Ueno, H., Feldhaus, R. W., & Metzler, D. E. (1982) *Biochemistry 21*, 4377–4386] for the inactivation of glutamate decarboxylase by serine *O*-sulfate (Scheme I, pathway b, R = COOH, X = OSO_3^-).

An important approach to the rational inactivation of specific enzymes is the use of mechanism-based enzyme inactivators (Silverman & Hoffman, 1984; Walsh, 1984; Abeles, 1983; Rando, 1984). These are unreactive compounds bearing a structural similarity to the substrate or product of an enzyme that are transformed by the target enzyme into reactive species; without prior release from the active site, these activated compounds react with active-site nucleophiles. A common approach to mechanism-based inactivation of pyridoxal 5′-phosphate (PLP)[1] enzymes has been the incorporation of a good leaving group positioned β to the amino group of an amino acid that acts as a substrate for the target enzyme. For example, β-haloalanines are valuable mechanism-based inactivators of aminotransferases (Morino et al., 1979), racemases (Wang & Walsh, 1978), and decarboxylases (Relyea et al., 1974). The usual mechanism of inactivation proposed is shown in Scheme I, pathway a (R = COOH, X = halogen). In 1982, however, Metzler and co-workers (Likos et al., 1982; Ueno et al., 1982) proposed an alternative mechanism for the inactivation of aspartate aminotransferase and glutamate decarboxylase by L-serine *O*-sulfate (Scheme I, pathway b, R = COOH, X = OSO_3^-). Their convincing substantiation for this proposal was the isolation of **4** (Scheme II, R = COOH), when the enzyme adduct **3** (R = COOH) was treated with base. Since this mechainsm, which will be referred to as the enamine mechanism, was revealed by Metzler and co-workers, two reports by Walsh and co-workers have indicated that the inactivations of alanine racemase from *Salmonella typhimurium dad B* (Badet et al., 1984) and an amino acid racemase from *Pseudomonas striata* (Roise et al., 1984) by β-fluoroalanine (**1**, R = COOH, X = F) also proceed by the enamine mechanism (Scheme I, pathway b, R = COOH, X = F) rather than the previously proposed elimination–Michael addition mechanism (Scheme I, pathway a). In all four of these confirmed examples of an enamine mechanism, a substituted α-amino acid is the inactivator. Furthermore, in all of these examples the partition ratios (the ratio of turnovers to give product per inactivation event) are quite high. It is not surprising that the partition ratios are high considering that the proposed intermediate **2** (Scheme I, pathway b, R = COOH) in the enamine mechanism is an unstable enamine that must undergo a rotation in order for the appropriate carbon–carbon bond formation to occur.

Several years ago Silverman and Levy (1980b) reported the inactivation of γ-aminobutyrate aminotransferase by (*S*)-4-amino-5-fluoropentanoic acid (**1**, R = CH_2CH_2COOH, X = F) and proposed an inactivation mechanism resembling that shown in Scheme I, pathway a. In this case a substituted γ-amino acid that exhibits a partition ratio of 0 is involved. In light of the findings of Metzler and co-workers (Likos et al., 1982; Ueno et al., 1982) and Walsh and co-workers (Badet et al., 1984; Roise et al., 1984), we have reinvestigated the inactivation of γ-aminobutyrate aminotransferase by **1** (R = CH_2CH_2COOH, X = F) and conclude that the enamine mechanism is operative. This is quite unexpected since every turnover of **1** (R = CH_2CH_2COOH, X = F) leads to inactivation of the enzyme.

Materials and Methods

Enzymes and Assays. γ-Aminobutyrate aminotransferase was purified to homogeneity from pig brains by the method of Churchich and Moses (1981). The enzyme showed one band on NaDodSO$_4$-PAGE at pH 7.0 and had a specific activity of 3.8 units/mg of protein. One unit is defined as the amount of enzyme that catalyzes the transamination of 1 μmol of GABA/min at 25 °C. Enzyme activity was measured as previously described (Silverman & Levy, 1981). Succinic semialdehyde dehydrogenase was prepared from Gabase (Sigma Chemical Co.) as described previously (Silverman & Levy, 1981).

