The CO₂ hydration and HCO₃⁻ dehydration activities of human red cell carbonic anhydrase isozymes B and C (HCAB and HCAC) have been studied as a function of temperature from 0° to 37°C. The Arrhenius plots of ln kcat versus 1/T are linear for both isozymes in both hydration and dehydration reactions, indicating that the rate-determining steps remain unchanged over this temperature range. The 37°C hydration kcat, at pH 7.5, is 13 × 10⁶ s⁻¹ for isozyme C and 0.71 × 10⁶ s⁻¹ for isozyme B. kcat for hydration, is 10 mM for C and 5 mM for B, and invariant with temperature. The uncatalyzed reactions are significantly affected by temperature, 30- to 40-fold rate enhancements being observed from 0° to 37°C. The enzyme-catalyzed processes are much less sensitive to temperature, the rate enhancements being 2- to 3-fold for HCAB and 5- to 6-fold for HCAC in this temperature range. These observations are consistent with a significant lowering of the free energy of activation by both isozymes. This effect is greater for C accounting for its higher catalytic power. The enthalpy of activation, at pH 7.5 and 8.2, in the rate-limiting step is considerably less for the B enzyme compared to C. This is, however, more than offset by a large negative entropy of activation in the case of HCAB. This observation indicates either a mechanistic difference in the rate-limiting events or a difference in the structural organizations of the active sites of the two isozymes, or both.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) catalyzes the reversible hydration of CO₂ to HCO₃⁻. The enzyme in human red cell occurs in two isozymes, designated B and C. Both isozymes are structurally well characterized, and their tertiary structures show considerable similarity (1–3). The catalytically essential unit in both isozymes is generally believed to be zinc-bound water, the metal being also liganded to three histidines. In spite of this critical similarity in the active sites, significant differences between the two isozymes exist. The most obvious and important of these is the kinetic difference, the C enzyme being a faster or catalytically more efficient enzyme than B. The Michaelis-Menten parameters, namely kcat and Km, for CO₂ hydration and HCO₃⁻ dehydration of these isozymes and their pH dependence have been extensively studied and reviewed (4–10). We now report work on the effect of temperature on CO₂ hydration and

HCO₃⁻ dehydration of HCAB and HCAC isozymes and attempt to provide a thermodynamic basis for the kinetic difference between the two enzymes. The 37°C data, reported fully for the first time, gives an important base for physiological calculations.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Human erythrocyte carbonic anhydrase isozymes B and C were commercially obtained (Worthington Biochemicals, Freehold, NJ) or isolated in pure form using an affinity chromatography technique (11). Concentrations of active enzymes were determined as described by Maren and Couto (12).

**Kinetic Methods**—The uncatalyzed and enzyme-catalyzed initial rates for both CO₂ hydration from 0° to 25°C and HCO₃⁻ dehydration from 0° to 37°C were determined using the methods of Maren and Couto (12). In hydration reactions, 7 mM barbital buffer was used and the mean pH (i.e., pH at which the buffer was half-titrated) was 7.53. For dehydration, the buffer was 7.5 mM or 5 mM phosphate, and the mean pH was 7.20. Phosphate was found not to have either inhibitory or activating effect on the carbonic anhydrase-catalyzed dehydration reaction at these concentrations. No external ion was added in reaction media to adjust the ionic strength. In dehydration reaction, ionic strength varied with substrate (HCO₃⁻) concentration. Earlier work in this laboratory showed that the initial rates are insensitive to ionic strength up to at least 0.25 M (12). The buffer factor at each temperature was determined by titrating barbital and phosphate buffers with standard H₂SO₄ and NaOH, respectively, over our experimental pH ranges. A few CO₂ hydration experiments were carried out in the pH range 8.5 to 8.0, the mean pH being 8.22. In these runs, 21 mM and 42 mM barbital buffers were used respectively for experiments at 1.5° and 21°C. The higher barbital concentrations did not affect the enzymes, but made the reaction times longer. The pH of the buffer solution was adjusted at each experimental temperature.

CO₂ hydration kinetics at 37°C were studied in a Durrum-Gibson D-110 stopped flow spectrophotometer coupled with a digital oscilloscope (Nicolet Instrument Corporation, Madison, WI). The buffer and the indicator used were barbital and phenol red in final concentrations (after mixing) of 0.025 M and 2 × 10⁻⁴ M, respectively. Initial rates of absorbance changes at 557 nm were measured and then converted to initial rates of CO₂ hydration by multiplication by a buffer factor, determined as described by Khalifah (4).

