acid was slowly added with stirring to disperse, by dissolution of the capsular substance, the stringy coagulum first formed, leaving a granular precipitate. The ethanolic supernatant, thoroughly filtered, was treated with NaOH solution until with neutralization maximum precipitation had occurred. After re-solution in acid 75% (v/v) ethanol, re-filtration and re-precipitation with NaOH, this precipitate, dissolved in a small volume of water, was subjected to dialysis against N/50 HCl and finally against distilled water—a precipitate forming during dialysis being removed by centrifugation. On evaporation the dialyzed solution became viscid as dryness was approached and the capsular material was eventually left as a tough vitreous layer on the floor of the containing dish from which it was parted with difficulty, emitting at separation an almost metallic 'cry'.

This material showed the general features of the products of Tomcsik and Ivánovics and their collaborators and, though reacting in high dilution with the only anti-P (anti-capsular substance) serum prepared against the whole organism, failed itself to engender a formation of antibodies in any of several rabbits injected with its solutions.

There was observed its ability to form precipitates with methylene blue and atebirin and Dr Harold King noted a similar reaction with diamidines. My colleague, Mrs R. V. Pitt Rivers, who further purified and freeze-dried a sample of the substance, from free amino-N determinations assessed the molecular weight at about 9000.

REFERENCES

- Ivánovics, G. & Erdős, L. (1937). *Z. Immunitäts.* **90**, 5.
 Tomcsik, J. & Szongott, H. (1933). *Z. Immunitäts.* **78**, 86.
 White, P. Bruce (1932). *J. Path. Bact.* **35**, 77.
 White, P. Bruce (1934). *J. Path. Bact.* **39**, 529.
 White, P. Bruce (1936). *J. Hyg., Camb.*, **35**, 498.

The Manometric Determination of the Activity of Carbonic Anhydrase under Varied Conditions

By F. J. W. ROUGHTON AND V. H. BOOTH (Ramsay Memorial Fellow), *Physiology Laboratory, University of Cambridge, England, and the Fatigue Laboratory, Harvard University, Boston, Mass., U.S.A.*

(Received 18 December 1945)

The activity of carbonic anhydrase was first measured by Meldrum & Roughton (1932) with the boat-manometric method which Brinkman, Margaria & Roughton (1933) had developed for study of the kinetics of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ in absence of enzyme. In this method a buffer solution is shaken violently with bicarbonate solution (or with a CO_2 -containing gas phase) in a boat-shaped vessel which is flexibly connected to a U-tube manometer. The rate of CO_2 output (or uptake) is followed by reading the pressure at various times after the shaking begins. The magnification of the rate, when carbonic anhydrase is added to the buffer, is taken as a measure of the activity of the enzyme. Variations and improvements in the manometric method have since been made by Stadie & O'Brien (1933), Van Goor (1934), Roughton & Booth (1938), Kiese & Hastings (1940), Kiese (1941), and Leiner (1941).

In all such work diffusion between the gas and liquid phases tends to become a limiting factor as the reaction velocity of the chemical process rises. The influence of diffusion can, however, be allowed for quantitatively by the theoretical and experimental methods recently worked out by Roughton

(1941) on the basis of the stationary film theory of gas-liquid interchange.

In another set of methods the time is measured for the reaction to bring about a certain change in pH in absence and in presence of the enzyme. Stadie & O'Brien (1933) followed the pH electrometrically, but most authors have preferred the much more simple methods with indicators (Brinkman, 1933; Van Goor, 1934; Philpot & Philpot, 1936). These methods are, however, in general much less powerful and definite than manometry as regards the range of conditions under which the activity of the enzyme can be investigated.

All the methods hitherto used are fairly adequate for determining the number of enzyme units present in a given preparation and have accordingly been much used in

(i) The study of the distribution of the enzyme in the blood, body fluids and tissues of a wide range of organisms with a view to unravelling its role in CO_2 transport, gastric secretion and other physiological processes.

(ii) The development of methods of purifying the enzyme, culminating recently in its crystallization by Scott & Fisher (1942).

(iii) The study of inhibitors and activators, though here much caution is needed in interpreting the results.

So far, however, no detailed study has been published of

(iv) The physico-chemical kinetics of the enzyme, e.g. the effect of substrate concentration, enzyme concentration, pH, salts, temperature, etc. Such knowledge is indispensable for working out the quantitative role of the enzyme in physiological processes (such as CO_2 removal into the expired air), as well as from the general viewpoint of enzyme chemistry.

Arbitrary units of enzyme activity are of no service for the projects listed under (iv), and it is instead essential to determine, in the ordinary chemical units of mol./l./sec., the extra rate of H_2CO_3 dehydration or CO_2 hydration brought about by the enzyme. This task has, for the following reasons, proved far more cumbersome than is usually the case in enzyme kinetics.

(a) CO_2 hydration and H_2CO_3 dehydration proceeded at appreciable rates in absence of the enzyme, and furthermore are catalyzed by inorganic buffers (Roughton & Booth, 1938; Kiese & Hastings, 1940).

(b) Progressive, and often appreciable, inactivation of the enzyme occurs during the manometric shaking.

(c) As mentioned already, the results have to be corrected for the limiting effect of diffusion on the speed of the manometric changes.

We have, however, extended the manometric method to cope with these difficulties, though at the cost of much complexity in the computations. In the following paper we describe the applications of the new procedure to the kinetics of the enzyme. But the extensions which we have made to the existing methods may well be necessary at times in work on the distribution, function, purification, inhibition, destruction and activation of the enzyme, and it therefore seems useful to present them separately here.

EXPERIMENTAL METHODS

For the attainment of accurate and repeatable results under varied conditions we have made some changes in the boat-manometric technique since our previous paper (Roughton & Booth, 1938), which should, however, be consulted for fuller details of the actual procedure. We have also had to take special care with the preparation of the buffer solutions and the handling of the enzyme preparations. Points under these three headings are discussed in the present section.

The boat-manometric technique. In all our recent experiments 4.2 ml. of liquid at 0° have been shaken violently at 290–320 times/min. with a 60 ml. gas phase at the same total air pressure within the manometric system as before,

namely $\frac{1}{2}$ atm. for CO_2 output and $\frac{1}{2}$ atm. for CO_2 uptake experiments. In our 1938 technique the 64 ml. experimental vessel was connected to one limb of the manometer, to the other limb of which was connected a 200 ml. thermo-compensating bottle fixed in one corner of the thermostat tank. Even with vigorous stirring of the tank a small variable irregularity traceable to thermal effects was observed in slow experiments. We have, therefore, replaced the fixed compensating bottle by a vessel identical in size and shape with the 'live' experimental vessel and held on the same shaker bar. The whole arrangement of manometric tubes and vessels is now symmetrical (as in the Barcroft differential manometer), and minute thermal and mechanical disturbances tend to balance out. The 'live' vessel is loaded with buffer solution $\pm$ enzyme, and with bicarbonate or with CO_2 gas according to whether CO_2 output or uptake is to be measured: the compensating vessel is freshly loaded with the same total volume of liquid (water) as the live vessel for each experiment. The buffer solutions are made up with water previously equilibrated with air at room temperature, and similar water is used in the compensating vessel. An almost equal amount of air therefore comes out of solution in each of the two vessels when shaking begins, so that manometric effects due to gases other than CO_2 are, in this way, practically eliminated.

