Assay of Carbonic Anhydrase by Titration at Constant pH

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1. A method is described for measuring accurately the initial velocity of the hydration reaction catalysed by the enzyme carbonic anhydrase (EC 4.2.1.1); the method depends on the titration of H^+ ions at constant pH. 2. Human erythrocyte carbonic anhydrase, isoenzyme C, was used to illustrate the method. Under the experimental conditions employed (0° , pH 7.0, in the presence of 45 mM-sodium chloride and 5 mM-sodium phosphate) isoenzyme C obeyed the Michaelis equation over the range of substrate concentration 1–16 mM-carbon dioxide. The kinetic constants found were: $K_m = 8.2$ mM; $V/[E_0] = 5.0 \times 10^4$ sec. $^{-1}$.

The reaction catalysed by carbonic anhydrase (EC 4.2.1.1) may be expressed, in a simplified form, as:

At or near neutral pH the equilibrium constant allows the reaction to be conveniently examined when proceeding in either direction. Reliable assays in current use mostly depend on rapid measurement of the change in extinction of a buffered reaction mixture containing an acid-base indicator, in a stopped-flow device (DeVoe & Kistiakowsky, 1961; Gibbons & Edsall, 1963, 1964; Ho & Sturtevant, 1963; Kernohan, Forrest & Roughton, 1963). Davis (1958) has introduced the use of a sensitive pH-meter that determines directly the production of H^+ ions, and Kernohan & Roughton (1966) have developed a flow technique that depends on the measurement of the heat of reaction and is particularly suitable for use with concentrated solutions of the enzyme.

The method for the determination of carbonic anhydrase activity that is presented below was evolved as part of an investigation of the enzyme in certain tissues of the reproductive tract (McIntosh & Lutwak-Mann, 1967). It depends on the titration at constant pH of H^+ ions produced in the hydration reaction. Compared with the spectrophotometric procedures, the titration method offers several definite advantages. It makes possible the maintenance of constant pH; it eliminates the specific ion effects arising from the use of highly concentrated buffers; it dispenses with the need for the determination of the relationship between the $[H^+]$ change and the buffer/indicator ionization; it also permits the

use of commercially available standard equipment.

Several authors in the past have used titration for the purpose of studying catalysis by carbonic anhydrase, particularly with regard to the influence of inhibitors (Holmgard, 1961; Leibman, Alford & Boudet, 1961; Leibman & Greene, 1967). However, none of these studies has been concerned primarily with the possibility of developing titration into a strictly quantitative and dependable method. Nor has the claim been made previously that methods based on titration can achieve the same standard of precision as the stopped-flow spectrophotometric technique. The present study describes a titration method for the quantitative assay of carbonic anhydrase that offers a satisfactory alternative to the spectrophotometric method and achieves the same standard of precision.

EXPERIMENTAL

Reagents. The chemicals used were all of A.R. grade unless otherwise stated. Bacteriological peptone was obtained from British Drug Houses Ltd., Poole, Dorset. The water was deionized. CO_2 (purity > 99.5%) at atmospheric pressure was used to saturate water at 0° in a gas absorption bottle. When a sample of the CO_2 -saturated water was added to $Ba(OH)_2$ solution back-titration indicated a concentration of CO_2 within 1–2% of that predicted by calculation based on the Henry's law constant (Harned & Davis, 1943). NaOH solutions were prepared by diluting (with degassed water) the supernatant obtained on centrifugation of a saturated NaOH solution from which the carbonate had been precipitated. Three solutions having concentrations between 0.3 N- and 2.5 N-NaOH were prepared; the bottles in which these solutions were kept were flushed with N_2 . The stock mixture of buffer and NaCl contained 45 mM- NaH_2PO_4 – Na_2HPO_4 and 0.405 M-NaCl at pH 6.89 (25°). At 0° the pH of this mixture was 6.99 and the ionic strength,

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after dilution in the assay, was 0.050 (5 mM concn. of phosphate ions).

Enzyme. Isoenzyme C (Rickli, Ghazanfar, Gibbons & Edsall, 1964) of human erythrocyte carbonic anhydrase was prepared from the erythrocytes of a single individual by the method of Armstrong, Myers, Verpoorte & Edsall (1966). This isolation entailed ion-exchange chromatography of the ethanol-chloroform extract from the haemolysate on DEAE-Sephadex (A-50). Chromatography was performed twice to ensure that the preparation was homogeneous. The concentration of the enzyme in the stock solution was determined by measurement of extinction at 280 m μ (Gibbons & Edsall, 1964). There was no contamination by haemoglobin.