**Calculation of kₐ and kₐₑ**—At each temperature, initial rates were obtained for a set of four to six different substrate concentrations. Fig. 1 shows typical double reciprocal plots of rate versus concentration for HCAB and HCAC. The solid lines represent weighted least square analysis of data, assuming each initial rate measurement has the same standard error (13). Kₐ and kₑ were usually obtained at each temperature for a set of three to six different experiments. The limits of standard deviations from the means were 12% for kₑ and 20% for Kₐ.

**Calculation of Activation Parameters**—The apparent activation energy (E_act) value for a particular pH was obtained from the slope of the Arrhenius plot of ln kₐ versus 1/T. The apparent enthalpy of activation (ΔH°) was calculated using the equation ΔH° = E_act − RT. Knowing ΔH°, the apparent entropy of activation (ΔS°) was calculated by using the equation ΔS° = k_B T / kₐₑ. The abbreviations used are: HCAB and HCAC, human erythrocyte carbonic anhydrase isozymes B and C.
Thermodynamics of Human Carbonic Anhydrases

RESULTS

Uncatalyzed Reactions

For the uncatalyzed reversible $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ process, the rate constants are defined as $k_1$ in the forward direction and $k_2$ (at a fixed pH) in the reverse direction. Fig. 2 shows the Arrhenius plots for the $k_1$ and $k_2$ (for pH 7.2). Each point represents several (at least six) determinations, agreeing within 5%. The plots are linear. For $k_1$, we obtain an activation energy of 17.9 kcal/mol. This agrees with earlier data (14, 15). For $k_2$, an activation energy of 15.5 kcal/mol is obtained at pH 7.2. Table I gives the corresponding $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta G^\ddagger$ values. Our $k_1$ data from 0° to 25°C agree with that reported and reviewed earlier (4, 14, 15). At 37°C, our $k_1$ is 0.110 s$^{-1}$. This is intermediate among data reported by several workers (5, 16, 17). The rate constant, $k_{-1}$, for $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$ is calculated from $k_2$, using the relation $k_{-1} = (k_{-2}/k_1)^{1/2}$, where $k_1$ = $[\text{H}^+][\text{HCO}_3^-]/[\text{H}_2\text{CO}_3]$ (see Table II). At 25°C, $k_1$ equals 2.5 × 10$^{-4}$ m (18). This yields $k_{-1}$ = 13.2 s$^{-1}$ which agrees well with the literature. Calculation of $k_1$ at other temperatures, however, remains an outstanding problem, since the temperature dependence of $k_1$ has not been established. This can only be done if one assumes the temperature effect on $k_1$ to be proportional to that on $K_1 = [\text{H}_2\text{CO}_3]/[\text{H}^+][\text{CO}_2]$ which is from $5 \times 10^{-7}$ m at 37°C to 2.6 × 10$^{-7}$ m at 0°C (19).

The physiologically important equilibrium ratio ($K$) for $\text{CO}_2/\text{H}_2\text{CO}_3$ can be calculated by using the $\Delta G^\ddagger$ values for $k_1$ and $k_2$. This is shown in Steps 4 to 7 of Table II and yields $K = 405$ for 25°C. This is in agreement with values reported in earlier studies (12, 14).

Catalyzed Reactions

Temperature Effect on $k_{cat}$—Fig. 3 shows Arrhenius plots from 0° to 37°C for both isozymes, for hydration and dehydration. This is the first complete study of the temperature problem, so we can only make comparisons with certain of the literature values. This will be attempted as follows. We exclude reference to work using 25 mm phosphate which inhibits hydration by B (20) and activates dehydration by B (5). The only previous work across the entire temperature range was by Kernohan, who plotted $V_{max}/K_m$ for $\text{CO}_2$ hydration by the bovine enzyme (21). Between 5° and 50°C, only a

![Image 1](image1.png)
thermodynamics of human carbonic anhydrases

The Arrhenius plots for CO₂ hydration (pH 7.5) and HCO₃⁻ dehydration (pH 7.2) kinetics of carbonic anhydrases B and C. The bars represent standard deviations of the mean in kₐₚ, where deviations are more than symbol heights.

