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,

unlike most other enzymes, catalyzes reactions which not only proceed at appreciable rates in absence of the enzyme but are also catalyzed by non-enzymic solutes, especially the more electronegative constituent of many buffer systems. Thus at pH below 8.0, the true overall rate of hydration of CO_2 is, according to Roughton & Booth (1938), Kiese & Hastings (1940*a, b*), and Roughton (1943), given by

$$-\frac{d[\text{CO}_2]}{dt} = \{k_u[\text{CO}_2] - k_0[\text{H}_2\text{CO}_3]\} \{1 + l[B]\}, \quad (a)$$

where $k_u = 0.0021$ at 0° ,

$k_0 = 2.0$ at 0° ,

B = catalytically active constituent, i.e. HPO_4^- in the case of phosphate buffer,

l = a catalytic coefficient of value, e.g. 8 for HPO_4^- , *c.* 1700 for SeO_3^- , *c.* 10,000 for H_2AsO_3^- , *c.* 30,000 for BrO^- .

Above pH 8.0 the rate of hydration begins to increase independently of the buffer concentration. The extra rate is proportional to the OH^- concentration, being given by

$$-\frac{d[\text{CO}_2]}{dt} = k'_u[\text{CO}_2][\text{OH}^-] - k'_0[\text{HCO}_3^-], \quad (b)$$

where k'_u is of the order of 1000 at 0° . Equation (b) may be equally well explained either by a direct reaction, $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$, or as due to a catalysis of the $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ reaction by OH^- ions, the ' l ' value of the latter being outstandingly large, namely, about 500,000. At present there is no reason to prefer one mechanism to the other, but on either view it seems unlikely that so intrinsically rapid a reaction would itself be further catalyzed by other substances. In our previous paper (1938) we did indeed show that phosphate, cacodylate and borate, though they markedly catalyze the $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ reaction, were all without effect on the process corresponding to equation (b). The same would probably have been found of the other buffers of our series had these too been investigated in similar detail.

Direct tests in the case of carbonic anhydrase are more elusive, though Kiese (1941) has brought forward definite theoretical and experimental arguments that process (b) is not catalyzed by the enzyme and this viewpoint will be adopted in the present paper. Even, however, with this simplification, the task of working out the catalytic action of carbonic anhydrase in buffer solutions is still a complicated one. Not only have there to be considered (i) the effect of pH upon the catalysis by the enzyme of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ but also (ii) the possible effect of the enzyme upon the catalysis of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ by

the more electronegative constituents of the buffer when such catalysis is but slight (as opposed to the large catalysis which OH^- may produce, as just mentioned), (iii) possible inhibitory or activating effects of the buffer upon the enzyme. Of these, (ii) and (iii) have been investigated by varying the total buffer concentration at constant enzyme and substrate concentrations. With the buffers used in the present work, over the range pH 5.5–10.0, the effects seem to be either negligible at buffer concentrations below 0.05M, or small enough to be allowed for by extrapolation to zero buffer concentration. Several buffers of lower pK and salts of strong acids, however, show marked depressions of enzymic activity even at concentrations of 0.01M and are therefore unsuitable.

EXPERIMENTAL METHODS

Apart from a few control experiments, in which the activity of the enzyme was measured by an all liquid indicator method (see later), all the results to be reported below were obtained at 0° by the boat-manometric technique described by Roughton & Booth (1938, 1946).

The buffer solutions were prepared and kept as free of CO_2 as possible, in the way described by Roughton & Booth (1946). The main buffers used were cacodylate, phosphate, veronal and dimethylglyoxaline, these covering the pH range to be studied, namely, pH 5.5–9.5. A few supplementary experiments were also done with pyrophosphate, allantoin and succinimide buffer solutions, prepared and treated in similar manner.

Most of our experiments have been made with the crude chloroform preparation of Meldrum & Roughton (1933), but some with solutions of lyzed red cells and a few data with the highly purified preparations of Mann & Keilin (1940). The crystalline preparation of Scott & Fisher (1942) was not available at the time of our work. The enzyme solutions were prepared and handled with the precautions described elsewhere (Roughton & Booth, 1946), and under these conditions the crude chloroform preparation used for the bulk of the experiments remained constant in activity (within experimental error) for at least a year, as has been the experience also of more recent observers. So far, no obvious differences have been found in the kinetics of the enzyme at various grades of purity save that the tendency to inactivation during manipulation and manometric shaking is increased by each stage of purification, especially the later ones.

