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Determination of Carbonic Anhydrase (E. C. 4.2.1.1.) Activity by Means of the pH-Stat-Technique¹⁾

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An improved pH-stat-technique for the determination of carbonic anhydrase is presented. As compared with common techniques the main advantages are:

1. The hydration reaction of CO_2 catalyzed by carbonic anhydrase can be determined at a constant and selected pH-value,
2. the substrate concentration can be kept constant,
3. the reaction volume amounts to only 2 ml, and
4. the reaction can be measured continuously for longer periods of time. The applicability of the method is demonstrated by several well known kinetic data. Instructions for performing the experiments and for the calculation of the results are presented in the Materials and Methods section of the paper.

Es wird eine verbesserte pH-stat-Methode zur Bestimmung der Carboanhydratase-Aktivität beschrieben. Die Vorteile gegenüber den bisher verwendeten Methoden bestehen im wesentlichen darin, daß die durch die Carboanhydratase katalysierte Hydratationsreaktion von CO_2 bei einem konstanten und wählbaren pH-Wert, bei gleichbleibender Substratkonzentration und in einem Reaktionsvolumen von nur 2 ml kontinuierlich über längere Zeit gemessen werden kann. Die Brauchbarkeit der Methode wird durch einige bekannte kinetische Daten belegt. Eine Anleitung zur Durchführung der Versuche und zur Berechnung der Ergebnisse ist im methodischen Teil der Arbeit wiedergegeben.

The commonly used procedures for measuring the activity of carbonic anhydrase are not quite satisfactory (1, 2). Most of them are simple but not sufficiently precise with respect to constancy of pH and of substrate concentration. On the other hand, the "stop flow" technique (3, 4, 5) requires extensive equipment hardly applicable to routine use.

In general, the pH-stat-technique has advantages, e. g. high consistency, and allows a great variability of experimental conditions. LEIBMAN et al. (6) were the first to determine the activity of carbonic anhydrase by means of this procedure. Later the hydration as well as the dehydration rates were estimated using the pH-stat-technique (7, 8). The determination, however, was limited to very short periods of time (15—30 sec) during which reliable measurements were possible. The simple pH-stat-technique described in the present paper allows the continuous determination of carbonic anhydrase activity for more than 5 min, thus rendering it suitable for routine use and kinetic investigations.

Materials and Methods

For the continuous pH-stat titrations the equipment of Radiometer-Copenhagen was used (see Apparatus). All measurements were performed at pH 7.75 with a proportional band adjustment of 0.05. By means of an ultracryostat (Colora FK 2300) the temperature of the jacketed reaction vessels and of the CO_2 tubing was adjusted to $5 \pm 0.2^\circ\text{C}$. Experiments conducted at 0°C did not yield adequate results, because of the sluggishness of the electrodes used. Daily prepared NaOH, 0.1 mol/l, served as the titrating agent (Titrisol Merck). A barbital-buffer, 30 mmol/l (pH 7.9), was used throughout the experiments, since this concentration did not impair the carbonic anhydrase activity in contrast to other buffer systems. Higher concentrations of the barbital-buffer inhibited the carbonic anhydrase activity (50 mmol/l = 18 %, 70 mmol/l = 47% inhibition). In order to stabilize the purified carbonic anhydrase (bovine erythrocytes carbonic anhydrase, Biochemica Boehringer, Mannheim), it was necessary to add 100—500 mg gelatine to 1 liter of the barbital-buffer. Only under these conditions was the enzymatic activity proportional to the concentration of the enzyme. Hemolyzed erythrocytes and tissue homo-

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genates in an appropriate dilution could be used in place of the purified carbonic anhydrase. The formation of foam, which interferes with the measurement, could be prevented by siliconization of the upper part of the reaction vessels.

CO₂ as the substrate was used in a mixture with O₂ or N₂ (35–124 ml CO₂/l O₂ and N₂ resp.). Higher concentrations of CO₂ should be avoided, because of the high rate of spontaneous and enzymatically catalyzed hydration-reaction occurring under this condition. The gas-mixture was instilled through a thin polyethylene tube, which passed by the electrodes. This tube ended in a bent cannula located at the bottom of the reaction-vessel between the glass-wall and the magnetic stirring bar. Thus a sufficient distribution of the gas-bubbles (about 50 ml/min) in the barbital-buffer was produced and a constant concentration of the substrate was guaranteed even at high enzymatic activities. Generally, the CO₂/O₂ mixtures were prepared in our laboratory and the particular partial-pressure of CO₂ was monitored by means of a pCO₂-electrode (see Apparatus) and adjusted as closely as possible to the required value. Identical results were obtained with calibrated CO₂/N₂-mixtures commercially available (Messer-Griessheim, Mannheim). The concentration of CO₂ physically dissolved at 5°C could be calculated according to HENRY's law, since the barbital-buffer does not influence the solubility of CO₂ (9).

