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A Spin Label Study of Human Erythrocyte Carbonic Anhydrases B and C

RONALD H. ERLICH, DONNAS K. STARKWEATHER, AND COLIN F. CHIGNELL

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY

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The topography of the active sites of human erythrocyte carbonic anhydrases (EC 4.2.1.1) B and C has been studied by means of a series of spin-labeled sulfonamide inhibitors. Electron spin resonance measurements indicated that the nitroxide group of 2,2,5,5-tetramethyl-3-[(*p*-sulfamoylphenyl)carbamoyl]-1-pyrrolidinyloxyl was highly immobilized when this inhibitor bound to the active site of human erythrocyte carbonic anhydrase C. As the chain length between the aromatic and the pyrrolidine rings was increased, the mobility of the nitroxide group of the enzyme-bound inhibitor progressively increased until, with 2,2,5,5-tetramethyl-3-[[[(*p*-sulfamoylphenyl)carbamoyl]methyl]carbamoyl]-1-pyrrolidinyloxyl, there was only minimal interaction between the free radical and the active site. These results suggest that the active site of human erythrocyte carbonic anhydrase C is a cleft approximately 140 nm deep. Similar experiments indicated that the active site of human erythrocyte carbonic anhydrase B had the same general shape as the C isozyme. Human erythrocyte carbonic anhydrase B, after reaction with a nitroxide analogue of bromacetamide, showed spectral evidence for the presence of fairly mobile and highly immobilized covalently bound spin labels. When spin labeling was carried out in the presence of the inhibitor acetazolamide, the highly immobilized component of the spectrum was abolished, which suggested that it arose from a nitroxide group attached to the active site of the enzyme. When a nitroxide analogue of *N*-ethylmaleimide reacted with human erythrocyte carbonic anhydrase C, the spin label exhibited a high degree of mobility. Since prior treatment of the enzyme with *p*-chloromercuribenzoate abolished all labeling by this reagent, it appeared that the spin label had reacted with a sulfhydryl group on the surface of human erythrocyte carbonic anhydrase C.

INTRODUCTION

Erythrocyte carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a metalloenzyme that reversibly catalyzes the hydration of CO_2 (1). This enzyme is specifically inhibited by aromatic sulfonamides that have the general formula $ArSO_2NH_2$, where Ar is either homocyclic or heterocyclic (2). X-ray crystallographic studies have shown that the active site of human erythrocyte carbonic anhydrase C is a cavity at the bottom of which is a single zinc atom (3, 4). When 3-acetoxymercuri-4-aminobenzenesulfonamide bound to human erythrocyte car-

bonic anhydrase C, the aromatic ring of the inhibitor occupied a narrow cleft at the bottom of the active site while the sulfonamide group complexed with the zinc atom (3).

Spin labels are stable free radicals that can be used as probes or reporter groups for biological macromolecules such as proteins, nucleic acids, and membranes (5, 6). Hsia and Piette (7, 8) have employed DNP-haptens[1] containing the nitroxide free radical as "molecular dipsticks" to study the combining sites of rabbit antibodies. By systematically increasing the chain length separating the haptenic group from the nitroxide radical, these workers were able to estimate that the average depth of the combining site in rabbit anti-DNP antibodies was 100 nm (7). These results were of particular interest, since electron microscopy studies had shown (9) that the dimension for the effective volume of the antibody combining site was 125 nm. In previous studies we have used the "molecular dipstick" approach to estimate the depth of the sulfonamide combining site of bovine erythrocyte carbonic anhydrase B (10, 11). This report describes the application of these same principles to a study of the active sites of human erythrocyte carbonic anhydrases B and C. In addition to the spin-labeled sulfonamides (Table 1) we have employed nitroxide analogues of bromoacetamide and *N*-ethylmaleimide which react covalently with the human erythrocyte carbonic anhydrases.

MATERIALS AND METHODS

Human erythrocyte carbonic anhydrases B and C were prepared from outdated blood bank blood by the method of Armstrong and co-workers (12). The isozymes were stored as a suspension in 30% ammonium sulfate at 4°. The salt was removed by dialysis at 4° against several changes of deionized water. The concentrations of the carbonic anhydrases were measured spectrophotometrically, taking $A_{280}^{1\%} = 16.3$ for isozyme B and $A_{280}^{1\%} = 17.8$ for isozyme C (12). All chemicals were of reagent grade and were used without further purification. The spin-labeled bromoacetamide (VII) and *N*-ethylmaleimide (VIII) analogues were purchased from Syva Associates, Palo Alto, Calif. We are indebted to Dr. Ira Ringler of Lederle Laboratories for gifts of 2-amino-1,3,4-thiadiazole-5-sulfonamide and acetazolamide.

[1] The abbreviation used is: DNP, 2,4-dinitrophenyl.

Preparation of Spin-Labeled Sulfonamides

3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was prepared by refluxing 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (Frinton Laboratories) with aqueous $Ba(OH)_2$ according to the procedure of Rozantsev and Krinitskaya (13). 3-Amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was prepared from the same intermediate by the method of Krinitskaya *et al.* (14). These compounds were allowed to react with the appropriate sulfonamide in the presence of either *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Aldrich Chemical Company) (15) or isobutyl (or ethyl) chloroformate (Eastman Kodak) (16), to produce the desired spin label (Table 1). Typical syntheses involving these reagents are given below. The preparation of the remaining spin-labeled sulfonamides will be reported elsewhere (17).

4-Succinamidobenzenesulfonamide. Sulfanilamide (2.0 g; 12 mmoles) and succinic anhydride (1.16 g; 12 mmoles) were dissolved in acetone (10 ml), and the reaction mixture was allowed to stand overnight. The precipitate (2.5 g) was filtered off and recrystallized from methanol to give colorless needle crystals, m.p. 208–210°.

