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THE DEHYDRATION KINETICS OF HUMAN ERYTHROCYTIC CARBONIC ANHYDRASES B AND C

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SUMMARY

1. The activity of Forms B and C of human erythrocytic carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) has been studied at 1.6°. The catalytic effect of the enzymes on the dehydration of carbonic acid was measured by means of a pH-stat method.

2. The dependence of the activity of Form C on the hydrogen ion activity has been studied between pH 6.9 and 7.9. The formation of the enzyme–substrate complex appears to be dependent on the acid form of a group which ionizes within this pH range, whilst the breakdown of the complex into free enzyme and the product, CO_2, proceeds independently of ionizing groups.

3. Conversely, the breakdown of the B enzyme–substrate complex into CO_2 and free enzyme appears to be dependent on the acid form of a group with $pK = 7.4$, whilst the formation of this enzyme–substrate complex appears to proceed independently of groups ionizing between pH 6.8 and 8.4.

4. Monovalent anions act as inhibitors for both enzymes. When listed in order of increasing inhibitory effectiveness towards the C enzyme, the following series is obtained: $Cl^- < Br^- < NO_3^- < I^- < SCN^-$. The inhibitory effect of anions is reversible and decreases with increasing pH. Sulphate, phosphate, and citrate do not affect the activity of the enzyme in concentrations below 0.1 M.

INTRODUCTION

In 1961 NYMAN[1] demonstrated that human erythrocytes contain 3 forms of the enzyme carbonic anhydrase, later denoted A, B and C (see ref. 2). The physical and chemical properties of the isolated forms have been studied by several groups of investigators[3–6]. The studies which mainly concern the B and C enzymes have revealed marked dissimiliarities between these two forms of carbonic anhydrase.

* Part of the experimental work has been performed at The Department of Blood Fractionation, Statens Seruminstitut.

GIBBONS AND EDSALL[7] have demonstrated that differences exist between the B and C forms with respect to the pH dependence of the catalytic effect of the enzymes on the hydration of CO_2. The present paper reports the results of a study of the dehydration kinetics of the enzymes. The measurements have been performed at 1.6° using a pH-stat method[8]. It is a characteristic feature of this method that the reaction product, CO_2, is removed from the reaction mixture as rapidly as it is formed during the course of the reaction. Consequently, corrections of the experimental data for the reciprocal reaction, which rapidly becomes significant if CO_2 is allowed to accumulate in the reaction mixture[9], are avoided.

MATERIALS

Purified enzyme B and enzyme C were prepared by Dr. P.-O. NYMAN[1] who kindly placed the preparations at our disposal for this study. The storage and handling of the enzyme preparations and the spectrophotometric determination of the molar concentration of the enzyme in the working solutions are described elsewhere[10]. Human albumin (Statens Seruminstitut) was used as a stabilizing agent and was added to the dilute enzyme solutions to a concentration of 0.5 g/l.

The reaction mixture used for the determination of carbonic anhydrase activity contained KH_2PO_4 and $NaHPO_4$ in a total concentration of either 10 or 13 mM, and EDTA in a concentration of 0.5 mM. In this concentration EDTA eliminates inhibition due to contamination by heavy-metal ions[10]. Solutions of $NaHCO_3$ were prepared on the day of use. Analytical grade chemicals dissolved in demineralized water were used throughout.

The equipment which was used for the pH measurements was manufactured by Radiometer, Copenhagen, and consisted of a pH meter type 26 in connection with a titrator type 11. Alternatively a titrator type TTT 1a was used. The glass electrodes were type G 202 C; the calomel electrodes were type K 4016 and K 101. The standard reference solution for the pH measurements contained 25 mM KH_2PO_4 and 25 mM Na_2HPO_4. The pH in this buffer is 6.97 at 2° (see ref. 11).

METHOD

The rate of formation of CO_2 in a solution containing $NaHCO_3$ (the dehydration reaction) has been determined at $1.6° \pm 0.3°$ by means of a pH-stat method[8]. The reaction is initiated by mixing a solution of $NaHCO_3$ with a phosphate buffer to a total volume of 10 ml in a reaction chamber. The reaction product, CO_2, is removed as rapidly as it is formed by bubbling CO_2-free air or pure N_2 through the contents of the reaction chamber at a flow rate of 700–1000 ml per min. H_2SO_4 is used as titrant. The amount of H^+ which must be added per l reaction mixture per unit time in order to keep pH constant is used as a measure of the rate of the dehydration reaction.