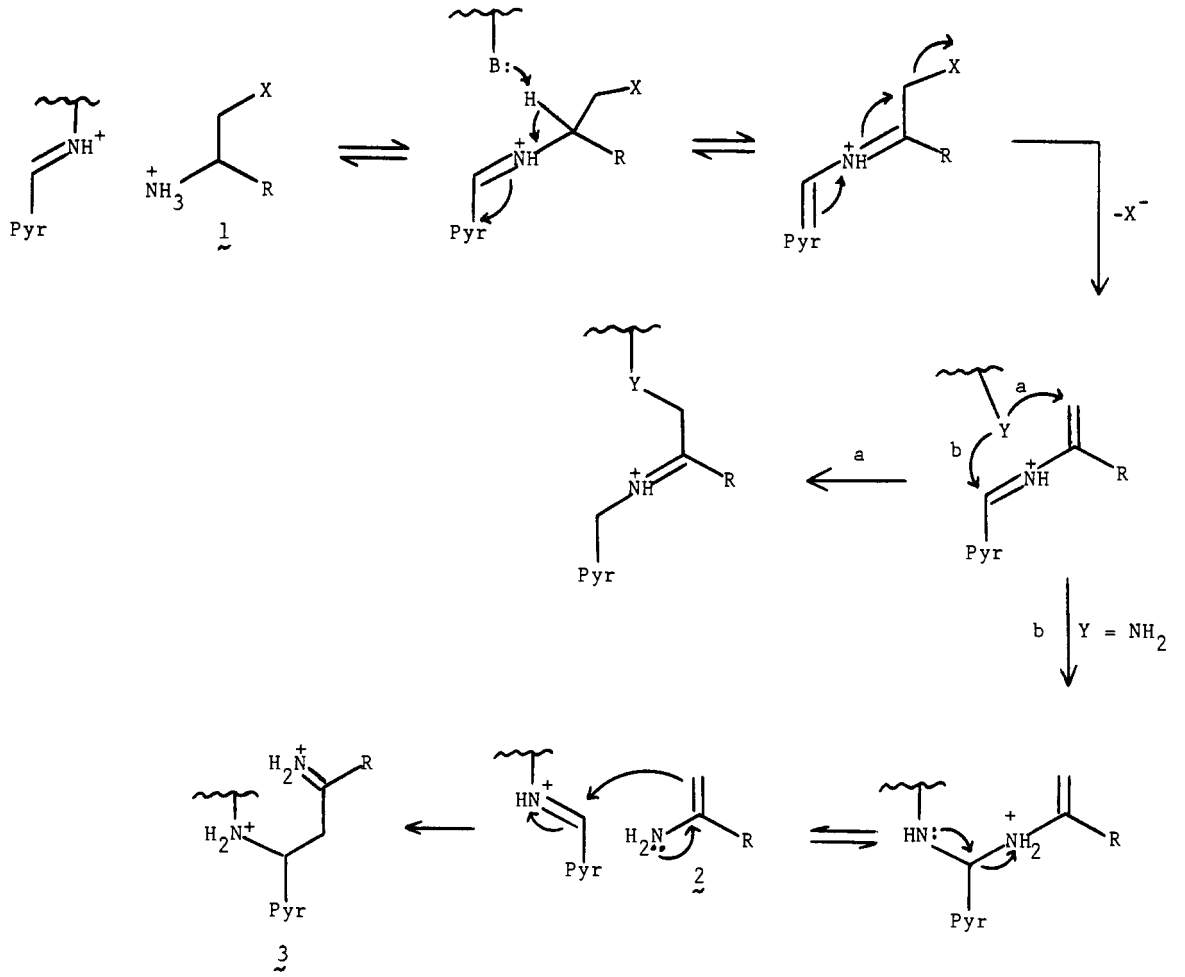
† This work was supported by Grant NS15703 from the National Institutes of Health. R.B.S. is the recipient of an Alfred P. Sloan Research Fellowship (1981–1985) and an NIH Research Career Development Award (1982–1987).

* Address correspondence to this author at the Department of Chemistry.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; GABA-T, γ-aminobutyric acid aminotransferase.