Titrator. The apparatus used for the continuous titration was a Radiometer Automatic Titrator TTT 1c together with a Recorder SBR 2c (V. A. Howe and Co. Ltd., London, W. 11), with the controls set for pH-stat operation, and the fastest motor (30 rev./min.) driving a syringe-burette of 0.5 ml. capacity. The glass electrode was a Radiometer G 202c type, recommended for use at 0°. A temperature compensator to correct the response of the instrument at 0° was supplied by the manufacturer of the titrator. pH calibration was performed by using a Beckman Instruments Inc. standard phosphate buffer.

The apparatus was connected to a second recorder that increased the speed of recording and amplified the movements of the syringe, thus making it possible to use more concentrated solutions of titrant. This additional recorder was a Servoscribe potentiometric instrument (Kelvin Electronics Co., Wembley, Middx.) linked to a precision 10-turn potentiometer that, mounted below the syringe-burette of the titrator and operated by the drive, indicated the position of the piston, thus giving a direct measure of the volume added. Good results could also be obtained without this additional recorder, but only when a second 30 rev./min. motor was used to move the chart paper of the main SBR 2c recorder.

The 20 ml. titrator cell was maintained at a constant temperature (0°) by a circulating refrigerant, which was passed first through a copper coil immersed in an insulated ice-containing bath, next to the titrator cell, and finally back to a refrigerator. With the refrigerator set at -1.5° and the insulated bath remaining at a temperature of 0-0.5°, the titrator cell had a temperature of 0 $\pm$ 0.1°. The gas absorption bottle and all solutions, as well as two pre-set syringes, were kept in the ice bath. A burette was connected to a coiled pipe of large volume and cooled by immersion in the bath, which terminated in a narrow-bore polythene tube and syringe needle. This assembly enabled a measured volume of cold water to be passed into the cell.

The temperature of the titrator cell was monitored with a calibrated miniature thermistor and bridge circuit whereby a temperature change of 0.05° could easily be detected. The volume of the cell, when positioned on the titrator, was adjusted by movement of the electrodes to exactly 18.0 ml. of liquid. The only access to the cell was by way of a small hole through which additions of liquid were made.

Assay procedure. The syringe-burette was filled with NaOH solution of concentration appropriate to the substrate concentration and air bubbles were carefully removed from the delivery tube. Both recorders were set to zero. A quantity of degassed water, equal to 18.0 ml. less the volume of the reacting solutions, was admitted from the burette

assembly into the cell, already in place on the titrator. A 2.0 ml. sample of the buffer-NaCl mixture was next added by means of the pre-set syringe, followed by 2.0 ml. of either 0.225 mM-EDTA or 0.09% (w/v) peptone. When the contents of the cell reached the correct temperature (usually in 5 min.), the enzyme was injected and the pH adjusted by adding a small quantity of 0.01N-HCl and titration by the instrument to pH 7.0. The second pre-set syringe was then used to inject rapidly the required volume of CO₂-saturated water, and at the moment of addition recording was begun. The initial part of the progress curve was recorded and after a suitable interval (approx. 10 half-lives of the reaction) the equilibrium value was obtained. At the end of the experiment the cell was emptied, rinsed and replaced rapidly so as not to alter the temperature of either the electrodes or of the alkali-delivery tube.

Treatment of experimental data. The rapid hydration reaction catalysed by carbonic anhydrase makes it convenient to determine a rate constant and calculate from it the initial rate, rather than to measure the rate directly as is usual in the study of enzymes. Though the uncatalysed reaction displays pseudo-first-order kinetics, it might be expected that in the presence of the enzyme zero-order behaviour would be observed, especially with substrate concentrations high in comparison with the Michaelis constant. However, under the conditions of the present study satisfactory results were obtained by treating the experimental data as though they obeyed first-order kinetics. A similar treatment of data was adopted by Gibbons & Edsall (1964) in their experiments with the spectrophotometric technique.

