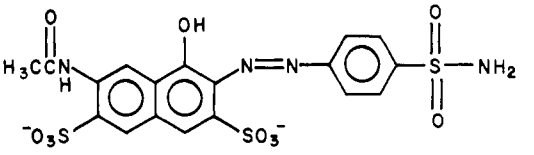
Evidence for Direct Metal–Nitrogen Binding in Aromatic Sulfonamide Complexes of Cadmium(II)-Substituted Carbonic Anhydrases by Cadmium-113 Nuclear Magnetic Resonance†

Jeffrey L. Evelhoch, David F. Bocian, and James L. Sudmeier*

ABSTRACT: ^{113}Cd NMR has been used to study the nature of the metal–sulfonamide interaction in complexes of ^{113}Cd-substituted carbonic anhydrases and the aromatic sulfonamides benzenesulfonamide and Neoprontosil. The ^{113}Cd chemical shifts of the complexes exhibit negligible dependence on the isotopic composition of the sulfonamide nitrogen. However, benzenesulfonamide and Neoprontosil, when 90% ^{15}N-enriched at the sulfonamide nitrogen, each split the ^{113}Cd resonance into a doublet ($J_{^{113}Cd-^{15}N} \simeq 200$ Hz). This constitutes evidence for metal–nitrogen bonds in these complexes. The chemical shifts of the complexes (~390 ppm) and their pH independence from pH 7.0 to 10.0 suggest the sulfonamides are bound in the anionic form. The resonance Raman (RR) spectra of ^{15}N-labeled and unlabeled Neoprontosil have been obtained to clarify the report of anomalous Neoprontosil binding in the nonionized form [Petersen, R. L., Li, T.-Y., McFarland, J. T., & Watters, K. L. (1977) *Biochemistry 16*, 726]—a report based on the assignment of a band at ~900 cm^{-1} to a sulfonamide S–N stretching vibration. We find the frequencies of Raman bands observed in the range 800–1700 cm^{-1} to be virtually identical for the ^{15}N-labeled and unlabeled molecules, indicating that none of the bands can be assigned to a S–N stretching vibration. The RR data unambiguously show the report of Petersen et al. (1977) is based on the misassignment of the band at ~900 cm^{-1}.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is an enzyme present in animals, plants, and certain bacteria, and its function is to catalyze the reversible hydration of CO_2 (Lindskog et al., 1971). A single equivalent of Zn(II), coordinated in distorted tetrahedral geometry to three histadyl nitrogens and presumably a water or hydroxide, is required for activity. It has been known for some time that halide ions and other monovalent anions inhibit carbonic anhydrases (Meldrum & Roughton, 1933). Aromatic sulfonamides are strong inhibitors of carbonic anhydrase (Maren, 1967) and are of pharmacological value because of their effects on various physiological reactions ultimately involving bicarbonate (Goth, 1972). Sulfonamides have been useful in studies of the physicochemistry and mechanism of carbonic anhydrase because of their highly specific interaction with the active site (Coleman, 1973; King & Burgen, 1976). Results from X-ray crystallographic studies indicate the sulfonamide function binds directly to the zinc ion (Fridborg et al., 1967; Kannan et al., 1977). However, it is not clear from these studies whether the sulfonamide is coordinated to the zinc via the oxygen (Taylor et al., 1970) or the nitrogen (Kannan et al., 1977), or both (Bauer et al., 1977; Kannan et al., 1977).

It was generally concluded on the basis of fluorescence emission (Chen & Kernohan, 1967), ultraviolet difference (King & Burgen, 1970), and resonance Raman (RR)[1] (Kumar et al., 1974, 1976) spectroscopic data that all sulfonamide derivatives bind directly to carbonic anhydrase in their ionized form (SO_2NH^-). However, Petersen et al. (1977) reported that the bulky azosulfonamide, 2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (Neoprontosil, I), binds in the nonionized form (SO_2NH_2) and suggested



I

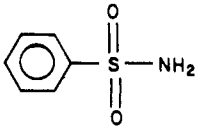
no direct coordination of the sulfonamide moiety to the zinc ion. This conclusion was based upon shifts observed in Raman bands assigned to the sulfonamide group which occur upon binding of Neoprontosil to the enzyme at various pH values. Carey & King (1979) later questioned the conclusions of Petersen et al. (1977), pointing out that because the pK_a values of the naphthol and sulfonamide functions in Neoprontosil are within 1 pH unit of each other, the ionization state of the sulfonamide cannot readily be correlated with the observed shifts in the Raman bands. It was apparent that additional experimental data were needed in order to better characterize the Neoprontosil–carbonic anhydrase complex.