In our recent work we have replaced the metal thermostat tank by a glass aquarium jar, so as to be able to see more easily inside the tank during necessary manipulations of the vessels and their connexions *in situ*. The ridged two-compartment vessels, which are indispensable for CO_2 output experiments, have been used also for CO_2 uptake so as to make conditions more comparable.

In uptake experiments at high CO_2 pressures an open-ended mercury manometer is used without any compensating vessel, the omission of which under these conditions leads to no appreciable loss of accuracy. Otherwise the manometer gauges are of simple U-shape without any bottom opening, and after having been cleaned with chromic acid and distilled water and dried with air, are generally filled through one side with de-aerated water. With water in the gauge no contamination of the enzyme by foreign vapour can occur. In a few experiments Clerici's fluid (sp.gr. 4.2) or *iso*-octane (sp.gr. 0.7) has been used in place of water. In experiments with CO_2 pressures of less than 3 cm. of water, the manometer is sloped at a gradient of 1 in 3.5, instead of being vertical, thereby giving about three times greater sensitivity per mm. movement of gauge fluid. With these various devices CO_2 pressures ranging from 0.005 to 0.5 atm. of an atmosphere can readily be used.

The manometers are calibrated by the calculation methods given by Dixon (1934), these being checked by liberation of known volumes of CO_2 from mixtures of HCl and NaHCO_3 . As an example we quote the details of the calibration for the majority of the experiments. Here the gauge is vertical and filled with water and the total gas pressure in the manometric vessels is initially $\frac{1}{2}$ atm.

Let x = total amount of CO_2 , measured in ml. at N.T.P., which is formed by, or disappears as the result of, the chemical reaction in the live vessel,

h = difference in cm. thereby produced between the liquid levels in the two limbs of the manometer,

θ = angle of inclination of U-tube to vertical (usually 0°),

A = area of cross-section of U-tube = 0.024 sq. cm.,
 V_g = volume of gas phase in live vessel = 59 ml.,
 V_g' = volume of gas phase in compensation vessel = 61 ml.,
 $V_L = V_L'$ = volume of liquid in each vessel = 4.2 ml.,
 P = total initial pressure in each vessel = 145 cm. water,
 p_w = aqueous vapour pressure at 0° = 4 cm. water,
 P_0 = the normal pressure = 76 cm. Hg = 1030 cm. water,
 α = solubility coefficient of CO_2 in water at 0° = 1.7,
 T = absolute temperature = 273° .

With vessels and tubes of these dimensions the following simplified form of Dixon's (1934) general equation (5) gives the calibration factor to less than 1%, namely,

$$x = h \left(\cos \theta + \frac{A(P - p_w)}{2V_g'} \right) \times \left(\frac{V_g \frac{273}{T} + V_L \alpha}{P_0} + \frac{A}{2} \times \frac{273}{T} \times \frac{P - p_w}{P_0} \right), \quad (1)$$

whence $x = h \times 0.0677$, i.e. a difference of level of 1 cm. corresponds to a total change of 0.0677 ml. CO_2 , of which $0.0677 \times 59 / (59 + 4.2 \times 1.7) = 0.0604$ ml. represents the change in CO_2 content of the 59 ml. gas phase, and $0.0677 \times 4.2 \times 1.7 / (59 + 4.2 \times 1.7) = 0.0073$ ml. represents the change in the amount of CO_2 dissolved in the 4.2 ml. liquid phase, as distinct from that in chemical combination.

Thus the change in molality of dissolved CO_2 corresponding to a difference of gauge level of 1 cm. = $0.0073 / 4.2 \times 22.4 = 7.76 \times 10^{-5} \text{ M-HCO}_3^-$ at 0° , whereas the change in the chemically bound CO_2 present in the solution

$$= 0.0677 / 4.2 \times 22.4 = 7.2 \times 10^{-4} \text{ M}. \quad (2)$$

Preparation of buffer solutions. The manometric method has proved suitable for CO_2 output experiments from pH 5.5 to 7.5 and for CO_2 uptake experiments from pH 6.5 to 10.0. The buffer systems which are available over this range are, however, limited, since in many cases one or other of the constituents of the buffer has an inhibitory effect on the enzyme and/or a marked effect on the reactions of CO_2 in water in absence of added enzyme. Experiments to be given elsewhere have, however, shown that the pH range 5.5–9.5 can be satisfactorily covered by the following systems, which, as emphasized below, must be kept free of all but minimal traces of CO_2 (i.e. $< 0.0001 \text{ M}$).

(i) Cacodylic acid-sodium cacodylate (pH 5.5–6.5). Solid cacodylic acid and CO_2 -free NaOH are dissolved in calculated amounts in CO_2 -free twice glass-distilled water, leaving only a small air space in the volumetric flask.

(ii) Phosphate (pH 6.3–7.7). A stock solution of $\text{m-KH}_2\text{PO}_4$ is made up by dissolving the calculated amount of the analytical grade salt in CO_2 -free twice glass-distilled water. A second stock solution m in total phosphate but containing KH_2PO_4 to K_2HPO_4 in the ratio 1 : 6 is similarly made up and the traces of CO_2 removed by warming the solution to 65° and evacuating at a powerful water-pump, shaking 1 min., letting in air through a dust filter and then repeating the process twenty times. The solution is made

up to volume and the ratio of KH_2PO_4 to K_2HPO_4 checked by titration. In many cases KHCO_3 has been used in place of K_2HPO_4 . Solutions having any desired ratio of KH_2PO_4 to K_2HPO_4 are made by mixing appropriate proportions of the two stock molar solutions.

(iii) Veronal (pH 7.6–8.5). Veronal and its sodium salt are weighed into a glass-stoppered volumetric flask and warm freshly boiled twice glass-distilled water is added so that only a small air space remains. Free veronal is used in place of the HCl customarily used in preparing veronal buffers from the sodium salt, since, below pH 8.0, Cl^- inhibits carbonic anhydrase.

(iv) Dimethylglyoxaline (pH 8.5–9.5). The crystalline hydrochloride of dimethylglyoxaline is hygroscopic. The contents of a sealed tube are therefore dissolved in water to make a solution of about 0.4 M strength, and the molarity found by titration. Portions of this stock solution are diluted with CO_2 -free NaOH.

Entry of CO_2 into the solutions after preparation is prevented by keeping them in glass-stoppered vessels with only a small air space. They are used only infrequently and usually only for one group of experiments. Concentrated stock solutions are made whenever solubility permits. The more alkaline solutions are poured quickly into a long burette with a greaseless tap, portions buretted directly into the manometer vessels as required and the upper portions of the liquid column discarded after a few hours. Even in the most alkaline solution used (namely, pH 9.5) the last portion remaining in the burette at the end of the day was found by Van Slyke analysis to contain only 0.00011 M-CO_2 .

The reason for such stringency is that appreciable traces of CO_2 would in output experiments raise the substrate concentration and alter the pH by a variable amount, while in uptake experiments the velocity of the back reaction would also be increased to an arbitrary degree. Furthermore, above pH 8.3, the CO_3^{2-} ions increasingly formed would have an inhibitory effect on the enzyme.

