min and freeze-dried. Paper chromatography of this tryptic hydrolysate showed the expected mixture[6] of the five tryptic peptides (R_f^B "a" = 0.12, "b" = 0.3, "c" = 0.3, "d" = 0.45, and "e" = 0.89). In some experiments also a small amount of peptide a and d (R_f^B 0.20) was observed. Better separation of this tryptic hydrolysate could be achieved by a two-dimensional combination of electrophoresis (pH 2; formic acid–acetic acid) and chromatography in the same solvent system.

Isolation of Peptide A from the Tryptic Digest. Samples (20 mg) of the heptacosapeptide amide (1000 clinical units/mg) and the free C-terminal tridecapeptide amide (sequence 15–27)[1] were digested with trypsin as described above. The mixtures were chromatographed on Whatman 3 MM sheets, and the band corresponding to peptide A (R_f^B 0.12) eluted with 0.1 *M* acetic acid. The materials thus obtained were compared by paper electrophoresis at three different pH values: 2.0 (formic acid, acetic acid), 6.4 (pyridine, acetic acid), and 8.5 (N-ethylmorpholine, acetic acid). A single band of identical mobility and ninhydrin color was observed in the three experiments for both preparations of peptide A. The purple ninhydrin color indicated α-aspartyl peptides rather than β-aspartyl derivatives.

An aliquot of these two preparations of peptide A was digested with leucine aminopeptidase[25] (Boehringer, Mannheim Corp., New York, N. Y.). Quantitative amino acid analyses of the digests gave the following ratios: peptide A from the tridecapeptide Asp, 1.10; Ser, 1.05; Ala, 1.00; Arg, 1.10; peptide A from the heptacosapeptide Asp, 1.05; Ser, 1.00; Ala, 0.90; Arg, 1.10.

Isolation of Peptide B from the Tryptic Digest. A sample (20 mg) of heptacosapeptide amide (1000 clinical units/mg) was digested with trypsin, as described above. The peptide mixture was fractionated by electrophoresis at pH 2 on Whatman 3 MM paper. The Pauly-positive band (peptide B) was eluted with 0.1 *M* acetic acid and freeze-dried (6.5 mg) To an aliquot of this preparation (3 mg) in 1% aqueous ammonium bicarbonate (0.3 ml), a 0.25% solution (24 μl) of chymotrypsin[26] in 1% ammonium bicarbonate was added, and the mixture incubated at 40° for 5 hr. A second 24-μl portion of chymotrypsin solution was added, and the digestion was allowed to proceed for a total of 24 hr. The digest was fractionated by preparative paper electrophoresis at pH 7 (collidine, acetic acid), and the single Pauly-positive band, which showed the mobility of an authentic sample of His-Ser-Asp-Gly-Thr-Phe,[27] was eluted with 0.1 *M* acetic acid. After removal of the solvent by freeze-drying, the residue was digested with leucine aminopeptidase.[25] Quantitative amino acid analysis of the digest gave the following molar ratios of amino acids: His, 1.00; Ser, 1.10; Asp, 0.51; Gly, 0.46; Thr, 0.60; Phe, 0.40.[16]

Digestion with Trypsin and Leucine Aminopeptidase. Samples (2 ml) of the crude and of the purified heptacosapeptide amide preparations were digested with trypsin as described above. The mixture of tryptic peptides thus obtained was then digested with leucine aminopeptidase.[25] Quantitative amino acid analyses of the resulting digests gave the following molar ratios: crude preparation (1000–1500 clinical units/mg): Asp, 1.32; Thr, 1.45; Ser, 2.7; Glu, 0.45; Gly, 1.45; Ala, 1.0; Val, 1.1; Leu, 5.2; Phe, 0.32; His, 0.73; Arg, 3.05. The purified preparation (about 4000 clinical units/mg) gave the following ratios: Asp, 1.9; Thr, 2.1; Ser, 3.6; Glu, 1.2; Gly, 1.9; Ala, 1.1; Val, 1.05; Leu, 5.6; Phe, 0.8; His, 1.06; Arg, 3.5.