Some of the results to be described were obtained from observing the reaction rate within the 1st min after initiation of the experiment (Procedure I). In this procedure the concentration of bicarbonate in the reaction mixture is calculated from the molarity of the $NaHCO_3$-working solution and the dilution which occurs upon mixing buffer and the $NaHCO_3$ solution in the reaction chamber. The titrant is delivered from a burette in increments of approx. 10 μl. The initial rate of the reac-

tion is calculated from the amount of acid added in a 30-sec measuring period[8,10].

During the present study the technique was changed (in collaboration with Dr. B. O. TURBECK) so that it became possible to determine the course of the dehydration reaction until more that 90% of the bicarbonate initially present had been converted to CO_2 and driven off (Procedure II). In this procedure the titrant H_2SO_4 is delivered to the reaction chamber by means of a syringe in increments of 0.1–0.6 μl. The number of increments which are delivered from the syringe during the course of the reaction is registered automatically at 60-sec intervals. The total amount of acid which has been added to the reaction mixture when the reaction has proceeded to completion is equivalent to the amount of $NaHCO_3$ initially present. The concentration of HCO_3^- at any time during the experiment can be calculated from this value and the amount of acid added at the given time. As an approximation, the substrate concentration in a 60-sec period has been taken to be the arithmetic mean of the concentrations of $NaHCO_3$ at the beginning and at the end of the period. The corresponding initial rate has been calculated from the amount of acid added within the given period. Thus the initial rate of the reaction at a number of bicarbonate concentrations can be obtained from a single experiment.

The carbonic anhydrase activity (v) is determined as the difference between the observed initial rate of the enzyme catalysed reaction (r_{obs}) and the initial rate of the spontaneous dehydration reaction (r_s). At pH$<$7.5 and ionic strengths below 0.1 M, r_s can be calculated from the equation[13]:

$$\log\left(\frac{r_s}{[HCO_3^-]}\right) = \log k_d^o - \mathrm{pH} + f_{HCO_3^-} \qquad (1)$$

in which $[HCO_3^-]$ is the molar concentration of bicarbonate and $f_{HCO_3^-}$, the activity coefficient, is calculated by means of the Debye–Hückel equation. k_d°, the 2nd order velocity constant for the dehydration reaction, is defined by the equation:

$$-\frac{d[HCO_3^-]}{dt} = k_d^o \cdot (H^+) \cdot [HCO_3^-] \cdot f_{HCO_3^-} \qquad (2)$$

in which $d[HCO_3^-]/dt$ is the amount of bicarbonate converted per l solution during the time dt, and (H^+) is the activity of the hydrogen ion as measured by the glass electrode. k_d° at 1.6° has been found to be 0.93×10^4 $M^{-1} \cdot sec^{-1}$ (see ref. 13). In reaction mixtures in which the ionic strength was $>$0.1 M or pH$>$7.5, the rate of the spontaneous reaction has been determined in separate experiments.

In all enzyme-catalysed runs the concentration of enzyme was chosen so that r_{obs} was at least twice r_s. Usually r_{obs}/r_s was between 3 and 5. Mean values of at least 2 determinations have been used. The coefficient of variation as calculated from duplicate determinations of r_{obs} is 5–7%.

RESULTS

Stability of the enzymes

The determination of the carbonic anhydrase activity has been performed in reaction mixtures which were turbulent due to mechanical stirring and to the stream of gas which passes through the reaction mixture during the measurements. In order to determine whether the enzyme preparations used were stable under these experi-

mental conditions, the activity of the enzymes was determined in solutions through which CO_2-free air had been bubbled and which had been stirred in the reaction chamber for various periods of time before the measurements were carried out. The results, which were obtained by means of Procedure I, are shown in Table I. No decrease in enzyme activity could be demonstrated in solutions through which gas had been bubbled for up to 5 min prior to the measurements. The C enzyme which has also been investigated by means of Procedure II, has been found to retain its catalytic activity in reaction mixtures through which gas had been bubbled for more than 15 min (unpublished data).