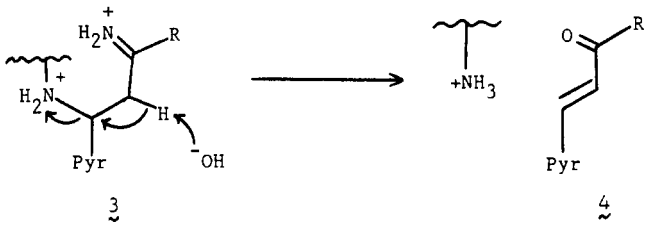
0006-2960/86/0425-6817$01.50/0

Scheme I: Inactivation Mechanisms for PLP-Dependent Enzymes by β-Substituted Alanines[a]



[a] Pyr represents the pyridine ring of PLP.

Scheme II: Formation of **4** by Base Treatment of Enzymes Inactivated by the Enamine Mechanism[a]



[a] Pyr represents the pyridine ring of PLP.

Analytical Methods. GABA aminotransferase assays were carried out on a Perkin-Elmer Lambda 1 UV/vis spectrophotometer. Radioactivity was measured in a Beckman LS-3100 scintillation counter using 10 mL of 3a70B scintillation fluid from Research Products International or Ready-Solv MP from Beckman. [U-^{3}H]Toluene, obtained from New England Nuclear, was used as an internal standard. Protein concentrations were estimated with the BCA protein assay reagent (Pierce Chemical Co.) with bovine serum albumin as a standard. High-performance liquid chromatography (HPLC) was peformed on a Beckman Model 330 HPLC with a Beckman Model 420 microprocessor controller and a Beckman Model 153 UV (254-nm) detector using an Alltech ultrasphere ODS 5-μm C_{18} silica gel column. All solvents were filtered and degassed prior to use. TLC was run on silica gel 60 coated plastic plates (Merck) or C_{18} silical gel coated glass plates (Whatman).

Reagents. PLP, PMP, pyridoxal, bovine serum albumin, β-mercaptoethanol, and α-ketoglutarate were purchased from Sigma Chemical Co. Trichloracetic acid and HPLC-grade methanol and acetonitrile were bought from Mallinckrodt; sodium boro[^{3}H]hydride was a product of ICN Radiochemicals. *Escherichia coli* alkaline phosphatase (code BAPF) was obtained from Cooper Biochemicals, and levulinic acid was a product of Crown-Zellerbach. (*S*)-4-Amino-5-fluoropentanoic acid was synthesized as previously reported (Silverman & Levy, 1980a).

[4-^{3}H]Pyridoxal 5'-Phosphate. This was synthesized by the method of Stock et al. (1966); further purification was achieved by cation-exchange chromatography (Dowex 50X-8, 200–400 mesh), as described by Lui et al. (1981) for [^{14}C]PLP, and by reversed-phase HPLC in two solvent systems. One system employed a linear gradient from pure acetonitrile to pure water (each containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL/min (T_R = 18 min). The second system involved isocratic elution with 100 mM sodium phosphate buffer, pH 7.0 (T_R = 16 min). The absorbance of the [4-^{3}H]PLP-containing eluent was measured at 388 and 330 nm in order to determine the concentration based on the extinction coefficients reported by Peterson and Sober (1954). Six aliquots of the [4-^{3}H]PLP obtained after purification were counted for radioactivity; the specific activity was 78 mCi/mmol (1.73×10^8 dpm/μmol). In addition to the product coeluting with PLP in the two HPLC systems, it also migrated with PLP by silica gel TLC (1-butanol:acetic acid:water = 12:3:5; R_f = 0.22) and by reversed-phase (C_{18}) silica gel TLC

(acetonitrile:water = 3:1, containing 0.1% trifluoroacetic acid; R_f = 0.64).