To avoid inactivation of the enzyme by metallic impurities, twice glass-distilled water and chemicals of analytical grade purity are used for making up the buffers wherever possible. All glassware used for the enzyme and other solutions is cleaned with chromic acid and then rinsed several times with twice glass-distilled water, contamination with dust and water which had been in contact with metal being sedulously avoided.

Two other solutions besides the stock buffers should also be mentioned:

(v) The bicarbonate solutions used in CO_2 output experiments. Solid KHCO_3 is freshly dissolved every few hours in water previously equilibrated with air, transferred to a greaseless burette and withdrawn from the bottom into the manometer vessel. The solution so prepared contains only 1% carbonate, which has but negligible effect on the pH immediately after mixing with the buffer, and is therefore preferred to the stable 5 : 1 bicarbonate : carbonate solution of Hodgson (1936) when used with very dilute

buffer solutions. No appreciable CO_2 is lost from the bicarbonate solution during its brief contact with air while equilibration proceeds.

(vi) CO_2 -free NaOH . This is obtained by centrifuging the saturated solution (about 18M- NaOH) in rubber-capped paraffined centrifuge tubes to remove carbonate. The supernatant solution is used directly, a long narrow pipette tip being placed well below the surface, the pipette filled, its outside quickly washed with water, its tip placed below the surface of the water in the volumetric vessel and the NaOH run in. This technique gives the greatest freedom from CO_2 contamination of any we have tried.

Handling of enzyme preparations. Most of our experience has been with the crude 'chloroform enzyme' of Meldrum & Roughton (1933) prepared as follows.

To 10 ml. of washed ox red cells are quickly added 8 ml. of 40% ethanol and 4 ml. of chloroform, the mixture is stirred in a centrifuge tube till the thick paste becomes a thin sludge (about 3 min.) and allowed to stand at least 20 min. before centrifuging. This slight modification in quantities gives a clearer and more colourless solution than the original method. We have also worked with crude laked blood and with the highly purified preparation of Keilin & Mann (1940), but have not yet had the opportunity to study the crystalline preparation of Scott & Fisher (1942).

The activity of the crude chloroform preparation remains constant for at least a year if stored at -10° . For testing it should be thawed at a little over 0° , diluted to 1 in 2000 with ice-cold water and then kept in the refrigerator, since the dilute solution, if allowed to warm up to room temperature under certain circumstances, soon loses its activity. The test portion of the diluted enzyme solution (often 0.1 ml.) is measured directly into the buffer solution in the manometric vessel, which is usually already immersed partially in the thermostat. The enzyme solution is now highly diluted, with the result that it has a marked tendency to inactivate during the shaking: this is allowed for in the way described later. In CO_2 uptake experiments there is also a preliminary period of shaking for the purpose of bringing the gases dissolved in the liquids into equilibrium with the gas phase before the CO_2 gas is introduced into the manometric vessel. By reducing this period to 5 sec. the inactivation produced should be inappreciable, whereas the equilibrium between the liquid and gas phases is so nearly reached that the residual exchange when the main shaking starts is mostly balanced by the compensating vessel. The stoppers of the manometric vessels are of rubber but are small and are situated at the top of long necks, so that only very slight contact is made by the enzyme solution with the rubber during the shaking. Some further points under this heading are taken up in other sections of this paper.

THE CALCULATION OF ENZYME ACTIVITY

Changes in the observed activity of the enzyme take place during a manometric determination owing to (i) the inactivation of the enzyme produced by the violent shaking, (ii) the changes during the progress of the experiment in the pH and concentrations of substrate and buffer. To avoid such difficulties we calculate the enzymic activity at a number of instants and extrapolate therefrom to zero time, thus obtaining an activity which is independent of all these changes. Correction is, however, still needed for the limiting effect of diffusion on the observed manometric rates, according to Roughton's (1941) equations, which, as shown below, need modification in certain cases. The requisite elaboration of the diffusion equations is described below (in small type) and may be omitted on a preliminary reading if desired.

Diffusion corrections in CO_2 uptake experiments. Equation (15) of Roughton (1941) is often accurate enough, especially at low CO_2 concentrations, but to define the accuracy precisely we must work out an amendment of his more general equation (29). The latter is not quite exact since it takes no account of the non-enzymic hydration of CO_2 which occurs simultaneously with the enzymic hydration. Roughton & Booth (1938) have shown that the extrapolated value of the non-enzymic hydration rate R_B^0 at zero time is given by

$$R_B^0 = k_u c_L (1 + l[B]) \text{ in mol. CO}_2/\text{l./sec.}, \quad (3)$$

where k_u = velocity constant of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ in absence of all catalysts = 0.0021 at 0° ,

c_L = concentration of dissolved CO_2 in the bulk of the liquid,

B = the catalytically active constituent of the buffer system,

l = catalytic coefficient of B .

Roughton's equation (28) should therefore be replaced by

R^0 = extrapolated observed rate of CO_2 uptake, at zero time, in presence of enzyme

$$= \frac{D_i A'}{\delta V_L} (c_i - c_L) = \frac{k_e E c_L}{c_L + K_{mu}} + v_u c_L, \quad (4)$$

where D_i = diffusion coefficient of dissolved CO_2 ,

δ = thickness of the stationary film of liquid at the gas/liquid interface,

A' = area of the stationary film,

V_L = volume of the liquid phase,

c_i = concentration of dissolved CO_2 at the outer surface of the stationary film = αp_i ,

α = solubility coefficient of CO_2 ,

p_i = pressure of CO_2 in gas phase,

K_{mu} = Michaelis constant of enzyme for CO_2 uptake,

k_e = velocity constant for dissociation of CO_2 -enzyme complex,

E = concentration of enzyme,

$v_u = k_u (1 + l[B])$.

Equation (14) of Roughton's paper, in the present symbols, reads

R_m = maximum rate of CO_2 uptake, at zero time, which the manometric apparatus can record (i.e. when diffusion is completely the limiting factor)

$$= \frac{D_i A'}{\delta V_L} c_i, \quad (5)$$

whilst by the Michaelis theory of enzyme action verified elsewhere for carbonic anhydrase (Roughton & Booth, 1946)

R = true rate of CO_2 uptake, at zero time, in presence of enzyme

$$= \frac{k_e E c_i}{c_i + K_{mu}} + v_u c_i. \quad (6)$$

From (4), (5) and (6) it follows that

$$\frac{R_m}{R^0} = \frac{c_i}{c_i - c_L}, \quad \frac{R_m}{R_m - R^0} = \frac{c_i}{c_L},$$

and

$$\begin{aligned} \frac{R_m R^0}{R_m - R^0} &= \frac{c_i}{c_L} R^0 = \frac{k_e E c_i}{c_L + K_{mu}} + v_u c_i \\ &= R \frac{c_i + K_{mu}}{c_L + K_{mu}} + v_u c_i \left(1 - \frac{c_i + K_{mu}}{c_L + K_{mu}} \right), \end{aligned}$$

whence

$$\begin{aligned} R &= \frac{R_m R^0}{R_m - R^0} \times \frac{c_L + K_{mu}}{c_i + K_{mu}} + v_u c_i \left(1 - \frac{c_L + K_{mu}}{c_i + K_{mu}} \right) \\ &= \frac{R_m R^0}{R_m - R^0} \left\{ 1 - \frac{c_i - c_L}{c_i + K_{mu}} \left(1 - \frac{R_m - R^0}{R_m R^0} v_u c_i \right) \right\} \\ &= \frac{R_m R^0}{R_m - R^0} \left\{ 1 - \frac{c_i}{c_i + K_{mu}} \frac{R^0}{R_m} \left(1 - \frac{R_m - R^0}{R_m R^0} v_u c_i \right) \right\}. \quad (7) \end{aligned}$$