TABLE I

THE STABILITY OF CARBONIC ANHYDRASES B AND C IN THE REACTION CHAMBER

CO_2-free air was bubbled through a 13 mM phosphate buffer (pH 7.2) containing carbonic anhydrase B or carbonic anhydrase C at a flow rate of 1 l per min for various periods of time. The dehydration reaction was then initiated by adding $NaHCO_3$ to a concentration of 40 mM. The rate of the reaction was determined during the following 30 sec. Temp., 1.6°; concn. of Form B: 7 mμM; concn. of Form C: 0.5 mμM.

Duration of bubbling through prior to measurements (min)	*Catalytic activity (v; $\mu M \cdot sec^{-1}$)*	
	Form B	*Form C*
0	30	27
1	30	28
3	31	28
5	29	27

The enzymic activity of solutions containing approx. 0.1 μM carbonic anhydrase and human albumin in a concentration of approx. 0.5 g/l has been found to be stable at pH values between pH 6 and 9 on standing for 4–6 h at 4°. Concentrated aqueous solutions of the enzymes (approx. 0.1 mM) have been stored for several months at 4° without significant loss of enzyme activity. Similar findings have been reported by other investigators[7,14].

Effect of anions

The effect of Cl^- on the catalytic activity of the B enzyme was studied in a 13 mM phosphate buffer at pH 7.3 by means of Procedure I. The initial rate of the reaction was determined at 6 concentrations of $NaHCO_3$ ranging from 10 to 35 mM. The results are shown in Fig. 1 as the reciprocal of the initial rate against the reciprocal of the concentration of $NaHCO_3$. Least square treatment yielded coefficients of correlation >0.99 and standard errors of estimate between 5 and 15% of the intercept on the ordinate[12]. The results suggest that NaCl acts on the B enzyme as a 'mixed type' inhibitor[15].

The catalytic activity of Form C is also affected by various anions. This is apparent from Fig. 2 which shows LINEWEAVER–BURK plots[16] of some results obtained by means of Procedure II. The experiments were performed in a 10-mM phosphate

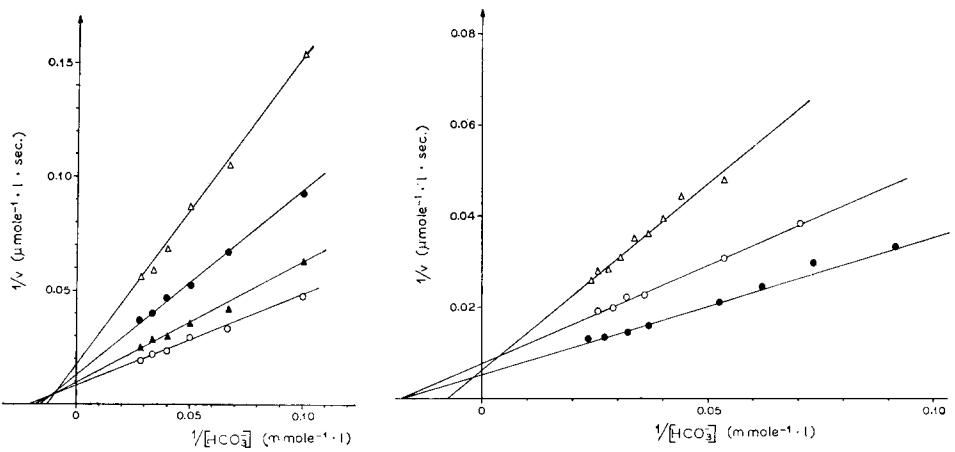


Fig. 1. Inhibition of carbonic anhydrase B by Cl^-. Ordinate: reciprocal activity ($1/v$, $\mu M^{-1} \cdot sec$); abscissa: reciprocal concentration of HCO_3^- (mM^{-1}). 13 mM phosphate buffer (pH 7.3). Enzyme concn.: 14 mμM. △, 80 mM NaCl; ●, 40 mM NaCl; ▲, 20 mM NaCl; ○, no NaCl added.

