

The Carbon Dioxide Hydration Activity of Carbonic Anhydrase

I. STOP-FLOW KINETIC STUDIES ON THE NATIVE HUMAN ISOENZYMES B AND C*

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SUMMARY

The kinetics of catalysis of CO₂ hydration by human carbonic anhydrases B and C (EC 4.2.1.1) has been reinvestigated with use of an improved pH indicator stop-flow approach that was checked by studying the uncatalyzed rate of hydration. The Michaelis-Menten parameters were determined between pH 5.8 and 8.8 in noninhibitory buffers. For both isoenzymes K_m was independent of pH, whereas V_{max} increased with increasing pH. These results are similar to the findings of others on bovine carbonic anhydrase, but differ from the earlier report of Gibbons and Edsall (*J. Biol. Chem.*, 239, 2539 (1964)) in the finding that K_m is pH independent.

Despite the differences between the low and high activity forms of carbonic anhydrase, there are close kinetic similarities between them that indicate underlying similarities in active site structure.

Imidazole inhibits the B enzyme, apparently competitively, but has no effect on the C enzyme. *N*-Methyl substitution of imidazole abolishes its inhibitory effect.

Nitrous oxide does not inhibit catalysis of CO₂ hydration by either of the two human isoenzymes, or by bovine carbonic anhydrase. The infrared absorption studies of Riepe and Wang (*J. Biol. Chem.*, 243, 2779 (1968)) had indicated that N₂O and CO₂ bind competitively to a site identified as the substrate-binding site. The present kinetic results are interpreted as representing a great specificity of carbonic anhydrase for the binding of its substrate CO₂.

It is proposed that the enzyme-catalyzed hydration of CO₂ requires, not only water activation by a basic group, but also charge neutralization in the transition state by an electron acceptor function. The zinc-bound water in carbonic anhydrase could be involved in both donor and acceptor roles.

Carbonic anhydrase (Carbonate hydro-lyase, EC 4.2.1.1) is a zinc metalloenzyme made up of one polypeptide chain. It occurs in human erythrocytes in the form of multiple isoenzymes, these being one high specific activity form designated carbonic anhydrase C, and two, nearly identical low specific activity forms designated A and B. The high and the low specific activity forms are also distinguishable from each other by properties such as amino acid composition, immunological specificity, and optical rotatory behavior, as recently reviewed by Edsall (1, 2). Other mammalian species have distinct carbonic anhydrase isoenzymes, although only high specific activity forms occur in bovine erythrocytes (3).

A preliminary study of the human carbonic anhydrases by Gibbons and Edsall (4) clearly established the kinetic differences between the B and C isoenzymes, but it also showed that for both human isoenzymes the pH dependence of the activity was qualitatively different from that of the bovine enzyme (5, 6). This implies possible fundamental differences in catalytic mechanism, and if true, it would be difficult to extend the conclusions derived from the many studies on the bovine enzyme to the human enzymes whose crystal structures are being determined by x-ray techniques (7).

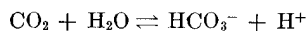
Since the study of Gibbons and Edsall (4) covered only a narrow pH range and utilized buffers subsequently shown to be inhibitory, a careful reinvestigation of the kinetics of the human isoenzymes seemed necessary. This provides a main object of our study. Such an investigation should delineate the similarities and differences between the high and low specific activity human isoenzymes, as well as those between the human enzymes and the corresponding isoenzymes from other species. In addition it should furnish a better basis for comparison between the CO₂ activity and the other catalytic activities that the mammalian carbonic anhydrases possess. These include the hydration of some carbonyl compounds (8, 9) and the hydrolysis of various substances, such as aliphatic and aromatic esters (10-12), cyclic sulfonate esters (13), and most recently 1-fluoro-2,4-dinitrobenzene and related compounds (14). It should be noted here that because of substrate solubility limitations, CO₂ hydration is the only activity for which individual Michaelis-Menten parameters can be reliably obtained at present.

The recent advances in the determination of the amino acid sequences of the human carbonic anhydrases (15-17) and their high resolution crystal structures (7) give good promise for the

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interpretation of their catalytic activity in terms of their three-dimensional structure. In addition, the simplicity of the over-all reaction catalyzed by carbonic anhydrase *in vivo*, which may be expressed as,



makes it an attractive model system for understanding the basis of enzyme action (18–22). It will be seen, however, that this same simplicity in structure, size, and properties can lead to much ambiguity, not frequently appreciated, in specifying their detailed interaction with the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Enzyme Preparations—Human carbonic anhydrases B and C were prepared from erythrocytes provided by the Blood Research Institute and the Massachusetts State Laboratories, Boston, Massachusetts. Separation of the crude carbonic anhydrase mixture from hemoglobin and the resolution of the individual isoenzymes were both done by chromatography on DEAE-Sephadex (Pharmacia) with procedures developed in this laboratory (23). The peaks corresponding to the two isoenzymes were separated by several liters of the eluant buffer, and no significant cross-contamination could be detected on polyacrylamide gel electrophoresis. Bovine carbonic anhydrase was prepared by chromatography of a commercial sample (Worthington) following the procedure of Lindskog (3), except that DEAE-Sephadex was substituted for DEAE-cellulose.

Buffers and Indicators.—Bis-tris¹ (24) was obtained from General Biochemicals and was used without further purification. Imidazole (Eastman) was recrystallized twice from acetone-petroleum ether before use. *N*-Methylimidazole, 1,2-dimethylimidazole, and 3,5-lutidine were all obtained from K and K Laboratories, Plainview, New York, the latter two being further purified by distillation under reduced pressure. *p*-Nitrophenol (Fisher) was recrystallized twice from water. Chlorphenol red and metacresol purple were obtained from Mann, and *p*-nitrosophenol from Eastman. These indicators were used without further purification.

Approximate acid pK' values of the protonated bases and indicators were determined at 25° and an ionic strength of 0.2, the former by titration with standardized acid and the latter by spectrophotometric titration (Cary 15) in solutions of known pH. The buffer-indicator pairs used in the kinetic studies, their approximate pK' values, and the wave lengths used in the spectrophotometric titration and kinetic studies were as follows: 3,5-lutidine (pK' 6.21) with *p*-nitrosophenol (pK' 6.2, 400 nm) or with chlorphenol red (pK' 6.3, 574 nm); imidazole (pK' 7.14) or *N*-methylimidazole (pK' 7.19) with *p*-nitrophenol (pK' 7.15, Reference 25, 400 nm); 1,2-dimethylimidazole (pK' 8.22) with metacresol purple (pK' 8.3, 578 nm); bis-tris (pK' 6.60) with chlorphenol red (pK' 6.3, 574 nm).

CO₂ and N₂O Solutions—Solutions of known concentration of CO₂ were made up either by bubbling analyzed mixtures of CO₂ and N₂ (Medical Technical Gases, Medford, Massachusetts) through thermostatted flasks containing deionized water (26), or by diluting a known volume of a CO₂ solution equilibrated with one atmosphere of CO₂ with a measured volume of carbon diox-

ide-free water (5). In the latter method, calibrated gas-tight syringes (Hamilton) of the luer type were used to contain the required volumes, and the dilution was done in absence of air by coupling the two syringes with a polyethylene coupler with luer-lock fittings (Hamilton), and then gently mixing by pushing the contents back and forth between the two syringes. In the case of the analyzed mixtures of CO₂ and N₂, the gas cylinders were routinely shaken or rolled before use so as to keep the contents homogeneous. Nitrous oxide was also obtained from Medical Technical Gases, and its solutions were prepared as described for CO₂. The solubility coefficients of CO₂ (27) and N₂O (28) were used in determining their concentrations in the equilibration flasks at the temperature and atmospheric pressure of the equilibration. A check on the final concentration of CO₂ in the reaction mixture was possible by the method described below.

Kinetic Method

Stop-Flow Apparatus—All kinetic determinations reported here were obtained with a Durrum-Gibson stop-flow spectrophotometer (Durrum Instrument Corporation, Palo Alto, California) equipped with a stainless steel flow system and a 20-mm optical path length observation chamber. It was found desirable, however, to replace the drive-syringe assembly supplied with the instrument. This was caused by excessive leakage past the Durrum drive-syringe plungers during normal stop-flow operation, probably because of the use of multift syringe barrels which are manufactured to be easily interchangeable with a variety of glass syringe barrels, and hence are loosely fitting. Besides leading to reaction traces with poor mixing qualities, this situation posed a critical problem for the use of gaseous substrates, which necessarily remain an appreciable time in the drive-syringes during thermal equilibration and subsequent reaction rate measurements. Following a suggestion by Dr. Q. Gibson, new drive-syringe plungers were therefore designed and constructed, in collaboration with Dr. P. L. Whitney, such that a teflon O-ring formed an adjustable seal with the wall of the drive-syringe barrels. A further modification was done to allow facile tightening of the seal between the plungers and the syringe barrels while the plungers are in place in the stop-flow apparatus. This design, permitting an easily adjustable seal, eliminated the leakage problem and made more convenient the use of the new drive-syringe plungers over a wide temperature range.