For 0°C, we agree fairly well with Christiansen and Magid (7), who studied both isozymes for hydration and dehydration in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or low (<10 mM) phosphate buffer. For 37°C, we are in fair agreement with the only other study.²

The Arrhenius plots for both B and C isozymes are linear in either hydration at pH 7.5 or dehydration at pH 7.2. This indicates that the rate-determining steps do not change in the catalytic pathways of HCA and HCB enzymes. Both isozymes cause a significant lowering of the activation energy barrier for CO₂ = HCO₃⁻ interconversion. This is reflected in the relative lack of temperature sensitivity of the enzyme-catalyzed reactions compared to the uncatalyzed reaction. While the uncatalyzed hydration and dehydration rate constants at 37°C are, respectively, 44-fold and 27-fold higher relative to the rate constants at 0°C, the turnover numbers of HCB are increased by only 2.4-fold in hydration at pH 7.5 and 2.9-fold in dehydration at pH 7.2. Over the same temperature range for HCA, these ratios are 5.5-fold in hydration and 5-fold in dehydration. The apparent enthalpy of activation of HAC is higher than that of HCB in the rate-limiting step. These differences are significant, HCA being 3.8 kcal/mol higher for ΔHₚ in the hydration reaction at pH 7.5, and 2.8 kcal/mol higher in dehydration at pH 7.2. When the apparent entropies of activation for the rate-limiting steps are calculated, it is found that the entropy of activation, e.g. in hydration reaction, is about the same for HCA as for the uncatalyzed reaction. The ΔSₚ for HCA catalysis is, on the other hand, about 18 e.u. more negative at pH 7.5 than for HCA. This more than offsets the lower enthalpy barrier in HCA catalysis, as reflected in the higher apparent free energy of activation (ΔGₚ) at pH 7.5 in the rate-limiting step of HCA compared to that of HCA. In HCO₃⁻ dehydration at pH 7.2, catalysis by HCA is entropically favorable compared to the uncatalyzed reaction. Catalysis by HCA is, however, again associated with a larger negative entropy of activation. In sum, ΔGₚ values for HCA at 25°C are 1.6 kcal/mol lower in hydration at pH 7.5 and 1.8 kcal/mol lower in dehydration at pH 7.2 than those for HCA. Table III summarizes these thermodynamic data for 25°C. These differences are consistent with those of the turnover numbers of the two isozymes, in accordance with the equation k = (kₑ⁻T/h) e⁻ΔG/RT, for a particular temperature.

The CO₂ hydration kinetics of HCB and HCA were also studied at pH 8.2 and at two temperatures, namely, 1.5° and 21°C. The kₑ values are indicated in Fig. 3. Calculation of the activation parameters from the high pH data again show that the apparent enthalpy of activation is 2.4-fold lower for HCB than for HCA. The apparent free energy of activation for the isozyme is, however, higher due to an unfavorable entropy contribution. These data, calculated for 25°C, are given in Table IV. The apparent enthalpies of activation for both isozymes are different at pH 8.2 from those at pH 7.5, indicating a pH-dependent contribution to ΔHₚ.

**Temperature Effect on kₑ**—The temperature insensitivity of kₑ for the substrates CO₂ and HCO₃⁻ against HCA and HCA is shown in Fig. 4. It is difficult to explain this observation, unless the meaning of kₑ is precisely known. One possibility is that kₑ is not a true equilibrium constant (Kₑ) representing enzyme-substrate dissociation, but a composite

### Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>ΔGₚ kcal/mol</th>
<th>ΔHₚ ± S.D. kcal/mol</th>
<th>ΔSₚ ± S.D. e.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>CO₂</td>
<td>19.45</td>
<td>17.30</td>
<td>7.43 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>HCO₃⁻</td>
<td>20.80</td>
<td>15.00</td>
<td>19.35 ± 0.15</td>
</tr>
<tr>
<td>HCA</td>
<td>CO₂</td>
<td>9.37</td>
<td>7.56 ± 0.14</td>
<td>6.07 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>HCO₃⁻</td>
<td>10.13</td>
<td>7.30 ± 0.18</td>
<td>9.46 ± 0.27</td>
</tr>
<tr>
<td>HCB</td>
<td>CO₂</td>
<td>9.07</td>
<td>3.79 ± 0.21</td>
<td>24.10 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>HCO₃⁻</td>
<td>11.96</td>
<td>4.48 ± 0.35</td>
<td>25.10 ± 0.21</td>
</tr>
</tbody>
</table>

ΔGₚ is calculated by using mean values of ΔHₚ and ΔSₚ.

Standard deviations in ΔHₚ reflect limits of variation when standard deviations in ln kₑ at each temperature are considered. For uncatalyzed reactions, these were insignificantly small.