THE CALCULATION OF THE ENZYMIC ACTIVITY

The activities of the enzyme are, throughout this paper, expressed as the additional rate of CO_2 uptake or output, when enzyme is added, over and above the rate of CO_2 uptake by or output from the same buffer solution in absence of enzyme. They are given in mol. CO_2 /l./sec., the calculations being

made by the methods already described in detail (Roughton & Booth, 1946): these allow not only for the changes in activity of the enzyme during the actual manometric shaking but also for the limiting effect of diffusion between the gas and liquid phases. In these respects, especially the latter, the results are superior to those of most previous manometric determinations, wherein furthermore the activity has often been expressed as a *magnification* of, rather than an *addition* to, the non-enzymic rate. The addition method of expressing activity is, however, indispensable when studying the effect of substrate concentration, and is likewise much more serviceable in disentangling the effects of pH and buffer concentration. Values given by it are, indeed, independent of any assumption of the physico-chemical mechanism of the enzyme reactions. The magnification method is useful in comparing the number of enzyme units in various preparations, but cannot cope properly with most of the questions dealt with in this paper.

EFFECT OF ENZYME CONCENTRATION

Previous workers have shown that the extra rate of reaction in presence of carbonic anhydrase is proportional to the concentration of the enzyme. Thus, Meldrum & Roughton (1933) found it so for blood solutions in $M/10$ phosphate buffer, over the narrow range in which corrections for the effect of diffusion are small, whilst, with allowance for the latter, Roughton (1941, Fig. 2) found that the relation between crude chloroform enzyme concentration and extra rate was linear to within $\pm 5\%$ over a 40-fold range when measured in $M/40$ phosphate, pH 7.5, 0° . We have made altogether 12 tests with blood solution and crude chloroform preparation as source of enzyme over the range pH 5.5–9.5 and with the whole available set of buffers. The enzyme concentration was varied over the useful range of the apparatus. In almost every case a linear relationship held good to $\pm 10\%$, the average divergency being only 4%, which is well within the limits of error of the activity measurements.

In about half of our preliminary experiments on the highly purified preparation of Mann & Keilin we found, however, an inflected curve like that shown in Fig. 1*B*. In this it is seen that low concentrations of enzyme produce only slight effect but that after a certain amount has been added the effect of further additions is practically linear. Fig. 1*B* represents the results obtained on a crude chloroform preparation in $M/40$ phosphate, pH 7.5, at an early stage of the work before all the necessary technical precautions (Roughton & Booth, 1946) had been fully mastered. For example, the solutions were prepared in water distilled from a metal, instead of an all-glass, still. Six weeks later, when the technique had been fortified, we obtained with

the same enzyme solution the linear relationship shown in Fig. 1*A*. It will be noted, however, that the steep linear portion of Fig. 1*B* is parallel to the straight line of Fig. 1*A*, so that if the activity had been calculated from the slope of the steep linear portion of Fig. 1*B* a correct result would have been

Fig. 1. Activity-enzyme concentration curve for carbonic anhydrase. *A*, the usual type of curve; *B*, occasional type.

obtained. This has been the basis of our activity calculation in those experiments with the highly purified preparation of Mann & Keilin, in which, in spite of all precautions, we obtained a curve of the type of Fig. 1*B*. As to the cause for such curves, one possibility is that in the experiment of Fig. 1*B*, there was some definite amount of metal or other enzyme poison present in the solutions and/or apparatus, and that an equivalent amount of enzyme was inactivated by combination with such impurity. After the latter had been removed thereby, further amounts of enzyme would have been free to exert their full proportional effects. In highly purified enzyme preparations the total amount of protein is so low that this phenomenon becomes increasingly difficult to guard against: another possibility is the adsorption of a definite amount of the enzyme at the solid/liquid or gas/liquid interfaces in the manometric vessel, these surfaces being preferentially occupied by other substances in the case of less pure enzyme preparations. Scott & Fisher (1942) and Scott & Mendive (1941*a, b*) have, indeed, found it necessary to add small quantities of peptone when measuring the activity of highly purified or crystalline preparations of the enzyme.