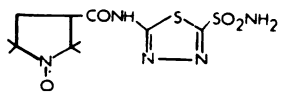
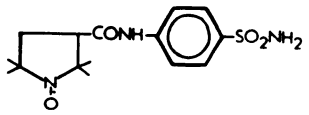
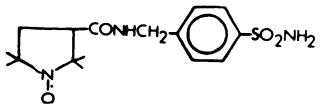
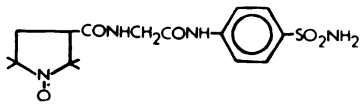
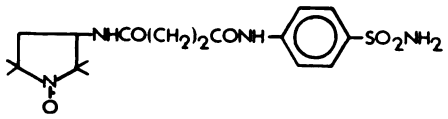
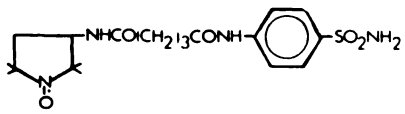
$C_{10}H_{12}N_2O_5S$

Calculated: C 44.11, H 4.44, N 10.29
Found: C 43.77, H 4.47, N 10.03

2,2,5,5-Tetramethyl-3-[3-[(p-sulfamoylphenyl)carbamoyl]propionamido]-1-pyrrolidinyloxyl, V (method 1). Freshly distilled isobutyl chloroformate (0.48 ml; 3.7 mmoles) was added dropwise to a solution of 4-succinamidobenzenesulfonamide (1.0 g; 3.7 mmoles) in dry tetrahydrofuran (20 ml) cooled in an ice-salt bath. After the mixture had been stirred for 2 hr, triethylamine (0.52 ml; 3.7 mmoles) was added, followed by a solution of 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (0.58 g; 3.7 mmoles) in dry

TABLE I

Structures of spin-labeled sulfonamides

No.	Structure	d^a	Synthetic method[b]
	←——*d*——→	*nm*	
I		96	2
II		100	1
III		108	1
IV		136	1, 2
V		145	1, 2
VI		152	1

[a] Calculated with the aid of Corey-Pauling-Koltun models for the fully extended conformations.

[b] See MATERIALS AND METHODS.

tetrahydrofuran (3 ml). The reaction mixture was then allowed to warm to room temperature and was stirred for 18 hr. After the reaction mixture had been evaporated to dryness, the residue was dissolved in water (10 ml) and then acidified to pH 3.0 with aqueous HCl and immediately extracted with three 10-ml portions of ethyl acetate. The ethyl acetate extracts were combined, dried (Na_2SO_4), and then evaporated to dryness. The residue (1.2 g) was recrystallized from methanol to give V as pale orange needle crystals, m.p. 208–209°.

$C_{18}H_{27}N_4O_5S$

Calculated: C 52.54, H 6.62, N 13.62
Found: C 52.55, H 6.75, N 13.63

2,2,5,5 - Tetramethyl - 3 - [(5 - sulfamoyl - 1,3,4 - thiadiazol - 2 - yl)carbamoyl] - 1 - pyrrolidinyloxyl, I (method 2). A mixture of 3 - carboxy - 2,2,5,5 - tetramethyl - 1 - pyrrolidinyloxyl (0.515 g; 2.8 mmoles), 2-amino-1,3,4-thiadiazole-5-sulfonamide (0.5 g; 2.8 mmoles), and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.69 g; 2.8 mmoles) in dry tetrahydrofuran (15 ml) was stirred at 37° for 24 hr. The pale yellow precipitate was filtered off and recrystallized from aqueous methanol to give pale yellow needle crystals of I (0.7 g), m.p. 254–255° (with decomposition).

$C_{11}H_{18}N_6O_4S_2$

Calculated: C 37.92, H 5.21, N 20.10
Found: C 38.20, H 5.38, N 19.90

Determination of Esterase Activity of Human Erythrocyte Carbonic Anhydrases B and C

The esterase activity of carbonic anhydrase was measured by the spectrophotometric technique of Armstrong and coworkers (12), using *p*-nitrophenyl acetate as the substrate. The K_I values for sulfonamide spin labels (Table 2) were calculated by the method of Armstrong *et al.* (12).

Electron Spin Resonance Measurements

ESR spectra were recorded at 25° with a Varian E-4 spectrometer equipped with a quartz aqueous sample cell. The ESR spectrum in Fig. 1 was obtained at a microwave power of 20 mW, using a modulation of 0.5 gauss. The remaining spectra were obtained with modulation settings of 1–2 guass at 10–20 mW microwave power. Care was taken to ensure that the ESR spectra were not artificially broadened by overmodulation or too high a microwave power setting. All samples that contained the spin-labeled sulfonamides and human erythrocyte carbonic anhydrase B or C (Figs. 3 and 4) were dialyzed at 25° against an equal volume of buffer in microcells purchased from the Chemical Rubber Company. After 24 hr, samples were removed from both sides of the dialysis cells and their ESR spectra were recorded at the same instrumental settings and stored in a Nicolet 1072 computer. Since the concentration of free spin-labeled sulfonamide was the same in both compartments of the microcell, it was possible to obtain the ESR spectrum of enzyme-bound spin label by spectral subtraction.

RESULTS AND DISCUSSION

Inhibition of Esterase Activity of Human Carbonic Anhydrases B and C by Spin-Labeled Sulfonamides

The spin-labeled sulfonamides inhibited the esterase activity of both carbonic anhydrases B and C in a noncompetitive manner (Table 2). While this result suggests that the sulfonamides do not bind to the active site of the enzyme, similar noncompetitive kinetics has been reported for the inhibition of the esterase activity of erythrocyte carbonic anhydrase by other sulfonamides, such as sulfanilamide (18), 5-dimethylaminonaphthalene-1-sulfonamide (19), and acetazolamide (12). Kernohan has suggested (18) that the apparent noncompetitive nature of the inhibition is due to a relatively slow rate of dissociation of the sulfonamide from the active site of carbonic anhydrase.