Acknowledgment. The authors express their gratitude to Professor J. Erick Jorpes, Dozent V. Mutt, Dr. M. I. Grossman, and Dr. S. L. Engel for biological assays; to Mr. J. Alicino and his group for the microanalyses; to Mr. O. Kocy for the electrophoretic studies; and to Mr. F. Russo-Alesi for the quantitative amino acid analyses.

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(26) Prepared from Worthington CD 1 α-chymotrypsin.

(27) This hexapeptide was prepared by Dr. John T. Sheehan of this laboratory.

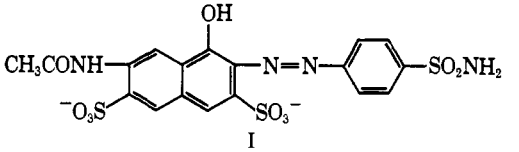
Communications to the Editor

Carbonic Anhydrase–Azosulfonamide Complexes. Optical Rotatory Dispersion and Circular Dichroism

Sir:

A variety of sulfonamides inhibit the zinc metalloenzyme carbonic anhydrase. The requirement for inhibition is the presence of an unsubstituted sulfonamide group attached to a ring system the structure of which can be varied widely without losing the relatively high affinity of the inhibitor for the enzyme.[1–3] These compounds have played an important role in studies of the active site of this enzyme since sulfonamide binding has been shown to be: (1) metal ion dependent,[4,5] (2) accompanied by changes in energy and optical activity of the d–d transitions of the Co(II) derivative of the enzyme,[6,7] and (3) limited to the metallocarbonic anhydrases containing Co(II) and Zn(II), the only first transition and IIb metal ions which restore significant enzymatic activity to apocarbonic anhydrase.[5,6]

The present communication reports spectral, optical rotatory dispersion (ORD), and circular dichroism (CD) studies of carbonic anhydrase–azosulfonamide complexes employing 2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (I) as the inhibitor. On binding of this sulfonamide to carbonic anhydrase, the strong absorption band of the azochromophore near 500 mμ (ϵ_{max} ~25,000) shows both marked



I

spectral shifts and induced optical activity. These features make this molecule an effective detector for changes in the environment or conformation of the bound sulfonamide and a sensitive probe for conformational features of the active center of carbonic anhydrase.

Carbonic anhydrase, isozyme B, was prepared from human or monkey erythrocytes by the methods previously reported.[7,8] The azosulfonamide was purchased