Method for Following Reaction—Since the hydration (or dehydration) of CO₂ at all slightly acidic, neutral, and alkaline pH ranges is rapid and is accompanied by the release (or removal) of protons, the stop-flow pH indicator technique (29) is the most versatile method that can at present be used for studies of the catalyzed or uncatalyzed reactions. The application of this method, especially for CO₂ reactions, involves an unusual number of difficulties and limitations, some of which have been previously noted (2, 26). It is imperative to recognize and to take into account all these problems. We discuss below, and in the "Appendix," some of the principles and procedures that we have followed in this respect.

If the ionization equilibria of the buffer and the indicator are instantaneous in comparison with the proton-releasing reaction under observation, then the rate of absorbance change of the indicator, dA/dt , is related to the rate of proton release, dx/dt , by the so-called buffer factor (26) as follows.

$$dA/dt = (\partial A/\partial x)_t (dx/dt) = (1/Q) (dx/dt)$$

¹ The abbreviation used is: bis-tris: 2,2-bis(2-hydroxymethyl)-2,2',2''-nitrilotriethanol.

where Q is the buffer factor and is defined by the above relation. In general, Q is a complicated function of the states of ionization of the buffer and indicator (see "Appendix"), and these quantities change to some extent during the course of the reaction. In such a case Q will be a function of x , and the time course of the absorbance change will not be linearly related to the time course of the actual proton-releasing reaction. It should be evident that if Q were kept constant, *i.e.* independent of x , during the reaction, the linearity between the absorbance kinetics and the actual kinetics that results will greatly enhance the accuracy of the rate determination. One example of this will be the elimination of the need for full absorbance-pH calibration curves (29, 30). The variation of the buffer factor can be minimized by using well buffered solutions to decrease the pH change occurring during the reaction, with the consequence, however, of decreasing the sensitivity of the measurement (26). It can be shown quite simply (see "Appendix") that a more general way to make the buffer factor independent of pH is to use together buffers and indicators which have nearly identical pK values. This principle was applied in this study, and we have already listed above the relevant pK data on the buffer-indicator systems that were employed. The need for good buffering capacity remains, of course, since the rate constants to be determined are generally pH dependent, and valid results cannot be otherwise obtained by this method.

Determination of Buffer Factors—The buffer factor is usually obtained from spectrophotometric titration of the buffer-indicator systems used for the kinetic studies. Aliquots of standardized acid or base are added and the resulting absorbance changes are measured, Q being the reciprocal of the slope of such titration plots (5, 26, 30). In this study it was possible to employ a different approach for the determination of the buffer factors, since the new method's accuracy relies on having the buffer factor independent of pH. The linear spectrophotometric titration plots are constructed from data obtained in the stop-flow apparatus during the kinetic determinations. At each substrate (CO_2) concentration, the full reaction trace is accurately determined, and this provides the value of the total absorbance change that occurs. The amount of CO_2 converted to HCO_3^- during the hydration represents the increment of acid that produced the recorded absorbance change.² At neutral and acid pH ranges there is incomplete conversion of CO_2 to HCO_3^- at equilibrium. It is easy to allow for this by consideration of the two equilibria that are involved. The first is the pH-dependent hydration equilibrium, whose equilibrium constant is closely approximated by the over-all first ionization constant of CO_2 in the following way (31).

$$K'_1 = \frac{[\text{H}^+]_f [\text{HCO}_3^-]_f}{[\text{CO}_2]_f + [\text{H}_2\text{CO}_3]_f} \approx \frac{[\text{H}^+]_f [\text{HCO}_3^-]_f}{[\text{CO}_2]_f}$$

where the subscript f indicates equilibrium conditions after the completion of the hydration reaction. Accurate values of K'_1 at different temperatures and ionic strength values have been obtained by Harned and Davis (32). The second equilibrium we consider is the ionization of the buffer. Since equal amounts of H^+ and HCO_3^- are released by the hydration reaction,² we can write the buffer ionization as follows.

² This will be valid only if the pH is such that at equilibrium the concentrations of CO_3^{2-} and H_2CO_3 are negligible in comparison to HCO_3^- .

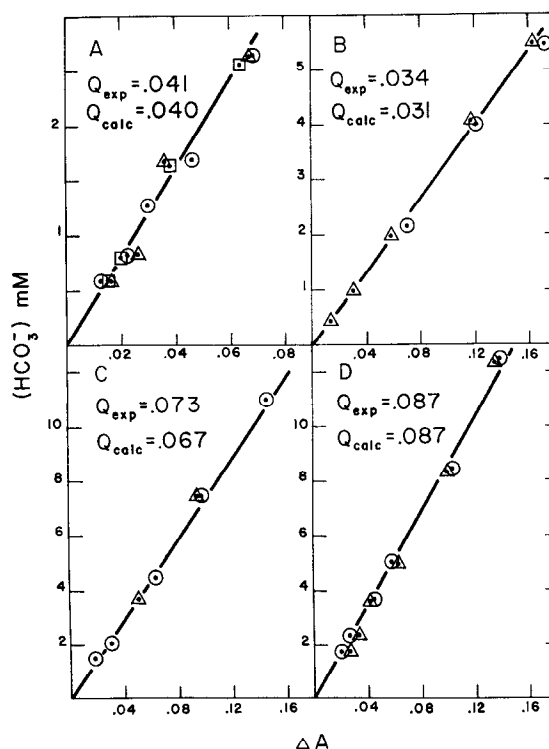


FIG. 1. Typical titration plots for buffer factor determination by using the method discussed in the text. The ordinate is the concentration of bicarbonate that is produced by the hydration reaction, and the abscissa is the corresponding total absorbance change that is observed, *i.e.* the amplitude of the reaction trace. The determinations were at 25° and 0.2 ionic strength, and the following buffer-indicator systems are shown above: A, 0.05 M 3,5-lutidine, 3.0×10^{-5} M *p*-nitrosophenol, pH 5.83; B, 0.05 M bis-tris, 2.7×10^{-5} M chlorophenol red, pH 6.21; C, 0.10 M *N*-methylimidazole, 4.0×10^{-5} M *p*-nitrophenol, pH 7.19; D, 0.10 M 1,2-dimethylimidazole, 3.0×10^{-5} M metacresol purple, pH 8.22. The slopes of the plots are the buffer factors (Q_{exp}), and these closely agree with the theoretical values (Q_{calc}) that can be calculated by using Equation 5. The initial rate determinations in these systems usually involved absorbance changes of 0.01 or less.

$$K'_{HB} = \frac{[\text{H}^+]_f [\text{B}]_f}{[\text{HB}^+]_f} = \frac{[\text{H}^+]_f ([\text{B}]_0 - [\text{HCO}_3^-]_f)}{([\text{HB}^+]_0 + [\text{HCO}_3^-]_f)}$$

where B and HB^+ refer, respectively, to the basic and the protonated buffer species, and the subscripts 0 and f refer, respectively, to the conditions at the beginning and the end of the hydration reaction. We can eliminate $[\text{H}^+]_f$ between the two equilibrium expressions above to obtain the relation

$$[\text{CO}_2]_f = \frac{K'_{HB} ([\text{HB}^+]_0 + [\text{HCO}_3^-]_f) [\text{HCO}_3^-]_f}{K'_1 ([\text{B}]_0 - [\text{HCO}_3^-]_f)}$$

and we note that the initial concentration of CO_2 is equal to the sum of the equilibrium concentrations of CO_2 and HCO_3^- . The above expression permits the calculation of the extent of hydration for any initial CO_2 concentration. $[\text{HB}^+]_0$ and $[\text{B}]_0$ are calculated from the experimentally found values of pK_{HB} and the initial pH. In practice it is often found convenient to construct for each buffer system a plot of $[\text{CO}_2]_f$ versus $[\text{HCO}_3^-]_f$ based on the above equation, and to use that plot to determine the extent of conversion for each substrate solution.

The kinetic measurements cover a sufficiently wide range of

CO₂ concentrations, and the absorbance changes for each CO₂ concentration are recorded frequently enough in the course of duplicate runs, to provide a sufficient amount of information for making the buffer plots at each pH. Typical results are shown in Fig. 1, where the expected linearity of the plots is apparent. There are two main advantages for obtaining buffer factors by the above procedure. The first one is that the determinations are carried out in the stop-flow apparatus with the same solutions on which kinetic observations are being carried out. The buffer factor and the kinetic information will thus be derived from data obtained under identical conditions of temperature, wave length, slit width, and phototube and amplifier sensitivity. The second and more important advantage is that once the plots of absorbance change *versus* proton release are constructed from the full data for a given buffer-indicator system, the concentration of each substrate solution can be checked by comparing the absorbance change it produces during the reaction with the expected value from the plot. It has not proved convenient previously to monitor *in situ* the substrate concentrations, although simultaneous Ba(OH)₂ titrations have sometimes been carried out (33). The buffer factor values obtained by the procedure we have followed have been compared with their calculated values with the equation given in the "Appendix," and close agreement was found. This is indicated by the examples shown in Fig. 1.

