Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Pigiet, V., and Schachman, H. K. (1972), *Biochemistry*, (in press).
Porter, R. W., Modebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1846.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Rosenbusch, J. P., and Weber, K. (1971a), *J. Biol. Chem. 246*, 1644.
Rosenbusch, J. P., and Weber, K. (1971b), *Proc. Nat. Acad. Sci. U. S. 68*, 1019.
Schachman, H. K. (1963), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7*, 4343.
Stein, E. A., and Fischer, E. H. (1960), *Biochim. Biophys. Acta 39*, 287.
Thiers, R. (1957), *Methods Biochem. Anal. 5*, 273.
Torriani, A. (1968), *J. Bacteriol. 96*, 1200.
Tyuma, I., Benesch, R. E., and Benesch, R. (1966), *Biochemistry 5*, 2957.
Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5*.
Weber, K. (1968), *Nature (London) 218*, 1116.
Weitzman, P. D. J., and Wilson, I. B. (1966), *J. Biol. Chem. 241*, 5481.
Wiley, D. C., and Lipscomb, W. N. (1968), *Nature (London) 218*, 1119.

Studies of the Histidine Residues of Carbonic Anhydrases Using High-Field Proton Magnetic Resonance*

Jack S. Cohen, Chung T. Yim,† Marianne Kandel, Allan G. Gornall, Stephen I. Kandel, and Murray H. Freedman‡

ABSTRACT: A comparative study of the titration behavior of the histidine residues of the B and C isoenzymes of human and the B enzyme of bovine carbonic anhydrase has been carried out using 220-MHz proton magnetic resonance spectroscopy. In the case of the bovine enzyme, at least five and up to eight imidazole C-2 protons are observed to titrate with the aid of computer curve fitting. These resonances give a fairly narrow range of pK values, with a highest value of 6.4. By contrast, four imidazole C-2 proton resonances are observed to titrate in the human B enzyme with widely spaced pK values (obtained by curve fitting) of 5.9, 6.1, 6.9, and 7.2. For the human C enzyme, seven C-2 proton titration curves are seen, with five having pK values in the range 5.9–6.6 and two others with values 7.1 and 7.2. While the number of titratable histidine residues found for the human B enzyme using the nuclear magnetic resonance (nmr) technique agrees with the number found using titrimetric methods, the nmr method shows a larger number of titrable histidines for the human C and bovine B enzymes than found by titration. The similarity of the two high pK values in the two human isoenzymes indicates similar unusual environments for two histidine residues in these protein structures, which are probably absent in the bovine enzyme. Several nontitrating and poorly resolved resonances are also present in the spectra of each protein. These may correspond to histidine residues which are unable to titrate or are rigidly held within the protein structure. The addition of the inhibitor acetazolamide (Diamox) in saturating amounts to human B, a histidine-alkylated human B, and human C enzymes produced several effects which indicate inhibitor-induced conformational changes. Consideration of these effects, as well as the data for the three native enzymes, tend to indicate that the group with p$K \simeq 7$ which is essential for catalysis is probably not a histidine residue.

Carbonic anhydrases are widely distributed enzymes that catalyze the reversible hydration of carbon dioxide and other hydrolytic reactions. They consist of a single polypeptide chain and contain a zinc atom which is essential for both the catalytic activity and the binding of anionic and sulfonamide inhibitors. In most mammalian erythrocytes carbonic anhydrases are present in at least two forms, which differ principally in their specific catalytic activity, one being characterized as the low- and the other as the high-activity form. The pH–rate profiles and the pH-binding curves indicate that the same group with a pK of approximately seven is involved in catalysis and binding of inhibitors for both the high- and low-activity forms (Edsall, 1968; see also discussion of Furth, 1968, and Bradbury, 1969).

It is believed that a water molecule bound to the zinc atom is the basis for the catalytic mechanism (Davis, 1961). However, the pK of a water molecule bound to a zinc atom in model compounds is significantly higher than 7 (p$K \simeq 10$) (Hunt, 1963). Also, a simple nucleophilic attack by a zinc-bound hydroxyl group which has been formed from the water cannot account for the observed high catalytic rate of the carbon dioxide hydration (Wang, 1969).

Several histidine residues have been shown to be present in the active-site region (Whitney *et al.*, 1967a,b; Kandel *et al.*, 1968; Bradbury, 1969), and it has been suggested that one or more of these might be involved in the catalytic or binding

* From the Physical Sciences Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland 20014, and from the Faculty of Pharmacy and the Department of Pathological Chemistry, University of Toronto, Toronto 5, Ontario, Canada. *Received September 2, 1971.* This investigation was supported by grants from the Medical Research Council of Canada (MA-3001, MT369, and MA-3057) and the Ontario Cancer Treatment and Research Foundation (245).

† Recipient of Fellowship from the Medical Research Council of Canada.

‡ To whom to address correspondence at the Faculty of Pharmacy, University of Toronto, Toronto 5, Ontario, Canada.

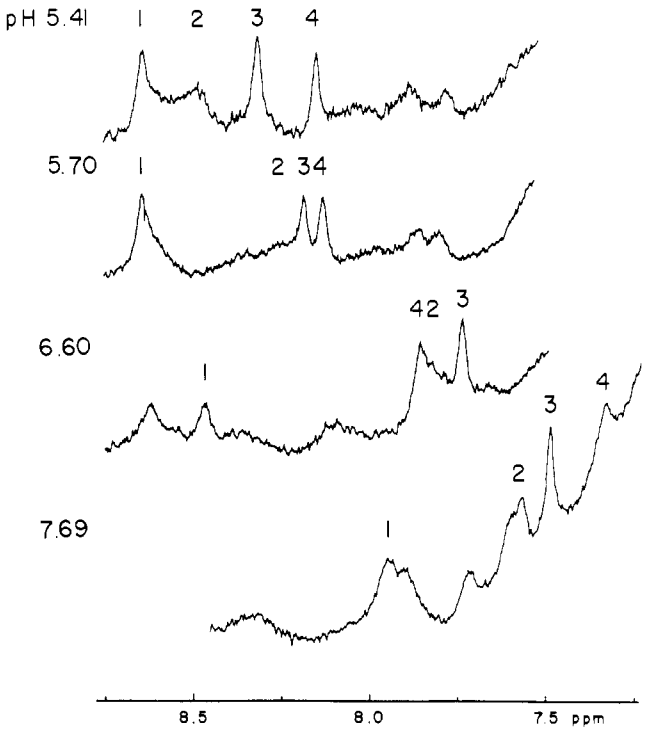


FIGURE 1: Histidine region of the 220-MHz nuclear magnetic resonance spectra of human carbonic anhydrase B at various pH values.

processes (Pocker and Storm, 1968; Wang, 1969). It was thought that investigation of the nuclear magnetic resonance (nmr) titration curves of histidine ring protons in carbonic anhydrases, and the effects of acetazolamide on these curves, might clarify this question.