6-[2-Hydroxy-3-methyl-6-(phosphonoxymethyl)-4-pyridinyl]-4-oxo-5-hexenoic Acid (**4**, R = CH_2CH_2COOH). PLP (275 mg, 1 mmol) was dissolved in 0.5 N aqueous potassium hydroxide (55 mL), and then levulinic acid (466 mg, 4 mmol) was added. The bright yellow solution was stirred at room temperature, protected from the light, for 100 h. The solution was titrated to pH 7.0 with concentrated perchloric acid, and the white solid that formed was removed by centrifugation. The amber supernatant was acidified with concentrated hydrochloric acid and then was extracted with ethyl acetate (3 × 25 mL). The product was obtained by evaporation of the solvent as a yellow solid: mp 217–220 °C dec; NMR (D_2O) δ 2.53 (s, 3 H), 2.57 (m, 2 H), 2.93 (m, 2 H), 4.50 (s, HDO), 4.80 (d, J = 6 Hz, 2 H), 6.79 (d, J = 17 Hz, 1 H), 7.50 (d, J = 17 Hz, 1 H), 8.07 (s, 1 H). One peak was observed by HPLC using the solvent system described under HPLC Analysis of Tritiated Products Released.

Reconstitution of apo-GABA-T with [4-^{3}H]PLP. The [4-^{3}H]PLP (0.07 μmol, 1.2 × 10^7 dpm) obtained from the second HPLC purification step (100 mM sodium phosphate, pH 7.0), was added directly to *apo*-GABA-T (2.04 mg). Once the enzyme activity had stopped increasing (about 9 h), the excess [4-^{3}H]PLP was removed by dialysis at 4 °C in the dark against four changes of 100 mM potassium phosphate buffer, pH 7.4, containing 0.25 mM β-mercaptoethanol (36 h). The specific activity, calculated from the average of six aliquots of [^{3}H]GABA-T and using a molecular weight of 100 000 (Beeler & Churchich, 1978), was 41 mCi/mmol.

Inactivation of [^{3}H]GABA-T by (S)-4-Amino-5-fluoropentanoic Acid. [^{3}H]GABA-T (0.11 mg) was incubated at 25 °C in 100 mM potassium phosphate buffer containing 0.5 mM α-ketoglutarate, 1 mM β-mercaptoethanol, and 2 mM (*S*)-4-amino-5-fluoropentanoic acid at pH 7.4. A control was run that was identical except that the inactivator was omitted. After 4.5 h, the control activity was 113%, but no activity remained in the sample containing the inactivator. The two enzyme solutions were incubated for an additional 3.5 h, and then each was dialyzed against four 2-L portions of deionized water or 10 mM potassium phosphate buffer, pH 7.0. Enzyme activity in the control was uneffected regardless of which dialysis solution was used. The dialyzed enzyme solutions was then raised to pH 11 or 12 with 0.1 N potassium hydroxide. After 15 h (pH 11) or 1 h (pH 12), solid trichloroacetic acid was added to give a final concentration of 10%, and then the enzyme mixture was centrifuged in a Beckman Microfuge B (9000*g*). The supernatant was separated from the pellet, which was washed three times (100 μL each time) with 10% trichloroacetic acid. The combined supernatants were lyophilized, and then the product was dissolved in 60 μL of deionized water. The protein pellet was taken up in 500 μL of 2 N KOH, diluted with 10 mL of scintillation fluid, and counted for radioactivity content.

HPLC Analysis of Tritiated Products Released. The product obtained above was analyzed by HPLC using as standards PLP, PMP, and **4** (R = CH_2CH_2COOH), which were pretreated as follows: each compound (1 mg) was added to water (1 mL), and the pH was raised to 12 with 1 N potassium hydroxide; then solid trichloroacetic acid was added to give a final concentration of 10%. A 25-μL aliquot of each standard was added to a 60-μL aliquot of redissolved product obtained from the radioactive experiment above, and HPLC was performed with a dual solvent system: solvent A was 67 mM potassium monobasic phosphate titrated to pH 2.6 with phosphoric acid; solvent B was methanol. The elution program used was solvent A (0.5 mL/min) for 30 min and then an increase in flow rate to 1 mL/min over 5 min. After 35 min, a solvent gradient to 15% solvent B was run over 15 min. This mixture of 85:15 solvent A to B was run for 60 min and then a reverse gradient to pure solvent A was run over 15 min. With this program, PMP and PLP were eluted at T_R = 8 min and T_R = 18 min, respectively, and **4** (R = CH_2CH_2COOH) was eluted at T_R = 102–107 min. Fractions of 1 mL were collected, mixed with 10 mL of scintillation fluid, and counted for radioactivity.