Determination of Initial Rates—Steady state initial rates were directly obtained from the initial, linear portions of the reaction traces (6). For time intervals that are small compared to the half-time, the substrate concentration is nearly invariant, so that the rate is constant and the reaction trace is linear. This will be true in either the high substrate ("zero order") or the low substrate ("first order") limits of the enzymatic reaction, provided that short enough times are considered. For each reaction trace, transmittance values were read off at nine equally spaced time intervals, by use of the storage mode of the oscilloscope and a finely calibrated graticule. The transmittance values were converted to absorbance values and were then plotted against time. The slopes of these linear plots represent the initial rate in absorbance per unit time, which is converted to moles per liter per unit time through multiplication by the buffer factor. The initial rates of the uncatalyzed reaction are determined by the same procedure. In previous studies, initial rates were obtained by using single exponential fits (5, 26). These would have given equivalent results, provided that the fits utilized the same data obtained from the initial, linear portion of the reaction trace. Measurements of initial rates were repeated four or five times with the same set of substrate and buffer or buffer-enzyme solutions present in the two syringes of the stop-flow apparatus, and the initial rate was taken as the average of these measurements. Initial rates for the enzyme-catalyzed reactions were obtained by subtracting the initial rate of the uncatalyzed reaction from the observed rate. This correction was usually 10% or less.

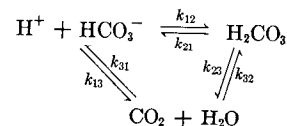
Calculation of Michaelis-Menten Parameters—Each set of Michaelis-Menten parameters was determined from data obtained with one enzyme-buffer solution and six different substrate solutions. Experiments were then run in duplicate by preparing another buffer-enzyme solution. The use of Lineweaver-Burk plots was avoided, since if statistical weights are neglected, these plots overemphasize the results at low substrate concentrations (34). Instead, the parameters were obtained

from the plots of v_0 *versus* v_0/s_0 , which are likely to give more valid results from linear plots (34). Least squares analysis was not attempted, since it was not clear how to assign weighting factors and whether systematic errors were completely absent. It may be noted that the initial rate determinations at the lowest substrate concentrations are more difficult, since the relative linearity of the reaction trace and the amplitude of the absorbance change are less than those at higher concentrations. The average reproducibility of the parameters was roughly 10% for $V_{\max}$ and 15% for K_m . However, it was evident from the results that the absolute errors in the values of K_m and $V_{\max}$ were greater than these limits, and greater than the limits that would have been derived from a least squares analysis of the plots. Typical Michaelis-Menten determination plots can be seen in Fig. 4 below.

RESULTS

Uncatalyzed Hydration Rate Constants

The kinetics of the uncatalyzed hydration of CO₂ was studied at 25° and 0.2 ionic strength during the course of this investigation. Table I shows the values we have obtained for the hydration rate constant k_{CO_2} , which pertains to the reaction scheme (36).



where the observed hydration rate constant at neutral pH is given by the following relation (26),

$$k_{\text{CO}_2} = k_{31} + k_{32}$$

The determination of this rate constant was of interest for several reasons. It was necessary to know its contribution to the observed rate in the enzymatic runs. It also provides a check on the consistency and reliability of the methods outlined above for measuring initial rates and for determining buffer factors over the relatively wide pH range and buffer-indicator systems used. Furthermore, the measurements provide additional information on the value of k_{CO_2} itself, since there still exist some discrepancies in its determination by different methods at 25°, as recently discussed by Edsall (31). In addition there have been some difficulties in detecting the slight catalysis exerted by some buffers on the hydration reaction (26, 36–38). With respect to the last point, it may be pointed out that small catalytic effects of buffers can be detected much more readily, if the uncertainties in the values of the buffer factors can be circumvented. Since we have used systems where buffer factors are essentially pH independent, buffer catalysis was studied by measuring the rate in different dilutions of the same buffer at constant ionic strength. The buffer factors at different dilutions will then be related to each other only by their dilution factors (see expression in "Appendix"), and they will not require independent determination. After correction for buffer and hydroxyl ion catalysis, Table I shows that the value of k_{CO_2} is 0.037 sec⁻¹ (S.D. = 0.002), which agrees to within ±0.002 with the results of Gibbons and Edsall (26), Ho and Sturtevant (37), Kernohan (6), and Magid and Turbeck (38). Thus all the recent determinations of this constant, in buffered media at 25°, are in close agreement.

TABLE I

CO_2 hydration rate constant k_{CO_2} at 25° and 0.2 ionic strength

The value of k_{CO_2} was obtained from the observed rate constant after making corrections for contributions from catalysis by hydroxyl ions and buffer species with the relation: $k_{\text{obs}} = k_{\text{OH}}(\text{OH}^-) + [1 + l_u(B)]k_{\text{CO}_2}$, where l_u is the catalytic coefficient of the basic form (B) of the buffer. The value of k_{obs} is defined by the relation: $k_{\text{obs}} = -[1/(\text{CO}_2)_0][d(\text{CO}_2)/dt]_{t=0}$. The initial rate of hydration is related to the initial rate of absorbance change through the "buffer factor" (see text), which was separately determined at each pH. The value of k_{OH} was that of Sirs (35), while l_u values were determined in this study as follows in parenthesis: *N*-methylimidazole (1.0), imidazole (2.0), 1,2-dimethylimidazole (5.1). An additional value was found for phosphate (5.6), but no determinations were made for bis-tris and 3,5-lutidine, the latter because of solubility limitations. Rate constants in these two buffers refer to values found at a total buffer concentration of 50 mM. The average value of k_{CO_2} was 0.037 sec⁻¹ (S.D. = 0.002), neglecting the last two entries at the highest pH, since these have large uncertainties resulting from the large corrections involved.

Buffer	Indicator	pH	k_{CO_2} sec ⁻¹
3,5-Lutidine.....	<i>p</i> -Nitrosophenol	5.83	0.038
3,5-Lutidine.....	<i>p</i> -Nitrosophenol	6.22	0.038
3,5-Lutidine.....	<i>p</i> -Nitrosophenol	6.72	0.039
Bis-tris.....	Chlorphenol red	6.21	0.039
<i>N</i> -Methylimidazole.....	<i>p</i> -Nitrophenol	6.73	0.035
<i>N</i> -Methylimidazole.....	<i>p</i> -Nitrophenol	6.85	0.041
<i>N</i> -Methylimidazole.....	<i>p</i> -Nitrophenol	7.19	0.034
<i>N</i> -Methylimidazole.....	<i>p</i> -Nitrophenol	7.34	0.038
<i>N</i> -Methylimidazole.....	<i>p</i> -Nitrophenol	7.55	0.040
Imidazole.....	<i>p</i> -Nitrophenol	6.35	0.037
Imidazole.....	<i>p</i> -Nitrophenol	7.03	0.035
Imidazole.....	<i>p</i> -Nitrophenol	7.66	0.033
1,2-Dimethylimidazole.....	Metacresol purple	7.80	0.036
1,2-Dimethylimidazole.....	Metacresol purple	7.87	0.037
1,2-Dimethylimidazole.....	Metacresol purple	8.22	0.036
1,2-Dimethylimidazole.....	Metacresol purple	8.74	0.030
1,2-Dimethylimidazole.....	Metacresol purple	8.75	0.030

Buffer and Indicator Effects on Enzymatic Activity

All the kinetic studies were done in buffers of ionic strength 0.2 in which noninhibitory SO_4^{2-} was the only anion in the buffering system. The majority of the enzymatic runs contained about 0.01% peptone added for stabilization (26, 39), and unless noted otherwise, the temperature was maintained at $25.0 \pm 0.2^\circ$. Since most monovalent anions inhibit carbonic anhydrase (5, 6, 33, 40–41), only cationic buffers could be used. In addition, the possibility of carbamate formation prevented the use of primary and secondary, nonaromatic amines. Table I includes all the buffers and indicators used. These were tested for any inhibitory effects that they might have towards both the B and C isoenzymes. In the range of concentrations used for the kinetic studies, the only significant inhibitory effect present was that of imidazole towards the B enzyme. This is discussed further below.

pH Dependence of Michaelis-Menten Parameters

K_m —The Michaelis-Menten parameters were determined between pH values 5.8 and 8.8. Fig. 2 shows that within experi-