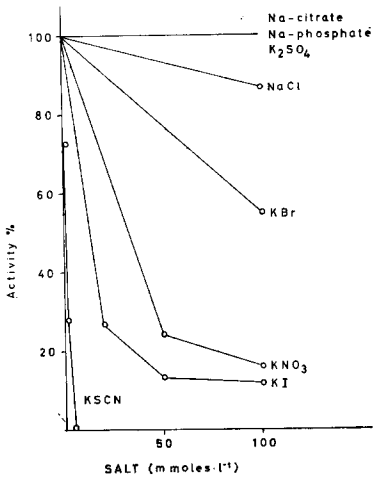
Fig. 2. Inhibition of carbonic anhydrase C by Br^- and SCN^-. Ordinate: reciprocal activity ($1/v$, $\mu M^{-1} \cdot sec$); abscissa: reciprocal concentration of HCO_3^- (mM^{-1}). 10 mM phosphate buffer (pH 7.3). Enzyme concn.: 1.0 mμM. △, 0.5 mM KSCN; ○, 50 mM KBr; ●, 100 mM K_2SO_4, is similar to results obtained in the absence of added salt.

buffer at pH 7.3. Some inhibition was apparent in the presence of 50 mM KBr, whilst KSCN in a concentration of 0.5 mM had a marked inhibitory effect. Na_2SO_4 in a concentration of 100 mM had no measurable effect.

The order of effectiveness with which different anions inhibit carbonic anhydrase C has been determined from measurements similar to those shown in Fig. 2. The slope of the line through points obtained from 10–12 consecutive 60-sec-measuring periods was determined by means of the method of least squares. The reciprocal value of the slope (v_{max}/K_m) which describes both competitive and non-competitive components of the inhibition, has been used as a measure of the enzyme activity. Least square analysis on the data from 8 experiments performed in the absence of inhibitory anions yielded coefficients of correlation >0.96. The individual values for v_{max}/K_m deviated less than 12% from the mean value. In Fig. 3 the activity (in percentage of the mean activity determined in the absence of inhibitor) is shown as a function of the concentration of added salt. Citrate, phosphate and sulphate in concentrations below 0.1 M had no measurable effect on the catalytic activity of Form C. Chloride, bromide, nitrate, and iodide exerted an inhibitory effect increasing in that order. Thiocyanate, which is by far the strongest inhibitor in this series, eliminated the catalytic activity in a concentration of only 5 mM.

The anionic inhibition of both forms of the enzyme increases with decreasing pH as demonstrated in Fig. 4. These results were obtained using Procedure I, by measuring the enzyme activity in a 13-mM phosphate buffer containing 50 mM $NaHCO_3$ in the absence of inhibitors (v) and in the presence of added salt (v_i) at pH 6.8, 7.4, and 8.0. The results have been plotted as log (v_i as percentage of v) against log (concentration of added salt).

The inhibitory effect of anions is reversible. This can be concluded from the



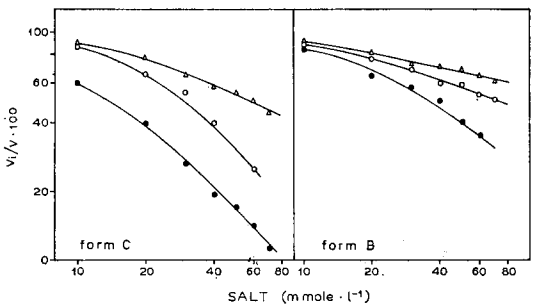


Fig. 3. Inhibition of carbonic anhydrase C by various anions. Ordinate: activity as percentage of activity in the absence of inhibitor; abscissa: concentration of added salt (mM). 10 mM phosphate buffer (pH 7.3). Enzyme concn.: 1.0 mμM.

Fig. 4. Anion inhibition of carbonic anhydrase C and B at various pH values. Ordinate: activity as percentage of activity in the absence of inhibitor; abscissa: concn. of added salt (mM). log — log scale. 13 mM phosphate buffer containing 50 mM $NaHCO_3$. KNO_3 was added in the case of enzyme C (left) and NaCl in the case of enzyme B. △, pH 8.0; ○, pH 7.4; ●, pH 6.8.

finding that the activity of form C was unaffected on dilution after incubation for 18 h in 0.5 M solutions of NaCl and KSCN. Incubation in 8 M urea caused an irreversible inactivation of approx. 90% of the enzyme (Table II).