^{113}Cd NMR has been very useful in the study of the coordination sphere of the metal ion in zinc metalloenzymes when ^{113}Cd is substituted for the zinc ion (Armitage et al., 1976, 1978; Sudmeier & Bell, 1977; Bailey et al., 1978; Jonsson et al., 1980). In this paper we report the study of the binding of two ^{15}N-labeled sulfonamides, Neoprontosil (I) and benzenesulfonamide (II), to ^{113}Cd-substituted carbonic anhydrases (HCAB, HCAC, and BCA). We conclude from our NMR data that both sulfonamides directly coordinate to the metal ion via the sulfonamide nitrogen in the Cd-substituted enzymes

† From the Department of Chemistry, University of California at Riverside, Riverside, California 92521. *Received January 19, 1981.* This research was supported by U.S. Public Health Service Grant 1R01 GM25877 and a grant from the University of California at Riverside Committee on Research. D.F.B. acknowledges support from the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the Cottrell Research Grants Program of the Research Corporation. The UCR Bruker WH90D-18 multinuclear Fourier transform NMR spectrometer was provided by Biomedical Sciences Grant 5S05RR 07010-09 from the National Institutes of Health and National Science Foundation Grant MPS75-06138. The Raman spectrometer was partially funded by Biomedical Sciences Grant 5507RR-07010-12.

[1] Abbreviations used: RR, resonance Raman; NMR, nuclear magnetic resonance; HCAB, human carbonic anhydrase B; HCAC, human carbonic anhydrase C; BCA, bovine carbonic anhydrase.

0006-2960/81/0420-4951$01.25/0



II

and suggest the sulfonamide moiety is bound in its anionic form. Finally, we resolve the contradiction between our conclusions and those of Petersen et al. (1977) by comparing the RR spectra of ^{15}N-labeled and unlabeled Neoprontosil.

Experimental Procedures

Enzymes. HCAB, HCAC, and BCA were prepared by affinity chromatography according to the Khalifah et al. (1977) modification of the method of Osborne & Tashian (1975). Metal-free enzymes were prepared by dialysis against 75 mM pyridine-2,6-dicarboxylic acid/0.2 M phosphate buffer according to Hunt et al. (1977). The chelator was removed by dialysis against six changes of deionized water. The ^{113}Cd-substituted enzymes were prepared by addition of stoichiometric $^{113}Cd(II)$ (as $^{113}CdSO_4$) to the apoenzyme at pH 6.5. The $^{113}CdSO_4$ was prepared by addition of a slight excess of concentrated H_2SO_4 to ^{113}CdO (96.3% enrichment, Oak Ridge National Laboratory, Oak Ridge, TN). The excess acid was neutralized by careful addition of dilute NaOH.

Sulfonamides. Nonlabeled Neoprontosil was a gift from Sterling Organics, Rensselaer, NY. Neoprontosil purchased from Sigma Chemical Co., St. Louis, MO, under the name Azosulfamide was found not to be Neoprontosil by a ^1H NMR pH titration, UV–vis spectroscopy, and thin-layer chromatography. Synthesis of ^{15}N-labeled Neoprontosil was as follows: (1) incorporation of ^{15}N into sulfanilamide by heating a mixture of $^{15}NH_4OH$ [prepared by trapping $^{15}NH_3$ produced upon adding concentrated $^{15}NH_4Cl$ (95% enrichment, Prochem Isotopes, Summit, NJ) to hot concentrated NaOH] and *N*-acetylsulfanilyl chloride (Aldrich Chemical Co., Inc., Milwaukee, WI), followed by acid hydrolysis of the acetamide function; (2) synthesis of 2-acetamido-8-naphthol-3,6-disulfonic acid by acetylation of 8-hydroxy-2-amino-3,6-disulfonic acid (Fluka AG, Buchs, Switzerland) with acetic anhydride and subsequent base hydrolysis of the *O*-acetyl function at room temperature; (3) diazotization of the [^{15}N]sulfanilamide with nitrous acid at 0 °C; (4) coupling to 2-acetamido-8-naphthol-3,6-disulfonic acid at room temperature. [^{15}N]Benzenesulfonamide was synthesized by addition of benzenesulfonyl chloride to a concentrated solution of $^{15}NH_4NO_3$ (95% enrichment, Prochem Isotopes, Summit, NJ) in the presence of excess base.