TABLE 2

Inhibition of esterase activity of human erythrocyte carbonic anhydrases B and C by spin-labeled sulfonamides

The incubations contained *p*-nitrophenyl acetate (1 mM) and sodium phosphate buffer, pH 7.4 (10 mM). The initial rate of the reaction was followed by absorbance measurements at 348 nm (12). The K_I values were calculated by the method of Armstrong and co-workers (12).

Spin-labeled sulfonamide	K_I	
	Isozyme B	Isozyme C
	μM	μM
I	0.17	0.16
II	0.31	0.08
III	0.26	1.89
IV	2.78	0.58
V	3.94	1.11
VI	1.82	0.50

The K_I values for the inhibition of the esterase activities of human erythrocyte carbonic anhydrases B and C by the spin-labeled sulfonamides covered a fairly wide range (Table 2). With the exception of the acetazolamide analogue (I) and one sulfanilamide derivative (III), all the sulfonamide spin labels were much better inhibitors of isozyme C than of isozyme B. Armstrong and co-workers have reported (12) that the K_I value of acetazolamide for the inhibition of the esterase activity of human erythrocyte carbonic anhydrase B was 0.3 μM at pH 7.0. However, Verpoorte has found[2] that the K_I value of acetazolamide for human erythrocyte carbonic anhydrase C is some 10–40-fold (depending on pH) lower than that for isozyme B. Since the K_I value of the spin-labeled acetazolamide analogue (I) for isozyme B was 0.17 μM (pH 7.4), it would appear that the pyrrolidine ring played a minor role in the binding of sulfonamide I to it. At the present moment there seems to be no satisfactory explanation why sulfona-

[2] J. A. Verpoorte, personal communication to T. H. Maren, quoted in ref. 1.

mide I was not a better inhibitor of carbonic anhydrase C. It should be pointed out, however, that the pyrrolidine ring is fairly bulky, so that steric interactions may have prevented sulfonamide I from binding as tightly as acetazolamide to the active site of isozyme C.

The K_I values of sulfanilamide for human erythrocyte carbonic anhydrases B and C are 25.7 and 2.4 μM, respectively (1). A comparison of these values with the data in Table 2 suggests that the pyrrolidine ring is responsible for the high affinities which many of the spin-labeled sulfanilamide analogues have for the isozymes. Since X-ray diffraction studies have shown that the active site of carbonic anhydrase C contains several polar groups (3), it does not appear that hydrophobic interactions involving the lipophilic pyrrolidine ring are of importance. However, Bergstén and co-workers have reported (3) that when highly active sulfonamide inhibitors bound to human erythrocyte carbonic anhydrase C they almost completely filled the active site and displaced all solvent molecules from it. These workers have suggested that solvent displacement from the active site increases the entropy of the interaction (3). It therefore seems possible that the bulky lipophilic pyrrolidine ring might aid in the expulsion of solvent molecules from the active site, thereby enhancing the affinity of the spin-labeled sulfanilamide analogues. This conclusion is supported by the fact that those sulfonamides in which the pyrrolidine ring was closest to the aromatic group tended to be the most potent inhibitors in the sulfanilamide series (Table 2).

Sulfonamide III was the only sulfanilamide analogue which was a better inhibitor of human erythrocyte carbonic anhydrase B than C (Table 2). While the precise cause of this anomalous behavior is unknown, it does seem likely that it involves some special interaction between the pyrrolidine ring of sulfonamide III and the active site of either carbonic anhydrase B or C.

Effect of Molecular Motion on ESR Spectrum of Nitroxide Group

When a nitroxide spin label is dissolved in a nonviscous solvent, such as aqueous buffer, its ESR spectrum consists of three sharp lines of nearly equal height (Fig. 1). However, when the motion of the nitroxide group is slowed by an increase in solvent viscosity, the spectral lines appear to exhibit unequal broadening (Fig. 2). The limiting line shape (Fig. 2, line *E*) is known as the rigid glass, powder, or polycrystalline spectrum of the nitroxide radical (5, 6). The rigid glass spectrum is characterized by the appearance of distinct low- and high-field peaks separated by approximately 64 gauss (Fig. 2, line *E*). Such a spectrum is observed when the spin label is randomly oriented with respect to the laboratory magnetic field and the molecular motion of the nitroxide group is slow on the ESR time scale (i.e., $\tau_c \gg 10^{-8}$ sec, where τ_c is the rotational correlation time of the spin label). Spectra approaching the rigid glass limit have been observed when nitroxide spin labels were tightly bound to macromolecules, such as proteins and membranes (5–8).