Effect of pH

The linearity of the plots in Figs. 1 and 2 indicates that the kinetics of the dehydration reaction catalysed by both forms of carbonic anhydrase are in accordance with the Michaelis–Menten reaction[16,17]:

TABLE II

THE ACTIVITY OF CARBONIC ANHYDRASE C AFTER INCUBATION WITH ANIONIC INHIBITORS

Carbonic anhydrase C was incubated at pH 7.0 and 4°. At the end of the incubation period the activity *(v)* was determined in a dilution 1:10 000. *v* is the initial rate of the enzyme-catalysed reaction in a 10 mM phosphate buffer (pH 7.3) and a concentration of $NaHCO_3$ of 35 mM. The reaction medium contained 1.4 mμM enzyme C.

Inhibitor	*Concn. of inhibitor in incubation medium (M)*	*Activity (v; $\mu M \cdot sec^{-1}$) after incubation for*	
		2 h	*18 h*
None	—	67	69
NaCl	0.5	—	67
KSCN	0.5	60	62
Urea	8.0	6	5

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \xrightarrow{k_{+2}} E + P \tag{3}$$

k_{-1} and k_{+2} are the 1st order rate constants for the dissociation of the enzyme–substrate complex (ES) into substrate (S) and product (P), which in the present context are assumed to be HCO_3^- and CO_2 respectively. k_{+1} is the 2nd order rate constant for the formation of the enzyme–substrate complex. The Michaelis constant for the dehydration reaction is defined as:

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \tag{4}$$

and the rate of the reaction at the substrate concentration which saturates the enzyme as:

$$v_{max} = k_{+2} \cdot E_0 \tag{5}$$

where E_0 is the concentration of the enzyme.

In order to evaluate the dependence of K_m and k_{+2} on the hydrogen-ion activity, the catalytic activity of Form B was studied at different pH values between 6.8 and 8.4, using Procedure I. At each pH the initial rate of the dehydration reaction was determined at 6 concentrations of $NaHCO_3$ (range 10–35 mM) in 13 mM phosphate buffers. The enzyme concentration was 13 mμM in all experiments. K_m and v_{max} at

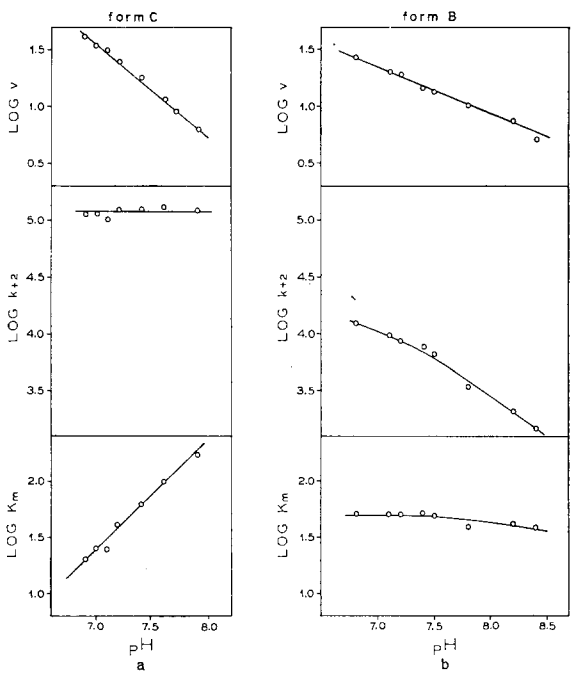


Fig. 5. The dehydration reaction catalysed by carbonic anhydrase C and carbonic anhydrase B; dependence on pH. Ordinate: log v (v in $\mu M \cdot sec^{-1}$), log k_{+2} (k_{+2} in sec^{-1}) and log K_m (K_m in mM); abscissa: pH. 13 mM phosphate buffer. Concn. of Form C: 1.0 mμM; concn. of Form B: 13 mμM. v (top of figure) was determined at an initial concentration of $NaHCO_3$ = 10 mM.

each pH value were calculated by means of least square analyses. The C enzyme in a concentration of 1.0 mμM was examined in the same manner in the pH range 6.9–7.9. The results are shown in Fig. 5.