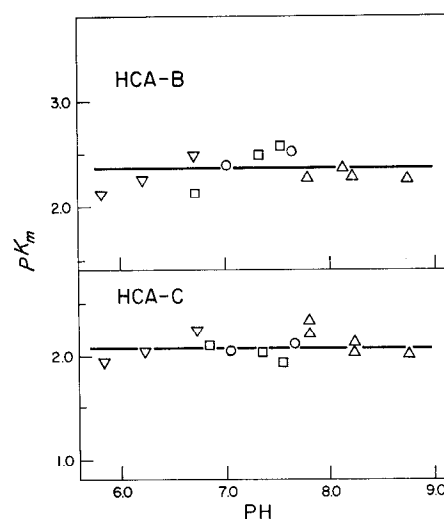


FIG. 2. pK_m values for human carbonic anhydrases B and C between pH 5.8 and 8.8 at 25° and 0.2 ionic strength. The following buffers were used: 3,5-lutidine (▽), imidazole (○), *N*-methylimidazole (□), 1,2-dimethylimidazole (△). The pK_m values for the B isoenzyme in the inhibitory imidazole system represent extrapolation of the apparent K_m values to zero buffer concentration (see Fig. 4). The average K_m values were 4 mM for the B isoenzyme and 9 mM for the C form.

mental uncertainties, pK_m is independent of pH over this interval for both the B and C isoenzymes. This behavior is identical with that of the bovine carbonic anhydrase (6). The earlier finding of Gibbons and Edsall (4) that K_m changes with pH for these two human isoenzymes is thus not substantiated when a wider pH range is considered. They reported that the K_m value of the B isoenzyme varied between 1.0 and 6.5 mM, while that of the C isoenzyme varied between 9 and 18 mM. The difference between the K_m values of the two isoenzymes that was noted by Gibbons and Edsall (4) is borne out by the present results. However, we found that the average K_m value of the B isoenzyme is 4 mM (S.D. = 2), while that for the C isoenzyme is 9 mM (S.D. = 2). McIntosh (39) has reported a K_m value of 8 mM for the C isoenzyme at 0° and pH 7. The K_m value for bovine carbonic anhydrase is about 12 mM (6).

V_{max} —Fig. 3 shows the pH dependence of the logarithm of k_{cat}/K_m , where k_{cat} is the turnover number, i.e. V_{max}/E_0 . Since K_m was found above to be independent of pH, the variation shown in Fig. 3 is the result of that of k_{cat} alone. Both human isoenzymes show a general increase in the k_{cat} value with increasing pH that is similar to the findings of Kernohan on the bovine enzyme (6). The turnover number thus appears to be dependent on the ionization of a group on the enzyme such that the basic form is active. The apparent pK of the variation can be estimated to be close to 7.0 for the C isoenzyme. Such an estimate could not be made for the B isoenzyme, since the activity at high pH did not level off. However, it must be higher than the pK of the C isoenzyme by at least 0.6 pH unit. It should be noted that the pH variation of k_{cat} for the B isoenzyme was not consistent with the titration of a simple, unperturbed ionizing group. The bovine enzyme has been found to have an apparent pK of about 6.4 under similar conditions (6).

Gibbons and Edsall (4) previously found an increase of k_{cat} with pH for both isoenzymes between the pH values of 6.3 and 7.5. They also reported that the k_{cat} value of the C isoenzyme

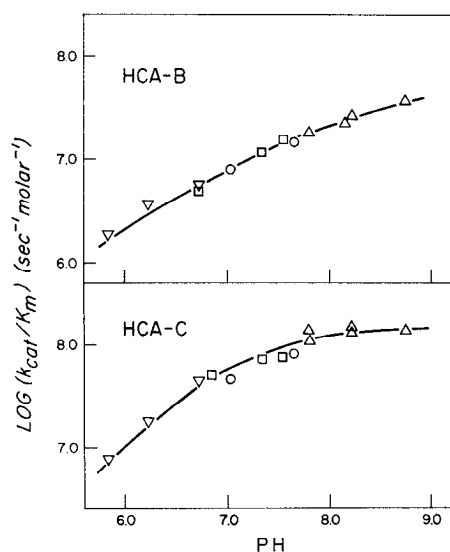


FIG. 3. The effect of pH on the turnover number (k_{cat}) of the human carbonic anhydrases B and C at 25° and 0.2 ionic strength. The buffer systems were the same as indicated in the legend to Fig. 2. The pH variation of k_{cat}/K_m is due to the variation of k_{cat} alone, since K_m is independent of pH (Fig. 2).

was from 25 to 50 times greater than that of the B isoenzyme in this pH interval. At the highest pH of the present study, k_{cat} for the B enzyme was still increasing, with a value of $2 \times 10^5 \text{ sec}^{-1}$, while that for the C enzyme had leveled off at a maximal value of $1.4 \times 10^6 \text{ sec}^{-1}$. Thus the ratio of the k_{cat} values of the two isoenzymes is reduced to about seven or less in the high pH limit, making the differences between the two human isoenzymes less marked than previously indicated. In comparison, the value of k_{cat} at high pH is about $1 \times 10^6 \text{ sec}^{-1}$ for the bovine enzyme (6).

Inhibition Studies

Imidazole—While investigating the effect of buffers on the enzymatic activity, we found that imidazole inhibits the B isoenzyme. Fig. 4 shows that this inhibition of the CO_2 hydration activity is probably competitive. The value of the apparent Michaelis constant extrapolated to zero inhibitor concentration is the same within the experimental error as the K_m value obtained in noninhibitory buffers. Imidazole did not show significant inhibition of the C isoenzyme at comparable concentrations, and the data of Kernohan (5) show that it has no effect on the high activity bovine carbonic anhydrase. The inhibition of the B isoenzyme was not affected by recrystallization of imidazole from organic solvents or by its passage through a mixed bed ion exchange resin. Studies at higher pH values indicate that the basic form of imidazole is the inhibitory species, with an inhibition constant of about 20 mM. This effect of imidazole is paralleled by characteristic changes it produces³ in the visible absorption spectrum of the active cobalt form of the B isoenzyme at high pH. To our knowledge, no competitive inhibitor of the CO_2 hydration activity of the human B isoenzyme has been previously reported. It is interesting to note that *N*-methyl substitution of imidazole removes its inhibitory effect, since *N*-methylimidazole and 1,2-dimethylimidazole did not inhibit the B isoenzyme.

³ P. L. Whitney, personal communication.

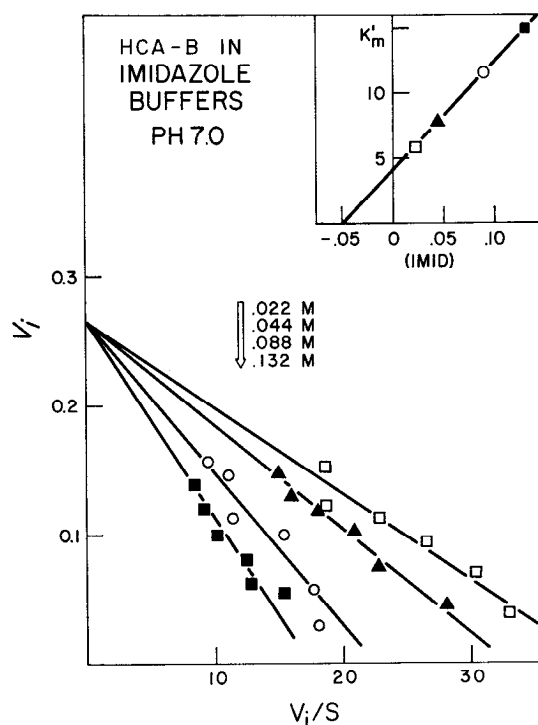


FIG. 4. The inhibition of human carbonic anhydrase B by imidazole at 25° and 0.2 ionic strength. The initial velocity of CO_2 hydration (arbitrary units) is plotted against the ratio of the initial velocity to the initial concentration of CO_2 (molar units). The total concentration of imidazole was varied as indicated. The slopes of the lines represent the apparent Michaelis constants (K'_m), and these were drawn to indicate that the data are consistent with competitive inhibition.

Nitrous Oxide—It was of interest to investigate whether nitrous oxide had any effect on the CO_2 activity of carbonic anhydrase, since it is the most similar molecule to CO_2 in size and physical properties. Riepe and Wang (42) had concluded from their infrared studies on the equilibrium binding of CO_2 to bovine carbonic anhydrase that N_2O competes with CO_2 for binding at the active site, and that both molecules had the same affinity for the enzyme. However, dehydration activity studies⁴ did not reveal any appreciable inhibition of carbonic anhydrase. This question could be resolved more directly by the study of the effect of N_2O on the CO_2 hydration activity. We have done such experiments on the human B and C isoenzymes, as well as on the major component of the high activity bovine enzyme. To achieve high concentrations of N_2O , the experiments were also carried out at 5° and N_2O was introduced in both the substrate and the buffer-enzyme solutions. Furthermore, the CO_2 concentrations were kept below the K_m values, so that the inhibition, if competitive, could be more readily detected. At low substrate concentrations, it would not be possible, of course, to distinguish the type of inhibition that may be encountered.

Table II gives the results in terms of the ratio of the hydration rate in the presence of the indicated concentrations of N_2O

⁴ E. Christiansen and E. Magid (42a) have investigated the effect of N_2O on the HCO_3^- dehydration activity of the B and C human isoenzymes. They have not found any appreciable inhibition by N_2O . We are indebted to Dr. Magid for communicating these results prior to publication.