The following relationships were derived for the uncatalysed reaction, but were equally applicable in the presence of the enzyme, where k_1' becomes the rate constant of the step in which the enzyme-substrate complex reacts. By using the nomenclature of Gibbons & Edsall (1963), the equilibrium between the reactants in solution may be expressed as:

Since at 0° and pH 7 the ratio $[\text{CO}_2]/[\text{H}_2\text{CO}_3] = 9.5 \times 10^2$ and the ratio $a_{\text{HCO}_3^-}/[\text{H}_2\text{CO}_3] = 2.5 \times 10^3$ (Roughton, 1941), $[\text{H}_2\text{CO}_3]$ is negligible. Therefore the expression can be simplified to:

where the primes denote values at a particular ionic strength. The experimentally observed rate includes a contribution from the reaction:

at pH values greater than 6-7. Pinsent, Pearson & Roughton (1956) report that $k_{\text{OH}^-} = 1.1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ at 0° and low ionic strength.

From eqn. (2) it is possible to derive the rate equation

describing the hydration of CO_2 , as expressed in terms of concentration:

$$-da/dt = k'_1 a - k'_{-1} [\text{H}^+] x \quad (4)$$

where $a = [\text{CO}_2]$ and $x = [\text{HCO}_3^-]$ at any time. This reaction is seen to be of pseudo-first-order at constant pH if the activity coefficient of H^+ remains unchanged. Such an assumption is justified by the fact that the ionic strength is approximately constant during the initial part of the reaction. Moreover, the rate constant is determined after extrapolation to zero time.

At equilibrium, $-da/dt = 0$ and:

$$k'_{-1} = k'_1 a_{\text{eq.}} / [\text{H}^+]_{\text{eq.}} x_{\text{eq.}} \quad (5)$$

Substitution of this expression in eqn. (4) gives:

$$-da/dt = k'_1 a - k'_1 a_{\text{eq.}} [\text{H}^+] x / [\text{H}^+]_{\text{eq.}} x_{\text{eq.}} \quad (6)$$

Again assuming constant H^+ activity coefficient, it follows by substitution of:

$$a_{\text{eq.}} = a_0 - x_{\text{eq.}} \quad (7)$$

where the subscripts indicate equilibrium and zero time, and rearrangement, that:

$$-da/dt = k'_{\text{app.}} (a - a_{\text{eq.}}) \quad (8)$$

Thus:

$$k'_{\text{app.}} = k'_{\text{app.}} (a_0 - a_{\text{eq.}}) / a_0 \quad (9)$$

where $a_{\text{eq.}}$ and a_0 represent the molar concentrations of CO_2 at equilibrium and at zero time, respectively, and $k'_{\text{app.}}$ ($k'_{\text{app.}}$) represents the rate constant of the pseudo-first-order reaction, as experimentally observed. At the beginning of the reaction, assuming that there is no HCO_3^- present:

$$(-da/dt)_0 = k'_1 a_0 \quad (10)$$

This enables one to calculate the initial rate of the hydration reaction.

The value of $(a_0 - a_{\text{eq.}}) / a_0$ is best found by experiment, though an estimate of its magnitude can be made by introducing the equilibrium constant $K = a_{\text{H}^+} a_{\text{HCO}_3^-} / a_{\text{CO}_2}$. This constant has been measured by Harned & Davis (1943) under conditions such that simple interpolation gives the value $2.7 \times 10^{-7} \text{ M}$ for the present case. However, the use of this constant to calculate $(a_0 - a_{\text{eq.}}) / a_0$ involves the substitution of the activity coefficient of H^+ and so the result, 0.78 at pH 7.0, is only approximately correct. The experimentally determined value at the same pH was 0.72.

The record of the volume of NaOH delivered against time was analysed by the usual method for a first-order reaction, which yielded a pseudo-first-order rate constant directly since the volume of NaOH titrated was proportional to the H^+ ions formed which equalled CO_2 hydrated. This rate constant was equal to the sum $k'_{\text{app.}}$ (catalysed reaction) + $k'_{\text{app.}}$ (uncatalysed reaction). The initial rate of the enzyme-catalysed hydration was calculated after subtracting $k'_{\text{app.}}$ for the reaction in the absence of enzyme.