Investigations of the histidine ring protons by nmr so far have been restricted to proteins of low molecular weight. This is a consequence of the relationship of resonance line width to the molecular correlation time. This parameter increases with increasing molecular size, giving rise to broad unresolvable resonances. The absence of disulfide bonds allows more molecular flexibility and undoubtedly facilitated the observation of resonances of Staphylococcal nuclease (mol wt $\simeq$ 17,500) (Cohen *et al.*, 1970b) and carbonic anhydrases (mol wt $\simeq$ 30,000) (King and Roberts, 1971) despite their higher molecular weights.

Materials and Methods

Acetazolamide was kindly supplied by the American Cyanamid Co. For nmr studies, it was dissolved in D_2O with addition of NaOD solution and was lyophilized (three times) from D_2O. Human and bovine carbonic anhydrases (EC 4.2.1.1) and bovine enzyme alkylated with bromoacetazolamide were prepared as reported previously (Kandel *et al.*, 1970). The preparation and purification as well as the properties of human carbonic anhydrase B alkylated with *N*-bromoacetylacetazolamide at the 1-nitrogen position of a histidine will be described elsewhere. The sample used was purified by liquid isoelectric focusing. On starch gel electrophoresis at pH 8.9, it migrated toward the anode as a single band ahead of the native enzyme (S.-C. C. Wong, S. I. Kandel, M. Kandel, and A. G. Gornall, unpublished data).

Protein samples were prepared for nmr studies as described previously (Cohen, 1969). In this case 0.1 M Na_2SO_4 was used, and the pH was adjusted with 0.05 M D_2SO_4 and NaOD. The protein solutions were 10% w/v in D_2O. Values of pH given are uncorrected glass electrode meter readings and the pK values are calculated from these figures. The pK values reported in this paper are individual apparent ionization constants, with the assumption that the deuterium isotope effect on the ionization equilibrium of histidines almost compensates for the isotope effect on the glass electrode of 0.4 pH unit. This is based on the previous observations on the deuterium isotope effect of imidazole (Sachs *et al.*, 1971), histidine (Li *et al.*, 1961), and protein-bound histidines (Roberts *et al.*, 1969).

Nmr spectra were recorded at 220 MHz on a Varian Associates HR 220 nmr spectrometer at 22 $\pm$ 0.5° equipped with a Varian C-1024 time-averaging computer. Chemical shift values are reported as parts per million downfield from Me_4Si used as an external reference.

Computer curve fitting of spectra and titration data by the MODELAIDE program was carried out as described previously (Cohen *et al.*, 1970a,b).

TABLE 1: Apparent Ionization Constants of Histidine Residues Observed by Nmr in Human Carbonic Anhydrases B and C.

	pK for Peak No.[a]						
	1	2	3	4	5	6	7
Human carbonic anhydrase B	7.23	5.88	6.09	6.93			
Human carbonic anhydrase B[b]	7.23	5.91	6.04	7.00			
Human carbonic anhydrase B + Diamox	7.23		6.09	6.93	6.04	6.73	
Apo human carbonic anhydrase B[b]	7.19	5.84	6.10	7.00	6.02		
Human carbonic anhydrase C	7.28	6.63	6.10	6.20	5.87	5.96	7.20

[a] Standard error of the curve fitting is $\pm$0.05 pH unit. [b] Data of King and Roberts (1971). Differences from the present work are: frequency of observation (100 MHz), concentration of protein (18%) and temperature (27.5°).

Results and Discussion

It is of interest to study the role of the histidine residues of carbonic anhydrases in the catalysis and binding of inhibitors by any available means for the following reasons (a). Both catalysis and inhibition have been related to a group in the enzyme with a pK around neutrality for all known carbonic anhydrases (Edsall, 1968; Bradbury, 1969). This has led to speculation that this group might be a histidine residue (Pocker and Storm, 1968; Wang, 1969). (b) Chemical modification has indicated the presence of histidine residues in the active-site region of several mammalian carbonic anhydrases (Whitney *et al.*, 1967a,b; Kandel *et al.*, 1968; Bradbury, 1969), although it is unsure whether any of these are related to catalysis or binding (Bradbury, 1969; Khalifah, 1971). (c). It has been shown that the chemical reactivity of the active site histidines are different between the high- and low-activity forms of carbonic anhydrases in different species, but are the

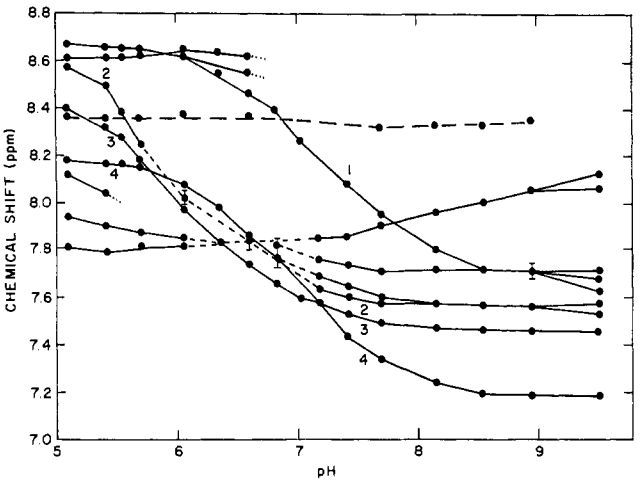


FIGURE 2: Titration data for the observable resonances in the histidine region of the spectra of human carbonic anhydrase B, with curves 1–4 corresponding to the numbered resonances in Figure 1. The lines joining points are hand drawn; the dashed line represents a very broad resonance; the short dashed lines indicate uncertain continuity.