TABLE II

CO₂ hydration activity in presence of nitrous oxide

The rate of hydration in the presence (v_i) and the absence (v_o) of N₂O were compared at the indicated concentrations of N₂O and the substrate CO₂. The K_m values are those found at 25° in the absence of N₂O and are given for reference. The CO₂ concentrations were kept below the K_m values so that the rate would be most sensitive to possible competitive inhibition. The K_m values for the human isoenzymes were determined in this study, while K_m for the bovine enzyme is that given by Kernohan (5). The buffer was *N*-methylimidazole, ionic strength 0.2, and the pH was 7.6 at the indicated temperatures.

	K_m	Tem- pera- ture	CO ₂	N ₂ O	v_i/v_o
	mM		mM	mM	
Human carbonic anhydrase B.	4	25	1.7	11	0.97
Human carbonic anhydrase C.	9	25	1.7	11	0.90
		25	1.7	22	0.99
		5	3.2	45	0.98
Bovine carbonic anhydrase B.	12	5	3.2	45	0.97

to that in the absence of N₂O. Within the experimental uncertainties, N₂O has no inhibitory effect on the CO₂ hydration activity of any of these isoenzymes. Slow equilibration of N₂O with the enzyme cannot be an important factor, since the two were in contact for as much as 30 min before the assay. These results show that if N₂O is an inhibitor of carbonic anhydrase, its dissociation constant must exceed 1 M, at least for the high activity isoenzymes. Experiments at 25°, where N₂O has lower solubility, also showed no effect of N₂O on the hydration activity.

DISCUSSION

Comparison of Isoenzymes—The occurrence of high and low specific activity isoenzymes in mammalian carbonic anhydrase now appears to be quite widespread (see discussion by Furth (43)). This was first detected by simple activity assays. The subsequent kinetic studies of Gibbons and Edsall (4) showed that definite and marked differences exist between the Michaelis-Menten parameters of the CO₂ activity of the human B and C isoenzymes. The results reported here provide further evidence of this distinct behavior of the human isoenzymes, although the differences in the K_m and k_{cat} values now appear to be less marked than was previously indicated.

Since there appears to be a close evolutionary relationship between the carbonic anhydrases from many different species, especially the mammalian ones, it was difficult to explain the data of Gibbons and Edsall (4) on the pH dependence of the Michaelis parameters. They found that the K_m values for both the B and the C human isoenzymes were pH dependent, whereas Kernohan (6) had found K_m to be independent of pH for the bovine enzyme. The more extensive results we have reported above indicate that the K_m values for both the B and the C isoenzymes are constant within the limits of error over a relatively wide pH range.⁵ In this respect, there is no funda-

⁵ It is not clear why the earlier study of Gibbons and Edsall (4) gave pH-dependent Michaelis parameters. The older stop-flow apparatus used in that work necessitated measurements of the catalyzed reaction rates that were only two or three times greater than those of the uncatalyzed rates that had to be subtracted. The probable errors in the enzymatic rates were thus substantially larger than in those conducted here. In addition,

mental difference in catalytic mechanism between the human and the bovine carbonic anhydrases.

The present results also support the frequent observation that, of the two human isoenzymes, the C form more closely resembles the high activity bovine carbonic anhydrase. These two isoenzymes have almost identical K_m and k_{cat} values, and their activities have apparent pK values which are very close. Amino acid sequences of the carboxyl (44)- and the amino (16)-terminal regions also show that the human C isoenzyme resembles the bovine enzyme more closely than it resembles the human B isoenzyme. Furthermore, chemical modification studies (45–47) have shown that the bovine enzyme and the high activity human and horse isoenzymes are all resistant to covalent modification by active site reagents that readily react with the low activity human and horse isoenzymes.

There have been many observations such as those mentioned above on the differences between high and low activity isoenzymes of carbonic anhydrase, and these have received much attention because of their interesting implications. The determination of the three-dimensional structures of the human B and C isoenzymes should permit an explanation of these differences, perhaps even the kinetic ones. It should not be overlooked, however, that there are general similarities among the carbonic anhydrases from many different species, regardless of the particular isoenzymes which are used in such comparisons. We exclude here the plant carbonic anhydrases, since they appear to form a distinct class so far (48). These similarities certainly extend to the catalytic properties. For example, whenever tested, the mammalian carbonic anhydrases have shown esterase and aldehyde hydration activity, in addition to the hydration of CO₂. Furthermore, although differences have been noted in the specific activities of various isoenzymes from the same or from different species (43, 49), these differences do not appear to be large from a thermodynamic point of view. There is a paucity of careful studies suitable for such a comparison, but consider the data presented above on the human carbonic anhydrases and those of Kernohan (6) on the bovine enzyme. The K_m values of any two isoenzymes differ by less than a factor of three, while the maximal k_{cat} values differ by a factor of seven or less. The esterase data for the monkey, bovine, and human isoenzymes given by Coleman (49) also show differences of less than an order of magnitude. It appears then that the *catalytically important residues* in the active sites of carbonic anhydrases must have been conserved to a considerable extent. The homologies already evident in the available amino acid sequences (15–17, 44) can be expected to be present particularly in the active site region, probably to a greater extent than has been noted for the serine proteases (50). One indication of this may well be the surprising binding specificity at the CO₂ substrate-binding site that is exhibited by the human and bovine carbonic anhydrases and is discussed below.

CO₂-binding Site of Carbonic Anhydrase

The elucidation of the mode of binding of the CO₂ substrate at the active site would be of much value in understanding the mechanism of catalysis by carbonic anhydrase. However,

the previous work covered a relatively narrow pH range, thus adding to the uncertainty in the trends of the kinetic constants. Complications from the use of phosphate buffers may also have occurred, since the inhibition by phosphate is expected to be pH dependent.

little is known about this aspect, but it is certain that the hypothesis of direct coordination of CO₂ to the zinc metal (18, 51) cannot be true, since kinetic studies have shown the noncompetitive nature of a variety of inhibitors known or believed to bind at the zinc. These include sulfide (52), monovalent anions (6), and some sulfonamides (53). The x-ray studies of the Uppsala group (7) have provided evidence for direct coordination of sulfonamides to the zinc atom of carbonic anhydrase.

More recent suggestions on the binding site of CO₂ have come from the infrared studies of Riepe and Wang (42). These authors have proposed that the CO₂-binding site of carbonic anhydrase is a hydrophobic surface or cavity which loosely binds CO₂ and other nonpolar molecules such as N₂O. The evidence for this was their observation that bound CO₂ in carbonic anhydrase has an asymmetric stretching frequency, ν_3 , not very different from that of CO₂ dissolved in water; indicating no distortion or strain in bound CO₂; and that N₂O competes with CO₂ for binding to the active site, with both having the same affinity for it (42).

Our kinetic studies on the effect of N₂O on the CO₂ hydration activity of carbonic anhydrase (Table II) have failed to reveal any significant inhibition for both human isoenzymes, as well as for the bovine enzyme used in the infrared work. For the latter enzyme, the N₂O concentration was approximately 4

TABLE III

Solubility comparison among CO₂, N₂O, and other gases in various solvents at 25°

The solubility coefficients (X_2) are on a mole fraction basis and were calculated from solubility data given in sources listed by Linke (27, 28) and by Battino and Clever (56). Some variation exists between the results of different investigators. The first three columns give the ratios of the solubility coefficients of CO₂ to those of N₂O, N₂, and O₂, while the last two columns give the actual solubility coefficients of CO₂ and O₂. A similar but shorter comparison not involving N₂O has been given by Edsall (31), while more extensive tabulations and discussion can be found in the monograph of Hildebrand and Scott (57), especially with regard to the comparison of CO₂ and N₂O. The X_2 coefficients represent the mole fraction of the dissolved gas when its partial pressure is one atmosphere.

Solvent	$\frac{(X_2)_{\text{CO}_2}}{(X_2)_{\text{N}_2\text{O}}}$	$\frac{(X_2)_{\text{CO}_2}}{(X_2)_{\text{N}_2}}$	$\frac{(X_2)_{\text{CO}_2}}{(X_2)_{\text{O}_2}}$	$X_2 \times 10^4$	$X_2^a \times 10^4$
Amyl acetate.....	0.86			245	
Methyl acetate.....	1.2	40	25	226	8.9
Acetone.....	1.2	38	24	207	8.5
Heptane.....	0.66	8.9	5.6	120	22 ^b
Chloroform.....	0.77	29	17	128	7.4
Carbon tetrachloride....	0.64	17	8.8	107	12
Benzene.....	0.70	22	12	95	8.2
3-Methyl-2-butanol.....	0.79	16		81	
Ethanol.....	1.1	20	12	71	6
Methanol.....	1.2	23	15	62	4
H ₂ O.....	1.4	50	26	6.1	0.23

^a The solubility trends of N₂ and CO closely follow that of O₂. They are less soluble than O₂ in the above solvents by a factor of 1.6 to 1.9 for N₂ and about 1.1 to 1.4 for CO.

