

## A Spectrophotometric Method for the Assay of Carbonic Anhydrase Activity

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A photometric method suitable for the routine determinations of the rate of the hydration reaction of carbon dioxide in the presence of Veronal buffer is described. The method utilizes a difference in ultra-violet absorption between the acidic and basic forms of the buffer. Thus, the change in composition of the buffer as a function of time can be measured, and this is related to the change in concentration of carbon dioxide in a manner which makes rate constant determinations quite simple.

The procedures described for the estimation of velocities of the reaction catalyzed by the enzyme carbonic anhydrase can be divided in two groups with respect to the reaction constituent studied. In the manometric method the rate of change in the carbon dioxide concentration is measured and the limitations of the usefulness of this method have been discussed by several authors<sup>1,2</sup>. The other approach consists of determinations of the rate of release or consumption of the protons involved in the reaction and are performed as measurements of the rate of titration of a buffer solution by electrometric methods or as colorimetry or photometry of a pH indicator present in the reaction mixture. In early colorimetric methods the time necessary for the reaction to reach an end point was determined and used as a measure of enzymic activity, while in other cases the reaction was followed continuously by the use of recording spectrophotometers<sup>3-5</sup> or recording electrometric equipment<sup>6,7</sup>. The latter techniques permit the estimation of substrate concentration at any point of the curve after calibration of the signals recorded either by titration of the buffer with strong acid or base<sup>7</sup> or by calculation of change in buffer composition from pH determinations using the  $pK$  value of the buffer<sup>3</sup>. With instruments equipped for the study of fast reactions, the photometric as well as the electrometric method has been utilized in kinetic studies of carbonic anhydrase<sup>3,5,7,8</sup>.

Similar to the earlier photometric and electrometric methods, the method described in this paper is a determination of the rate of a titration reaction. The measurements are performed on the hydration reaction of carbon dioxide

in the presence of Veronal buffer. Instead of using the indirect way of measuring the change in absorption of a pH indicator, the change in concentrations of the buffer constituents is measured by utilization of a spectral difference between the basic and the acidic forms of the buffer in such a manner that a linear relation between substrate concentration and absorbancy is obtained. The restrictions inherent in the method make it unsuitable for detailed studies of the kinetic properties of the enzyme but its simplicity offers convenience in routine determinations of enzyme activity.

#### PRINCIPLES AND EXPERIMENTAL

The following equation is derived by Perrin<sup>9</sup> for the hydration of carbon dioxide in a solution of the buffer  $B^- - HB$  with an ionization constant  $K$ :

$$[CO_2]_t = [CO_2]_0 - [B^-]_0 + \frac{[B^-] + [HB]}{1 + [H^+]_t/K} \quad (1)$$

The brackets with subscript  $t$  refer to molar concentrations at time  $t$  and those with subscript 0 to initial molar concentrations. This equation which is valid under conditions where  $[CO_3^{2-}]$  is negligible in comparison with  $[HCO_3^-]$  is equivalent to

$$[CO_2]_t = [CO_2]_0 - [B^-]_0 + [B^-]_t \quad (2)$$

At  $t = \infty$ , *i.e.* when equilibrium has been reached

$$[CO_2]_\infty = [CO_2]_0 - [B^-]_0 + [B^-]_\infty \quad (3)$$

By combination of (2) and (3) the following expression is derived:

$$[CO_2]_t = [B^-]_t - [B^-]_\infty + [CO_2]_\infty \quad (4)$$

The quotient  $[CO_2]_0/[CO_2]_\infty$  is a function of the equilibrium pH, and the following relation, which can be deduced from the equilibrium constants of the carbonic acid system, is a valid approximation for equilibrium  $pH < 9$ :

$$\frac{[CO_2]_0}{[CO_2]_\infty} = 1 + \frac{K_1}{[H^+]_\infty} \quad (5)$$

$K_1$  in eqn. (5) is defined as in Ref.<sup>10</sup> By the use of (3) and (5), eqn. (4) can be transformed in the following way:

$$[CO_2]_t = [B^-]_t - [B^-]_\infty + \frac{[H^+]_\infty}{K_1} ([B^-] - [B^-]_\infty) \quad (6)$$