TLC Analysis of Tritiated Products Released. An aliquot (5 μL) of each of the pretreated standards used for HPLC was added to an aliquot (1300 cpm) of the redissolved radioactive products (see Inactivation of [^{3}H]GABA-T by (*S*)-4-Amino-5-fluoropentanoic Acid). This was spotted on a silica gel TLC plate with fluorescent indicator and eluted in the dark with 2-butanone–water–ethanol–aqueous ammonia (15:5:5:5) (Ahrens & Korytnyk, 1970). The standards were located by UV (R_f^{PLP} = 0.33, R_f^{PMP} = 0.41, R_f^{4} = 0.23), cut out, soaked in water (600 μL) for 30 min, then diluted with 10 mL of scintillation fluid, and counted for radioactivity.

Dephosphorylation of Tritiated Products Released. An aliquot (17 000 cpm) of the product obtained under Inactivation of [^{3}H]GABA-T by (*S*)-4-Amino-5-fluoropentanoic Acid and an aliquot from the corresponding control reaction were lyophilized, redissolved in water (250 μL), and incubated for 28 h with alkaline phosphatase (5 μL, 1.3 units) in 1 M Tris-HCl buffer, pH 8.0, at 25 °C. An additional 1.3 units of alkaline phosphatase was added, and incubation at 25 °C continued for 24 h more, after which time a third 1.3-unit aliquot of alkaline phosphatase was added. After an additional 120 h, the products were analyzed by HPLC as described in HPLC Analysis of Tritiated Products Released except with the following elution program at 1 mL/min: solvent A was pumped for 20 min, and then a gradient to 70:30 solvent A to B over 30 min was run. The 30% solvent B mixture was pumped for 10 min followed by a reverse gradient to pure solvent A over 30 min.

Results and Discussion

Silverman and Levy (1980b) reported that (*S*)-4-amino-5-fluoropentanoic acid **1**, R = CH_2CH_2COOH, X = F) was a time-dependent, irreversible inactivator of GABA-T. The mechanism proposed is that shown in Scheme I, pathway a (R = CH_2CH_2COOH, X = F). If the enamine mechanism of inactivation (Ueno et al., 1982; Likos et al., 1982), however, is relevant, then treatment of the inactivated enzyme (Scheme I, pathway b, **3**, R = CH_2CH_2COOH) with base should release the PLP analogue **4** (Scheme II, R = CH_2CH_2COOH). This compound was synthesized as a standard by the aldol condensation of PLP and levulinic acid; only the (*E*) isomer was obtained, as evidence by the *trans*-vinyl coupling constant (J = 17 Hz) in its NMR spectrum. *apo*-GABA-T was reconstituted with [4-^{3}H]PLP, inactivated with (*S*)-4-amino-5-fluoropentanoic acid, treated with base at pH 12, and denatured with trichloroacetic acid. All of the radioactivity was released from the protein under these conditions. HPLC of the radioactive metabolites showed that 80–95% (three experiments) of the released radioactivity coeluted with **4** (R = CH_2CH_2COOH), and less than 3% of the radioactivity coeluted with PLP and PMP. On the other hand, HPLC of the radioactive metabolites from the control reaction, which was identical with the inactivation experiment except that the enzyme was not treated with inactivator, resulted in 60–75% of the radioactivity coeluting with PLP and/or PMP and none

with **4** (R = CH_2CH_2COOH). These results support the enamine inactivation mechanism in this case (Scheme I, pathway b, R = CH_2CH_2COOH). In addition to HPLC, silica gel TLC of the released metabolites showed that 85–90% of the radioactivity comigrates with synthetic **4** (R = CH_2CH_2COOH) and none with either PLP or PMP. Two other radioactive products (4% and 6% of the radioactivity) were detected; the larger of these did not migrate from the origin, and the smaller moved near the solvent front. It is known that PLP derivatives are very sensitive to light (Ahrens & Korytnyk, 1970; Reiber, 1972), and these may be photodegradation products.