Apparatus

- Automatic titration device (Radiometer Copenhagen): pH-meter — PHM-26c, titrator — TTT1b, titrigrph — SBR 2, auto-burette — ABU 12b (0.25 ml), titration assembly — TTA 31, reaction-vessel — V 516, glass electrode — G 2222c, calomel electrode — K 4112 (filled with KCl-solution saturated at 5°C).
- Ultracryostat (e. g. Colora FK 2300) for the regulation of the temperature of the reaction-vessel and the gas-supply.
- Mixtures of CO₂ with O₂ or N₂. If the mixtures of CO₂ required could not be produced in our own laboratory, calibrated gas-mixtures were obtained commercially (e. g. Messer-Griessheim, Mannheim).
- Device for the determination of the pCO₂ by means of a pCO₂-electrode (e. g. blood-gas-analyzer Eschweiler + Co, Kiel or Astrup, Radiometer Copenhagen). The determination of pCO₂ can also be omitted, since a calculation of the CO₂-concentration in the reaction mixture is possible, based on the spontaneous hydration of CO₂.

Procedure

- Filling of the burette with NaOH (100 mmol/l)
- Calibration of the electrodes at 5°C
- Determination of spontaneous hydration of CO₂. Reaction-vessel filled with 2 ml of barbital-buffer (5°C). Perfusion with different concentrations of CO₂. Begin recording after the pH had dropped from 7.9 to 7.75.
- Determination of the enzymatically catalyzed hydration of CO₂. Adequate dilutions of tissue-homogenates or of purified carbonic anhydrase in barbital-buffer made up to a final volume of 2 ml in the reaction-vessel with barbital-buffer; otherwise identical with c). The carbonic anhydrase activity equals the total activity minus the spontaneous hydration-rate of CO₂.
- Determination of the activity in presence of an inhibitor (e. g. acetazolamide). Preincubation of carbonic anhydrase and inhibitor in the reaction-vessel filled with 2 ml barbital-buffer. After 5 min CO₂ as the substrate is instilled; otherwise identical with c) and d). Acetazolamide can also be added in a small volume (10–100 µl) during the course of recording, because the final degree of inhibition was obtained in less than 15 s. For this particular time-interval the direct addition caused an interruption of the registration.

Data analyses

- Determination of pCO₂ according to the concentration of CO₂ in O₂ and N₂ respectively.

pCO₂ = volume fraction CO₂ (barometric pressure minus vapour pressure of H₂O at 5°C).

- Determination of CO₂ molality at 5°C using pCO₂ according to HENRY's law

$$\text{Molality CO}_2 = \frac{2.774 \cdot \text{pCO}_2}{44 \cdot \text{barometric pressure}} \left[\frac{\text{mol}}{\text{kg}} \right]$$

- Determination of the hydration rate of CO₂. The amount of NaOH (mol) consumed per unit of time equals the amount of H⁺ produced by the hydration of CO₂ in a total volume of 2 ml in the reaction-vessel.

Burette: 0.25 ml NaOH (100 mmol/l = 25 µmol NaOH = 100%)

$$\frac{\text{mol (OH}^-) \cdot 1000 \text{ (ml)}}{\text{min} \cdot 2 \text{ (ml)}} = \frac{\text{mol (H}^+)}{\text{min}}$$

Results and Discussion

Spontaneous hydration of CO₂

The spontaneous, non enzymatic hydration was proportional to the CO₂ concentrations (2.2–7.8 mmol/l) yielding a regression-line $y = 0.0041x - 0.005$ (y = mmol H⁺/s, x = mmol CO₂/l) with a correlation coefficient $r = 0.999$. The slope of the line reflects the rate constant of the hydration, since other rate constants can be neglected under the present experimental conditions (10, 11). The dissociation constant of 0.0041/s, determined at 5°C, is in good agreement with the values obtained by MAREN (9) at 1°C (0.0027/s) and at 8.2°C (0.0078/s).