^b The solubilities of O₂ and N₂ in 2-methylheptane, nonane, octane, and hexane are the same within $\pm 14\%$ as their solubilities in heptane.

times that found by Riepe and Wang (42) to cause a 50% decrease in CO₂ binding. Christiansen and Magid⁴ (42a) have also failed to detect significant inhibition by N₂O in the dehydration reaction. Evidently, the CO₂ at the site identified by Riepe and Wang (42) as being the true substrate-binding site represents nonproductive binding of CO₂ close to the zinc in the active site.⁶

These results show that carbonic anhydrase has a remarkable specificity at the binding site of its natural substrate CO₂. It is difficult to imagine 2 molecules that are more alike than N₂O and CO₂ in their physical properties. They are isoelectronic, linear molecules, the sum of their bond lengths being the same to within 0.01 Å. However, N₂O (NNO) is asymmetric, with slightly unequal bond lengths (NN = 1.126 Å, NO = 1.186 Å), and it has a small residual dipole moment of 0.17 Debye unit (55), whereas CO₂ has none. The solubility properties of these 2 molecules are also remarkably similar in a wide range of solvents (Table III), as was noted by Hildebrand and Scott (57).

This binding specificity shown by carbonic anhydrase is quite surprising, since the enzyme is now known to catalyze several diverse reactions (see "Introduction"), in addition to CO₂ hydration. The above results, however, suggest that the specific site for CO₂ is distinct from those of the other substrates, although all the different catalytic activities are associated with the same active site region that includes the essential zinc. It is perhaps significant here that no general class of competitive inhibitors of the CO₂ hydration activity of carbonic anhydrase has been found to date. Imidazole is an example, since it appears to be a competitive inhibitor of the human B isoenzyme (Fig. 4), but not of the C form. A more typical example is the behavior of the potent sulfonamide inhibitors, which have been discussed at length by Lindskog (53).

The explanation of such a specificity in molecular terms will certainly prove to be a difficult task. Little is known, for example, about the hydrogen-bonding properties of CO₂ or about similar types of interaction that may be capable of producing some specificity. However, Hildebrand and Scott (57) noted a long time ago that the solubility properties of CO₂ in various solvents show considerable evidence of solvation. This was most striking in solvents containing the carbonyl group (31, 57). This can be seen in the data of Table III, where the solubility properties of CO₂ and N₂O are compared with those of the more inert gases O₂, N₂, and CO, as was done by Edsall (31). The latter gases are most soluble in the pure hydrocarbons, whereas CO₂ has a distinct preference for the more polar solvents that contain the carbonyl group. The data of

⁶ Riepe and Wang (42) reported two types of binding sites for CO₂, designated Sites A and B by Riepe (54). The A site was identified (42, 54) as being the specific (substrate) CO₂-binding site, since the bound CO₂ in it was displaced by inhibitors of the enzyme, such as ethoxzolamide, azide, and NO₃⁻. They reported that partial inhibition of the activity of the enzyme caused a corresponding decrease in binding at Site A. This type of experiment shows that the displaced CO₂ was probably bound in the active site, but it does not distinguish between productive and nonproductive binding of CO₂, since none of the inhibitors used has been shown to be a competitive inhibitor of carbonic anhydrase. In general, sulfonamides have not yielded a consistent pattern of inhibition (53). However, Kernohan (6) has clearly shown that NO₃⁻ is a noncompetitive inhibitor of the CO₂ hydration activity. Our experiments on N₂O, and those of Magid and Christiansen,⁴ show no inhibition within experimental error, whereas Riepe and Wang (42) find N₂O to be a competitive inhibitor of CO₂ binding at Site A.

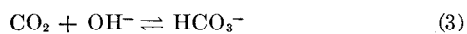
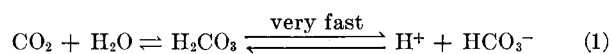
Table III do not, of course, provide an explanation for the greater preference of carbonic anhydrase for CO₂ over N₂O, but they do indicate that CO₂ may exist in an environment that could include some polar groups usually associated with specific interaction.

It may be interesting in this regard that the infrared results of Riepe and Wang are compatible with a polar environment for CO₂ bound at either of the two sites that they have detected in bovine carbonic anhydrase.^{6,7} They have suggested (42) that the value of ν_3 for bound CO₂ may reflect environmental influences and have given ν_3 values for CO₂ dissolved in several solvents. Their values (42, 54), along with those in the literature (58), permit the construction of the following series of frequency values for CO₂ in various solvents or binding sites according to decreasing values of ν_3 : H₂O (2343.5 cm⁻¹); bovine carbonic anhydrase-Site A⁷; (2341); acetone (2341); bovine carbonic anhydrase-Site B⁷ (2340.5); methanol (2339.8); *n*-pentane (2338.5); 1-propanol (2337.8); *n*-hexane (2337); cyclopentane (2336.5) benzene (2336); cyclohexane (2336). These data suggest that all the CO₂-binding sites of carbonic anhydrase detected by Riepe and Wang may be more polar than previously suspected.

The CO₂-binding specificity, although not yet understood, indicates the possibility that the binding of the substrate may play an important role in the catalytic mechanism of the enzyme. This could occur, for example, if there was an orientation requirement in the hydration reaction. A fuller appreciation of such requirements can be obtained only when the mechanism of catalysis, discussed below, is elucidated in detail.

Catalytic Mechanism—The mechanism of catalysis of CO₂ hydration-dehydration by carbonic anhydrase is not yet known with any certainty. This is largely because of the lack of knowledge of the detailed structure of the enzyme and its complexes with its substrates. While rapid progress is being made in the x-ray investigation of the human carbonic anhydrases (7), other difficulties remain, since there is some uncertainty about fundamental aspects of the CO₂ hydration reaction.

Consider, for example, the three paths usually postulated for CO₂ hydration-dehydration (26, 36, 37).



The first two paths (Equations 1 and 2) are kinetically indistinguishable under ordinary conditions, since the ionization of H₂CO₃ is known to be extremely rapid (59). However, Eigen *et al.* have stated (60) that the second pathway (Equation 2) is the more plausible one, especially from the viewpoint of catalysis. This appears to be true for the reaction catalyzed

⁷ Riepe (54) and Riepe and Wang (42) have given the ν_3 frequencies for CO₂ bound at the two sites detected⁶ in bovine carbonic anhydrase. It is interesting to note that CO₂ is bound to Site B ($K_{\text{diss}} = 42$ mM) more tightly than to Site A ($K_{\text{diss}} = 75 \pm 25$ mM) (54), the former value being closer to the Michaelis constant (12 mM) for the bovine enzyme (6). The K_m is believed on kinetic grounds to reflect the true substrate dissociation constant. While it is not possible to tell whether Site B is the true substrate-binding site, it is difficult to eliminate this possibility on the available evidence, and it must be kept in mind. Note that Riepe (54) has found that NO₃⁻ and ethoxzolamide do not inhibit CO₂ binding at Site B.

by carbonic anhydrase. Devoe and Kistiakowsky (61) have shown that H₂CO₃ cannot be a substrate in the dehydration reaction, otherwise rates exceeding the diffusion limit would have to be postulated. Another ambiguity is associated with the hydroxide reaction, since a direct attack of OH⁻ on CO₂, as written in Equation 3, is formally indistinguishable from a hydroxyl ion catalysis of the other two pathways (Equations 1 and 2). It is interesting in this respect that no catalysts of the hydroxyl ion reaction have apparently been established, and in fact, it has sometimes been possible to argue against their existence in some studies (62). This is what one would expect if the hydroxyl ion itself was a catalyst, *i.e.* if the mechanism of the reaction of Equation 3 was not a simple attack of OH⁻ on CO₂. Other interpretations are, of course, possible.

Evidence has now accumulated to suggest that the catalysis of CO₂ hydration-dehydration by carbonic anhydrase is intimately dependent on the water molecule believed to be bound to the zinc at the active site (52). A variety of studies have indicated that the ionization of this zinc-bound water, with pK near 7, is the basis of the pH dependence of the activity. It is presumed that the basic form (ZnOH) is active in the hydration reaction, while the acid form (ZnOH₂⁺) is active in the dehydration reaction and in the binding of monoanion inhibitors (see discussion by Bradbury (63)). This has suggested to a number of investigators that the catalytic mechanism of carbonic anhydrase involves primarily the attack of the zinc-bound hydroxide or water on CO₂ in the hydration reaction, with the resulting product, HCO₃⁻, presumably being bound to the zinc (20–22, 42, 52, 64, 65). One particular view, for example, holds that an essential imidazole side chain, acting as a base, must facilitate the attack of the coordinated hydroxide or water on CO₂ (21, 22, 66). Wang (20) has thus compared the nucleophilicity of the zinc-bound hydroxide with that of free OH⁻ and concluded that, on the basis of their reactivities in CO₂ hydration, the bound OH must be made more reactive by a factor of about 1×10^8 to account for the observed enzymatic rate. This led to the suggestion (21, 22) that there must be an essential imidazole in the active site that increases the nucleophilicity and rate of reaction by hydrogen bonding to the zinc hydroxide.