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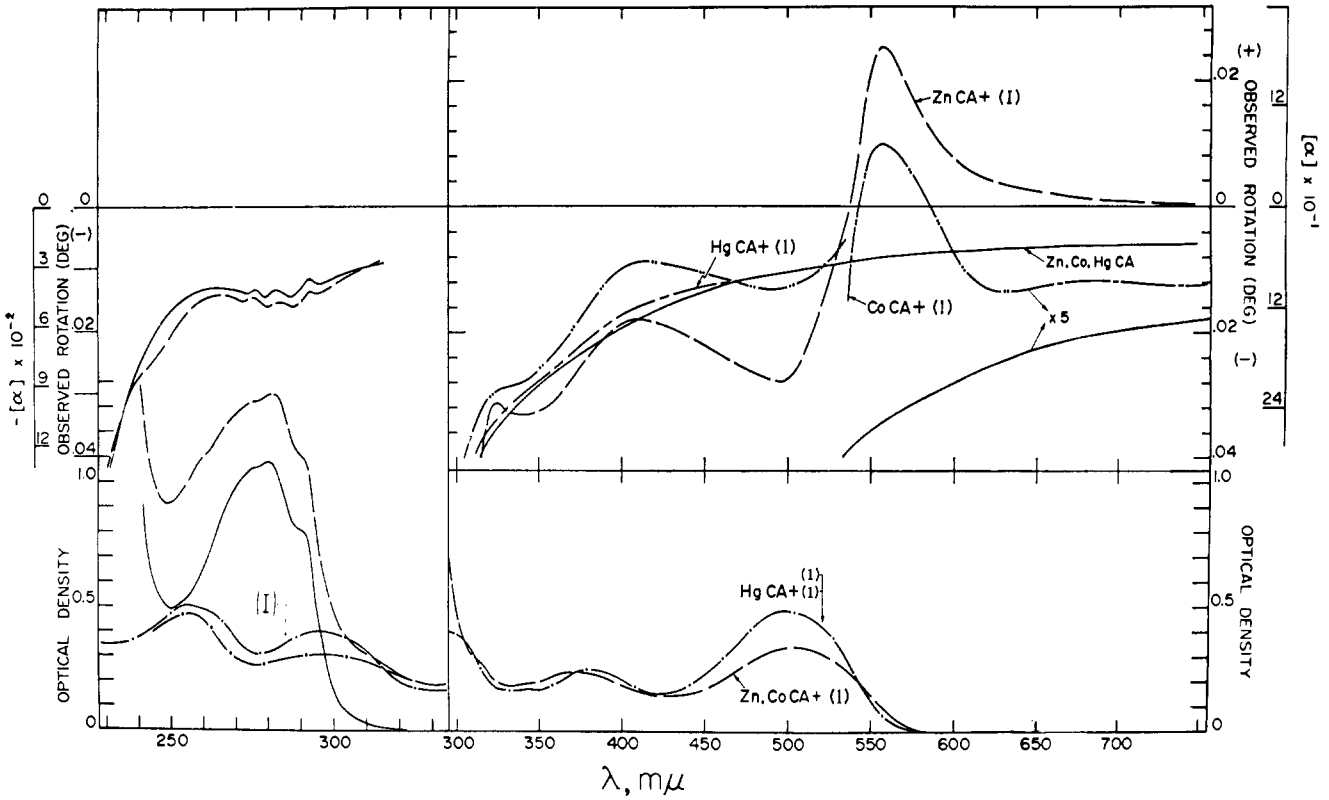

Figure 1. Absorption and ORD spectra of human carbonic anhydrase B-azosulfonamide complexes. ORD spectra: (———) 4.5×10^{-5} *M* Zn(II), Co(II), and Hg(II) enzymes; (— — —) 4.5×10^{-5} *M* Zn(II) enzyme plus equimolar I; (—· ·—) 4.5×10^{-5} *M* Co(II) enzyme plus equimolar I; (— - —) 4.5×10^{-5} *M* Hg(II) enzyme plus equimolar I. Above 520 mμ, the Co(II) enzyme and its complex are shown at 2.16×10^{-4} *M* to improve detail. The curves shown are traces of the recordings from samples with a 1-cm path length in the visible and a 0.2 cm path length in the ultraviolet. The observed rotation is indicated on the inner ordinates (precision = ±0.002° or less throughout the region pictured). Specific rotation, [α], refers to all curves except the more concentrated Co(II) enzyme solution. Absorption spectra: (———) 2×10^{-5} *M* Zn(II) enzyme; (— · —) 2×10^{-5} *M* compound I or 2×10^{-5} *M* Hg(II) enzyme plus equimolar I; (— — —) 2×10^{-5} *M* Zn(II) and Co(II) enzyme plus equimolar I; (—×—) difference spectrum of 2×10^{-5} *M* Zn(II) enzyme complex minus Zn(II) enzyme. ORD measurements were made with a Cary Model 60 recording spectropolarimeter as previously described.[7] All samples were run at several protein concentrations and path lengths (0.1–1.0 cm). A typical set of tracings is shown. Base lines were determined using the same concentration of free azosulfonamide as was present in the sample. All solutions were 0.025 *M* in Tris buffer, 25°, pH 7.5 for the Zn(II) enzyme, pH 8.5 for the Co(II) enzyme. Ultraviolet spectra are shown only for the Zn(II) enzyme and its complex. Similar curves are observed for the Co(II) enzyme. Abbreviation used: CA, carbonic anhydrase with prefixed metal symbol designating the metal ion inserted at the active site, *e.g.*, CoCA = cobalt carbonic anhydrase.

from Winthrop Laboratories. Esterase activity was determined with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone as described by Lo and Kaiser.[9] Compound I is a potent inhibitor of the enzyme, *ca.* 1×10^{-6} *M* producing 50% inhibition of the esterase activity of both the Zn (II) and Co(II) enzymes.