NMR Spectra. The ^{113}Cd NMR spectra were obtained at ~19.97 MHz on a modified multinuclear Bruker WH90-18 (18-in. 2.11-T magnet) NMR spectrometer. The probe used an insert designed for 15-mm (outside diameter) sample tubes containing 5-mL samples. An Avantek wide-band preamplifier, Locus image-reject mixer, and quarter-wave line made of semirigid coaxial cables were used. The spectrometer used quadrature phase detection for all nuclei. Normally we used the following conditions: sweep width, 15 kHz; acquisition time, 0.27 s (4K data in real spectrum); exponential multiplication to produce line broadening of 10 Hz; a pulse flip angle of 30°. The 90° pulse was 12.5 s for 25 W of pulse power. Chemical shifts are reported vs. external 0.20 M $^{113}CdSO_4$.

Resonance Raman Spectra. The RR spectra were recorded on a Spex Industries Ramalog 6 spectrometer equipped with a cooled Hamamatsu R955 phototube and a photon-counting system. The 4880-Å line of a Spectra-Physics Model 164-05 Ar ion laser was used for monochromatic excitation. The spectra were obtained by using melting point capillaries at a sample concentration of 5×10^{-5} M. The incident laser power was ~150 mW and the spectral resolution was 4 cm^{-1}. No deterioration of the sample occurred during the recording of the spectra.

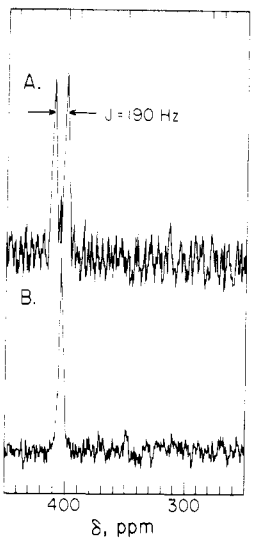


FIGURE 1: ^{113}Cd NMR spectrum of (A) 3.7×10^{-3} M BCA plus 1 equiv of ^{15}N-labeled Neoprontosil, pH 8.9; (B) 5.3×10^{-3} M BCA plus 1 equiv of unlabeled Neoprontosil, pH 9.1; 12-h data collection for both (A) and (B).

Table I: ^{113}Cd Chemical Shifts and ^{113}Cd–^{15}N Coupling Constants in Complexes of Aromatic Sulfonamides and Carbonic Anhydrases

enzyme	inhibitor	$\delta^{113}Cd$	$J^{113}Cd$–^{15}N (Hz)
HCAB	benzenesulfonamide	355 ± 1	210 ± 3
HCAC	benzenesulfonamide	390 ± 1	190 ± 3
BCA	benzenesulfonamide	387 ± 1	190 ± 3
BCA	Neoprontosil	403 ± 1	190 ± 3

Results and Discussion

The ^{113}Cd NMR spectra of BCA with ^{15}N-labeled and unlabeled Neoprontosil are shown in parts A and B, respectively, of Figure 1. In both spectra the ^{113}Cd chemical shift is 403 ppm; however, in Figure 1A the ^{113}Cd resonance is a doublet with a coupling constant of 190 Hz. The doublet is due to spin–spin coupling of ^{113}Cd with the ^{15}N of Neoprontosil. The large magnitude of the coupling constant is indicative of a direct metal–nitrogen interaction. In this regard, the complex of HCAC with [^{13}C]cyanide yields a coupling constant of 1040 Hz (Jonsson et al., 1980), whereas [^{15}N]-cyanide produces no observable splitting (J. L. Sudmeier, unpublished results). It appears the metal–ligand coupling is rapidly attenuated, allowing observation of spin splitting only for direct coordination. The ^{113}Cd spectra of complexes of [^{15}N]benzenesulfonamide with HCAB, HCAC, and BCA exhibit doublets as well. Table I gives the ^{113}Cd chemical shifts and ^{113}Cd-^{15}N spin coupling constants for the complexes. It is apparent that both the small aromatic sulfonamide, benzenesulfonamide, and the bulky azosulfonamide, Neoprontosil, form a metal–nitrogen bond in their complexes with Cd-substituted carbonic anhydrases.