Chemical modification studies on carbonic anhydrase do not show that the basic side chains required by the above type of mechanism, *e.g.* imidazole⁸ or amino groups (46), play an essential role in the catalytic mechanism. This is not too surprising, since there are fundamental considerations based on the nature of the CO₂ hydration reaction itself that indicate that theories based solely on an "activated water" hypothesis are unlikely to provide a full explanation of the catalytic mechanism of carbonic

⁸ The specific, covalent modification of histidines in the active sites of various carbonic anhydrases causes a large decrease in catalytic activity (45–47, 63). This has frequently been taken as supporting the participation of an *essential* histidine side chain in the catalytic mechanism of the enzyme (9, 21, 67). With one exception involving a very bulky reagent (45), all the modified enzymes possess residual activity of roughly one order of magnitude below that of the native enzymes. As previously noted (46, 63), this does not warrant assigning an essential role to these modifiable histidine side chains, although their presence in the active site may account for some of the observed differences in specific activity between various isoenzymes. We have carried out kinetic studies on the residual CO₂ hydration activity of some of these modified carbonic anhydrases, and these will be reported in a following publication.

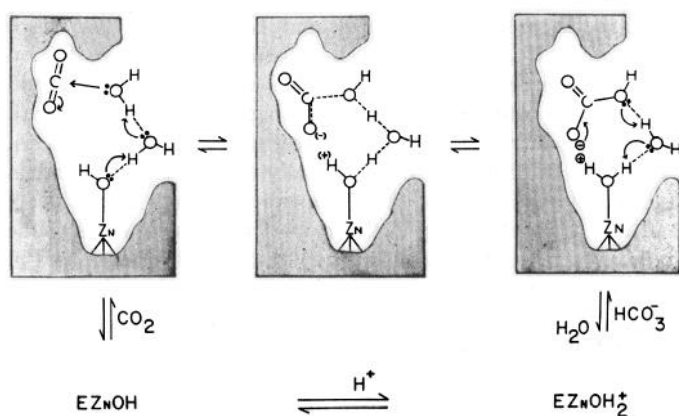


FIG. 5. Schematic illustration of a catalytic mechanism for CO_2 hydration-dehydration by carbonic anhydrase in which the zinc-bound water participates in both the electron donor and acceptor requirements of the reaction (see text). It is not intended to imply that the CO_2 and HCO_3^- bind competitively to the enzyme. The protonation-deprotonation reactions of the enzyme in absence of bound substrates are believed to be unusually rapid, and represent an important aspect of the over-all catalysis.

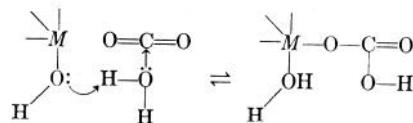
anhydrase. As noted above, there is no certainty that even the nonenzymatic hydration at high pH involves *only* a nucleophilic attack of a hydroxyl group on CO_2 . The strongest evidence on this point comes from the study of Breslow on the catalysis of CO_2 hydration by copper-peptide complexes whose activity is linked to an ionizable water ligand of the metal (62). Breslow noted that the 1:1 complex of Cu(II) and glycylglycine has a pK of about 9, *i.e.* the coordinated OH is about 1×10^7 times less basic than free OH^- , yet it is only about 20 times less effective than free OH^- in the catalysis of CO_2 hydration. The studies of Breslow and especially of Dennard and Williams (68) have also indicated that general base catalysis cannot be sufficient to account for the catalytic efficiency of a wide range of catalysts, including organic bases and oxyanions. No simple correlation could be observed between the catalysis of these substances and their base strengths.

The above considerations indicate that in both the enzymatic and the nonenzymatic catalysis, there are important requirements in addition to the activation of the water substrate.⁹ One such requirement becomes evident when it is noted that the attack of an OH^- group on the carbon of CO_2 is necessarily accompanied by the development of electronic charge on one or both of the oxygens of the molecule. Considerable stabilization of the transition state in the hydration can thus occur if there were means of efficiently neutralizing the developing charge on the incipient HCO_3^- . Dennard and Williams (68) have noted that good catalysts have not only electron donor properties, but also acceptor ones, *i.e.* "polyfunctional" catalysis is involved.

⁹ The activation of the other substrate, CO_2 , by the induction of "strain" because of its distortion in the enzyme-substrate complex appears a little unlikely at present. The small size of the CO_2 molecule and its almost diffusion-limited rate of combination with the enzyme (at least 1×10^7 to $1 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$, which is the value of k_{cat}/K_m) do not seem to favor this possibility. Nevertheless, it cannot be ruled out, and as pointed out by Edsall (2), the bending of the CO_2 molecule is expected to greatly facilitate its rate of hydration. This view is strongly supported by the observation that the hydration of the bent molecule SO_2 proceeds approximately 10^8 times as rapidly as that of the linear CO_2 molecule in the absence of catalysts (Reference 59, p. 1038).

Breslow (62) reached a similar conclusion, and she suggested that the metal ion could provide the acceptor function. Such a charge neutralization may be particularly important in the active site of carbonic anhydrase, especially if CO_2 were bound in an environment that hinders solvation of intermediates or lacks the possibility of proper charge neutralization, since in that case the activation energy would be prohibitively raised.

The mechanism suggested by Breslow for the catalysis of CO_2 hydration has interesting implications for the enzymatic catalysis, and it can be expressed as follows.



An analogous concerted mechanism was suggested by Dennard and Williams (68) for polyfunctional catalysis by oxyanions. The attractive feature of the above mechanism is that both donor and acceptor functions revolve around the metal ion (M), so that it is unnecessary to invoke the participation of other functional groups for which no evidence exists in carbonic anhydrase. However, it does require a change in metal coordination number, at least in the transition state, so that the zinc in the enzyme must be capable of accepting a fifth ligand with a minimum of distortion and bond rearrangement. The metal-ligand bond rearrangements could be slow, since they might be linked to changes in the conformation of the protein. The mechanism also implies that the CO_2 substrate would have to be bound rather close to the zinc, and it is already known that many zinc ligands, *e.g.* monoanions, are noncompetitive inhibitors of CO_2 hydration.

These difficulties, if they actually exist in carbonic anhydrase, may be overcome by the general type of mechanism shown in Fig. 5 that retains all the essential requirements for catalysis discussed above, as well as the structural information already available about carbonic anhydrase. In this mechanism, the donor and acceptor functions originate on the zinc-bound water. These two functions can operate by a "push-pull," concerted mechanism that minimizes the development of uncompensated charge in the active site throughout the course of the transformation. It can also utilize water bridges, depending on the distance of the bound CO_2 to the metal-bound hydroxide. Proper orientation of the substrates could lead to a stereochemistry in which the proton transfers occur along existing or potential hydrogen bonds, and hence could be quite rapid. The inherent advantages of such cooperative mechanisms in acid-base catalysis of fast reactions have been pointed out by Eigen (69) and others. A rather different cyclic mechanism involving water bridges has been suggested previously by Kaiser and Lo (13).

Since ligand substitution processes are fast for zinc complexes (59), it is not essential that the normal coordination of HCO_3^- be as an outer sphere ligand. However, such binding does not appear to be incompatible with existing evidence, and, in fact, Pocker and Dickerson have previously suggested similar binding for some monoanion inhibitors of carbonic anhydrase (67), although not for HCO_3^- (70). It is well known, for example, that the binding affinity of monoanion inhibitors of carbonic anhydrase does not parallel their ligand field strength, but follows instead the so-called Hofmeister series (33, 41, 70). This indicates an unusual coordination mode for anion binding. The x-ray studies on carbonic anhydrase provide direct evidence

that in the case of the iodide inhibitor, the distance between the bound anion and the zinc is greater than expected for direct coordination (7). An outer sphere coordination need not hold for all the anionic inhibitors, but it is certainly more plausible for HCO_3^- in view of the spectroscopic studies on the effect of inhibitors on the visible spectrum of the catalytically active cobalt form of the enzyme. The data of both Lindskog (33) and Coleman (64, 71) show that the visible spectrum of cobalt carbonic anhydrase in the presence of HCO_3^- is very similar to that of the uninhibited enzyme at low pH, where it presumably exists as Co(II)OH_2^+ . Coleman has also noted that this similarity extends to the comparison of the optical rotatory properties of cobalt carbonic anhydrase (71).