If $v_u c_i$ is neglected, equation (7) then reduces to equation (29) of Roughton's (1941) paper, namely,

$$R = \frac{R_m R^0}{R_m - R^0} \left(1 - \frac{c_i}{c_i + K_{mu}} \frac{R^0}{R_m} \right).$$

In many cases (see later) sufficient accuracy is attained by the use of the simpler equation

$$R = \frac{R_m R^0}{R_m - R^0}. \quad (8)$$

Of the five quantities appearing in equation (7):

(a) R^0 is determined as described below;

(b) v_u , c_i are obtained by the procedure of our previous paper (Roughton & Booth, 1938);

(c) R_m is given by Roughton's (1941) method. This is based either on the manometric rate of solution of CO_2 when 4.2 ml. of water are shaken in the usual way with a gas phase containing CO_2 , or on the initial rate of CO_2 uptake by buffer solutions containing a high concentration of carbonic anhydrase (the observed manometric rate tends to a maximum as the enzyme concentration is increased). At 0° and with the 4.2 ml. of solution shaken under the usual conditions, both methods agree in giving $D_i A'/V_L = 0.63/4.2 = 0.15$ (see Roughton, 1941, pp. 131, 135), so that $R_m = 0.15 c_i$ mol. CO_2 /l./sec.

(d) K_{mu} is first obtained roughly from a series of determinations at different substrate concentrations, the effect of diffusion being allowed for by the approximate equation (8). This rough value of K_{mu} is used in equation (7), and the corresponding values of R at the different substrate

concentrations then give a more accurate value of K_{mu} . By such process of successive approximation the value of K_{mu} is finally fixed within experimental error.

In the following paper it is shown that at 0° , $K_{mu} = 0.009$ over the whole pH range 6.0–9.5. In most of our experiments a standard c_i value of 0.0014 M has been used; R^0/R_m has been kept below 0.2, and since $v_u > 0.0021$ it follows that the expression $\frac{c_i}{c_i + K_{mu}} \frac{R^0}{R_m} \left(1 - \frac{R_m - R^0}{R_m R^0} v_u c_i \right)$ is not greater than 0.03, so that equation (8) is accurate enough.* Outside this range the more elaborate equation (7) must be used.

Theoretically a small diffusion correction (of the order of 3%) is also necessary for R_B^0 , namely,

R_B = true rate of CO_2 uptake, at zero time, in absence of enzyme

$$= \frac{R_m R_B^0}{R_m - R_B^0}. \quad (9)$$

Finally, we have

R_{eu} = true rate of CO_2 uptake due to enzyme alone = $R - R_B$. (10)

Calculation of R_{eu} in a typical example at pH < 8.0. Fig. 1A shows the rate of CO_2 uptake at 0° by a 0.024 M-phosphate buffer solution, pH 7.5, at

Fig. 1. Rate of CO_2 uptake by 0.024 M-phosphate buffer, pH 7.5, at 0° . A, without enzyme; B, with enzyme.

the CO_2 pressure used in most of our uptake experiments, namely, 20 cm. H_2O at zero time, whereas Fig. 1B shows the effect of adding thereto 14.3 parts per million (p.p.m.) of crude enzyme solution. The curves are as usual diphasic, the first rapid uptake being due to physical solution of CO_2 . The slow portion of each curve is extrapolated back to zero time reaching the pressure axis at $p_B^0 = p^0 = 18.2$ cm. This is the normal figure, but a range of ± 0.6 cm. H_2O is possible. The times taken for the CO_2 pressure to drop by 3% of the initial value, i.e. by 0.6 cm. H_2O , are then read off for a series of pressure intervals beginning 0.8 cm. below p^0 .

* Even in the very fast rate represented by the top point of Fig. 2 in Roughton's (1941) paper, equation (8) is accurate to 8%, since $R^0/R_m = 0.6$ and $c_i/(c_i + K_{mu}) = 0.14$.

Now a pressure change of 0.6 cm. H_2O corresponds by equation (2) to $0.6 \times 7.2 \times 10^{-4} = 4.32 \times 10^{-4} M$ - HCO_3^- formation, so the reciprocals of these times multiplied by $4.32 \times 10^{-4} M$ give the mean values of the over-all enzymic rate, R' , and the non-enzymic rate, R'_B , both in mol. $HCO_3^-/l./sec.$ (The calculation of R' and R'_B in the present case is exactly the same as in the detailed example at pH > 8.0 in Table 1.)

Fig. 2 shows the plot of R'/R'_B against t_m , the mean time from the beginning of shaking for each pressure interval, for a duplicate pair of experiments of this kind. The appreciable decay of R'/R'_B with

Fig. 2. Curve showing fall in activity of carbonic anhydrase during an experiment at pH < 8.0. ● First experiment; ○ duplicate experiment.

time is only to a minor extent due to the lowering of pH through uptake of CO_2 (the enzyme activity is sensitive to pH in this range) and is mainly accounted for by the appreciable rate of inactivation of the enzyme with shaking at pH < 8.0. Extrapolation to zero time gives $R^0/R'_B = 3.3 (\pm c. 10\%)$. To obtain R'_B we note that c_L , the extrapolated value of the concentration of dissolved CO_2 , at zero time = $18.2 \times 7.75 \times 10^{-5} = 1.41 \times 10^{-3} M$, and since in the present phosphate buffer, the concentration of the catalytically active constituent, HPO_4^- , = 0.018 M and its l value = 8 (Roughton & Booth, 1938), we have

$$R'_B = 0.0021 \times 1.41 \times 10^{-3} (1 + 8 \times 0.018) \\ = 3.39 \times 10^{-6} \text{ mol. } CO_2/l./sec.$$

Since

$$R_m = 0.15c_i = 0.15 \times 1.41 \times 10^{-3} = 2.11 \times 10^{-4},$$

by equation (8),

$$R_B = \frac{2.11 \times 10^{-4} \times 3.39 \times 10^{-6}}{2.11 \times 10^{-4} - 3.39 \times 10^{-6}} = 3.45 \times 10^{-6}.$$

Now $R^0 = 3.3R'_B = 1.14 \times 10^{-5}.$

In this case equation (8) is also applicable, therefore

$$R = \frac{2.11 \times 10^{-4} \times 1.14 \times 10^{-5}}{2.11 \times 10^{-4} - 1.14 \times 10^{-5}} = 1.21 \times 10^{-5},$$

whence

$$R_{eu} = R - R_B = 8.6 \times 10^{-6} \text{ mol. } CO_2/l./sec.$$

The value of R_{eu} so computed is usually accurate to $\pm 10\%$, and, as mentioned above, is independent of the inactivation of the enzyme and of the changes in pH, substrate and buffer concentrations during the shaking. In the present example the diffusion corrections only amount in the end to about 7.5% owing to the low enzyme concentrations used, but at higher values the corrections may become considerable.