SUBSTRATE CONCENTRATION AND THE MICHAELIS CONSTANT

According to the Michaelis theory of enzyme reaction, the extra rate of CO_2 uptake in presence of the enzyme should be given by

$$R_{eu} = \frac{k_{eu} cE}{c + K_{mu}}, \quad (1)$$

where c = concentration of dissolved CO_2 ,

E = concentration of the enzyme,

k_{eo} = velocity constant for dissociation of the enzyme complex $E \cdot \text{CO}_2$,

K_{mu} = Michaelis constant for CO_2 uptake.

Similarly the extra rate of CO_2 output in presence of the enzyme should be given by

$$R_{eo} = \frac{k_{eo}xE}{x + K_{mo}}, \quad (2)$$

where x = concentration of H_2CO_3 ,

k_{eo} = velocity constant for dissociation of the enzyme complex $E \cdot \text{H}_2\text{CO}_3$,

K_{mo} = Michaelis constant for CO_2 output.

Let K = equilibrium constant of the reaction
 $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$

= about 900 at 0° (Roughton, 1943),

c_∞ , x_∞ = the respective concentrations of CO_2 and H_2CO_3 at equilibrium.

Then $c_\infty = Kx_\infty$.

Since at equilibrium the rates of CO_2 uptake and CO_2 output must both be equally catalyzed by the enzyme

$$\begin{aligned} \frac{k_{eu}Ec_\infty}{c_\infty + K_{mu}} &= \frac{k_{eo}Ex_\infty}{x_\infty + K_{mo}} \\ &= \frac{k_{eo}Ec_\infty/K}{c_\infty/K + K_{mo}} = \frac{k_{eo}Ec_\infty}{c_\infty + KK_{mo}}. \end{aligned} \quad (3)$$

Equation (3) is true whatever value of c_∞ be selected. This can only be so if

$$k_{eu} = k_{eo} = k_e \quad (4)$$

and

$$K_{mu} = KK_{mo}. \quad (5)$$

Equations (4) and (5) have already been made use of for calculating enzymic activity from CO_2 output experiments (Roughton & Booth, 1946).

Determination of K_{mu} . Equation (1) has been tested at three to four CO_2 concentrations, ranging from 0.001 to 0.02M, (i) at pH 7.5 (M/40 phosphate), (ii) at pH 8.0 (M/24 veronal), (iii) at pH 9.3 (M/15 dimethylglyoxaline), in the case of the crude chloroform enzyme solution, the final dilution of the latter being 14.3 parts per million (p.p.m.). For the low CO_2 pressures the manometer gauge was filled as usual with water, but for the higher ones an open-ended mercury gauge was used, or Clerici fluid was placed in the U-tube gauge. In correcting for the limiting effect of diffusion, the simple equation $R = R_m R^0 / (R_m - R^0)$ was generally adequate, but in a few cases the more detailed method of approximation described in our adjoining paper (1946) was used.

From equations (1) and (4) it follows that

$$\frac{c}{R_{eu}} = \frac{c + K_{mu}}{k_e E}. \quad (6)$$

The circles in Fig. 2 show the plot of c/R_{eu} against c in each of the above three cases. The points almost all fall on straight lines within experimental error as equation (6) requires. K_{mu} is given by the distance from the origin at which each line intercepts

Fig. 2. Effect of substrate concentration on activity of carbonic anhydrase. $\circ$ = chloroform enzyme solution, 14.3 p.p.m. $\square$ = Red cell solution, 7.2 p.p.m.

the c -axis and $k_e E$ by the cotangent of the angle made with the c -axis. It is clear from Fig. 2 that k_e varies markedly with pH, but that the value of K_{mu} , namely 0.009 ± 0.001 M at 0° , is roughly constant. Kiese & Hasting's finding (1940b) that the pH activity curve of carbonic anhydrase is the same when measured at two different CO_2 pressures (100 and 230 mm. Hg) over the more extended range pH 6.1–10.0 leads to the same conclusion. We did not attempt to determine K_{mu} directly below pH 7.0, since the total CO_2 uptake at low CO_2 pressures then becomes too small for accurate measurement.