Interaction of Spin-Labeled Sulfonamides with Human Erythrocyte Carbonic Anhydrases B and C

Sulfonamide I is a spin-labeled analogue of acetazolamide, a potent inhibitor of erythrocyte carbonic anhydrase (1, 2). The ESR spectrum of sulfonamide I bound to carbonic anhydrase B is broad and asymmetrical and has a splitting of 60.5 gauss between the

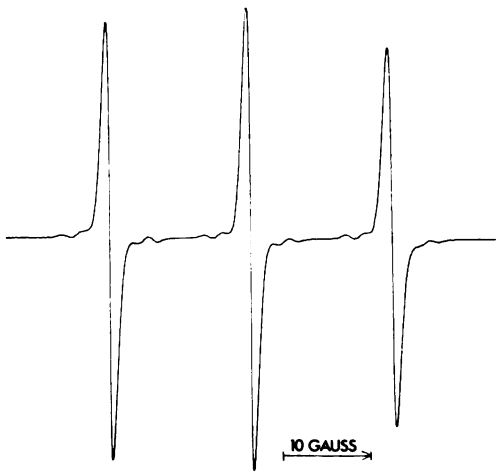


FIG. 1. *ESR spectrum of sulfonamide I (20 μM) in 0.1 M sodium phosphate buffer, pH 7.4*

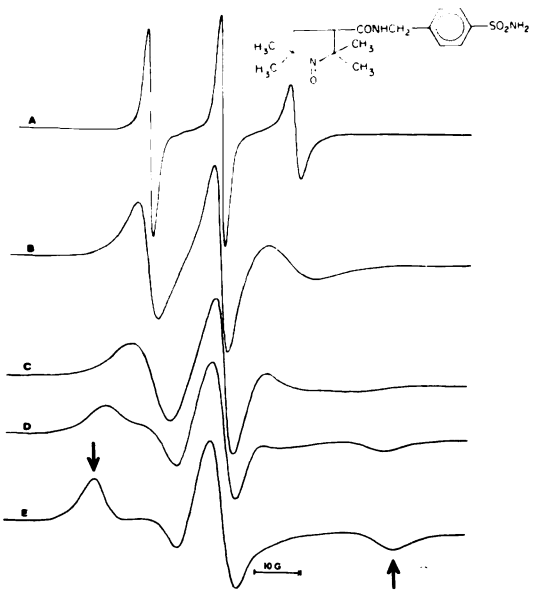


FIG. 2. *ESR spectrum of sulfonamide III (0.1 mM) dissolved in glycerol containing 10% (v/v) methanol*

A, 60°; *B*, 30°; *C*, 20°, *D*, 10°; *E*, 0°. The arrows indicate the positions of the low- (left) and high- (right) field peaks, which are characteristic of a highly immobilized nitroxide radical.

low- and high-field peaks (Fig. 3). Since this spectrum resembles the rigid glass spectrum of the nitroxide group (Fig. 2, line *E*), there can be little doubt that the pyrrolidine ring of sulfonamide I is highly immobilized when this inhibitor binds to isozyme B. The ESR spectrum of sulfonamide I bound to carbonic anhydrase C has a splitting of 59 gauss between the low- and highfield extrema (Fig. 4), which is also characteristic of a highly immobilized nitroxide group.

Sulfonamides II–VI are spin-labeled analogues of the carbonic anhydrase inhibitor sulfanilamide, in which the length of the chain connecting the aromatic and pyrrolidine rings has been progressively increased (Table 1). Although the ESR spectrum of sulfonamide II bound to human erythrocyte carbonic anhydrase B was broad and asymmetrical, the splitting between the low- and high-field peaks was only 53 gauss (Fig. 3). However, when sulfonamide II bound to carbonic anhydrase C, the low- and high-field extrema were separated by 59 gauss (Fig. 4). These results are in good agreement with the recently published findings of Mushak and Coleman (19), who have found that the nitroxide group of 2,2,6,6-tetramethyl-4-oxopiperidinooxyl *p*-sulfamylphenylhydrazone, an inhibitor in which the spatial relationship between the sulfonamide group and the nitroxide radical is approximately the same as it is in sulfonamide II, was more highly immobilized when bound to carbonic anhydrase C than it was when bound to isozyme B.

When a single methylene group was inserted between the aromatic ring and the amino group of sulfonamide II to give sulfonamide III (Table 1), the nitroxide radical showed increased mobility at the active site of both isozymes (Figs. 3 and 4). In the complex between sulfonamide IV and carbonic anhydrase B the free radical still showed some evidence of interaction with the active site of the enzyme (Fig. 3). However, when sulfonamide IV bound to the active site of carbonic anhydrase C, the nitroxide group of the inhibitor exhibited considerable freedom of movement (Fig. 4). As the distance between the aromatic and pyrrolidine rings was further increased to give sulfonamide V, both isozymes B and C permitted fairly free rotation of the nitroxide group at their respective active sites (Figs. 3 and 4). In their spin-label study of hapten binding to rabbit immunoglobulins, Hsia and Piette (7) obtained ESR spectra that were closely similar to those shown in Figs. 3 (V and VI) and 4 (IV and V). They suggested that such spectra were characteristic of spin-labeled ligands bound to rather narrow combining sites in such a way that their nitroxide groups were just outside the site (7).

X-ray diffraction studies have shown (3, 4) that when 3-acetoxymercuri-4-aminobenzenesulfonamide binds to human erythrocyte carbonic anhydrase C the sulfonamide group is coordinated to the zinc atom (through the nitrogen or one of the oxygens) while the phenyl ring is located in a narrow crevice. The crevice lies at the bottom of a deep cavity, and undoubtedly it is this cavity region which is being probed by the spin-labeled sulfonamide. Sulfonamide IV was the first of the sulfanilamide spin labels in which