All our CO_2 uptake experiments below pH 8.0 have been calculated in this way, pressure intervals equal to 3% of the total initial CO_2 pressure being generally used for the computations. Pressure changes occurring earlier than 25 sec. from the start of the reaction should, if possible, be avoided for calculation, since the irregularities which are apt to occur just after shaking begins may not have had time to die out. In the instance plotted in Fig. 2 the enzyme has lost about one-quarter of its activity after 200 sec. shaking. In some cases (notably when inhibitory salts are present) we have found losses of a half or even more, and have then plotted $\log R'/R'_B$ against t instead. The points so obtained have fallen closely on a straight line, so that extrapolation to zero time can be made with confidence.

Determination of R_{eu} at pH > 8.0. In this range the velocity of the back reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ is inappreciable and therefore there should be but little change, during the progress of the experiment, in R'_B/p_m , where p_m = the mean CO_2 pressure for the interval over which R'_B is calculated. This is found to be the case, and it is also found that above pH 8.0 the drift in R'/p_m is usually but slight. More satisfactory extrapolations to zero time are therefore obtained in this range by plotting R'_B/p_m and R'/p_m against time. From such graphs we thus obtain R'_B and R^0 . We then have

$$R'_B = p_B^0 \times (R'_B/p_m \text{ at zero time}) \quad (11)$$

and $R^0 = p^0 \times (R'/p_m \text{ at zero time}). \quad (12)$

R_B and R are calculated as above, and R_{eu} is finally obtained. A further advantage of this method is that it avoids (i) any assumption as to the ' l ' value of the catalytically active constituent of the buffer, (ii) complications due to the velocity of the reaction $CO_2 + OH^- \rightarrow HCO_3^-$, which becomes significant above pH 8.0.

A typical example of the calculation for 0.038 M-dimethylglyoxaline solution, pH 8.8, 0° , $p_0 = 18.6$ cm. H_2O , ± 14.3 p.p.m. crude enzyme solution, is given in Table 1. In this case the diffusion correction finally amounts to about 13%.

Table 1. Calculation of enzymic activity at pH > 8.0 and 0°

Absence of enzyme					Presence of enzyme				
p_B (cm. H ₂ O)	p_m	Time (sec.)	$10^6 R_B'$	$10^7 R_B'/p_m$	p (cm. H ₂ O)	p_m	Time (sec.)	$10^6 R'$	$10^7 R'/p_m$
17.8	—	96	—	—	17.8	—	32.5	—	—
—	17.5	—	5.93	3.38	—	17.5	—	18.8	10.7
17.2	—	170	—	—	17.2	—	55.9	—	—
—	16.9	—	5.87	3.47	—	16.9	—	18.9	11.2
16.6	—	246	—	—	16.6	—	79.2	—	—
—	16.3	—	5.40	3.31	—	16.3	—	16.7	10.2
16.0	—	327.3	—	—	16.0	—	105.5	—	—
—	15.7	—	5.07	3.23	—	15.7	—	16.4	10.4
15.4	—	414	—	—	15.4	—	132.2	—	—
$R_B^0/p_B^0 = 3.56 \times 10^{-7}$.					$R^0/p^0 = 11.5 \times 10^{-7}$.				
$p_B^0 = 18.6$ cm.					$p^0 = 18.6$.				
$R_B^0 = 0.66 \times 10^{-5}$ mol. CO ₂ /l./sec.					$R^0 = 2.14 \times 10^{-5}$ mol. CO ₂ /l./sec.				
$R_B = 0.68 \times 10^{-5}$ mol. CO ₂ /l./sec.					$R = 2.36 \times 10^{-5}$ mol. CO ₂ /l./sec.				
$R_{eu} = 1.68 \times 10^{-5}$ mol. CO ₂ /l./sec.									

Corrected equations for the effect of diffusion in CO₂ output experiments

Equation (30) of Roughton's (1941) paper is also at fault through neglect of the velocity of the non-enzymic reaction, the effect of which is quite significant at pH 6.0 and below. Taking this into account we have, on the basis of the treatment given on p. 136 of his paper, at zero time,

$$\frac{DA'}{\delta V_L} c_L = \frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L - \frac{k_e E c_L}{c_L + K_{mu}} - v_u c_L = R^0, \quad (13)$$

where x_L = concentration of H₂CO₃ in the bulk of the liquid phase,

K_{mo} = Michaelis constant for CO₂ output,

k_e = velocity constant for the dissociation of the enzyme-H₂CO₃ complex = velocity constant for the dissociation of the enzyme-CO₂ complex (Roughton & Booth, 1946),

$v_0 = k_0(1 + l[B])$,

k_0 = velocity constant of the H₂CO₃ → CO₂ + H₂O reaction in absence of catalyst,

K = equilibrium constant of the reaction H₂CO₃ ⇌ CO₂ + H₂O = $k_0/k_u = v_0/v_u$.

As shown elsewhere (Roughton & Booth, 1946) $K_{mu} = KK_{mo}$. Therefore equation (13) reduces to

$$R^0 = \frac{DA'}{\delta V_L} c_L = \frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L - \frac{k_e E c_L}{c_L + KK_{mo}} - \frac{v_0}{K} c_L. \quad (14)$$

As $t \rightarrow 0$, we have

$$R_B = v_0 x_L, \quad (15)$$

$$R = \frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L, \quad (16)$$

R_{mo} = maximum output velocity rate at very high enzyme concentration

$$= \frac{DA'}{\delta V_L} x_L K, \quad (17)$$

for in this case the CO₂ and H₂CO₃ are always in equilibrium in the body of the liquid and therefore $c_L = x_L K$.

From (14) and (17),

$$\frac{R^0}{R_{mo}} = \frac{c_L}{x_L K} \quad \text{or} \quad c_L = x_L K \frac{R^0}{R_{mo}}.$$