From equations (2), (4) and (5) of this paper and equation (29) of the adjoining paper it follows that

$$\begin{aligned} R_{eo} &= \frac{k_e E}{1 + K_{mo}/x} = \frac{k_e E}{1 + K_{mu} K'_1 / f_{\text{HCO}_3} \alpha_{\text{H}} [\text{HCO}_3^-]} \\ &= \frac{k_e E [\text{HCO}_3^-]}{[\text{HCO}_3^-] + K_{mu} K'_1 / f_{\text{HCO}_3} \alpha_{\text{H}}}, \end{aligned} \quad (7)$$

whence

$$\frac{[\text{HCO}_3^-]}{R_{eo}} = \frac{[\text{HCO}_3^-] + K_{mu} K'_1 / f_{\text{HCO}_3} \alpha_{\text{H}}}{k_e E}. \quad (8)$$

K_{mu} may thus be also obtained indirectly from CO_2 output experiments with mixtures of NaHCO_3 and buffer in the pH range in which the uptake is too small for accurate measurement. The value of

$f_{\text{HCO}_3} a_{\text{H}}$ is obtained by means of equations (24) or (25) of Roughton & Booth (1946), and R_{eo} calculated as therein described.* $[\text{HCO}_3^-]/R_{eo}$ is then plotted against $[\text{HCO}_3^-]$, and the value of $K_{mu}K_1/f_{\text{HCO}_3} a_{\text{H}}$ is given as before by the intercept on the $[\text{HCO}_3^-]$ axis, whereas the cotangent of the angle made with the latter gives $k_e E$. In this treatment the concentration of the actual substrate, H_2CO_3 , disappears, which is an advantage since our estimate of it depends on the value of K which is only approximately known.

We have done several experiments of this kind with the chloroform enzyme solution and at 0.0025 to 0.02M-NaHCO₃ both in M/20-M/50 cacodylate buffer, pH 6.2, and in M/30 phosphate buffer, pH 6.3. The results, however, show a rather wide scatter owing mainly to the diffusion corrections being so large in this range, not only for the enzymic but also for the non-enzymic reactions. Drawing the best lines through the data gives, however, values of $K_{mu} = 0.009$ ($\pm$ about 40%). With NaHCO₃ concentrations of 0.01–0.03M in M/40 phosphate buffer, pH 7.0 (29 p.p.m. of chloroform enzyme), the reaction rates are much slower, and yield the value of 0.009 to within ± 0.001 , as in Fig. 2. On the whole, it seems safe to assume therefore that K_{mu} is independent of pH over the range 6.0–10.0.

That K_{mu} is also independent of partial purification is shown by comparison of the squares with the circles in Fig. 2, the squares representing results obtained in M/24 veronal, pH 8.0, 0° with blood solution (7.1 p.p.m. of red cells) in place of the chloroform enzyme solution. On this basis we might expect that the value of K_{mu} would be the same in pure solutions of carbonic anhydrase, but the lesser stability of the latter makes it very difficult to obtain accurate enough measurements by the manometric technique. Since this work was completed, however, Kiese (1941) has reported values of K_{mu} for a highly purified preparation from horse red cells. After the usual ethanol-chloroform procedure, the enzyme was further purified by fractional precipitation with ammonium sulphate and dialysis. His value at pH 7.4 and 1° was only 0.0012, i.e. about one-seventh of our value and was furthermore affected by pH, rising to 0.0022 at pH 9.3. Kiese knew of, and took, some of the precautions in estimation listed above and by Roughton & Booth (1946), but it is not clear that he allowed for them all—a matter of great importance with pure solutions. Apart from this, we have no explanation of

* In the allowance for diffusion by equation (18) of the adjoining paper, a value for K_{mu} is assumed. A 100% variation in the assumed value of K_{mu} , however, makes a change of only about 10% in the calculated value of R_{eo} ; hence it seems fair provisionally to assume $K_{mu} = 0.009$ in this calculation and use the results so obtained to test equation (8).

the difference from our results other than the special purity of his enzyme solutions. Leiner (1943), working with a highly purified enzyme preparation in presence of peptone as stabilizer at pH 7.39, 0°, gives some data on uptake rates at several CO₂ pressures. His results only permit an approximate calculation of K_{mu} , but the value we find from them, namely 0.0075 ($\pm 20\%$), is in the same range as our own values of K_{mu} rather than the values given by Kiese.

Experimental proof that the enzyme catalyzes both phases of the reaction equally, i.e. that $k_{eu} = k_{eo} = k_e$. Kiese & Hastings (1940b) have shown, as expected, that at equilibrium carbonic anhydrase catalyzes both CO₂ output and CO₂ uptake equally: in their determinations, however, no allowance was made either for the limiting effect of diffusion, which was probably but slight in many instances, or for the change in activity of the enzyme during the manometric shaking, which may sometimes have been quite large. We have made two special tests with chloroform enzyme solution in phosphate buffer, under conditions where the diffusion correction is not more than 15%.