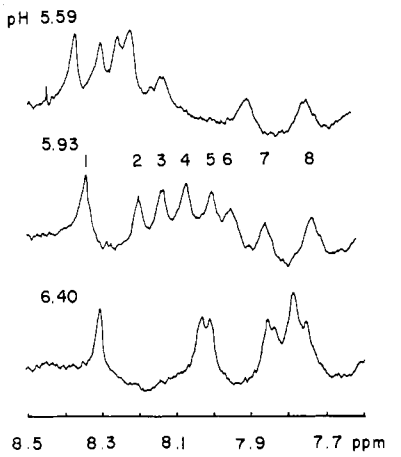


FIGURE 3: Histidine region of the 220-MHz nuclear magnetic resonance spectra of human carbonic anhydrase C at various pH values.

same within each group (Kandel *et al.*, 1970). (d) X-Ray crystallography revealed that in human carbonic anhydrase C three histidine residues are liganded to the zinc atom, and two others are in the active-site region (Liljas, 1971).

We have therefore carried out a comparative nmr study of the histidine region of two high-activity carbonic anhydrases (human enzyme C and bovine enzyme B) and one low-activity carbonic anhydrase (human enzyme B) including their complexes with acetazolamide.

Titration Curves of the Three Carbonic Anhydrases. During the course of this work, King and Roberts (1971) reported the titration of histidine residues of human carbonic anhydrase B with and without the presence of inhibitors using nmr spectroscopy. A comparison of the spectra which they obtained at 100 MHz with ours obtained at 220 MHz clearly demonstrates the improved signal-to-noise ratio and resolution in the case of our data obtained at the higher field strength (Figure 1). Nevertheless, the spectral analysis and the pK values determined (Table I) are remarkably similar, with a maximum difference of only 0.07 pH unit. This speaks well for the accuracy of the nmr method, notwithstanding the many differences in the two studies. These include a different method of sample preparation, different concentration of protein solutions used (18%), and different temperature (27.5°) (King and Roberts, 1971). The major difference in terms of spectral analysis is our ability at 220 MHz to observe the broad resonance 2 at low pH, and to resolve several other resonances at low and high pH values which may describe incipient titration curves (Figure 2).

A maximum of nine resonances can be distinguished at low pH for human carbonic anhydrase B (Figure 2), but several of these are broad and do not titrate. One peak unusually shifts downfield with increasing pH, with what may be a pK value at about 8.2. It is not possible to ascribe these resonances definitively to histidine C-2 protons since their areas cannot be measured with sufficient accuracy, and they do not titrate normally (Cohen *et al.*, 1970b). Several may be broad amide resonances which also absorb in this region. In any case the clear observation of only four titrating resonances is consistent with the conclusion of Riddiford (1964) from titrimetric analysis of only four titrating histidines in human carbonic anhydrase B. The other seven histidines must be sufficiently restricted in motion to be broadened beyond detection (Cohen and Jardetzky, 1968; Cohen, 1969), or if observed, must be incapable of protonation, possibly as a result of ion complexation as described by McDonald and Phillips (1963) for histidine–cobalt complexes. Nontitrating histidine resonances have been described for myoglobin (Sheard *et al.*, 1970). It is known from the X-ray crystallographic studies of human carbonic anhydrase C that three histidine residues are bound to the Zn^{2+} ion (Liljas, 1971). It is also known from spectrophotometric studies of the cobalt-substituted derivatives that the environment of the metal ion is very similar for the three enzymes studied here (Lindskog, 1963; Duff and Coleman, 1966; Coleman, 1968).

In the case of human carbonic anhydrase C the spectra are extremely well resolved (Figure 3). It is possible to follow the continuities of eight resonances with change in pH (Figure 4) with little ambiguity. Seven imidazole C-2 protons were seen to titrate. This result is clearly inconsistent with the report of Riddiford *et al.* (1965) of only five titrating histidine residues from titrimetric measurements. The electron density map of

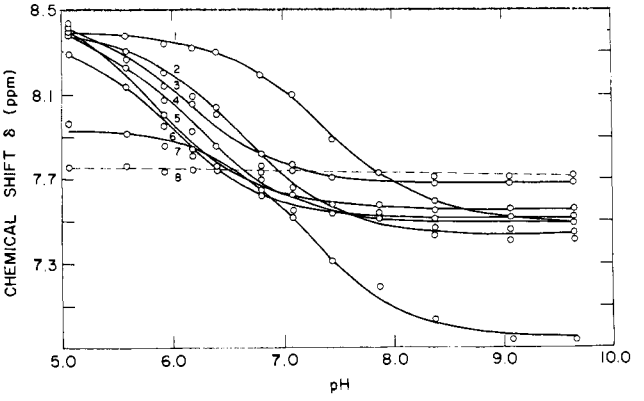


FIGURE 4: Titration data for the resolved resonances in the histidine region of the spectra of human carbonic anhydrase C, with curves 1–8 corresponding to the numbered resonances in the pH 5.93 spectrum of Figure 3. Solid lines are the best fits to the data using the simple titration equation (Cohen *et al.*, 1970b). The dashed line represents a nontitrating resonance.