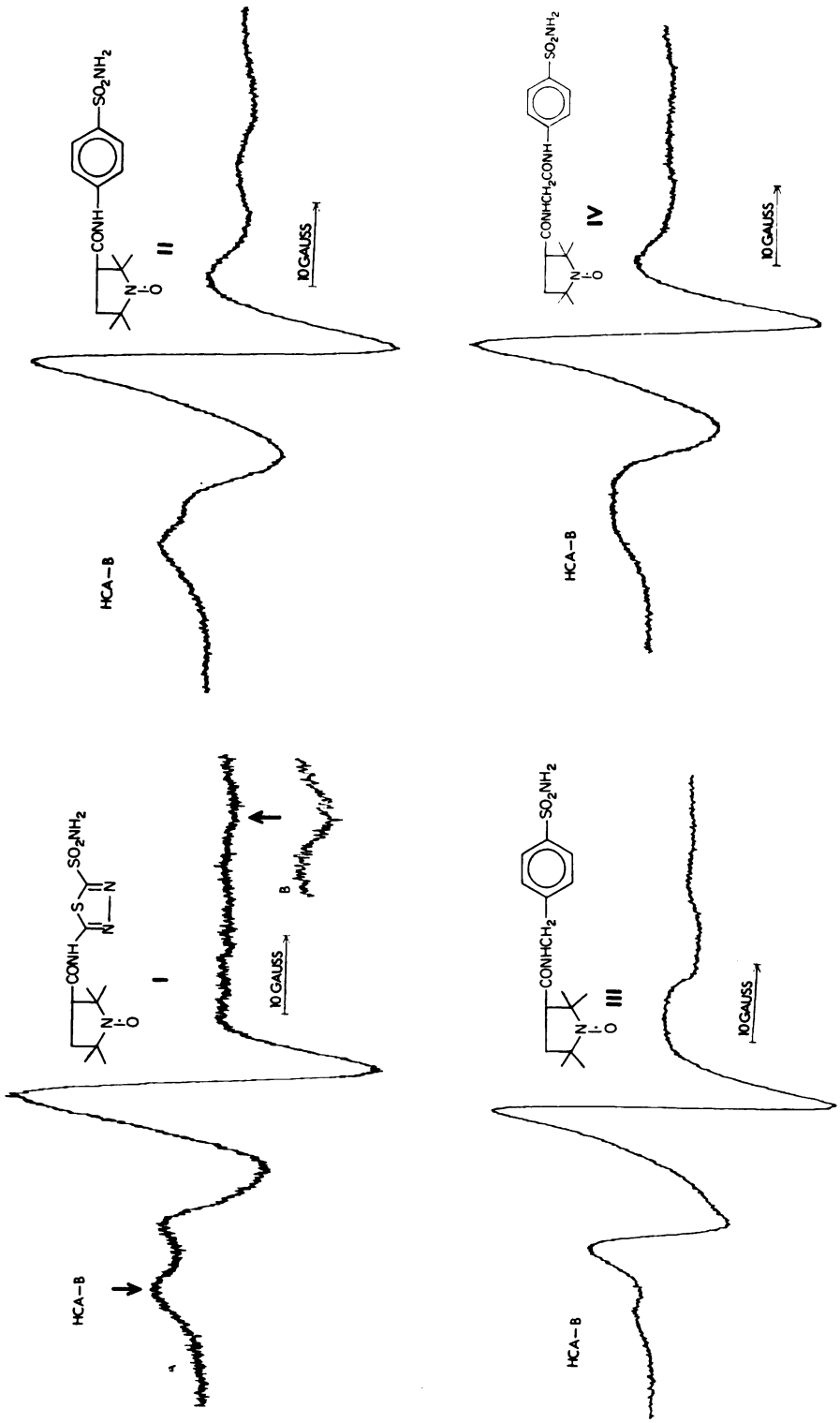

HCA–B
I
10 GAUSS
B
HCA–B
II
10 GAUSS
HCA–B
III
10 GAUSS
HCA–B
IV
10 GAUSS

Fig. 3 (I–IV)

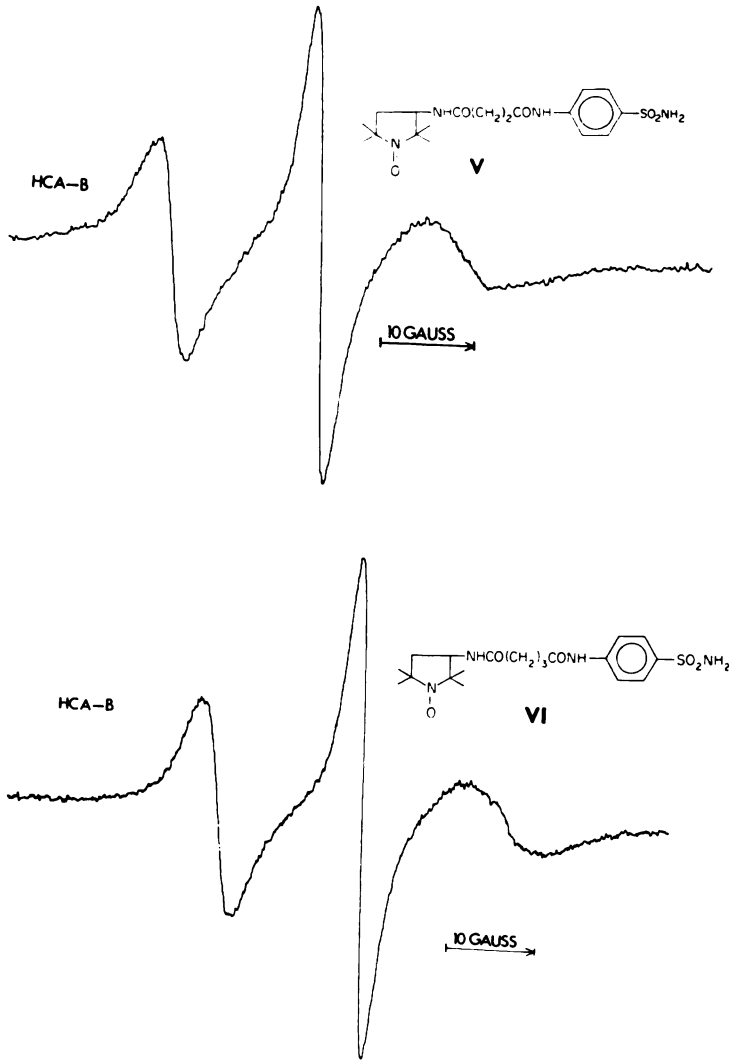


FIG. 3. *ESR spectra of sulfonamides I–VI (20 μM) in the presence of human erythrocyte carbonic anhydrase (HCA-B) (0.1 mM) and 0.1 M sodium phosphate buffer, pH 7.4*