(a) At pH 7.3:

(i) CO₂ uptake experiment:

$$[\text{CO}_2] = 0.00143 \text{ M; enzyme} = 57 \text{ p.p.m.}$$

$$R_{eu} = 3.7 \times 10^{-3} \text{ mol. CO}_2/\text{l./sec.}$$

Then by equation (1)

$$\frac{k_{eu} E \times 0.00143}{0.00143 + 0.009} = 3.7 \times 10^{-3},$$

whence

$$k_{eu} E = 2.70 \times 10^{-4}.$$

(ii) CO₂ output experiment:

$$[\text{HCO}_3^-] = 0.02 \text{ M; enzyme} = 28.6 \text{ p.p.m.}$$

$$f_{\text{HCO}_3} a_{\text{H}} = 4.25 \times 10^{-3}; R_{eo} = 3.2 \times 10^{-5} \text{ mol. CO}_2/\text{l./sec.}$$

Then by equation (7)

$$\frac{k_{eo} E \times 0.02}{0.02 + 0.009 \times 2.6 \times 10^{-7}/4.25 \times 10^{-3}} = 3.2 \times 10^{-5},$$

whence

$$k_{eo} E = 1.2 \times 10^{-4}.$$

Thus

$(k_{eu} E \text{ in CO}_2 \text{ uptake experiment})/$

$$(k_{eo} E \text{ in CO}_2 \text{ output experiment}) = 2.24$$

as against a value of 2.0 for the ratio of the enzyme concentration in the two experiments.

(b) At pH 7.5:

In a similar pair of experiments we had (i) $[\text{CO}_2] = 0.00149 \text{ M}$, enzyme = 14.3 p.p.m. and (ii) $[\text{HCO}_3^-] = 0.032 \text{ M}$, enzyme concentration = 28.5 p.p.m. From the values of R_{eu} and R_{eo} we found

$(k_{eu} E \text{ in CO}_2 \text{ uptake experiment})/$

$$(k_{eo} E \text{ in CO}_2 \text{ output experiment}) = 0.465,$$

whereas (enzyme concentration in CO₂ uptake experiment)/(enzyme concentration in CO₂ output experiment) was equal to 0.5.

Thus in these two tests we find that $k_{eu} = k_{eo} = k_e$ to within $\pm 10\%$ on the average. This is well within experimental error.

EFFECT OF BUFFER CONCENTRATION

Table 1 summarizes results for the four main buffer systems used. The values of $k_e E$ listed in the last column are calculated as in the examples just given, a constant value for K_{mu} of 0.009M at 0° being assumed throughout. With the two more alkaline buffers, veronal and dimethylglyoxaline, $k_e E$ is constant within the experimental error ($\pm 10\%$) over the range 0.015 to 0.08M-buffer concentration, and its actual values therefore probably depend only on the effect of pH on the activity of the enzyme. A similar result was found in preliminary experiments with two other alkaline buffers, namely, succinimide and allantoin. As regards the effect of phosphate, Exp. D of Table 1 clearly shows a depressing effect at concentrations of total buffer ≥ 0.048 M (i.e. $[\text{HPO}_4^-] > 0.036$ M). Part, but not nearly all, of this depression is due to the lowered pH at higher concentrations of phosphate (see below). At lower concentrations of HPO_4^- (i.e. 0.024M and less), Exps. B and C show practical constancy, though in Exp. D the value at 0.0096M appears to be definitely below those at 0.015 and 0.024M-total phosphate. Exp. D was, however, done at an early stage in the development of the technique: in Exp. C, which was carried out nearly at the end of the research, the low range of phosphate concentration was carefully reinvestigated, and the value of $k_e E$ was found to be constant within experimental error. This constancy would best be confirmed by working at concentrations below 0.0096M-phosphate, but the total CO_2 uptake then becomes too small for accurate measurement.

The effect of cacodylate concentration has proved very hard to establish satisfactorily. Of several difficulties the worst is that in CO_2 output experiments below pH 6.4 the correction for the limiting effect of diffusion becomes very large, i.e. 50% or more, and correspondingly doubtful. The values given in Exp. A of Table 1 seem, as they stand, to show a significant activating effect of this buffer at concentrations above 0.048M. A similar result was found in two other experiments, but in view of the experimental and computational uncertainties, the activation by cacodylate cannot be regarded as definitely established.