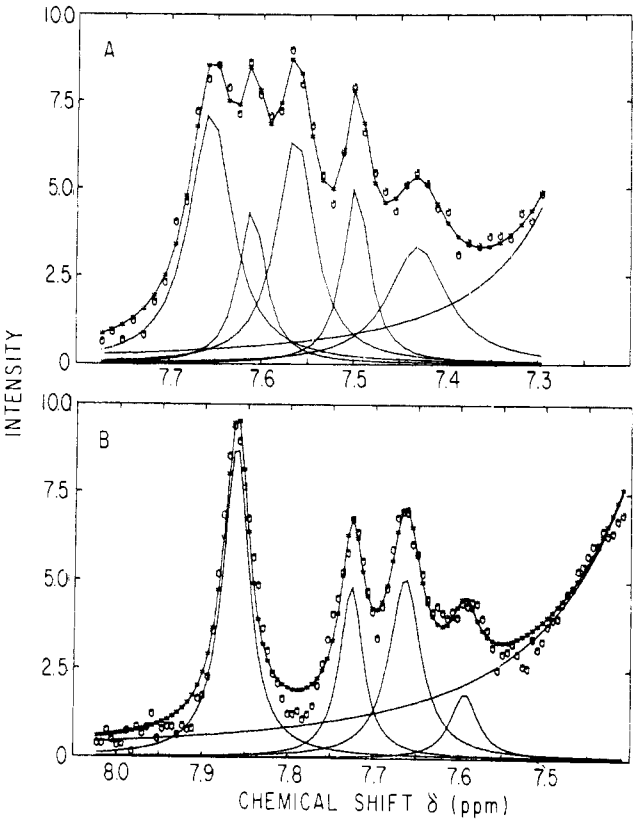


FIGURE 5: Computer generated plots of the histidine region of the 220-MHz nuclear magnetic resonance spectra of bovine carbonic anhydrase B; A, pH 5.91; B, pH 6.36. The circles are observed points, the asterisks are calculated points which are joined by a line representing the sum of the individual component peaks drawn below.

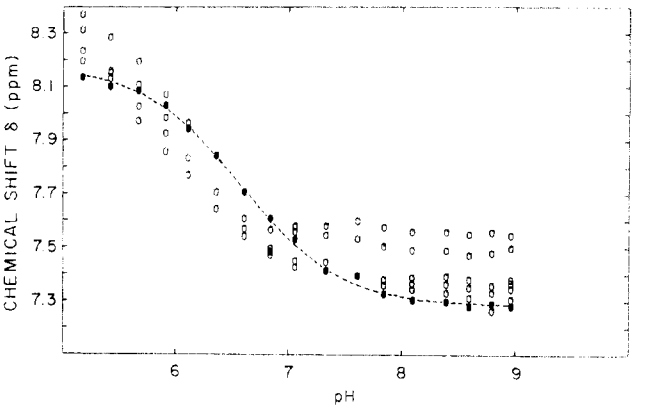


FIGURE 6: Titration data for the resonances in the histidine region of the spectra of bovine carbonic anhydrase B. The data points represent all reasonable peak maxima which are resolved in this region. The dashed line is the best fit to the points (closed circles) chosen in order to estimate the highest pK value (6.4).

human carbonic anhydrase C, on the other hand, revealed that six of the twelve histidine residues are on the surface of the molecule (Liljas, 1971) and hence are accessible for titration.

Two of the histidine residues, represented by curves 1 and 7, of human carbonic anhydrase C and two of those, 1 and 4 for human carbonic anhydrase B, have high pK values ($\simeq$7). Histidine titration curves with such values have been reported previously (Cohen, 1969; Rüterjans and Pongs, 1971). Such a value could result from a neighboring negative charge or high concentration of electron density (Cohen, 1969; King and Bradbury, 1971).

The maximum and minimum values of the chemical shift of the C-2 proton titration curve for histidine itself are 8.84 and 7.79 ppm (Sachs *et al.*, 1971). These correspond to the fully protonated and fully unprotonated forms of imidazole, respectively. While there are small differences from the normal values for several of the histidine titration curves in the carbonic anhydrases, only curve 4 of human carbonic anhydrase B and 7 of human carbonic anhydrase C deviate significantly. These have maximum and minimum chemical shift values for human carbonic anhydrase B, 4 of 8.18 and 7.18 ppm and for human carbonic anhydrase C 7 of 7.92 and 6.93 ppm, respectively. An upfield shift could result from a nearby negative charge, this is consistent with the previously noted high pK value for these groups. Alternatively, the upfield shifts in these curves could result from diamagnetic anisotropy, as shown for example by carbonyl groups. It is therefore conceivable that an adjacent carboxyl group could produce both the upfield shifts and high pK values observed for these histidine titration curves.

However, any more detailed analysis would be speculative since the distance dependence of electronic and charged effects on histidine ring proton chemical shifts and pK values are not known. We are in the process of preparing model histidine peptides to clarify these effects, which should shed light on the nature of protein environments for which imidazole acts as a nondisturbing probe. Nevertheless, it can be concluded that these titration curves represent similar and unusual environments for two histidine residues in the two human isoenzymes.

A comparison of the other histidine titration curves of the two human enzymes indicates that two further curves (2 and 3 for human carbonic anhydrase B, 5 and 6 for human carbonic anhydrase C) are very similar, with very close pK values (Table I).

By contrast to the results for human enzyme B and C the histidine C-2 proton curves for bovine carbonic anhydrase B cluster closer together (Figures 5 and 6). The distinctive high pK and shifted curves described above are absent in this case, and it is possible to conclude that the structural features which produce them are absent in the bovine enzyme, unless they are in such an environment that they are broadened beyond detection. It is of interest to note that there is a cysteine residue in both the human isoenzymes which is absent in bovine carbonic anhydrase B (Nyman and Lindskog, 1964). Such a group might give rise to electron shielding and a high pK value for a histidine in close proximity to it, as suggested for the case of human lysozyme (Cohen, 1969).

Because of the lack of resolution of the histidine C-2 proton resonances in bovine carbonic anhydrase B there was ambiguity in the continuities to be used to delineate the titration curves. By choice of appropriate points it is possible to obtain by curve fitting an estimate of the maximum pK value of 6.4 (Figure 6). Curve fitting of the digitized spectra with Lorentzian peaks was used to determine the relative areas of peaks (Cohen *et al.*, 1970b). As shown in Table II this indicated the number of titrating protons to be between 6 and 8. There is, of course, some inaccuracy in this method since the spectral peaks clearly represent overlapping resonances, and the sum of two Lorentzians not completely superimposed is not itself a Lorentzian. Other limitations of this method are discussed elsewhere (Cohen *et al.*, 1970a). In one spectrum (Figure 5A)

TABLE II: Areas of Peaks in Spectra of Bovine Carbonic Anhydrase B.

pH of Spectrum	Relative Areas of Peaks 1	2	3	4	5	Total Area[a]
5.17	1.4	1.7	1.6	1.0	1.6	6
5.91	2.6	1.0	2.1	1.1	1.7	9
6.11	1.8	2.3	1.0	1.3		6
6.36	2.1	1.0				3 or 6
6.61	2.8	2.3	1.0	2.1		8
7.61	1.0	1.1	4.6			7

[a] Total area represents the sum of each relative peak area rounded to the nearest integer.