From (14) and (16),

$$\begin{aligned} \frac{R}{R^0} &= \frac{\frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L}{k_e E \left(\frac{x_L}{x_L + K_{mo}} - \frac{c_L}{c_L + KK_{mo}} \right) + v_0 \left(x_L - \frac{c_L}{K} \right)} \\ &= \frac{\frac{k_e E x_L}{x_L + K_{mo}} + v_0 x_L}{k_e E \left(\frac{x_L}{x_L + K_{mo}} - \frac{x_L K R^0 / R_{mo}}{x_L K R^0 / R_{mo} + KK_{mo}} \right) + v_0 (x_L - x_L R^0 / R_{mo})} \\ &= \frac{\frac{k_e E}{x_L + K_{mo}} + v_0}{\frac{R_{mo} - R^0}{R_{mo}} \left\{ \frac{k_e E K K_{mo}}{(x_L + K_{mo})(x_L K R^0 / R_{mo} + KK_{mo})} + v_0 \right\}}, \end{aligned}$$

whence

$$\begin{aligned}
 R &= \frac{R_{mo}R^0}{R_{mo}-R^0} \left\{ \frac{k_e E(x_L KR^0/R_{mo} + KK_{mo}) + v_0(x_L + K_{mo})(x_L KR^0/R_{mo} + KK_{mo})}{k_e EKK_{mo} + v_0(x_L + K_{mo})(x_L KR^0/R_{mo} + KK_{mo})} \right\} \\
 &= \frac{R_{mo}R^0}{R_{mo}-R^0} \left\{ 1 + \frac{x_L R^0/R_{mo}}{K_{mo} + \frac{v_0}{k_e E}(x_L + K_{mo})(x_L R^0/R_{mo} + K_{mo})} \right\} \\
 &= \frac{R_{mo}R^0}{R_{mo}-R^0} \left\{ 1 + \frac{x_L R^0/R_{mo}}{K_{mo} + \frac{R_B(x_L R^0/R_{mo} + K_{mo})}{R - R_B}} \right\}.
 \end{aligned} \quad (18)$$

Since, as may be shown from equations (15) and (16),

$$\frac{v_0}{k_e E}(x_L + K_{mo}) = \frac{R_B}{R - R_B}.$$

If v_0 is neglected, equation (18) reduces to equation (30) of Roughton's (1941) paper, namely,

$$R = \frac{R_{mo}R^0}{R_{mo}-R^0} \left(1 + \frac{x_L R^0}{K_{mo}R_m} \right).$$

The value of R is calculated from equation (18) by successive approximation, $R_B/(R - R_B)$ first being placed $= R_B^0/(R^0 - R_B^0)$, which is given directly by extrapolation from the experiments. From the value of R so obtained, a new value of $R_B/(R - R_B)$ is derived, inserted in equation (18), R recalculated and so on. Even in the most unfavourable case, three such successive approximations have proved enough to fix R to within 2%: often a single approximation process is sufficient. We also have

$$R_B = \frac{R_{mo}R_B^0}{R_{mo}-R_B^0}, \quad (19)$$

whence $R_{eo} = R - R_B$ is obtained.

Determination of R_{mo} . Roughton (1941) has shown theoretically, and confirmed by experiment, that

$$R_{mo} = \text{constant} \times \frac{b}{1 + K'_1/f_{HCO_3} a_H}, \quad (20)$$

where b = total CO_2 in all forms at zero time

$$= [CO_2] + [H_2CO_3] + [HCO_3^-],$$

K'_1 = apparent first ionization constant of carbonic acid

$$= f_{HCO_3} a_H [HCO_3^-] / ([CO_2] + [H_2CO_3])$$

$$= 2.61 \times 10^{-7} \text{ at } 0^\circ \text{ (Shedlovsky \& MacInnes, 1935),}$$

f_{HCO_3} = activity coefficient of bicarbonate ion,

a_H = hydrogen-ion activity of solution.

The value of this constant, R_{mo} , according to Roughton's treatment, is the same as that of the constant in equation (5) for R_m , namely, 0.15 for the particular apparatus and shaking conditions used by us.

Determination of $f_{HCO_3} a_H$. In cacodylate buffer solutions we have

$$a_H = K_C [HCac] / f_C [Cac^-], \quad (21)$$

where K_C = ionization constant of cacodylic acid,

f_C = activity coefficient of cacodylate ion.

Therefore

$$f_{HCO_3} a_H = K_C [HCac] f_{HCO_3} / f_C [Cac^-]. \quad (22)$$

Now because HCO_3^- and Cac^- are both univalent anions, it follows from the Debye-Huckel theory of dilute solutions that

$$f_{HCO_3} = f_C = \text{antilog} (-0.5 \sqrt{\mu}), \quad (23)$$

where μ = ionic strength of the solution. This relationship is experimentally confirmed by the electrometric determinations of Hastings & Sendroy (1925) on CO_2 - H_2CO_3 mixtures, and of Morton (1928) on cacodylate buffer solutions. Therefore

$$f_{HCO_3} a_H = K_C [HCac] / [Cac^-]. \quad (24)$$

The numerical value of K_C at 0° is taken as $10^{-6.29}$. (See note at the end of this paper.)

Most of our CO_2 output experiments have been done in cacodylate and phosphate buffer solutions. In the latter the activity corrections are complex owing to the presence of bivalent HPO_4^- ions and no such simple equation as (24) can be used. Instead, we determine the value of $f_{HCO_3} a_H$, at zero time, closely enough by comparing the observed rate of CO_2 output, R_p^0 , as $t \rightarrow 0$ in the phosphate buffer experiment with the observed rate of CO_2 output, R_C^0 , in a corresponding cacodylate buffer experiment. We then have

$$\begin{aligned}
 \frac{R_p^0}{R_C^0} &= \frac{(f_{HCO_3} a_H [HCO_3^-]) \text{ in phosphate experiment} \times (1 + l_p [HPO_4^-])}{(f_{HCO_3} a_H [HCO_3^-]) \text{ in cacodylate experiment} \times (1 + l_C [Cac^-])} \\
 &\quad (25)
 \end{aligned}$$

$l_p = 8.0$, $l_C = 9.0$ and, all the other quantities in equation (25) being known, $f_{HCO_3} a_H$ for the phosphate solution is then calculable. A similar procedure has been used in the few experiments with other buffer solutions.

Determination of K_{mo}/x_L . This is the remaining quantity still required before equation (18) can be applied. We have

$$f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-] = K_1 [\text{H}_2\text{CO}_3] = K'_1 ([\text{CO}_2] + [\text{H}_2\text{CO}_3]), \quad (26)$$

where K_1 = true first ionization constant of carbonic acid. Therefore

$$K_1 = K'_1 (1 + K) = K K'_1, \quad (27)$$

since K is of the order of 900 at 0° (see Roughton, 1941), and since

$$K = k_0/k_u, \quad (28)$$

$$\text{So } x_L = [\text{H}_2\text{CO}_3] = f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-] / K_1 = f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-] / K K'_1.$$

Now $K_{mo} = K_{mu}/K$. Therefore

$$\begin{aligned} K_{mo}/x_L &= K_{mu} K'_1 / f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-] \\ &= 9 \times 10^{-3} \times 2.61 \times 10^{-7} / f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-] \\ &= 2.35 \times 10^{-9} / f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-] \text{ at } 0^\circ. \end{aligned} \quad (29)$$

Calculation of R_{eo} in cacodylate experiments. As an example we shall describe the evaluation of R_{eo} in case of CO_2 output at 0° from a solution consisting initially of 0.0025M-NaHCO₃, 0.0192M-HCac, 0.0048M-NaCac ± 57 p.p.m. of crude enzyme. The total CO_2 evolved gave rise to a gauge level difference of 10 cm. and Table 2 shows the respective times taken over four pressure intervals of 1 cm. starting after a 2 cm. difference of level has been produced. R'/R'_B is thence plotted against the mean time corresponding to each interval just as in the CO_2 uptake procedure at pH < 8.0, and extrapolation to zero time gives $R^0/R'_B = 2.6$.