The arrows indicate the positions of the low- (left) and high- (right) field peaks of sulfonamide I bound to the enzyme. Spectrum *B* was obtained at a 5-fold higher gain than spectrum *A* to emphasize the high-field peak.

the pyrrolidine ring appeared to be outside the active site of isozyme C (Fig. 4). The distance between the nitrogen atom of the sulfanilamide group and position 3 of the pyrrolidine ring was estimated to be 138 nm for the fully extended conformation of sulfonamide IV (Table 1). This would suggest that the active site of carbonic anhydrase C is about 140 nm deep. This value is in good agreement with an estimate of 150 nm obtained by Bergstén and co-workers (3) from their X-ray diffraction studies of human erythrocyte carbonic anhydrase C. An examination of the ESR spectra obtained with carbonic anhydrase B indicates that sulfonamide V was the first spin label in which the pyrrolidine ring appeared outside the enzyme active site (Fig. 3). Thus it would appear that the active site of isozyme B is approximately 145 nm deep (Table 1).

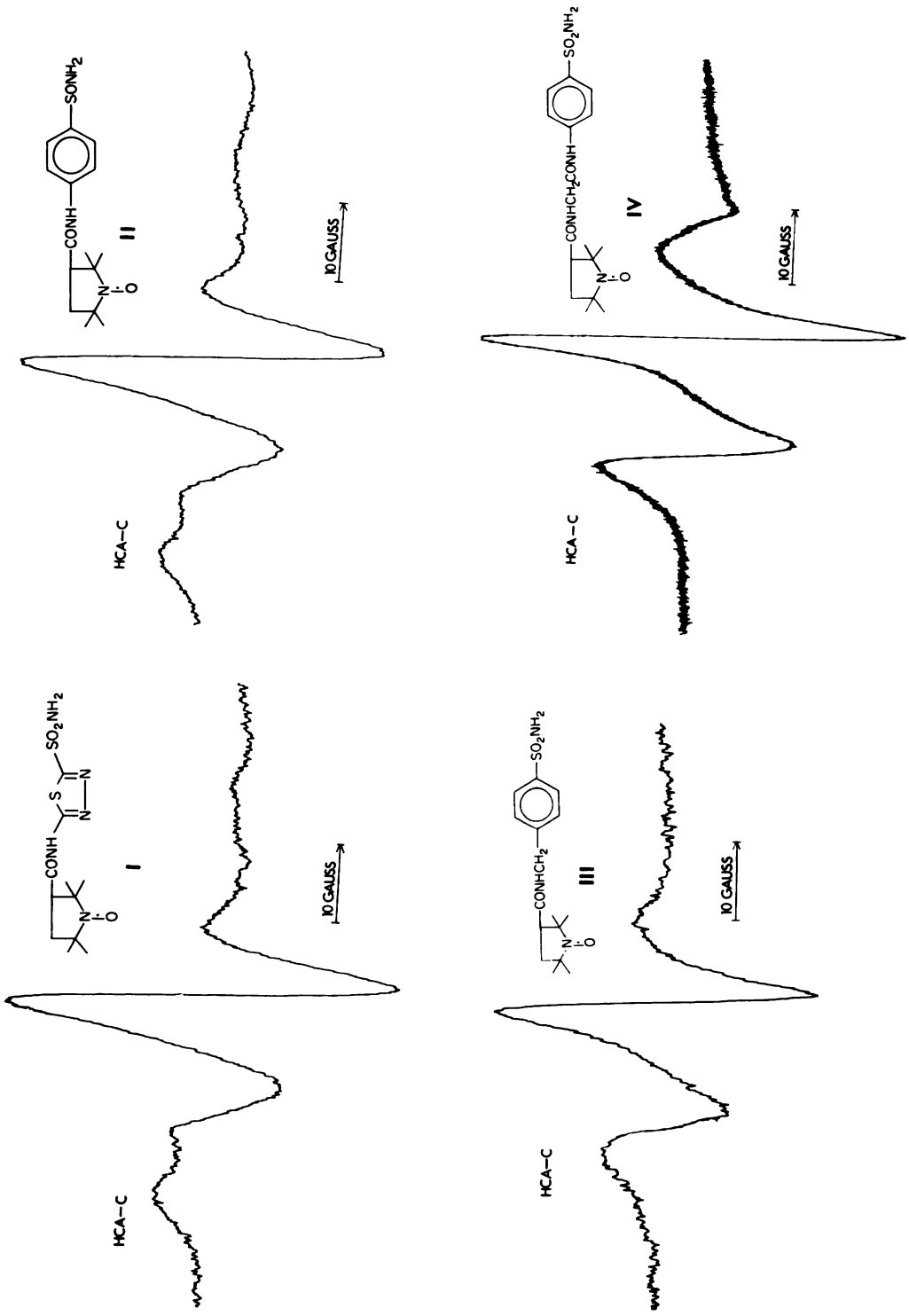


FIG. 4 (I–IV)