k_{+2} for carbonic anhydrase C was approximately constant within the pH range 6.9–7.9 (Fig. 5a). The mean value of k_{+2} was found to be $1.1 \cdot 10^5$ sec^{-1} (range $0.9 \cdot 10^5$–$1.3 \cdot 10^5$ sec^{-1}). This finding indicates that the dissociation of the enzyme–substrate complex into enzyme and CO_2 proceeds independently of groups which ionize in the pH range investigated. K_m varied from 20 mM (pH 6.9) to 180 mM (pH 7.9). A plot of log K_m against pH yields a straight line with the slope +1. The findings are in accordance with the assumption that HCO_3^- reacts with an acid form of the enzyme which ionizes in this pH range. It can be excluded that H_2CO_3 is the substrate, as the rate constant for a reaction between carbonic acid and the C enzyme (approx. 10^{10} $l \cdot M^{-1} \cdot sec^{-1}$) is larger than possible for the diffusion of a small molecule to a protein (*cf.* discussion by DEVOE AND KISTIAKOWSKY[9] and KERNOHAN[14]).

k_{+2} for carbonic anhydrase B (Fig. 5b) decreased with increasing pH from $1.3 \cdot 10^4$ sec^{-1} (pH 6.8) to $1.5 \cdot 10^3$ sec^{-1} (pH 8.4), K_m was almost constant (54–50 mM) between pH 6.8–7.5 and insignificantly lower (41–37 mM) between pH 7.5–8.4. Thus a 9-fold change in k_{+2} had little effect on K_m which can be considered to be almost equal to k_{-1}/k_{+1}, the dissociation constant for the HCO_3^--binding site. The rate limiting reaction is the breakdown of the enzyme–substrate complex. This reaction appears to be dependent on the acid form of a group with a pK value close to 7.4.

Carbonic anhydrase C has a higher catalytic activity per unit weight than the B form as first demonstrated by NYMAN[1]. In a reaction mixture containing 10 mM $NaHCO_3$, the C form was found to be 22 times as active at pH 6.9 and 9 times as active at pH 7.9 as the B enzyme (Fig. 5, top).

DISCUSSION

The results in Figs. 1–4 show that both forms of human carbonic anhydrase are inhibited by monovalent anions. The findings are in general agreement with the results obtained with semipurified preparations of human and bovine carbonic anhydrase[9,14,18,19]. When the anions are listed according to their inhibitory effect towards the C enzyme (Fig. 3), a sequence is obtained which is frequently referred to as the lyotropic series of Hofmeister[20]. Comparable series of anions have been obtained on the basis of their inhibitory effectiveness towards several enzymes[21] and in connection with a variety of other phenomena which are due to changes in the physicochemical properties of macromolecules[22,23]. In the case of carbonic anhydrase the anionic inhibition is assumed to be due to a combination of anions with an anion-binding site, leading to inactivation of the enzyme as first suggested by ROUGHTON AND BOOTH[19]. The pH-dependency of the anionic inhibition (Fig. 4) and the fact that the effect of inhibitory salts is freely reversible (Table II) are in accordance with this assumption which is further supported by the results of the investigations performed by LINDSKOG[24] on cobalt-carbonic anhydrase.

The effect of pH on the dehydration kinetics of bovine erythrocytic carbonic anhydrase has been studied by DEVOE AND KISTIAKOWSKY[9] who demonstrated that v_{max} and K_m were affected by changes in (H^+) in a manner similar to what has been found to be the case for the human C enzyme in the present study (Fig. 5a). In order

to account for the finding that K_m varied in inverse proportion to (H^+) while v_{max} was almost constant in the pH range investigated (pH 7.0–7.4), these authors proposed an enzymic mechanism which assumes that the enzyme contains binding sites, possibly on the zinc atom, for which hydroxide ion and bicarbonate ion compete. The results of the extensive studies later performed by KERNOHAN[14,18] on the kinetics of the bovine enzyme are consistent with this mechanism. KERNOHAN performed the determinations in imidazole buffers containing Cl^- in a concentration of 80 mM. No appreciable substrate binding was found in the dehydration reaction in contrast to the results in Fig. 5. The discrepancy may be due to the absence of inhibitory anions in the reaction medium which was used in the present study.

The results obtained with the human B enzyme (Fig. 5b) according to which no measurable changes occur in K_m between pH 6.8 and 8.4 seem to exclude the possibility that the enzymic mechanism of this form of carbonic anhydrase is identical to the mechanism referred to above. Two different enzymic mechanisms may exist, one belonging to the bovine and the human C enzyme, and the other to the human B enzyme. This assumption is in alignment with the fact that the two former enzymes, according to NYMAN AND LINDSKOG[5], have almost the same specific catalytic activity while the latter enzyme is a less efficient catalyst.

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