Choosing the conditions of the measurements in order to make  $[H^+]_\infty/K_1$  small, together with calculating the rate constant from the beginning of the reactions, *i.e.* at high values of  $[B^-]_t - [B^-]_\infty$ , allows the use of eqn. (6) in the following simplified form:

$$[CO_2]_t = [B^-]_t - [B^-]_\infty \quad (7)$$

With a difference in spectral properties between the basic and the acidic forms of the buffer ( $\epsilon_B \neq \epsilon_{HB}$ ),  $[CO_2]_t$  in the equations above can be expressed

in molar extinction coefficients ( $\epsilon_{B^-}$  and  $\epsilon_{HB}$ ) and absorbancies in a 1-cm cell ( $A$ ). Thus, eqn. (7) becomes:

$$[\text{CO}_2]_t = \frac{1}{\epsilon_{B^-} - \epsilon_{HB}} (A_t - A_\infty) \quad (8)$$

From Fig. 1, which illustrates the type of spectral difference utilized, it is evident that in the wavelength region examined the molar extinction coefficient for the basic form of Veronal ( $\epsilon_{B^-}$ ) is greater than that of the acidic form ( $\epsilon_{HB}$ ). The difference between the two coefficients increases when going from longer to shorter wavelengths. The determinations of reaction velocity are performed on a slope in a differential absorption spectrum between Veronal buffers of different pH similar to that in Fig. 1.

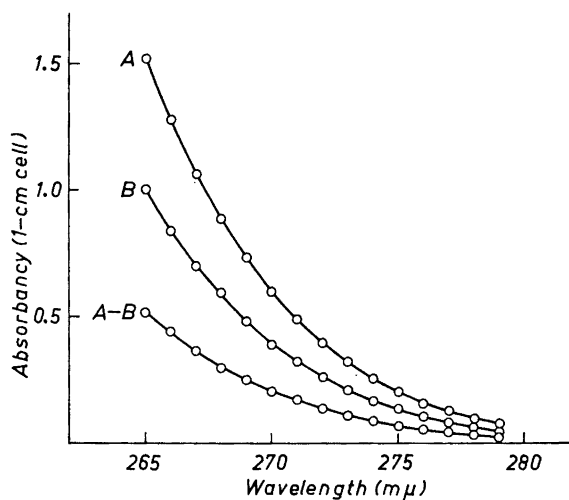
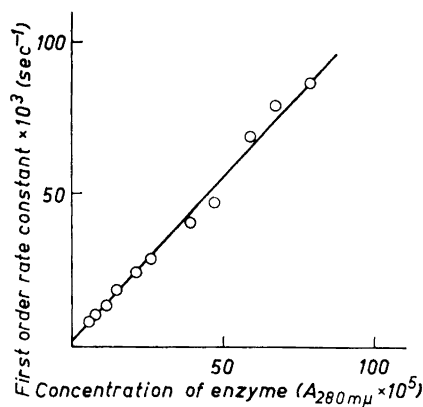


Fig. 1. Partial ultraviolet absorption spectra of 0.004 M Veronal-HCl buffers measured at 1.1–1.3° with water as a blank. A and B refer to buffers of pH 8.78 and 8.02, respectively (measured at 20°). A–B is the differential spectrum between the buffers.

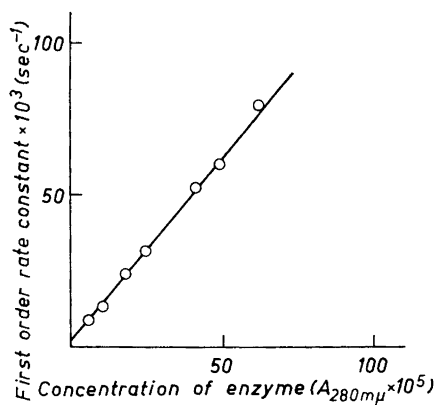
Veronal buffers were made from pharmaceutical grade sodium salt of 5,5-diethylbarbituric acid and reagent grade hydrochloric acid in deionized water. For some of the experiments the sodium barbiturate was purified by recrystallization<sup>11</sup> but no differences in properties between solutions from purified and unpurified salts were observed.