The assumption that the uncatalysed reaction followed first-order kinetics was justified by the linear form of the plots of $\log(\text{titrant vol.}_{\text{eq.}} - \text{titrant vol.}_t)$ against time. In addition, it was found that the first third at least of the catalysed reaction closely followed this rate law. There was no zero-order behaviour observed in the initial part of the progress curves, even at the highest substrate concentrations used. Therefore good estimates of the initial values of

Fig. 1. Relationship between the concentration of carbonic anhydrase isoenzyme C and the sum of the rate constants of the catalysed and uncatalysed hydration reactions, which is proportional to the sum of the initial hydration rates. The substrate concentration was $2.0 \text{ mM-}\text{CO}_2$. $\circ$, In the presence of $25 \mu\text{M-EDTA}$; $\bullet$, in the presence of 0.01% (w/v) peptone.

Fig. 2. Relationship between substrate concentration and initial velocity of the hydration reaction catalysed by carbonic anhydrase isoenzyme C, illustrated by plotting $[\text{initial substrate}] / (\text{initial rate})$ against $[\text{initial substrate}]$. The enzyme concentration was $3.6 \mu\text{M}$ and the conditions were 0° , pH 7.0, 45 mM-NaCl and $5 \text{ mM-sodium phosphate}$. $\circ$, In the presence of $25 \mu\text{M-EDTA}$; $\bullet$, in the presence of 0.01% peptone.

$k'_{\text{app.}}$ were obtained by extrapolation of the log plots to zero time. Approximate values of the initial velocity, made by examination of the progress curves at zero time, gave satisfactory agreement with those calculated as described.

Variation of initial velocity with enzyme concentration. Fig. 1 shows that the sum of the catalysed and uncatalysed rate constants of the hydration reaction, and hence the

initial velocity, was a linear function of the concentration of the enzyme. The assays on which Fig. 1 is based were performed in the presence of $25\text{ }\mu\text{M}$ -EDTA or 0.01% (w/v) peptone, with a substrate concentration of 2.0 mM - CO_2 . Each point represents the results of a single determination. The useful upper limit of enzyme concentration was reached under the present experimental conditions when the total rate was 15 times the uncatalysed velocity.

Rate constant of the uncatalysed reaction. Extrapolation to zero enzyme concentration (Fig. 1) yielded an uncorrected value of $k'_1(\text{uncatalysed}) = 0.0024\text{ sec}^{-1}$. Subtraction of the contribution of OH^- catalysis mentioned above left 0.0023 sec^{-1} . Finally, it was necessary to allow for the catalytic effect of phosphate ion. The data provided by Roughton & Booth (1938) and Gibbons & Edsall (1963) indicate that this correction amounts to a reduction of 4% and so the final value of $k'_1 = 0.0022\text{ sec}^{-1}$.

Catalytic constants of human erythrocyte carbonic anhydrase isoenzyme C. The initial velocity of the hydration reaction catalysed by isoenzyme C at five initial substrate concentrations was measured, and the results are shown in Fig. 2 as a graph in which $[\text{initial substrate}]/(\text{initial rate})$ is plotted against $[\text{initial substrate}]$. Each point represents the average of three determinations. The average deviation from the mean of the individual measurements was 2% . However, the error in the absolute value of each determination was probably greater than this, since the errors in CO_2 concentration and in the volumes of the CO_2 and enzyme solutions added were of the order of $\pm 2\%$ and $\pm 1\%$ respectively. Moreover, the temperature control of $\pm 0.1^\circ$ would be expected to lead to an uncertainty in k'_{app} of $\pm 2\%$ (Benson, 1960), and the accuracy of the pH-meter was probably not greater than ± 0.05 pH unit. It was concluded that an absolute error of $\pm 10\%$ could reasonably be expected in the measured value of K_m , though the results obtained on separate occasions agreed within $\pm 5\%$. The uncertainty in V was likely to be greater owing to the difficulty of estimating $[\text{E}_0]$ with accuracy and to systematic errors in the initial velocities and the determination of $(a_0 - a_{\text{eq}})/a_0$.

It can be seen that over the 16-fold range of initial CO_2 concentration examined, in the presence of both peptone and EDTA, isoenzyme C closely obeyed the Michaelis equation. Consequently, it was possible to assign to it the values $K_m = 8.2\text{ mM}$, $V/[\text{E}_0] = 5.0 \times 10^4\text{ sec}^{-1}$, under the conditions of this experiment.

DISCUSSION

In the present investigation only the hydration reaction catalysed by carbonic anhydrase was examined. The apparatus used for that purpose could equally well be employed to study the dehydration reaction, though in this case certain precautions would have to be observed for the following reasons. The K_m of dehydration appeared to be considerably greater than that of the hydration reaction when both were examined by Gibbons & Edsall (1964). Therefore, to make reliable measurements, higher concentration of the substrate, which is HCO_3^- , would have to be used and this would induce much variation in the initial ionic strength.