Precision

For the spontaneous hydration rate of CO₂, as determined for a period of more than 25 days, a variation-coefficient (CV) of $\pm 7.2\%$ was determined.

Fig. 1

Proportionality between amount of carbonic anhydrase and hydration rate of CO₂

Abscissa: Amount of carbonic anhydrase present in 2 ml
Ordinate: Enzymatic activity expressed as MAREN-units. 1 unit according to MAREN equals that amount of enzyme which doubles the spontaneous hydration rate of CO₂.
Each point represents the mean ($\bar{x} \pm s\bar{x}$) of enzymatic activity in the presence of 9 different concentrations of CO₂ (2.2–7.8 mmol CO₂/l)

Fig. 2

Hydration of CO_2 in the presence of 4 different amounts of carbonic anhydrase, plotted acc. to LINEWEAVER and BURK

Carbonic anhydrase concentration (ng/2 ml)	regression coefficient (b)	correlation coefficient (r)
125.0	1.46	0.988
62.5	2.78	0.973
31.3	5.42	0.988
15.6	10.67	0.879

Enzymatic catalyzed hydration of CO_2

A proportionality between different concentrations of an enzyme and its enzymatic activity is a prerequisite for the application of the MICHAELIS-MENTEN kinetics. As can be seen in Figure 1, the described method fulfilled these conditions. A correlation was established between four different concentrations of the enzyme (15.6–125 ng carbonic anhydrase/2 ml) and MAREN-units. One MAREN-unit is defined as the amount of carbonic anhydrase which doubles the spontaneous hydration rate of CO_2 . The experimental data obtained from 9 different concentrations of substrate (2.2 to 7.8 mmol CO_2 /l) were used to calculate the MAREN-units. Plotting the MAREN-units versus the corresponding amounts of the enzyme (Fig. 1), 47.5 ng of the carbonic anhydrase-preparation equaled 1 MAREN-unit.

For each of the four enzyme concentrations the LINEWEAVER-BURK plots yielded straight lines with a common intercept at the abscissa (Fig. 2). The mean intercept based on the four regression lines corresponded to a K_m of 34.5 mmol CO_2 /l. Taking into account the differences in the experimental approaches, the K_m -value does not significantly differ from those obtained by DE VOE and KISTIAKOWSKY (4) ($K_m = 9$ –13 mmol CO_2 /l, conditions: carbonic anhydrase from bovine erythrocytes, pH = 7.0, 0.5°C, phosphate-buffer) and by MAREN (9) ($K_m = 23.0$ mmol CO_2 /l, conditions: carbonic anhydrase from canine erythrocytes, pH = 7.8, 0–12°C, barbital-buffer).

Fig. 3

Inhibition of carbonic anhydrase by acetazolamide plotted acc. to DIXON

CO_2 concentration (mmol/l)	regression coefficient (b)	correlation coefficient (r)
1.5	279.2	1.0
3.2	121.0	1.0
5.3	91.8	1.0

The maximal rate of hydration ($V_{\max}$) can be calculated from the K_m -value and the slope of the straight lines. The international enzyme unit ($\text{U} = \mu\text{mol substrate/min}$) can be derived from $V_{\max}$ and the corresponding enzyme concentration. About 5 pg of the used carbonic anhydrase-preparation are equivalent to 1 U.

Precision

The variation-coefficient (CV) for the carbonic anhydrase activity was $\pm 3.9\%$ for a series of one day, and $\pm 6.6\%$ for a period of more than 25 days.

Accuracy

Due to differences in the determination procedures as well as in the definitions of carbonic anhydrase-units, data concerning the accuracy of the present technique cannot be given. According to the supplier (Biochemica Boehringer, Mannheim) the carbonic anhydrase preparation used in our experiments contained 1 MAREN-unit/500 ng (determined by chromometric changing pH-technique). According to the pH-stat technique this preparation contained 1 MAREN-unit/47.5 ng (see Fig. 1).

Inhibition of carbonic anhydrase with acetazolamide

The carbonic anhydrase was inhibited noncompetitively but reversibly by acetazolamide. The dissociation constant (K_i) of this classical inhibitor amounted to 3.9 nmol/l according to the DIXON-plot (Fig. 3). This value is lower than that obtained by LEIBMAN et al. (6) (8.5 nmol/l). Under our experimental conditions K_i proved to be independent of the CO_2 concentration and was not influenced by the preincubation time (range investigated: 15 s to 5 min).

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