In the standard boat method of Meldrum & Roughton (1933) for measuring anhydrase activity, M/5 NaHCO_3 is mixed with an equal volume of M/5 phosphate buffer, pH 6.8, $\pm$ enzyme, and the rate of CO_2 output followed. According to Table 1, an inhibition of the order of 40% is produced by this concentration of phosphate, and it is therefore

Table 1. *Effect of buffer concentration on activity of enzyme*

Exp.	Type	Buffer		pH	$k_e E$
		Acid/ salt	Conc.		
Substrate 0.005M-HCO ₃ ⁻ . Enzyme 47.6 p.p.m.					
A	Cacodylate	1.0	0.096	6.18	1.79 × 10 ⁻⁴
			0.048	6.21	1.24 × 10 ⁻⁴
			0.024	6.23	1.43 × 10 ⁻⁴
			0.012	6.24	1.07 × 10 ⁻⁴
	Extrapolation		0.0		1.10 × 10 ⁻⁴
Substrate 0.010M-HCO ₃ ⁻ . Enzyme 28.6 p.p.m.					
B	Phosphate	1.0	0.048	6.93	8.55 × 10 ⁻⁵
			0.024	7.01	8.45 × 10 ⁻⁵
Substrate 0.00149M-CO ₂ . Enzyme 14.3 p.p.m.					
C	Phosphate	0.36	0.024	7.48	5.77 × 10 ⁻⁵
			0.0154	7.52	6.28 × 10 ⁻⁵
			0.0096	7.56	5.6 × 10 ⁻⁵
D	Phosphate	0.33	0.096	7.37	4.48 × 10 ⁻⁵
			0.048	7.44	5.45 × 10 ⁻⁵
			0.024	7.51	7.0 × 10 ⁻⁵
			0.0154	7.55	7.5 × 10 ⁻⁵
			0.0096	7.59	6.19 × 10 ⁻⁵
E	Veronal	0.33	0.044	8.21	9.1 × 10 ⁻⁴
			0.015	8.25	8.0 × 10 ⁻⁴
F	Veronal	0.20	0.083	8.48	9.5 × 10 ⁻⁵
			0.050	8.51	9.5 × 10 ⁻⁴
			0.024	8.54	10.2 × 10 ⁻⁴
G	Dimethylgly-oxaline	3.0	0.038	8.41	8.4 × 10 ⁻⁵
			0.019	8.38	7.65 × 10 ⁻⁵
H	Dimethylgly-oxaline	0.33	0.067	9.40	12.3 × 10 ⁻³
			0.029	9.36	12.0 × 10 ⁻⁵

necessary, in comparing the activity of the enzyme from different sources or under different conditions (e.g. added 'activators'), to be sure that the inhibitory effect of the phosphate is the same in the various cases. This precaution has not been taken in most previous work (see later).

THE EFFECT OF pH

Fig. 3 summarizes our complete data as to the effect of pH upon anhydrase activity in the various buffer solutions for a standard concentration of 14.3 p.p.m. of crude chloroform enzyme in each case. The output method was used between pH 5.5 and 7.5, and the uptake method between pH 6.5 and 10. The size of the rectangles denotes the probable experimental error ($\pm 10\%$), whilst the enclosed letter indicates the nature of the buffer used. The concentration of the latter was below the limit at which, according to Table 1, there is a significant effect of the buffer on the observed activity (e.g. 0.03M in case of phosphate).

The results between pH 6.2 and 9.0 are clear-cut and satisfactory. There is a fairly broad minimum

between pH 6.2 and 6.7, and thereafter the curve rises almost linearly with pH. In pH zones in which different buffers overlap (namely pH 6.2–6.5 for cacodylate and phosphate, pH 7.5–8.0 for phosphate and veronal, pH 8.2–8.7 for veronal and dimethylglyoxaline, and pH 8.8–9.0 for dimethylglyoxaline and pyrophosphate) there is good agreement which—ever buffer is used, in spite of the great diversity in the chemical nature of the various buffers, especially

compounds (e.g. ammonia and glycine). Succinimide does not suffer from any of these disadvantages, but it is unstable in solution and the three points obtained with this buffer between pH 9.0 and 10.0 are presented therefore only tentatively. They do, however, suggest a steady rise of the curve above pH 9.0 rather than a plateau.

The shape of the curve given in Fig. 3 differs from that put forward by Kiese (1941), who reports that

Fig. 3. Activity-pH curve for chloroform enzyme preparation.

in their ionizing group. This suggests that the relationship shown in Fig. 3 is dependent only upon pH and is not clouded by activating or inhibitory effects of the buffers.