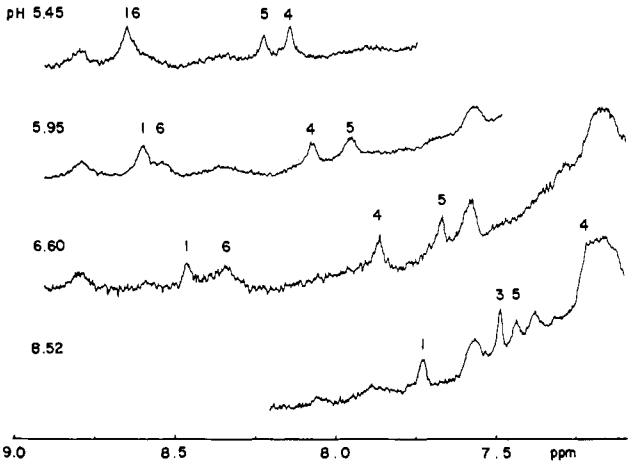
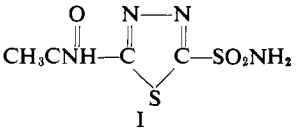


FIGURE 7: Histidine region of the 220-MHz nuclear magnetic resonance spectra of human carbonic anhydrase B at various pH values in the presence of excess Diamox.

we were able to resolve five resonances in the middle of the titration range, which we feel is the minimum number of titrating histidines, and seven resonances at pH 9. By contrast, Nilsson and Lindskog (1967) reported only four titrating histidines from titrimetric experiments with bovine carbonic anhydrase B. This is clearly an underestimate, with a probable actual number closer to seven.

Effects of Inhibitor. It is well known that sulfonamides are potent inhibitors of activity of carbonic anhydrases (Maren, 1967). We chose Diamox (acetazolamide, I) in this study since its inhibitory properties are well known (Maren, 1967),



I

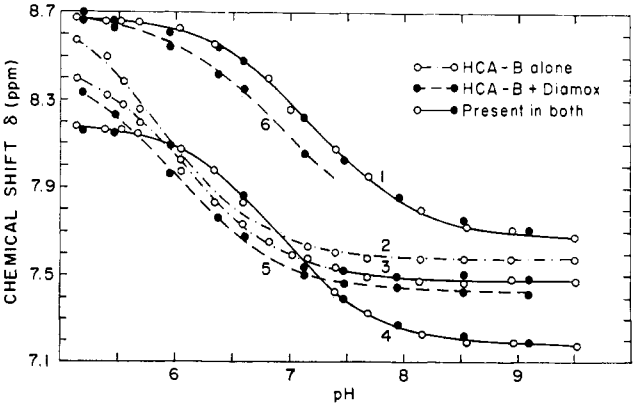
and it has no protons which absorb in the region under study (exchangeable positions were deuterated). It has a high association constant (10^7–10^8 M^{-1} at pH 8, Pocker and Stone, 1965; Verpoorte *et al.*, 1967) and sufficient excess was used (2.5-fold molar ratio) to ensure full occupancy of the site in the pH range studied.

Spectra of the human carbonic anhydrase B–Diamox complex are shown in Figure 7 and the effect of bound Diamox on the titration curves of human carbonic anhydrase B are shown in Figure 8, with the results for native human carbonic anhydrase B alone for comparison. From this the following observations can be made: (a) curves 1 and 4 are unaffected; (b) an extra titration curve (5) is seen, due to a histidine residue with pK = 6.04; (c) curve 2 is not seen; (d) an extra broad titrating resonance (6) is seen below pH 7.2; (e) peak 3 is seen clearly only above pH 7.5. These results indicate local Diamox-induced conformational change(s) affecting histidine residues. It is interesting to note that curve 6 is not clearly observable in alkaline pH while curve 3 is only observable in this range. It is conceivable that these curves correspond to a single histidine residue which exists in two environments related by slow exchange for Diamox with a transition around pH 7. In this connection it should also be noted that the binding of Diamox decreases in alkaline pH, and 7 is the pK value of the group on the enzyme responsible for binding.

Our results differ somewhat from those of King and Roberts (1971). They observed most notably an extra titration curve with both the inhibitors that they used, as well as several other effects. Their results may be summarized as follows:

Inhibitor	New or Altered Curve	pK Value
p-Carboxybenzenesulfonamide	7	7.30
	7′	7.00
	8	6.77
Iodide	9	6.80

The extra curve (5) that we observed with Diamox does not coincide with any of the curves 7–9, neither in pK nor chemical shift values. However, a comparison with the data of King and Roberts (1971) for the apoenzyme of human carbonic anhydrase B reveals a remarkable resemblance. Thus, they describe an extra titration curve (5 in their Figure 4) which runs parallel just to higher field than our curve 5, with a pK value of 6.02 (Table I). There is also an extra titrating peak (6) in the apoenzyme that can only also be observed at low pH. We feel that the consistency with the rest of our data allows us to conclude that curve 5 in the human carbonic anhydrase



FIGUVE 8: Titration data for resonances in the histidine region of the spectra of human carbonic anhydrase B with and without Diamox. All curves are calculated best fits to the appropriate data points. The numbering system is derived from that in Figure 7 and is common to Figures 1 and 2.