The ^{113}Cd chemical shifts of complexes of benzenesulfonamide and Neoprontosil with HCAB, HCAC, and BCA are independent of pH from pH 7.0 to pH 10.0 and further downfield than expected for Cd(II) with four nitrogen ligands. (The sulfonamide nitrogen replaces water in the sulfonamide carbonic anhydrase complexes.) Below pH 9 the complex is in slow exchange with free enzyme, and two peaks are ob-

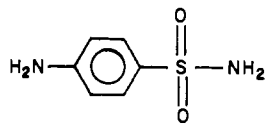
Table II: ^{113}Cd Chemical Shifts in Complexes of Cd(II) with Nitrogen Ligands and in Complexes of HCAC

compound	δ^{113}Cd
1.0 M $[Cd(NH_3)_6]^{2+}$ [a]	287 ± 1
0.3 M $[Cd(IM)_6]^{2+}$ [b]	201 ± 1
0.3 M $[Cd(MeHis)_2]^{2+}$ [b]	224 ± 1
0.3 M $[Cd(MeHis)_3]^{2+}$ [b]	254 ± 1
HCAC-Br^- [d]	240 ± 1
HCAC-Cl^- [b]	239 ± 1
HCAC-CN^- [c]	354 ± 1
HCAC-SH^- [d]	374 ± 1
HCAC-OH^- [c]	275 ± 2
HCAC-H_2O [c]	229 ± 2

[a] From Cardin et al. (1975). [b] From Sudmeier & Bell (1977). [c] From Jonsson et al. (1980). [d] Unpublished results.

served. The chemical shift of free enzyme (~220 ppm) is a function of pH and bicarbonate [see Jonsson et al. (1980)], and the chemical shift of the complex (~390 ppm) is pH independent. The pH independence of the chemical shift of the complex indicates the sulfonamide is bound in the same form from pH 7.0 to pH 10.0. In Table II, the ^{113}Cd chemical shifts of Cd^{2+} with several nitrogen ligands and several HCAC complexes are presented. The chemical shifts of Cd(II) complexes with nitrogen ligands are normally in the range of 200–275 ppm (Cardin et al., 1975; Sudmeier & Bell, 1977). The chemical shifts of complexes of ^{113}Cd-substituted HCAC with halides are ~240 ppm, whereas the chemical shifts of complexes of HCAC with cyanide and bisulfide are at a much lower field (Sudmeier & Bell, 1977; J. L. Sudmeier, unpublished results). The low-field peaks are expected for the bisulfide complex, since the cysteine sulfur ligands in metallothionein give ^{113}Cd(II) a chemical shift of >600 ppm (Otvos & Armitage, 1980). Cyanide has π-antibonding orbitals available for back-bonding to Cd(II), forming a bond more covalent in nature than halides and deshielding the Cd(II) to produce a low-field chemical shift. If the sulfonamide moiety is ionized in the complex, the S–N bond would have some π character. This would form a more covalent bond and a lower field chemical shift than for sulfonamide bound in the nonionized form. Thus, the pH independence and position of the chemical shift suggest the sulfonamide binds to carbonic anhydrase in the anionic form.

The ^{113}Cd–^{15}N coupling constants and ^{113}Cd chemical shifts in complexes of benzenesulfonamide with HCAC and BCA are similar to each other, yet different from that in the benzenesulfonamide–HCAB complex (see Table I). The chemical shift and magnitude of the coupling constants reflect differences in the coordination geometries of the Cd-substituted enzyme complexes. It is known that native HCAB and HCAC have structurally different active sites resulting in different coordination geometries in complexes of these isozymes with sulfanilamide (III) (Notstrand et al., 1975). On the other