On the binding of one molecule of the optically inactive compound I to the Zn(II) or Co(II) enzymes, a series of Cotton effects is induced corresponding to the visible and near-ultraviolet absorption bands of the sulfonamide (Figure 1). The largest, centered near 525 mμ, has an amplitude of 120,000°, expressed as molar rotation. In addition, the azochromophore exhibits a red shift and a marked hypochromicity of the absorption band in the visible (Figure 1). Visible and near-ultraviolet absorption bands of aromatic azo compounds, *e.g.*, azobenzene, have been resolved into a large number of transitions.[10] Band intensities and position are sensitive to substituents on the rings, to the symmetry surrounding the –N=N– group, and particularly to change from the *cis* to the *trans* form.[11] A preference for one of the latter configurations or, more specifically, a fixed angle between the planes of the two ring systems seems likely in the enzyme-bound azosulfonamide and may account for the observed spectral shifts. The phenylazonaphthols also exist in solution as equilibrium mixtures of the azo and hydrazone tautomers,[12] an equilibrium which is sensitive to the polarity of the solvent and may be altered on binding with consequent spectral change. The dissymmetry induced in these originally symmetrical transitions must occur by fixation of the molecule in the dissymmetric molecular environment of the protein through interactions with the protein backbone or side chains and probably the metal ion.[5] Electron density difference maps constructed from X-ray diffraction data show the sulfonamide group of acetoxymercurisulfanilamide to occupy a position very close to the Zn(II) ion in its complex with human carbonic anhydrase C.[13]

The Cotton effects characteristic of the carbonic anhydrase–azosulfonamide complexes can be used to ex-

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amine features of sulfonamide binding directly related to the presence of the metal ion in this enzyme. Only the Zn(II) and Co(II) enzymes induce the hypochromic–bathochromic shift and anomalous dispersion in the visible absorption bands of the azosulfonamide. Although the metal ions Mn(II), Ni(II), Cu(II), Cd(II), and Hg(II) have been shown to bind at the active site,[7,13,14] they do not induce the specific binding of the azosulfonamide which results in the visible Cotton effects. The ORD of Hg(II) carbonic anhydrase plus azosulfonamide given in Figure 1 is a typical example of this group. There is a small perturbation of the ORD in the near-ultraviolet region which may reflect weak binding of the aromatic portion of the molecule. Slight binding of [^{3}H] acetazolamide to the inactive metallocarbonic anhydrases as well as the apoenzyme has been observed to begin at inhibitor concentrations of *ca.* 2×10^{-5} *M*.[5] The Hg(II) enzyme, however, induces no changes in the visible absorption bands above 350 mμ (Figure 1). These observations support the findings with [^{3}H]acetazolamide.

While Zn(II) and Co(II) carbonic anhydrase induce identical shifts in the azosulfonamide absorption bands, large differences in the magnitude of the 525-mμ Cotton effect indicate subtle differences in the binding of the sulfonamide to the two metallocarbonic anhydrases (Figure 1). Both the amplitude and the position of the Cotton effects of the carbonic anhydrase–azosulfonamide complexes prove extremely sensitive to small changes in protein structure. Carbonic anhydrase isozymes B and C from three different species each show different visible ORD patterns for their complexes with the azosulfonamide. One of these, the azosulfonamide complex of the monkey enzyme B, is illustrated in Figure 2 by the CD spectrum of the complex compared to that of the native enzyme.[15] As is not immediately apparent from the ORD, most if not all of the bands of the bound azosulfonamide show induced optical activity. In contrast to the human isozyme B, the complex of this isozyme has several ultraviolet bands which have rotatory strengths comparable to or greater than those of the protein itself. Hence, the small chromophore with optical activity induced only by the dissymmetric molecular environment dominates the entire rotatory dispersion curve of the large protein complex. The phenomenon is particularly striking in carbonic anhydrase, since the rotatory powers of all the dichroic bands of the protein above 215 mμ are relatively weak.