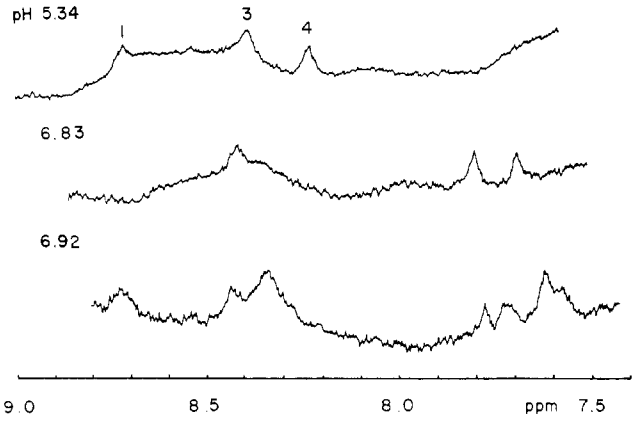


FIGURE 9: Histidine region of the 220-MHz nuclear magnetic resonance spectra of human carbonic anhydrase B alkylated with *N*-bromoacetylacetazolamide and lower trace after the addition of excess Diamox.

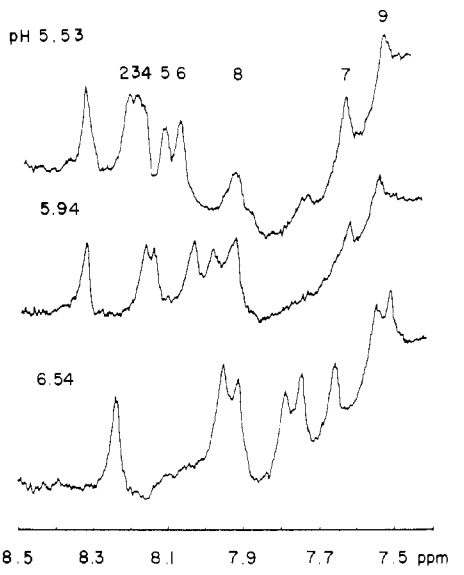


FIGURE 11: Histidine region of the 220-MHz nuclear magnetic resonance spectra of human carbonic anhydrase C at various pH values in the presence of excess Diamox.

B–Diamox complex (Figure 8) corresponds to the same histidine residue giving rise to curve 5 in the data for the human carbonic anhydrase B apoenzyme of King and Roberts (1971). They interpreted this to derive from a histidine residue which is released into a much less physically restricted environment on the removal of the zinc atom. Such a conformational change cannot be extensive, since curves 1–4 in their data are unchanged for the apoenzyme. Therefore, it must correspond to a local conformational change, involving one of the histidine residues possibly close to or liganded to the zinc atom. If this is so, then it can likewise be concluded that the addition of Diamox to human carbonic anhydrase B does not result in a major conformational change, but does selectively displace a histidine residue. This, however, cannot be a histidine liganded to the zinc atom, and must therefore be restricted in the native enzyme in another manner. The X-ray crystallographic structure of human carbonic anhydrase C shows two histidine residues (63 and 128) that are hydrogen bonded as a result of organized water structure in the active site (Liljas, 1971). Therefore, curve 5 may correspond to a histidine residue in a similar environment in human carbonic anhydrase B.

The differences observed for the three inhibitors reflect differences in the effects on the histidine residues on binding. This is not surprising for iodide in that it binds very weakly (K_a = 5.0 × 10^2 M^{-1} at pH 6.5) compared to the two sulfonamides, and was used in high molar excess (King and Roberts, 1971), which might lead to some nonspecific binding. One might expect similarities and dissimilarities between the effect of Diamox and *p*-carboxybenzenesulfonamide on the histidine curves due to the similarities and dissimilarities in the structure of these two inhibitors. The presence of an aromatic ring with a carboxyl group in *p*-carboxybenzenesulfonamide and not Diamox would be expected to result in some differences in their binding properties, as well as difference in electronic shielding effects when bound. Thus, it is tempting to conclude that the extra titration curve observed by

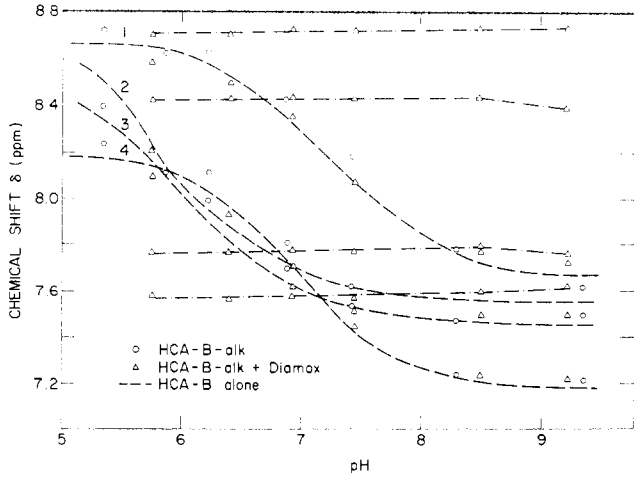


FIGURE 10: Titration data for resonances in the histidine region of the spectra of alkylated human carbonic anhydrase B and after the addition of Diamox. The four nontitrating resonances in the latter case are indicated by dot–dash lines; the curves observed for native human carbonic anhydrase B are shown as dashed lines for comparison.

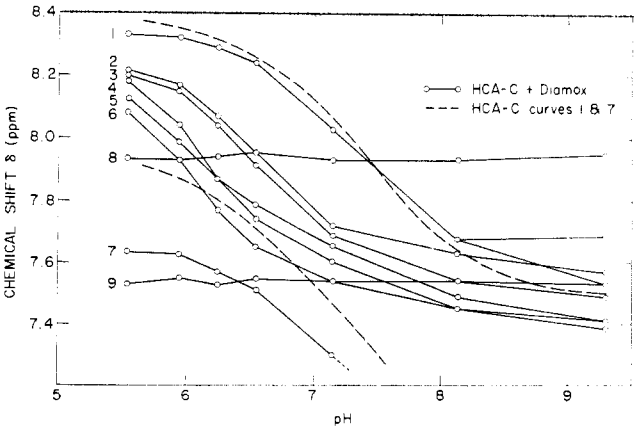


FIGURE 12: Titration data for resonances in the histidine region of the spectra of human carbonic anhydrase C with Diamox. The numbering system is derived from the pH 5.53 spectrum in Figure 11. The dashed lines are curves 1 and 7 for native human carbonic anhydrase C from Figure 4 for comparison. The solid lines are drawn to connect points.