Standard deviations are deviations, from the mean, of ΔSₚ values calculated from data at four temperatures, using the mean values of ΔHₚ e.u. = cal deg⁻¹ mol⁻¹.

### Table IV

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ΔGₚ kcal/mol</th>
<th>ΔHₚ kcal/mol</th>
<th>ΔSₚ e.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCA</td>
<td>9.07</td>
<td>11.7</td>
<td>8.81</td>
</tr>
<tr>
<td>HCB</td>
<td>10.58</td>
<td>4.87</td>
<td>−19.15</td>
</tr>
</tbody>
</table>

² Data presented by P. J. Wistrand and S. Lindahl (Department of Medical Pharmacology, University of Uppsala, Sweden) at the Sixth International Congress of Pharmacology, July 20 to 25, 1975, in Helsinki.
standard deviations
hydrases
ratios, we corrected our hydration error of our method. 

determining rates. At 25°C, the agreement between the Hal-dane constants for the two isozymes and the equilibrium constant for temperatures and the calculated equilibrium constant for which yields \( \text{pK}_a \) values of 7.0 and 7.6 at 25°C, respectively, in constant containing kinetic parameters. 

constant containing kinetic parameters. Alternatively, if \( K_n \) is equal to \( K_a \), then the enthalpy of substrate binding must be close to zero and cannot be determined beyond the experimental error of our method.

Haldane Relationship—Table V gives a comparison between our kinetically determined Haldane ratios at different temperatures and the calculated equilibrium constant for \( \text{HCO}_3^- / \text{CO}_2 \) at pH 7.2, based on our direct measurements of uncatalyzed hydration and dehydration rate constants \( k_1 \) and \( k_2 \). The \( k_1 / k_2 \) ratios agree with the \([\text{HCO}_3^-] / [\text{CO}_2^-] \) equilibrium values of Harned and Davis (19). For calculating Haldane ratios, we corrected our hydration \( k_{\text{cat}} \) data at pH 7.5 to pH 7.2. This correction is based on Khaliliah's pH rate profile (4), which yields \( \text{pK}_a \) values of 7.0 and 7.6 at 25°C, respectively, in the C and B enzymes for ionizations of the catalytic groups determining rates. At 25°C, the agreement between the Haldane constants for the two isozymes and the equilibrium \( \text{HCO}_3^- / \text{CO}_2 \) ratio is good. At other temperatures, pH correction of our hydration \( k_{\text{cat}} \) data included the change in \( \text{pK}_a \) with temperature, -0.02 per degree, determined for the bovine enzyme using the esterase activity (24). This was used in our calculation in absence of equivalent data for \( \text{CO}_2 \) hydration by HCAB and HCAC. No correction in \( K_n \) values was made since these are pH independent (4).

**TABLE V**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Enzyme</th>
<th>( H^\circ )</th>
<th>( \text{HCO}_3^- )</th>
<th>( k_{\text{cat}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3°C</td>
<td>HCAC</td>
<td>12</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>13°C</td>
<td>HCAB</td>
<td>11</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>HCAC</td>
<td>12</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>HCAB</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* \( H^\circ = k_{\text{cat}} \) (hyd) \cdot K_n (dehyd) \cdot K_{\text{cat}} (dehyd) / K_n (hyd) *

\( k_1 \) and \( k_{\text{cat}} \) are uncatalyzed \( \text{CO}_2 \) hydration and \( \text{HCO}_3^- \) dehydration rate constants at pH 7.2.

Discussion

Our data show that at physiological pH human red cell carbonic anhydrase B has a lower activation enthalpy than the C isozyme. However, a significantly unfavorable entropy contribution is responsible for a higher apparent free energy of activation and lower catalytic efficiency of the B isozyme. A similar conclusion follows from our pH 8.2 hydration data.