Sulfonamides have complex ultraviolet spectra associated with the variety of ring structures that have been synthesized. Some of the bands are associated with very large Cotton effects induced on binding of the sulfonamides to carbonic anhydrase. These may be positive, negative, or absent depending on the structure of the inhibitor. With the number of structures

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(15) The CD spectrum of the monkey isozyme B is similar to that for the human isozyme B, as previously reported by S. Beychok, J. M. Armstrong, C. Lindblow, and J. T. Edsall, *ibid.*, **241**, 5150 (1966). The origins of the ellipticity bands of the native protein have been discussed in detail by these authors. The molecular ellipticity, [θ], has been expressed per mole of protein rather than per mole of amino acid residue in view of the fact that the ellipticity bands of the complex arise largely from the incorporation of 1 mole of azosulfonamide. The values in Figure 2 can be compared to those calculated on a mean residue basis by dividing by 256.

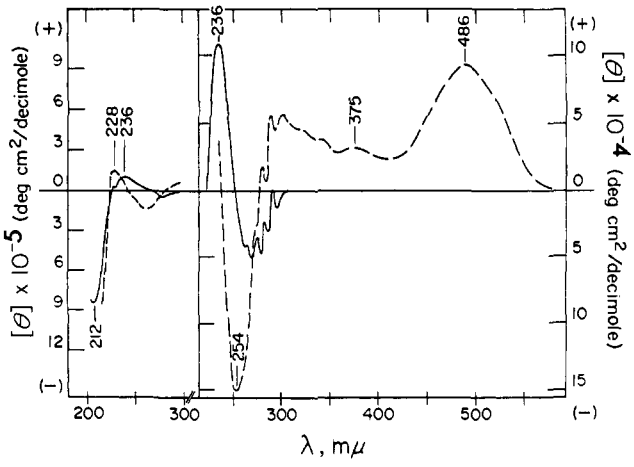

Figure 2. CD spectra of *Macaca mulata* carbonic anhydrase B (———) and its 1:1 azosulfonamide complex (— — —). Protein concentration was 4.5×10^{-5} *M* in 0.025 *M* Tris, pH 7.5, 25°. Measurements were made with a Durrum–Jasco ORD/UV-5 spectropolarimeter equipped with the CD attachment. Maximum deflection was ±0.002° with a maximum deviation between runs of ±0.00005° above 230 mμ and ±0.0001° at 215 mμ. The slit width above 300 mμ was 0.3 mm or less. Path lengths varied from 0.2 to 1.0 cm. Ellipticity is expressed as degrees square centimeter per decimole. Concentrations were expressed as decimoles of protein per cubic centimeter and values of ellipticity are uncorrected for the index of refraction of the medium. Spectral positions of the main dichroic bands in millimicrons are indicated by the numbers on the figure.

and incorporated chromophores available in these molecules, spectral and ORD studies of their carbonic anhydrase complexes provide a sensitive probe for the topography surrounding the active site of this enzyme.[16] The determination of the structure of some of these carbonic anhydrase–sulfonamide complexes seems feasible in light of progressing work on the structure of the crystalline enzyme by X-ray diffraction[13] and may provide information on the sort of protein interactions that induce the dissymmetry in the sulfonamide molecules.

Acknowledgment. I thank Dr. Thomas H. Maren of the University of Florida for many helpful discussions and suggestions concerning the properties of the sulfonamide inhibitors. The circular dichroism instrument was kindly made available by Dr. Yash P. Myer of the State University of New York, Albany, N. Y. This work was supported by Grant No. AM-09070-03 from the U. S. Public Health Service.

(16) J. E. Coleman, in preparation.

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Received May 8, 1967

A Novel Oxidative Rearrangement with Manganese Dioxide

Sir:

During an investigation of the possible use of manganese dioxide as an oxidizing agent for quadricyclanol (**1**), a very interesting reaction and rearrangement were discovered which are also potentially significant mechanistically both in the study of manganese dioxide oxidations and in carbonium ion chemistry.