III

hand, the active sites and sulfanilamide coordination geometries of native BCA and HCAC are expected to be similar, based on a comparison of their specific activities, affinity for inhibitors, and chemical reactivity toward modifying agents (Wyeth & Prince, 1977). The sulfanilamide coordination geometries in native HCAB, HCAC, and BCA correlate directly with the ^{113}Cd–^{15}N coupling constants and ^{113}Cd chemical shifts in complexes of [^{15}N]benzenesulfonamide with

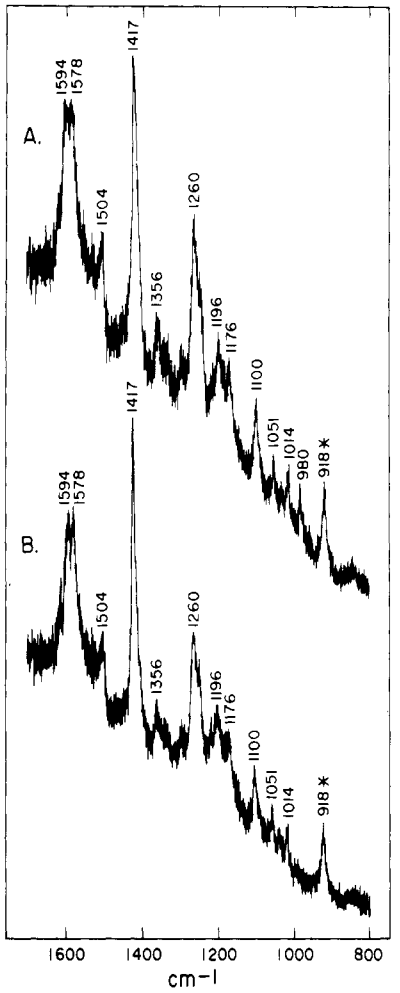


FIGURE 2: Resonance Raman spectrum of (A) 8×10^{-5} M unlabeled Neoprontosil, pH 9.0; (B) 5×10^{-5} M ^{15}N-labeled Neoprontosil, pH 9.0. The band labeled by the asterisk was previously assigned as the S–N stretching vibration (Petersen et al., 1977) but is unshifted upon isotopic substitution. The band at 980 cm^{-1} in (A) is most likely due to an impurity.

the ^{113}Cd-substituted isozymes. This correlation suggests the nature of the aromatic sulfonamide-enzyme complex is similar for the two metal ions.

Our ^{113}Cd NMR data clearly indicate that Neoprontosil is directly bound to the metal ion in carbonic anhydrase via the sulfonamide nitrogen and suggests the sulfonamide moiety is bound in its anionic form. This is in direct opposition to Petersen et al. (1977), who concluded on the basis of RR data that Neoprontosil binds in the nonionized form. The conclusion of Petersen et al. (1977) is based primarily on the frequency differences observed at various pH values of a Raman band at ~900 cm^{-1} assigned by these workers to the sulfonamide S–N stretching vibration. Since these arguments concerning the nature of the Neoprontosil–enzyme complex hinge on the correct assignment of the 900-cm^{-1} Raman band, we have compared the RR spectra of ^{15}N-labeled and unlabeled Neoprontosil in order to determine whether the assignment is correct. We find that the RR spectra of the ^{15}N-labeled and unlabeled molecules are virtually identical in the frequency range 800–1700 cm^{-1} (see Figure 2). In particular, the band assigned by Petersen et al. (1977) to the sulfonamide S–N stretching vibration *does not* shift upon substitution of ^{15}N for ^{14}N. (The difference in reduced mass of the S–N group for the two nitrogen isotopes should result in a shift of ~20 cm^{-1} to lower frequency for the ^{15}N-labeled molecule.) Our RR data incidate that none of the Raman bands observed for Neoprontosil can be assigned to a sulfon-

amide S–N stretching vibration. Thus, the conclusions reached by Petersen et al. (1977) concerning the state of ionization of the sulfonamide group based on such an assignment are clearly invalid. In addition, Carey & King (1979) have pointed out that because the pK_a values of the naphthol and sulfonamide functions are within 1 pH unit of each other, the ionization state of the sulfonamide moiety cannot readily be correlated with the observed shifts in the Raman bands.