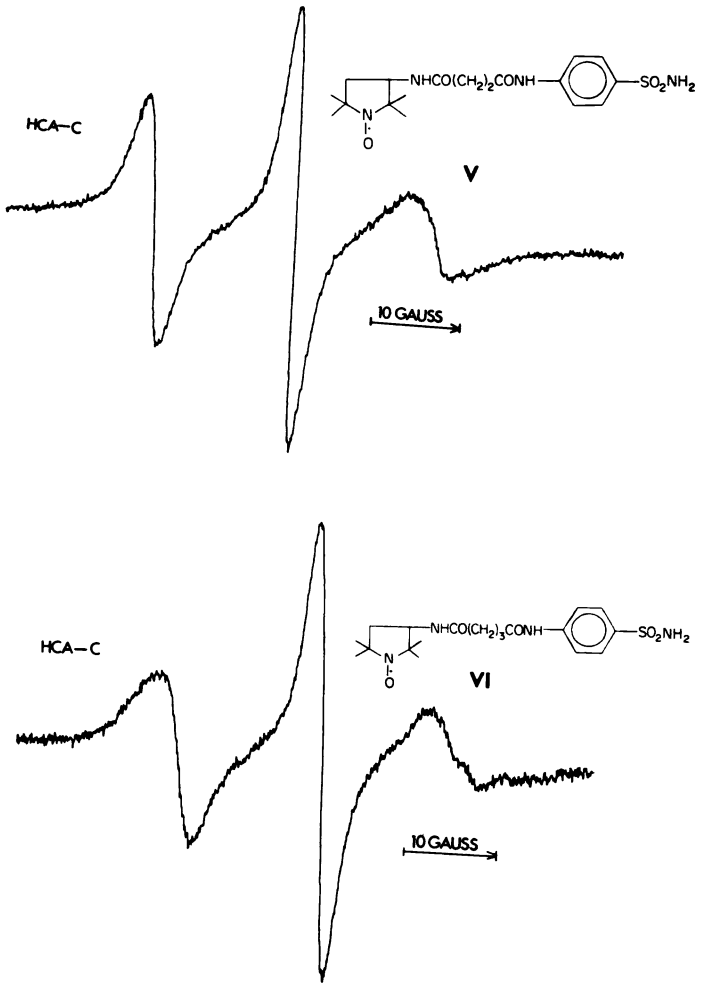


FIG. 4. *ESR spectra of sulfonamides I–VI (20 μM) in the presence of human erythrocyte carbonic anhydrase C (HCA-C) (0.1 mM) and 0.1 M sodium phosphate buffer, pH 7.4*

The most likely source of error in the estimation of the depth of a combining site by means of flexible spin labels is the existence, at or near the combining site, of a subsidiary site with a high affinity for the pyrrolidine ring. Thus it might be possible for the pyrrolidine ring of an enzyme-bound sulfonamide spin label to turn back and interact with the subsidiary site instead of remaining unhindered outside the active site. However, in our present investigation, as well as in a previous study of bovine erythrocyte carbonic anhydrase (11), we have found that if the chain length that separates the aromatic and pyrrolidine rings is increased by small increments, such interactions can be detected. A good example of this phenomenon is sulfonamide IV, which exhibits greater immobilization at the active site of human erythrocyte carbonic anhydrase B than does sulfonamide III (Fig. 3). Although these observations suggest that the pyrrolidine ring of IV may interact with a subsidiary binding site in the active site of isozyme B, it is also possible that the active site region being probed by sulfonamide IV is narrower than that sensed by sulfonamide III. At the present moment it is not possible to distinguish between these alternatives. In contrast to sulfonamide IV, the next sulfonamide in the series (V) showed little interaction with the active site of carbonic anhy-

drase B (Fig. 3). Thus the depth estimate for the active site of this isozyme (145 nm) should perhaps be regarded as an upper limit, since it was calculated from the molecular dimensions of sulfonamide V.

When the spin-labeled sulfanilamide analogues bound to human erythrocyte carbonic anhydrase C there was a smooth transition from the highly immobilized sulfonamide II to the fairly mobile sulfonamide IV (Fig. 4). Thus it would appear that the active site of isozyme C does not contain any subsidiary sites capable of interacting with the pyrrolidine group. The depth estimate of 140 nm, calculated from the molecular dimensions of sulfonamide IV, should therefore provide a reliable measure of the true size of the active site. The good agreement between the depth values obtained from our spin-labeling studies and the X-ray diffraction measurements of Bergstén *et al.* (3) supports this contention.

It is of interest to note, however, that the nitroxide group of sulfonamide VI shows greater interaction with human erythrocyte carbonic anhydrase C than does the nitroxide group of sulfonamide V (Fig. 4). This suggests that the side chain connecting the aromatic and pyrrolidine rings of VI may be sufficiently flexible to allow the alicyclic ring to turn back and interact with the active site of carbonic anhydrase C. Similar observations have been made when sulfonamide VI binds to bovine erythrocyte carbonic anhydrase B (11). These findings further emphasize the importance of increasing the distance separating the aromatic and pyrrolidine rings in small increments, since if sulfonamide VI had been the next in the series after sulfonamide III, an erroneous depth estimate for the active site of isozyme C would have been obtained.