In conclusion, attention should be drawn to one paradoxical aspect of the carbonic anhydrase catalysis of CO_2 hydration. The assumption that HCO_3^- is the substrate in the dehydration reaction leads to the interpretation of the pH rate profiles as requiring the basic form of the enzyme to be active in CO_2 hydration and the acid form in the dehydration of HCO_3^- . Since the enzyme undergoes a change in its state of protonation during substrate turnover, it must subsequently undergo the reverse protonation in order to return to its active form. However, the ionization processes for groups with pK near 7 commonly have rate constants of around $1 \times 10^8 \text{ sec}^{-1}$ (59, 60, 72), well below the 1×10^5 to $1 \times 10^6 \text{ sec}^{-1}$ turnover rates encountered with carbonic anhydrase (Fig. 3). The latter values imply that proton transfer from and to the active site must be unusually fast. The enzyme must then have special means of facilitating proton transfer between the active site and solvent molecules in absence of bound substrates. This aspect of the catalysis by carbonic anhydrase appears just as important as the requirement for rapid, intramolecular proton transfer between the enzyme and the bound substrates. The possible explanation that both of these fast proton transfer processes are facilitated by protein side chains (72) is not supported by the available evidence.⁸ Accurate determination of the rates of ionization of the water bound to the zinc or cobalt in carbonic anhydrases should be of much value in understanding this aspect of the catalysis.¹⁰

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APPENDIX

Note on Use of pH Indicator Technique, with Special Reference to CO_2 Hydration Reactions

pH Dependence of Buffer Factor—The buffer factor discussed above can be derived, following DeVoe (30) and Gibbons and Edsall (26), by using relations such as

$$Q = \left(\frac{\partial x}{\partial A} \right)_t = \left(\frac{\partial x}{\partial \alpha_B} \right) \left(\frac{\partial \alpha_B}{\partial \alpha_{In}} \right) \left(\frac{\partial \alpha_{In}}{\partial A} \right) \quad (4)$$

¹⁰ Dr. J. E. Coleman has also emphasized the problem of the unusually fast proton transfer that was mentioned above. He has furthermore proposed an attractive explanation in terms of the bound water at the active site that is suggested by the x-ray studies of the Uppsala group (see contribution by J. E. Coleman in *Progress in Bioorganic Chemistry* (73)).

where x represents the amount of acid added to the buffer solution, A is the absorbance at a given wave length, α_B and α_{In} are the buffer and indicator fractions in the basic form. If the indicator is assumed not to contribute to the buffering capacity of the solution, one can derive the following expression that is equivalent to that given by Gibbons and Edsall (26),

$$Q = \left(\frac{C_B}{L \cdot E \cdot C_{In}} \right) \left(\frac{K_B}{K_{In}} \right) \left(\frac{K_{In} + H^+}{K_B + H^+} \right)^2 \quad (5)$$

Here L is the optical path length, E is the difference in extinction coefficients of the basic and acid forms of the indicator at the wave length of interest, and C_B and C_{In} are the total concentrations (acid + base forms) of the buffer and the indicator species, respectively, whose ionization constants are K_B and K_{In} . Inspection of the above equation immediately reveals that when K_B is equal to K_{In} , the buffer factor Q will be independent of pH for any pH or buffer value of the system. In this respect, the use of the absorption bands of the buffer would be ideal. In other cases, Q will depend on pH in the useful pH range of the buffer system, this dependence being greater with increasing difference between K_B and K_{In} . This can be visualized by making plots of the quantity y in

$$y = \frac{QC_{In}LE}{C_B} = \left(\frac{K_B}{K_{In}} \right) \left(\frac{K_{In} + H^+}{K_B + H^+} \right)^2 \quad (6)$$

as a function of pH for different values of the ratio K_B/K_{In} . The resulting family of curves can be conveniently normalized to intersect at $y = 1$, by choosing the abscissa variable as $\text{pH} - 0.5(\text{p}K_B + \text{p}K_{In})$ instead of pH. This follows because Equation 6 can be rewritten as

$$y = \left(\frac{k + h}{1 + kh} \right)^2 \quad (7)$$

where $k = \sqrt{K_{In}/K_B}$, and $h = (H^+)/\sqrt{K_B K_{In}}$. Another way in which the sensitivity of Q (or y) to pH can be appreciated is to calculate the fractional change in Q per unit change in pH, i.e. the quantity f defined by

$$f = \frac{1}{Q} \left(\frac{\partial Q}{\partial \text{pH}} \right)$$

for several values of the ratio K_{In}/K_B , by use of Equation 7. Since this quantity will be somewhat dependent on pH, the calculations can be made at one reference pH, which can be taken for convenience to be $\text{pH} = 0.5(\text{p}K_B + \text{p}K_{In})$, i.e. midway between the pK values of the buffer and the indicator. For these conditions, it is easy to show that f will be given by

$$f = \frac{1}{Q} \left(\frac{\partial Q}{\partial \text{pH}} \right) = \frac{4.6 [\sqrt{K_{In}/K_B} - 1]}{\sqrt{K_{In}/K_B} + 1} \quad (8)$$

One thus finds that if $\text{p}K_B - \text{p}K_{In} = 0.3$, then $f = 0.8$, i.e. Q changes by about 8% for each 0.1 change in pH. For $\text{p}K_B - \text{p}K_{In} = 1.0$, $f = 2.3$. The importance of matching the buffer and the indicator pK values should be quite evident. It is commonly assumed that if the pK of the indicator is close to the pH under study, then the variation of the buffer factor with pH is minimized. This is not true, as can be seen from calculations at $\text{pH} = \text{p}K_{In}$, for which f is given by

$$f = \frac{1}{Q} \left(\frac{\partial Q}{\partial \text{pH}} \right) = \frac{2.3 [(K_{In}/K_B) - 1]}{(K_{In}/K_B) + 1} \quad (9)$$

We thus find that for $pK_B - pK_{In} = 0.3$, then $f = 0.74$, and for $pK_B - pK_{In} = 1.0$, then $f = 1.8$.

Buffer Factor for CO_2 Hydration at High and Low pH—At neutral pH the hydration of each CO_2 molecule leads to the formation of only HCO_3^- , so that 1 proton (x) is released in the reaction. In that case the rate of absorbance change is related to the rate of hydration by the relation

$$\frac{d(CO_2)}{dt} = \left(\frac{\partial(CO_2)}{\partial A} \right)_t \left(\frac{dA}{dt} \right) = \left(\frac{\partial x}{\partial A} \right)_t \left(\frac{dA}{dt} \right) \quad (10)$$

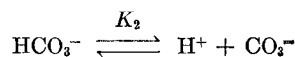
The expression for Q derived above in Equations 4 and 5 is then applicable for the CO_2 hydration reaction, and one can determine Q by titration with a standard acid. However, at high pH values CO_3^{2-} formation becomes important, so that more than one proton is released for each CO_2 molecule hydrated. The relevant buffer factor is then Q_c in the expression

$$\left(\frac{1}{Q_c} \right) = \left(\frac{\partial A}{\partial(CO_2)} \right)_t$$

and Q_c does not correspond to the expressions of Equations 4 and 5, since $\partial A / \partial(CO_2)$ no longer is equal to $\partial A / \partial x$. Instead it must be calculated with the relation

$$Q_c = \left(\frac{\partial(CO_2)}{\partial \alpha_B} \right) \left(\frac{\partial \alpha_B}{\partial \alpha_{In}} \right) \left(\frac{\partial \alpha_{In}}{\partial A} \right) \quad (11)$$

Experimentally, Q_c can be derived only from titrations with CO_2 as the added acid. The titration plots will not be linear, however, even if $K_B = K_{In}$, since the CO_3^{2-} ionization equilibrium



will produce an additional dependence of Q_c on pH. This can be evaluated from a straightforward derivation that leads to the following expression for Q_c

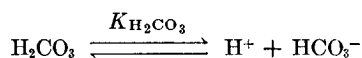
$$\left(\frac{Q_c}{Q} \right) = 1 + \left\{ \frac{(H^+ + K_B)(H^+ - H_0^+)K_2}{(H_0^+ + K_B)} - (H^+ + 2K_2)K_2 \right\} \frac{1}{(H^+ + 2K_2)^2} \quad (12)$$

where Q is given by Equation 5, and H_0^+ and H^+ refer to the hydrogen ion concentrations in solution before and after the addition of CO_2 . For small increments of added CO_2 or in well buffered solutions, one can use the approximation that $H^+ = H_0^+$, so that Equation 12 simplifies to

$$\frac{Q_c}{Q} = 1 - \left(\frac{K_2}{H^+ + 2K_2} \right) \quad (13)$$

At the high pH limit, each CO_2 molecule hydrated releases 2 protons, and as expected Equation 13 leads to Q_c/Q of one-half. At low pH, Q_c/Q is unity, and CO_3^{2-} can be ignored. A plot of Q_c/Q versus pH would yield a sigmoidal curve that varies between 1.0 and 0.5, with a midpoint at a pH a little below pK_2 .

In the present study, the pH was always sufficiently below pK_2 , so that complications arising from carbonate formation were avoided. At low pH analogous problems can arise, because of contributions from the ionization,



The relevant Q value for the hydration reaction is then expected to increase to infinity in the low pH limit. This again was not a problem in this study.

All the complications arising from the use of buffer factors could be entirely avoided if a method were available for directly following changes in CO_2 concentration. The most reasonable way to achieve this would be to combine the stop-flow technique with infrared absorption detection, taking advantage of the strong infrared absorption bands of CO_2 . Such an approach should also allow the study of buffer effects at much higher buffer concentrations than can be tolerated by the pH indicator method.

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