Kernohan (1964) has suggested that results may be wrongly interpreted if insufficient attention is paid to the maintenance of constant ionic strength and anion concentration. In his kinetic study of the bovine erythrocyte enzyme, he found K_m of the dehydration to be dependent on the anion concentration, becoming very large concomitantly with an increase in the anion concentration. Moreover, he found that the initial velocity decreased as the anion concentration increased, and it was therefore presumably affected in this way by the substrate itself.

The value of k'_1 for the uncatalysed reaction, found by the titration method used in the present study, is in good agreement with previous determinations at 0° , which averaged 0.0021 sec^{-1} (Edsall & Wyman, 1958). Roughton & Booth (1938), Mills & Urey (1940) and Gibbons & Edsall (1963) have all shown that this rate constant is independent of ionic strength. In addition, Roughton & Booth (1938) have demonstrated that HCO_3^- ions have no significant catalytic action on the hydration rate.

On the other hand, the enzymic hydration rate may be influenced purely by ionic strength and there is no doubt that most anions exhibit a specific inhibitory effect, as in the case of the reverse reaction. Thus, in his experiments with the bovine erythrocyte enzyme, Kernohan (1964) found anion inhibition also in the hydration reaction, though he presented no evidence that K_m was influenced by anions. For these reasons care was taken in the present work to minimize the increase in ionic strength during the reaction by adding sodium chloride and by extrapolating the log plots to zero time when measuring k'_{app} .

With deionized water the initial velocity was not a function of the enzyme concentration unless peptone or EDTA was included in the assay. Instead, the slope of the plot of velocity against enzyme concentration increased with rising enzyme concentration and appeared to approach a maximum. A similar effect was observed by Roughton & Booth (1946) and also by Clark & Perrin (1951). The addition of peptone or EDTA completely eliminated this behaviour. It is assumed that some impurity, perhaps a heavy-metal ion, was responsible for causing an inhibition, and that when sufficient enzyme was available to chelate the inhibitor the effect was abolished. In the present work relatively small amounts of EDTA were required to produce a linear relationship: $1\text{ }\mu\text{M}$ -EDTA was found to be sufficient. A plot of initial velocity against the EDTA or peptone concentration indicated that the amounts used as a routine were ample and that neither substance led to inhibition when present in large quantities. If peptone was replaced by EDTA in the assay there

was no difference in the initial velocity and no change was detected under such conditions in the kinetic constants of isoenzyme C. Gibbons & Edsall (1964) reported a similar finding and state that neither one nor the other substance affected the uncatalysed reaction. Davis (1959), too, showed that EDTA had no inhibitory action on the human erythrocyte enzyme.

Glass-distilled water, unlike deionized water, did not lead to an effect suggesting inhibition. When glass-distilled water was used almost as great a rate was obtained in the presence as in the absence of peptone. Nevertheless, in all routine experiments, including several not reported here, in which carbonic anhydrase from sources other than human erythrocytes was investigated, deionized water was employed and peptone, rather than EDTA, added to the assay. This procedure was followed because it has been observed by Ho & Sturtevant (1960) that EDTA causes a sixfold activation of the bovine erythrocyte enzyme.

Experimenting with an electrometric assay method in a flow system, DeVoe & Kistiakowsky (1961) obtained erratic results that they explained by postulating that the enzyme was being adsorbed on the surface of the glass electrode. No such effect was observed in the present work, but the presence of the peptone may have helped to prevent it.

When compared with the values of the kinetic constants of isoenzyme C obtained at 25° by Gibbons & Edsall (1964), the results found in the present work show that K_m decreased from 12mM at 25° to 8mM at 0°, and likewise $V/[E_0]$ decreased from $2.8 \times 10^5 \text{sec}^{-1}$ to $5.0 \times 10^4 \text{sec}^{-1}$. In both investigations the experiments were performed at approx. pH 7.0, but in the present one the ionic strength was twice as great and resulted mainly from the presence of sodium chloride, whereas the sodium phosphate concentration was fivefold lower. This difference in the composition of the assay mixture makes it difficult to compare the values of $V/[E_0]$ found in the two investigations.

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