Chemical Modification of Human Carbonic Anhydrases B and C with Spin Labels

When carbonic anhydrase B was incubated with bromoacetamide spin label VII, the enzyme became covalently labeled by the reagent. The ESR spectrum (Fig. 5A) revealed the presence of at least two populations of bound spin labels, which differed markedly in their mobilities. The highly

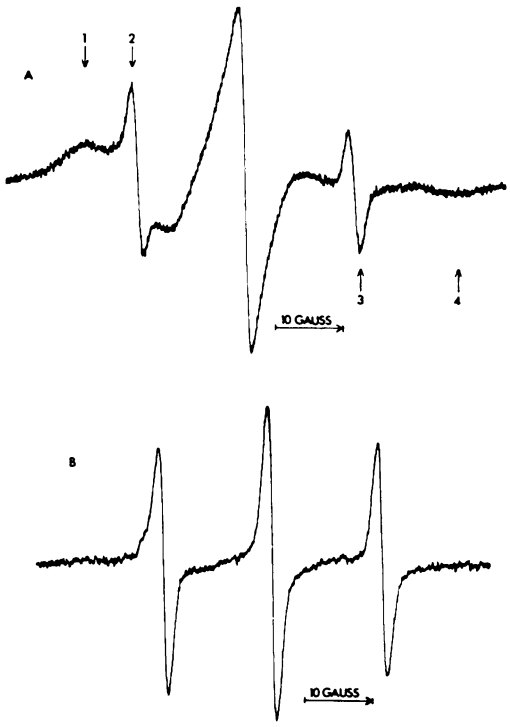
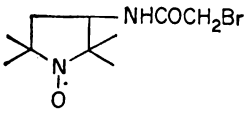


FIG. 5. *Human erythrocyte carbonic anhydrase B (0.2 mM) labeled with VII in the absence (A) and presence (B) of acetazolamide*

Arrows *1* and *4* indicate the positions of the low- and high-field peaks, respectively, of the highly immobilized spin label. Arrows *2* and *3* indicate the positions of the low- and high-field peaks of the fairly mobile spin label. All solutions contained 0.1 M sodium phosphate buffer, pH 7.4.

immobilized component of the ESR spectrum (Fig. 5A, lines *1* and *4*) was eliminated by carrying out the alkylation in the presence of acetazolamide (Fig. 5B). Whitney and co-workers have reported (20) that human erythrocyte carbonic anhydrase B was inactivated by iodoacetamide and that sulfanilamide protected against the inactivation. Their experiments demonstrated that iodoacetamide reacted at the 3′-imidazole nitrogen of a histidine located in the active site of isozyme B. It therefore seems reasonable to assume that the highly immobilized component of the spectrum shown in Fig. 5A is due to spin label VII covalently attached to a histidine at the active site of carbonic anhydrase B. The high degree of immobilization (56 gauss splitting between



VII

low- and high-field peaks) suggests that the histidine residue modified by VII is at the bottom of the active site. The mobility of the second component in the spectrum (Fig. 5A, peaks *2* and *3*; Fig. 5B) suggests that the groups modified are on the surface of the protein, where the spin label can rotate unhindered.

The human erythrocyte carbonic anhydrase C was readily labeled by incubation with the spin-labeled analogue of *N*-ethylmaleimide, VIII (Fig. 6). The ESR spectrum of the spin-labeled enzyme indicated that the nitroxide group had a high degree of mobility (Fig. 6). Prior treatment of carbonic anhydrase C with *p*-chloromercuribenzoate prevented VIII from spin-labeling the enzymes. These results suggest that VIII reacts with a sulfhydryl group (or groups) present in isozyme C. The high mobilities of the nitroxide group in carbonic anhydrase C spin-labeled with VIII suggest that the sulfhydryl group is present on the surface of this enzyme. The X-ray diffraction studies of Bergstén and co-workers (3) have shown

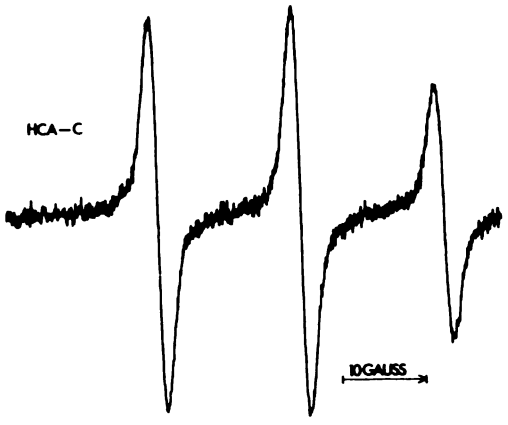
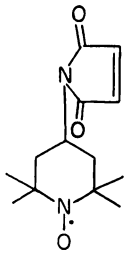


FIG. 6. *Human erythrocyte carbonic anhydrase C (HCA-C) (80 μM) labeled with VIII*

All solutions contained 0.1 M sodium phosphate buffer, pH 7.4.



VIII

that human erythrocyte carbonic anhydrase C has a single sulfhydryl group on its surface which is very close to the cleft that forms the active site.

A consideration of the distances involved suggested that the nitroxide group of either sulfonamide V or VI bound to carbonic anhydrase C should be close enough to the spin-labeled sulfhydryl group to permit spin-spin interactions between the two free radicals (21). However, experiments showed that when either V or VI bound to isozyme C spin-labeled with VIII the resultant ESR spectrum was the sum of the two component spectra. No evidence was found for spin-spin interactions, which should produce a spectrum with five lines (21). The most likely explanation for this result is that the two radicals do not spend sufficient time close to one another to permit spin-spin interactions to take place.

These studies, together with those of other workers (19, 22), have shown how spin-labeled inhibitors can be used to examine the topography of an enzyme active site. The present work validates the "molecular dipstick" technique of Hsia and Piette (7) for the estimation of the depth of an active site. The good agreement between the present spin-labeled sulfonamide studies of human erythrocyte carbonic anhydrase C and the previously published X-ray diffraction pattern of the enzyme (3, 4) suggests that there is very little difference between the conformation of the active site in the crystal state and in aqueous solution. The topographies of the active sites of human erythrocyte carbonic anhydrases B and C appeared to be quite similar. Thus the spin-labeled sulfonamide studies did not reveal any structural feature at the active site of carbonic anhy-

drase C which might explain the higher tunover rate of this isozyme.

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