Two sets of conditions of substrate and buffer concentration were used; the details of these are quoted in the captions to Fig. 2 and 3, respectively. The reaction proceeded from pH 8.9 to 8.1 and 8.3, respectively (these values refer to 1°; for the temperature dependence of p*K* of Veronal, see Ref.<sup>11</sup>). These values of the equilibrium pH were regarded as sufficiently high ( $[\text{H}^+]_\infty/K_1 < 0.03$ ) to permit the use of eqn. (7). For both sets of conditions the linear relation between absorbancy and concentrations of the buffer constituents was tested in the following manner. The absorbancy (1-cm cell)



*Fig. 2.* Relation between first order rate constant and concentration of a highly purified preparation of carbonic anhydrase component CA V (see Ref.<sup>18</sup>). The enzyme concentration is expressed as absorbancy at 280  $m\mu$  in the reaction cuvette (1-cm cell). The values are not corrected for the nonenzymatic hydration. The rate constant for this reaction is equal to the intercept of the vertical axis. Conditions used: 2.0 ml 0.05 M Veronal-HCl, pH 8.6 (at 20°), 0.50 ml substrate solution. Temperature:  $1.7 \pm 0.5^\circ$ . The reaction was followed at 276  $m\mu$  with a slit width of 0.1 mm.

of a series of buffers of the actual concentration but of different pH, including the pH range for the reaction, was measured at a number of wavelengths. A buffer with approximately the same pH as the equilibrium pH for the reaction was used as the blank and the measurements were performed at the same temperature as the determinations of reaction rate. The pH of the buffers was carefully determined and the concentrations of buffer constituents were calculated by the use of the ionization constant<sup>11</sup>. For the determination of



*Fig. 3.* Relation between first order rate constant and concentration of carbonic anhydrase component CA III (see Ref.<sup>18</sup>). Conditions used: 2.5 ml 0.005 M Veronal-HCl, pH 8.6 (at 20°), 0.050 ml of substrate solution. Temperature: 1.0–1.5°. The reaction was followed at 265  $m\mu$  with a slit width of 0.3 mm. For further explanations, see the caption to Fig. 2.

the equilibrium pH of the reaction, a reaction mixture at equilibrium was also included in the photometric measurements. Plots of absorbancy against  $[B^-]$  gave straight lines for a number of wavelengths. One of these giving the linear relation together with a suitable absorbancy range for the reaction ( $A_0 - A_\infty = 0.6 - 0.7$  absorbancy units), was selected for use. The deviations from Beer-Lambert law observed were present at the shorter wavelengths where the absorption in the blank cuvette gradually becomes very high.

The photometer used was a Beckman DU equipped with double thermostats and an energy recording adapter connected to a Varian or Speedomax recorder with a sensitivity of 10 mV and a balancing time of 1 sec. A suitable amount of Veronal buffer was put into each of two 1-cm cuvettes. To both cuvettes an appropriate amount of the enzyme solution to be tested was then added (usually 5 to 25  $\mu$ l). The cuvettes were placed in the cuvette housing of the photometer and left there until temperature equilibrium (1.0–1.3°) had been reached. One of the two cuvettes (the reference) was by means of a cold syringe or micropipette rapidly supplied with the suitable amount of deionized water saturated with carbon dioxide at a temperature of 0–0.5°. When the reaction in the reference cuvette had reached equilibrium, the recorder was zeroed with this cuvette as a blank. Then the reaction was started in the test cuvette and the decrease in absorption with time at the wavelength used was recorded. The absorbancy values from the curve obtained in this way are equivalent to  $A_t - A_\infty$  in eqn. (8), and in a semilogarithmic plot a straight line is obtained over a wide range from which the first order rate constant is calculated.