A comparison of the pH 7.5 and 8.2 data (Tables III and IV) indicates that our activation enthalpy and entropy values are pH dependent. The pH-dependent contributions probably come from the temperature effect on the \( K_n \) for the active site ionizations of the two isozymes. It is widely believed that both B and C isozymes have a zinc-bound water molecule essential for catalytic activity (see Ref. 33 for a recent discussion). The deprotonated form or the so-called zinc hydroxide form is responsible for \( \text{CO}_2 \) hydration, while the protonated form of the same group causes \( \text{HCO}_3^- \) dehydration activity. The very low \( \text{pK}_a \) of \(~5\) for this group in HCAC reflects a special, possibly hydrophobic, environment at the active site. The \( \text{pK}_a \) of this catalytically active group is at least 0.6 unit higher at 25°C in the B enzyme (4), indicating a difference in the environments of zinc-water in the two isozymes. To obtain activation enthalpy and entropy values independent of any contribution from the temperature effect on \( K_n \), one has to consider the effect of temperature on the pH-independent turnover number \( (k_{\text{cat}})^\circ \). In hydration, \( k_{\text{cat}}^\circ \) may be considered to be equivalent to the \( k_{\text{cat}} \) when the enzyme is in fully basic form and should be related to the apparent \( k_{\text{cat}} \) at a particular pH by the relation \( k_{\text{cat}}^\circ = k_{\text{cat}} / (1 + H^\circ / K_n) \). We are unable, at this point, to calculate \( k_{\text{cat}}^\circ \) at different temperatures since the temperature dependence of the \( K_n \) values of the two isozymes are not known for \( \text{CO}_2 \) hydration. Calculation of \( k_{\text{cat}}^\circ \) at 25°C from our pH 7.5 data, using \( \text{pK}_a = 7.0 \) for HCAC and 7.6 for HCAB (4), yield 1.1 \( \times \) 10¹⁰ s⁻¹ and 1.2 \( \times \) 10¹⁰ s⁻¹, respectively, for the C and B isozymes. These numbers are only 9% higher for HCAC and 20% higher for HCAB than our experimental values at pH 8.2 and 21°C. Our data are, then, consistent with the expected 94% ionization of HCAC and 80% ionization of HCAB at pH 8.2 and 25°C. The \( K_n \) dependent contributions to \( \Delta H^\circ \) must then be substantially reduced at this pH, unless the temperature effects on the active site ionizations are unusually large. Consequently, our pH 8.2 data indicate that the lower \( \Delta H^\circ \) and more negative \( \Delta S^\circ \) situation for HCAB, compared to HCAC, may be valid for the completely basic forms of the two isozymes in \( \text{CO}_2 \) hydration, that is to say when the data would be independent of temperature perturbation of \( \text{pK}_a \).

It is conceivable that the large negative \( \Delta S^\circ \) for HCAB may be due to freezing out of water molecules at the active site in the transition state. In comparison, the active site of HCAC may be more organized in the ground state, so that the loss in entropy is smaller in forming the transition state. This situation would be consistent with a looser active site structure of HCAB, accessible to more water molecules, in analogy to the proposal of Roberts and Elmore (25) for \( \alpha \)-trypsin compared with \( \beta \)-trypsin. The presence of water molecules in the enzyme-active site would be of mechanistic significance if the rate-determining step is an internal proton transfer mechanism (8, 26-30) mediated through water molecules (34).

We are not certain, however, whether the activation en-
thalpies and entropies represent the same reaction steps in the two isozymes. While a proton transfer step is thought to be rate-limiting in catalysis by bovine and human C isozymes (8, 26-30), chemical identity of the rate-limiting step for the human B enzyme has not been established. There are indications that, in the presence of buffer, the proton exchange mechanism in the B enzyme is different from the mechanism in human C and bovine enzymes. This is based on proton NMR relaxation studies by Fabry (31) and an oxygen-18 exchange investigation by Tu and Silverman (32).

The kinetic meaning of $K_m$ is not clear from our data. For human C and bovine carbonic anhydrase, $k_{cat}$ and $K_m$ show similar magnitudes of isotope effects, so there is no isotope effect on $k_{cat}/K_m$ (8, 26). This is consistent with $k_{cat}$ and $K_m$ being composed of the same kinetic steps (8). However, since we now find that $K_m$ is temperature independent and $k_{cat}$ is not, $k_{cat}/K_m$ (which is the lower limit for the second order enzyme-substrate association rate constant) varies with temperature in the same way as $k_{cat}$. $K_m$ and $k_{cat}$ do not, then, appear to be measurements of identical pathways.

Finally, we have established the $37^\circ C$ kinetic parameters which may be used in physiological work. Since C is the chiefly active isozyme in red cells and is (or is very closely akin to) the carbonic anhydrase in most secretory tissues, these values are summarized. For hydration, $k_{cat} = 13 \times 10^5 s^{-1}$ and $K_m = 13$ mm. For dehydration, $k_{cat} = 3.5 \times 10^5 s^{-1}$ and $K_m = 53$ mm.

Acknowledgment—We thank Ms. Elsa Couto for help with experiments in the early part of this project.

REFERENCES