We have by equation (24), at zero time,

$$f_{\text{HCO}_3} a_{\text{H}} = 10^{-6.29} \times 0.0192 / 0.0048 = 2.1 \times 10^{-6}.$$

Therefore by equation (20),

$$\begin{aligned} R_{mo} &= (0.15 \times 0.0025) / (1 + 2.6 \times 10^{-7} / 2.1 \times 10^{-6}) \\ &= 3.3 \times 10^{-4} \text{ mol. CO}_2/\text{l./sec.} \end{aligned}$$

Table 2. Calculation of R_{eo} in cacodylate buffer solution

Presence of enzyme		Absence of enzyme		R'/R'_B
p_B (cm H ₂ O)	Time (sec.)	p_B (cm. H ₂ O)	Time (sec.)	
2	10.7	2	21.4	2.74
3	14.2	3	31.0	2.72
4	18.2	4	41.9	2.91
5	23.0	5	55.6	2.96
6	28.4	6	73.2	

$$\frac{R'}{R'_B} = \frac{\text{time to traverse pressure interval in absence of enzyme}}{\text{time to traverse same pressure interval in presence of enzyme}}$$

From Roughton & Booth's (1938) data,

$$\begin{aligned} R_B^0 &= k_0(1 + l_c [\text{Cac}^-]) [\text{HCO}_3^-] f_{\text{HCO}_3} a_{\text{H}} / K_1 \\ &= k_u(1 + l_c [\text{Cac}^-]) [\text{HCO}_3^-] f_{\text{HCO}_3} a_{\text{H}} / K_1 \\ &= 0.0021(1 + 9 \times 0.0048) \times 0.0025 \times 2.1 \\ &\quad \times 10^{-6} / 2.6 \times 10^{-7} \\ &= 4.43 \times 10^{-5}. \end{aligned}$$

Therefore

$$R^0 = 2.6 \times 4.43 \times 10^{-5} = 1.15 \times 10^{-4} = 0.35 R_{mo},$$

$$R_B^0 / (R^0 - R_B^0) = 0.628,$$

and by equation (29),

$$K_{mo}/x_L = 2.35 \times 10^{-9} / (2.1 \times 10^{-6} \times 0.0025) = 0.45.$$

Substituting these values in equation (18) we get

$$R = 2.42 \times 10^{-4}.$$

Since

$$\begin{aligned} R_B &= (3.3 \times 10^{-4} \times 4.43 \times 10^{-5}) / (3.3 \times 10^{-4} \\ &\quad - 4.43 \times 10^{-5}) = 5.12 \times 10^{-5}, \end{aligned}$$

$R_B / (R - R_B) = 0.268$ to a first approximation.

Substituting in equation (18) we get $R = 2.69 \times 10^{-4}$ to a second approximation. A third approximation gives $R = 2.73 \times 10^{-4}$. Therefore

$$\begin{aligned} R_{eo} &= 2.73 \times 10^{-4} - 5.12 \times 10^{-5} \\ &= 2.22 \times 10^{-4} \text{ mol. CO}_2/\text{l./sec.} \end{aligned}$$

If no correction for diffusion had been applied, the enzymic activity would have been taken as equal to $(R^0 - R_B^0)$, i.e. to 0.71×10^{-4} , which is only one-third of the corrected value for R_{eo} . The influence of diffusion in this instance is thus very large, owing to the high speed of the non-enzymic as well as of the enzymic reaction at the relatively acid pH of 5.6; larger corrections than this seem to be of questionable validity, and we have therefore felt it unsafe to push the manometric method to more acid pH than 5.6. As the pH is raised the diffusion correction rapidly decreases: thus in cacodylate buffer at pH 6.4 and with the same enzyme concentration as that in the above experiment the diffusion correction is only 25%.

The theoretical treatment and experiments quoted by Roughton (1941, pp. 138-40) indicate that in output experiments the initial irregularities after shaking begins should disappear in about 10 sec., and hence times as early as this have been utilized in Table 2. The reason that the initial irregular period is so much shorter than in CO_2 uptake experiments, wherein a minimum of 25 sec. should be allowed before readings are used, is that in the latter case a much greater formation of dissolved CO_2 occurs in the initial period just after shaking begins.

Exactly the same method of calculation has been used in all our output experiments. The value of R'/R'_B is often found to rise appreciably with time

during the progress of the experiment, the inactivation brought about by the shaking being more than compensated for by the increase in relative activity of the enzyme as the solution becomes more alkaline through loss of CO_2 and as the substrate concentration decreases (see Discussion).

DISCUSSION

The above methods of computing carbonic anhydrase activity have been cumbersome to describe, but after some practice become rapid in actual use. Of the necessity of using them properly pointed instances are given in the following paper (Roughton & Booth, 1946); e.g. (i) Our first experiments on the pH activity curve of the enzyme were done before the diffusion corrections had been completely worked out: when these were correctly applied to the data, a curve of quite different shape was obtained. In our first calculation we had underestimated the size of the corrections at acid pH. (ii) In an early set of experiments on the activity of a crude chloroform enzyme preparation kept at 0° we found an apparent rise in enzymic activity during storage: in these we have, however, calculated the activity of the enzyme from its magnifying effect on the rate of CO_2 exchange during the second quarter of the manometric process, as was done originally by Meldrum & Roughton (1933), but we had made no allowance for the inactivation of the enzyme during shaking. When, however, the activity was extrapolated to zero time as above, it was found to be the same within experimental error; the older preparations, therefore, had not increased in absolute activity, but had apparently suffered some change which depressed the rate of inactivation during the manometric shaking.

The activity of the enzyme in a given buffer solution should theoretically be proportional to the amount added: we have sometimes found, especially in our earlier work before full cleanliness precautions had been adopted (see Fig. 1 of Roughton & Booth, 1946), that with small amounts of enzyme the observed effect is proportionally much less than with large amounts. This is probably due to traces of metals, or other inactivating impurities, in the apparatus or solutions, the first amounts of added enzyme combining with and removing the impurity thus enabling further enzyme to exert its full activity. Frequent controls should therefore be made as to proportionality between activity and amount of enzyme added.

In the following paper we show that the activity of the enzyme, as measured manometrically, agrees fairly closely with that measured colorimetrically in an all-liquid system, wherein no inactivation, adsorption or other effects at the gas/liquid interface could have occurred. This result gives some reply

to the most obvious criticism that can be levelled against the manometric method of measuring carbonic anhydrase activity. The other methods of measuring the enzyme activity are also more appropriately discussed in that paper (Roughton & Booth, 1946) in light of the new data given there on the effect of various ions and of substrate concentration on the activity of the enzyme.

Note on the ionization constant of cacodylic acid at 0°

Faurholt's (1924) value for the ionization constant of cacodylic acid (K_C) at 0° was based on the equation

$$\frac{K_C}{K_1} = \frac{[\text{HCac}][\text{HCO}_3^-]}{[\text{Cac}^-][\text{CO}_2]},$$

where the symbols in brackets represent the concentrations of the substances at equilibrium, when known amounts of CO_2 or bicarbonate are added to cacodylate buffer solutions. Faurholt measured the value of $[\text{CO}_2]$ at equilibrium by his carbamino method, and deduced the concentrations of the remaining substances from their initial values and the found value of $[\text{CO}_2]$. A small error in $[\text{CO}_2]$ would therefore give rise to a several times larger error in K_C/K_1' . Faurholt's actual value for K_C/K_1' was 2.34 ± 0.2 , and assuming Kendall's value for K_1' of 2.24×10^{-7} at 0° he thence calculated $K_C = 10^{-6.29}$.