## RESULTS AND DISCUSSION

From 17 measurements on a sample of carbonic anhydrase giving a mean rate constant of  $29 \times 10^{-3} \text{ sec}^{-1}$ , the standard deviation was calculated to be  $\pm 4\%$ . The standard deviation, even when expressed as percent, may, however, be a function of the rate constant value.

The first order rate constant for the non-enzymatic hydration of carbon dioxide in 0.04 M Veronal buffer at  $1.7 \pm 0.5^\circ$  was found to be  $3.68 \times 10^{-3} \text{ sec}^{-1}$  (mean value of 13 measurements). After correction for catalytic effect of the Veronal buffer<sup>12</sup> the corresponding value is  $3.0 \times 10^{-3} \text{ sec}^{-1}$ . This latter value can be compared with earlier determinations of the rate constant for the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ . Interpolation (in a semilogarithmic plot of  $k_{\text{CO}_2}$  against  $1/T$ ) of earlier values from different kinds of measurements<sup>13–16</sup> to the reaction temperature used here gives as a mean  $2.59 \times 10^{-3} \text{ sec}^{-1}$  which is in rather good agreement with the value obtained by the present method. The difference may partly be due to the error introduced by the approximation leading to eqn. (7) which, under the experimental conditions used, is estimated to make the calculated first order rate constants a few percent too high. Probably to the major part, however, the difference is due to the occurrence of the reaction  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$  which in the pH region used is known to contribute to the hydration of carbon dioxide<sup>17</sup>.

The conditions quoted in the caption to Fig. 2 have been used in the routine assay of enzyme activity throughout the development of a purification method

for human erythrocyte carbonic anhydrase<sup>18</sup> and the experience thus obtained may be of interest from a practical point of view. The specific enzyme activity of a hemolysate is so low that the suitable amount in the cuvettes is high enough to cause appreciable absorption. This has to be canceled out by the use of the same amount of hemolysate in both the reference and the test cuvette. With enzyme solutions from later stages of purification, the ultra-violet absorption is negligible (Figs. 2 and 3). By means of such solutions it is very easy to introduce in the reference cuvette an amount of enzyme high enough to cause a rapid equilibration without getting any disturbing ultra-violet absorption. Such a procedure makes the measurements more convenient. The relation between first order rate constant and enzyme concentration for purified carbonic anhydrase components is shown in Figs. 2 and 3. In contrast to the findings of Datta and Shepard<sup>4</sup>, the relation found here is linear even at the higher concentration of Veronal used.

It seems reasonable to believe that the present method for enzyme activity measurements can be performed also with other concentrations of Veronal buffer and substrate than those tested here. Conditions permitting the use of eqn. (7) makes the calculation of rate constants simple but there seems to be no other argument against the utilization of a pH region where eqn. (6) has to be used. The latter case necessitates an evaluation of the last term in eqn. (6) in absorbancy units. This can be obtained from initial absorbancy and knowledge of the equilibrium pH; the latter probably easiest to determine photometrically as described in the previous section. However, it seems advisable, when adapting the method to other conditions than those tested here, to check the relation between absorbancy and concentrations of the components of the buffer.

Regardless of the value of the equilibrium pH, the choice of wavelength depends upon the actual value of  $[B^-]_0 - [B^-]_\infty$ . Small values of  $[B^-]_0 - [B^-]_\infty$  necessitates the use of shorter wavelengths to give suitable absorbancy ranges for the reaction than the greater values of  $[B^-]_0 - [B^-]_\infty$ . This follows from the increase of  $\epsilon_{B^-} - \epsilon_{HB}$  towards shorter wavelengths pointed out in the previous section and may be illustrated by the two sets of conditions used here (captions to Figs. 2 and 3). The possibilities of utilization of a given value of  $[B^-]_0 - [B^-]_\infty$  is probably dependent of the value of  $[B^-]_\infty$  as indicated by the deviations from Beer-Lambert law observed at high absorption in the blank cuvette. This may be of importance in attempts to minimize the pH range for the reaction and for the location of the reaction in the titration curve of Veronal.

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