As further confirmation of the structure of the released radioactive product, dephosphorylation with alkaline phosphatase was carried out. Half of the radioactivity, analyzed by HPLC, coeluted with the product of alkaline phosphatase treatment of synthetic **4** (R = CH_2CH_2COOH). The remainder of the radioactivity eluted in the void volume and was not identified. Because of the length of the incubation time (7 days) with alkaline phosphatase, it is not surprising that much decomposition could have occurred. Nonetheless, this experiment provides additional support for the formation of **4** (R = CH_2CH_2COOH).

In conclusion, it has been shown that the mechanism of inactivation of GABA-T by 4-amino-5-fluoropentanoic acid is that shown in Scheme I, pathway b (R = CH_2CH_2COOH). Since all of the radioactivity of [^{3}H]GABA-T is released from the enzyme by inactivation, base treatment, and denaturation, and essentially none is detected as PLP or PMP, it suggests that this is the exclusive pathway. The partition ratio for inactivation is 0 (Silverman & Levy, 1981); therefore, the binding interactions of the inactivator with GABA-T must be sufficiently tight to prevent release of intermediate **2** prior to its conversion to **3**. Presumably, the carboxylate of the inactivator is anchored to the active site by an electrostatic interaction. The added flexibility in the side chain of **2** (R = CH_2CH_2COOH) relative to **2** (R = COOH) may be responsible for the efficiency of the C–C bond formation to give **3** (R = CH_2CH_2COOH) without release from the active site. This is the first example of a γ-amino acid that undergoes this type of inactivation mechanism and also is the first example of an inactivator that proceeds by this mechanism and still inactivates the enzyme with every turnover. With the use of [4-^{3}H]PLP containing GABA-T, we will be able to screen many known GABA-T inactivators to determine whether the enamine mechanism is the prominent inactivation pathway for other inactivators as well.

References

Abeles, R. H. (1983) *Chem. Eng. News 61* (Sept. 19), 48–56.

Ahrens, H., & Korytnyk, W. (1970) *Methods Enzymol. 18A*, 489–493.

Badet, B., Roise, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5188–5194.

Beeler, T., & Churchich, J. E. (1978) *Eur. J. Biochem. 85*, 365–371.

Churchich, J. E., & Moses, U. (1981) *J. Biol. Chem. 256*, 1101–1104.

Likos, J. J., Ueno, H., Feldhaus, R. W.. & Metzler, D. E. (1982) *Biochemistry 21*, 4377–4386.

Lui, A., Minter, R., Lumeng, L., & Li, T. K. (1981) *Anal. Biochem. 112*, 17–22.

Morino, Y., Kojima, H., & Tanase, S. (1979) *J. Biol. Chem. 254*, 279–285.

Peterson, E. A., & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169–175.

Rando, R. R. (1984) *Pharmacol. Rev. 36*, 111–142.

Reiber, H. (1972) *Biochim. Biophys. Acta 279*, 310–315.

Relyea, N. M., Tate, S. S., & Meister, A. (1974) *J. Biol. Chem. 249*, 1519–1524.

Roise, D., Soda, K., Yagi, T., & Walsh, C. T. (1984) *Biochemistry 23*, 5195–5201.

Silverman, R. B., & Levy, M. A. (1980a) *J. Org. Chem. 45*, 815–818.

Silverman, R. B., & Levy, M. A. (1980b) *Biochem. Biophys. Res. Commun. 95*, 250–255.

Silverman, R. B., & Levy, M. A. (1981) *Biochemistry 20*, 1197–1203.

Silverman, R. B., & Hoffman, S. J. (1984) *Med. Res. Rev. 4*, 415–447.

Stock, A., Ortanderl, F., & Pfleiderer, G. (1966) *Biochem. Z. 344*, 353–360.

Ueno, H., Likos, J. J., & Metzler, D. E. (1982) *Biochemistry 21*, 4387–4393.

Walsh, C. (1984) *Annu. Rev. Biochem. 53*, 493–535.

Wang, E., & Walsh, C. (1978) *Biochemistry 17*, 1313–1321.