In manometric experiments on the rate of CO_2 output at 0° from a solution containing 0.016M-HCac, 0.024M-NaCac, 0.005M- NaHCO_3 , we found

$$\frac{d[\text{CO}_2]}{dt} = 3.40 \times 10^{-3} [\text{HCO}_3^-].$$

Theoretically

$$\begin{aligned} \frac{d[\text{CO}_2]}{dt} &= \frac{k_o f_{\text{HCO}_3} a_{\text{H}} [\text{HCO}_3^-]}{K_1} (1 + l[\text{Cac}^-]) \\ &= k_u \frac{K_C}{K_1'} \frac{[\text{HCac}]}{[\text{Cac}^-]} [\text{HCO}_3^-] (1 + l[\text{Cac}^-]). \end{aligned}$$

Since $k_u = 0.0021$, $[\text{HCac}]/[\text{Cac}^-] = 0.67$, $l = 9$, $[\text{Cac}^-] = 0.024$, we get $K_C/K_1' = 1.98$.

Taking the more recent and better established value of Shedlovsky & MacInnes (1935) for K_1' of 2.61×10^{-7} at 0° , we find $K_C = 5.17 \times 10^{-7} = 10^{-6.29}$. This value by coincidence agrees exactly with that of Faurholt. We believe it to be correct to within $\pm 10\%$, and have used it in all CO_2 output calculations. At 30° Morton (1928) reports that $K_C = 5.66 \times 10^{-7} = 10^{-6.25}$.

SUMMARY

1. Improvements are described in the technique and calibration of the manometric apparatus for measuring carbonic anhydrase activity. For accurate results special precautions are needed in the preparation of the buffer solutions and for the handling of the enzyme preparations.

2. To study the kinetics of carbonic anhydrase under varied conditions it is necessary to express the activity of the enzyme, not in some arbitrary units, but in terms of the actual extra rate, due to the presence of the enzyme, of CO_2 uptake or output in mol. CO_2 /l. solution/sec. Directions are given for

calculating the extra rate in this unit at a series of instants during the course of the manometric experiment. These extra rates are then extrapolated to zero time so as to eliminate changes in the activity of the enzyme during the actual experiment owing to (i) progressive alterations in pH, substrate and buffer concentrations as CO_2 is taken up or evolved, (ii) destruction brought about by the violent shaking. The extrapolated rates are finally corrected for the limiting effect of diffusion by means of extensions

of the equations previously developed by Roughton, on the basis of the application of the stationary film theory to gas-enzymic reactions.

3. Detailed examples of the computation of enzymic activity are given for CO_2 uptake experiments both below and above pH 8.0 and also for CO_2 output experiments.

4. Applications of the methods to the study of the kinetics of carbonic anhydrase under varied conditions are given in the following paper.

REFERENCES

- Brinkman, R. (1933). *J. Physiol.* **80**, 171.
 Brinkman, R., Margaria, R. & Roughton, F. J. W. (1933). *Philos. Trans. A*, **232**, 65.
 Dixon, M. (1934). *Manometric Methods*. Cambridge.
 Faurholt, C. (1924). *J. chim. Phys.* **21**, 400.
 Hastings, A. B. & Sendroy, J. (1925). *J. biol. Chem.* **65**, 445.
 Hodgson, T. H. (1936). *Brit. J. exp. Path.* **17**, 75.
 Keilin, D. & Mann, T. (1940). *Biochem. J.* **34**, 1163.
 Kiese, M. (1941). *Biochem. Z.* **307**, 207, 400.
 Kiese, M. & Hastings, A. B. (1940). *J. biol. Chem.* **132**, 267.
 Leiner, M. (1941). *Biochem. Z.* **311**, 119.
 Meldrum, N. U. & Roughton, F. J. W. (1932). *J. Physiol.* **75**, 15P.
 Meldrum, N. U. & Roughton, F. J. W. (1933). *J. Physiol.* **80**, 113.
 Morton, C. (1928). *J. chem. Soc.* p. 1401.
 Philpot, F. J. & Philpot, J. St L. (1936). *Biochem. J.* **30**, 2191.
 Roughton, F. J. W. (1941). *J. biol. Chem.* **141**, 129.
 Roughton, F. J. W. & Booth, V. H. (1938). *Biochem. J.* **32**, 2049.
 Roughton, F. J. W. & Booth, V. H. (1946). *Biochem. J.* **40**, 319.
 Scott, D. A. & Fisher, A. M. (1942). *J. biol. Chem.* **144**, 371.
 Shedlovsky, T. & MacInnes, D. A. (1935). *J. Amer. chem. Soc.* **57**, 1705.
 Stadie, W. C. & O'Brien, H. (1933). *J. biol. Chem.* **103**, 521.
 Van Goor, H. (1934). M.D. Thesis, Groningen.

The Effect of Substrate Concentration, pH and other Factors upon the Activity of Carbonic Anhydrase

By F. J. W. ROUGHTON AND V. H. BOOTH (Ramsay Memorial Fellow), *Physiology Laboratory, University of Cambridge, England and the Fatigue Laboratory, Harvard University, Boston, Mass., U.S.A.*

(Received 18 December 1945)

This paper presents data obtained between 1936 and 1940 on the effects of substrate concentration, pH and salts on the activity of carbonic anhydrase at 0° . A brief summary of the more important findings has already been given (Roughton, 1943). Completion of the work has been much delayed, in part by difficulties in working out the results, but in the main by preoccupation of both authors with research related to the war. During the interim, important advances have been made in other directions, e.g. the demonstration of zinc as the active metal of the enzyme (Keilin & Mann, 1940), the crystallization of the enzyme by Scott & Fisher (1942), the claim of the existence of activators by Leiner (1940), and the discovery of the inhibitory effect of sulphanilamide (Mann & Keilin, 1940). Most of our data, however, seem still to retain their value, and after presenting them we shall discuss their significance in the light of the more recent work.

The topics to be dealt with are routine ones in the study of the kinetics of an enzyme, and it might well be asked why such results were not published within a year or so of the discovery of carbonic anhydrase by Meldrum & Roughton (1932). The answer is that in the case of this enzyme a number of unusual difficulties were encountered which had first to be resolved. As regards substrate concentration Meldrum & Roughton's (1933) preliminary observations at 15° suggested that the Michaelis constant in the case of CO_2 uptake by the enzyme might be so high that for the enzyme to be largely saturated with substrate it would be necessary to work at CO_2 pressures of several atmospheres. At 0° , however, the Michaelis constant is much lowered and adequate data can be obtained with CO_2 pressures ranging merely from 0.01 to 1.0 atm., which are well within the scope of ordinary manometric technique. As regards the effect of pH and salts the main difficulty has been that carbonic anhydrase,