In conclusion, the ^{113}Cd NMR spectra of complexes of ^{113}Cd-substituted carbonic anhydrases with aromatic sulfonamides which are 90% ^{15}N enriched at the sulfonamide nitrogen have been used to unambiguously determine the direct coordination of that nitrogen to the cadmium ion. In addition, on the basis of the ^{113}Cd NMR and RR results presented here and the earlier studies of other aromatic sulfonamide–carbonic anhydrase complexes, we infer that aromatic sulfonamides which inhibit carbonic anhydrase are bound with the sulfonamide moiety ionized.

Acknowledgments

We thank Dr. Edward W. Schurick of Sterling Organics for providing us with very helpful information and guidance in the synthesis of ^{15}N-labeled Neoprontosil. We also thank Drs. Nalle B.-H. Jonsson and Lena A. E. Tibell for preparing the enzymes, Jeffrey J. Roy for synthesizing [^{15}N]benzenesulfonamide, and Karen L. Wilson for experimental assistance.

References

Armitage, I. H., Pajer, R. T., Schoot-Uiterkamp, A. J. M., Chlebowski, J. F., & Coleman, J. E. (1976) *J. Am. Chem. Soc. 98*, 5710.

Armitage, I. H., Schoot-Uiterkamp, A. J. M., Chlebowski, J. F., & Coleman, J. E. (1978) *J. Magn. Reson. 29*, 375.

Bailey, D. B., Ellis, P. D., & Cardin, A. D. (1978) *J. Am. Chem. Soc. 100*, 236.

Bauer, R., Limkilde, P., & Johansen, J. T. (1977) *Carlsberg Res. Commun. 42*, 325.

Cardin, A. D., Ellis, P. D., Odom J. D., & Howard, J. W. (1975) *J. Am. Chem. Soc. 97*, 1672.

Carey, P. R., & King, R. W. (1979) *Biochemistry 18*, 2834.

Chen, R. F., & Kernohan, J. C. (1967) *J. Biol. Chem. 242*, 5813.

Coleman, J. E. (1973) *Inorg. Biochem. 1*, 488.

Fridborg, K., Kannan, K. K., Liljas, A., Lundin, J., Strandberg, B., Strandberg, R., Tilander, B., & Wirēn, G. (1967) *J. Mol. Biol. 25*, 505.

Goth, A. (1972) in *Medical Pharmacology*, 6th ed., p 413, C. V. Mosley, St. Louis, MO.

Hunt, J. B., Rhee, M. J., & Storm, C. B. (1977) *Anal. Biochem. 79*, 614.

Jonsson, N. B.-H., Tibell, L. A. E., Evelhoch, J. L., Bell, S. J., & Sudmeier, J. L. (1980) *Proc. Natl. Acad. Sci. USA 77*, 3269.

Kannan, K. K., Vaara, I., Notstrand, B., Lövgren, S., Borell, A., Fridborg, K., & Petef, M. (1977) in *Drug Action at the Molecular Level* (Roberts, G. C. K., Ed.) p 73, University Park Press, Baltimore, MD.

Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241.

King, R. W., & Burgen, A. S. V. (1970) *Biochim. Biophys. Acta 207*, 276.

King, R. W., & Burgen, A. S. V. (1976) *Proc. R. Soc. London, Ser. B 193*, 107.

Kumar, K., King, R. W., & Carey, P. R. (1974) *FEBS Lett. 48*, 283.

Kumar, K., King, R. W., & Carey, P. R. (1976) *Biochemistry 15*, 2195.

Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes, 3rd Ed. 5*, 587.

Maren, T. H. (1967) *Physiol. Rev. 47*, 595.

Meldrum, N. U., & Roughton, F. J. W. (1933) *J. Physiol. (London) 80*, 113.

Notstrand, B., Vaara, I., & Kannan, K. (1975) *Isozymes, Int. Conf., 3rd., 1974 1*, 575.

Osborne, W. R. A., & Tashian, R. E. (1975) *Anal. Biochem. 64*, 297.

Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094.

Petersen, R. L., Li, T.-Y., McFarland, J. T., & Watters, K. L. (1977) *Biochemistry 16*, 726.

Sudmeier, J. L., & Bell, S. J. (1977) *J. Am. Chem. Soc. 99*, 4499.

Taylor, P. W., King, R. W., & Burgen, A. S. V. (1970) *Biochemistry 9*, 3894.

Wyeth, P., & Prince, R. H. (1977) *Inorg. Perspect. Biol. Med. 1*, 37.