King and Roberts (1971, Figure 5, curve 7) on the addition of *p*-carboxybenzenesulfonamide might correspond to the same histidine residue that is released on the addition of Diamox (Figure 8, curve 5). Its p*K* value might be shifted from 6.04 to 7.30 by the *p*-carboxyl group in close proximity to it. One further similarity in the effects of the inhibitors is that on curve 2 of human carbonic anhydrase B. It is unresolvable for both the sulfonamides, and broadened and shifted by iodide (King and Roberts, 1971). It would appear that this histidine residue is more restricted when each of these inhibitors is bound.

For comparison we prepared and obtained spectra for a derivative of human carbonic anhydrase B alkylated with *N*-bromoacetylacetazolamide on one histidine residue in the active site. We also added Diamox in excess to this material, since there is kinetic evidence that it still binds inhibitors (S.-C. C. Wong, S. I. Kandel, M. Kandel, and A. G. Gornall, unpublished data). As shown in Figures 9 and 10, curves 1, 3, and 4 were essentially unaffected in both cases. However, peak 2 was unobservable in both cases, and on the addition of Diamox four nontitrating peaks appeared. The conformational change(s) which must produce these effects indicates that Diamox is binding to the alkylated derivative of human carbonic anhydrase B. Although peak 2 was broad in human carbonic anhydrase B itself it is unlikely that it is purely fortuitous that it is unobservable in all cases of inhibitor binding and alkylation of human carbonic anhydrase B, indicating that it corresponds to a histidine residue at or near the sulfonamide binding site.

Addition of Diamox to human carbonic anhydrase C resulted in one extra nontitrating resonance (9, Figures 11 and 12). Most of the curves (1–6) are shifted somewhat upfield (below pH 7.5) and to higher p*K* values. However, the magnitude of these effects is quite small. Curve 7 for native human carbonic anhydrase C (Figure 4) is changed on the addition of inhibitor, and does seem to have shifted further upfield to the position of curve 7 in Figure 12. It is also unresolved above pH 7.2, which is in contrast to human carbonic anhydrase C without inhibitor. We can conclude that this histidine residue is selectively more restricted on the binding of Diamox. The presence of titrating peaks which become unresolvable in alkaline pH in both the human isoenzyme B– and C–Diamox complexes is a feature which may reflect upon the nature of the active site in the complex, as described above.[1]

There are differences between human carbonic anhydrase B and human carbonic anhydrase C in that no "extra" titration curves are observed in the latter case on the addition of inhibitor. However, this may result from less restricted environments of histidine residues in human carbonic anhydrase C (probably for curves 2, 3, and 4, Figure 4) compared to human carbonic anhydrase B. So that even if a histidine residue near the active site of the former was displaced on inhibitor binding, as long as there were no major changes in its environment or p*K* value it would not be detectably different in terms of line width. Thus, there is either no comparable local conformational change for human carbonic anhydrase C (apart from the effect on curve 7) or it is undetectable.

The important question then arises as to the potential involvement of a histidine residue in the activity of carbonic anhydrases since a group with a p*K* around neutrality has been implicated. Knowing the high degree of sequential homology between bovine enzyme B and human enzyme C (Nyman *et al.*, 1968), one would expect to see a histidine in a similar magnetic environment or at least with similar p*K* values in these two enzymes provided that the group in the enzyme which controls the catalysis is a histidine residue. Although in human enzyme C there is a histidine (7) with a p*K* of 7.2 which is affected by acetazolamide indicating that it is probably located at or around the active site, no titratable histidine with a p*K* higher than 6.4 was found in bovine enzyme B. Even if it is argued that the low-activity carbonic anhydrases (like human enzyme B) are different from the high-activity forms (such as human enzyme C) (Furth, 1968; Kandel *et al.*, 1970), it is assumed that they operate by the same basic mechanism. In human enzyme B; however, the two histidines (1 and 4) and in human enzyme C the other histidine (1) with p*K* values corresponding to that of the catalytically essential group are not affected by Diamox suggesting that they are not located in the active-site region. These observations tend to argue against the direct involvement of a histidine in the catalytic mechanism unless it is sufficiently rigid to be unobservable with the nmr techniques currently available. The other possibility that there is a critical histidine which does not titrate seems to be unlikely.

[1] No inhibitor binding studies were performed with bovine carbonic anhydrase B due to the ambiguity of the titration curves of the enzyme itself, although several spectra of the bromoacetazolamide alkylated derivative (Kandel *et al.*, 1968) did indicate extensive changes.

References

Bradbury, S. L. (1969), *J. Biol. Chem. 244*, 2002.

Cohen, J. S. (1969), *Nature (London) 223*, 43.

Cohen, J. S., and Jardetzky, O. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 92

Cohen, J. S., Shrager, R. I., McNeel, M., and Schechter, A. N. (1970a), *Biochem. Biophys. Res. Commun. 40*, 144.

Cohen, J. S., Shrager, R. I., NcNeel, M., and Schechter, A. N. (1970b), *Nature (London) 228*, 642.

Coleman, J. E. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 123.

Davis, R. P. (1961), *Enzymes 5*, 545.

Duff, T. A., and Coleman, J. E. (1966), *Biochemistry 5*, 2009.

Edsall, J. T. (1968), *Harvey Lect. 62*, 191

Furth, A. J. (1968), *J. Biol. Chem. 243*, 4832.

Hunt, J. P. (1963), Metal Ions in Aqueous Solution, New York, N. Y., Benjamin, p 50.

Kandel, M., Gornall, A. G., Wong, S.-C. C., and Kandel, S. I. (1970), *J. Biol. Chem. 245*, 2444.

Kandel, S. I., Wong, S.-C. C., Gornall, A. G., and Kandel, M. (1968), *J. Biol. Chem. 243*, 2437.

Khalifah, R. G. (1971), *J. Biol. Chem. 246*, 2561.

King, N. L. R., and Bradbury, J. H. (1971), *Nature (London) 229*, 404.

King, R. W., and Roberts, C. K. (1971), *Biochemistry 10*, 558.

Li, N. C., Tang, P., and Mathur, R. (1961), *J. Phys. Chem. 65*, 1074.

Liljas, A. (1971), Acta Universitatis Upsaliensis Dissertation from the Faculty of Science, Uppsala.

Lindskog, S. (1963), *J. Biol. Chem. 238*, 945.

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

McDonald, C. C., and Phillips, W. D. (1963), *J. Amer. Chem. Soc. 85*, 3736.

Nilsson, A., and Lindskog, S. (1967), *Eur. J. Biochem. 2*, 309.

Nyman, P. O., and Lindskog, S. (1964), *Biochim. Biophys. Acta 85*, 141.

Nyman, P. O., Strid, L., and Westermark, G. (1968), *Eur. J. Biochem. 6*, 172.

Pocker, Y., and Stone, J. T. (1965), *J. Amer. Chem. Soc. 87*, 5497.

Pocker, Y., and Storm, D. R. (1968), *Biochemistry 7*, 1202.

Ridiford, L. M. (1964), *J. Biol. Chem. 239*, 1079.
Ridiford, L. M., Stellwagen, R. H., Mehta, S., and Edsall, J. T. (1965), *J. Biol. Chem. 240*, 3305.
Roberts, G. C. K., Meadows, D. H., and Jardetzky, O. (1969), *Biochemistry 8*, 2053.
Rüterjans, H., and Pongs, O. (1971), *Eur. J. Biochem. 18*, 313.
Sachs, D. H., Schechter, A. N., and Cohen, J. S. (1971), *J. Biol. Chem. 246*, 6567.
Sheard, B., Yamane, T., and Shulman, R. G. (1970), *J. Mol. Biol. 53*, 35.
Verpoorte, J. A., Mehta, S., and Edsall, J. T. (1967), *J. Biol. Chem. 242*, 4221.
Wang, J. H. (1969), *in* CO_2 Chemical, Biochemical and Physiological Aspects, Forster, R. E., Edsall, J. T., Otis, A. B., and Roughton, F. J. W., Ed., Washington, D. C., Natural Aeronautics and Space Administration, p 157.
Whitney, P. L., Fölsch, G., Nyman, P. O., and Malmström, B. G. (1967a), *J. Biol. Chem. 242*, 4206.
Whitney, P. L., Nyman, P. O., and Malmström, B. G. (1967b), *J. Biol. Chem. 242*, 4212.

Solution Electron Paramagnetic Resonance Spectra of Hemoproteins at Room Temperature*

Toshio Asakura,† George H. Reed, and John S. Leigh, Jr.

ABSTRACT: Electron paramagnetic resonance (epr) spectra of high-spin ferrihemoproteins including hemoglobin, myoglobin, cytochrome *c* peroxidase, and horseradish peroxidase were measured in solutions at ambient temperatures. The spectra are similar to those measured for frozen samples at low temperatures, indicating that no gross changes in the electronic environment of the heme occur in the frozen state. At 15° the peak-to-peak line widths of the "*g* 6" signal of the acidic and basic forms of ferrihemoglobin are over 500 G while that of the fluoride complex is only 136 G. The line width of the *g* 6 signal varied among the different hemoproteins examined. Epr spectra for ferrihemoglobin were altered when the protein conformation was changed by guanidine hydrochloride. The temperature dependence of the high-spin to low-spin equilibrium in alkaline ferrimyoglobin was detected by changes in the intensity of the *g* 6 signal with changes in sample temperature. The ample epr signals obtained for ferrihemoproteins at room temperature opens the possibility of utilizing room-temperature epr measurements in mechanistic studies of hemoproteins in solutions.

Electron paramagnetic resonance studies of hemoproteins have provided important information about the site symmetry of the heme iron, the nature of chemical bonding of the iron to the surrounding ligand atoms, and the orientation of the heme plane in the protein molecule (Ingram, 1969). Since the transition metal atom is intimately involved in the specific biological activity of these molecules, a study of the electronic properties of the metal ion moiety may provide vital clues to the reaction mechanisms. Most epr[1] studies of hemoproteins have been carried out on frozen samples at liquid nitrogen or lower temperatures for sensitivity reasons. However, aqueous solutions are noted for solvent–solute segregation phenomena which occur during freezing (see, for example, Ross, 1965, and Leigh and Reed, 1971), and these effects may not only expose the protein to unusually high-salt or pH gradients (Taborsky, 1970) but may also produce magnetically concentrated samples (Ross, 1965). Deleterious effects have been observed when hemoproteins were frozen (Yonetani and Schleyer, 1967; Iizuka and Kotani, 1969b). Furthermore, temperature-dependent transitions between high- and low-spin states have been observed for hemoproteins both above and below 0° (George *et al.*, 1964; Iizuka and Kotani, 1969a,b), and sharp transitions from high-spin to low-spin states have been observed for ferrihemoproteins at the freezing point, (Yonetani *et al.*, 1966; Ehrenberg, 1966; Iizuka and Kotani, 1969b). These observations pose some questions as to whether structural information obtained from epr studies on frozen samples of hemoproteins is entirely relevant to studies of biological activity of these molecules at physiological temperatures.

Ehrenberg (1962) has reported a broad epr signal at *g* 6 for ferrimyoglobin at 20°. Yonetani and Leigh (1971) have reported room-temperature epr measurements for single crystals of the fluoride complexes of hemoglobin and myoglobin. The present paper describes *solution* epr spectra for several ferrihemoproteins in various liganded states.

Experimental Section

Hemoglobin was crystallized from fresh human blood according to the method of Drabkin (1946). Methemoglobin was prepared by treatment with potassium ferricyanide; excess ferricyanide was removed by successive dialyses against cold water and then 0.1 M potassium phosphate buffer (pH 7.0). Sperm-whale myoglobin was purchased from Calbiochem. Horseradish peroxidase was the product of Sigma (type VI, RZ = 3.0). Cytochrome *c* peroxidase was kindly supplied by Dr. T. Yonetani.

Mixed-state hemoglobins (αFe^{3+}–$\beta Fe^{2+}O_2$ and $\alpha Fe^{2+}O_2$–

* From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 16, 1971.* This investigation was supported by Research Grants GM-12202 and GM-12446 from the National Institutes of Health.

† Recipient of a Career Development award (1-K4-GM-47,463) from the U. S. Public Health Service; to whom to address correspondence.

[1] Abbreviation used is: epr, electron paramagnetic resonance.