Below pH 6.0 the curve seems to rise again, though it must be admitted that (i) there is still some suspicion that even at low concentrations cacodylate may have an activating effect, and we have not yet found any other convenient and non-inhibitory buffer with which to check the results in this range, (ii) as already mentioned the corrections for the limiting effect of diffusion become uncomfortably large.

There is likewise a dearth of satisfactory buffers above pH 9. The usual systems inhibit the enzyme (e.g. phenate, borate, and carbonate), catalyze the non-enzymic reaction too greatly (e.g. borate), or react independently with CO_2 to form carbamino

the pH curve has a plateau below pH 6.0 and also above pH 9.0, whilst between these limits it has the same shape as the titration curve of a weak acid of $\text{pK} = 7.2$. We had reached the same conclusion at an earlier date as Kiese (1941) courteously acknowledged. The differences of our present results from those of Kiese are, we believe, partly due to more accurate methods of observation and calculation, and partly to the fact that Kiese assumed that K_{mu} increases with pH, whereas, according to our finding, K_{mu} is independent of pH.

We have also made some study of the effect of pH on the enzyme in other degrees of purification. Fig. 4 shows a much smaller series of experiments upon the effect of pH on the anhydrase activity of ox red cell solutions. All the points except that at pH 8.5 fall to within 12% upon the same pH activity curve as in Fig. 3. The same concordance

in the overlapping regions of phosphate and cacodylate buffer, phosphate and veronal buffers, is again noted.

Towards the end of the research we carried out a few experiments upon an enzyme preparation highly purified by the method of Keilin & Mann, to whom we are indebted for this sample. Of the six

pH 7.50. The latter buffer solution, it will be recalled, has apparently no inhibitory effect. The results with chloride and nitrate show the same diminution in activity for the three cations Na^+ , K^+ and NH_4^+ ; the anions on the other hand have marked specific effects, the series running $\text{SO}_4^- < \text{acetate}^-$, Cl^- , $\ll \text{Br}^-$, NO_3^- , I^- . Except possibly in

Fig. 4. Activity-pH curve for lyzed ox red cell solution.

points obtained, one fell right off the previous pH-activity curves of Figs. 3 and 4, but the remaining points were all fairly close. Two different concentrations of the enzyme were used in all cases, and a fair-to-good proportionality between concentration and activity was observed except in two instances, namely, at pH 6.2 and 8.2, wherein results similar to those of Fig. 1B were found.

Our provisional conclusion is that purification is without significant effect on the pH-activity curve of carbonic anhydrase, as has also been found for yeast saccharase by Euler, Josephson & Myrbäck (1924).

EFFECT OF CERTAIN SALTS

The study of the effect of salts is only preliminary and limited in scope. Table 2 gives the effect of adding a small series of neutral salts (analytical grade purity, final concentration mostly 0.024M) on the activity of 14.3 p.p.m. crude chloroform enzyme solution in 0.024M-potassium phosphate,

the case of sulphate, the effects observed are much larger than could be accounted for by the changes in pH produced by adding the salts.

Table 2. *Effect of some neutral salts on anhydrase in 0.024M-phosphate, pH 7.5*

Salt	Molarity	Activity in salt + phosphate
		Activity in phosphate
NH_4Cl	0.024	0.70
KCl	0.024	0.61
NaCl	0.024	0.69
NaCl	0.096	0.33
KBr	0.024	0.196
KI	0.024	0.172
KNO_3	0.024	0.182
NaNO_3	0.024	0.177
K_2SO_4	0.072	0.565
Na acetate	0.024	0.60
Na cacodylate	0.048	0.97

The results at the two NaCl concentrations conform with a reversible reaction of Cl^- with the enzyme, i.e. $E + \text{Cl}^- \rightleftharpoons E \cdot \text{Cl}^-$, the complex being

inactive and having at pH 7.5 a dissociation constant of about 0.045. The affinity of the enzyme for Cl^- is found to decrease markedly with increase of pH, the inhibition by 0.05M- Cl^- being barely detectable in veronal and dimethylglyoxaline buffers above pH 8.4. On this account it has been possible to utilize the dimethylglyoxaline-dimethylglyoxaline hydrochloride buffer systems over almost their whole pH range, but in the case of the veronal series with their lower pH range we had to prepare the buffer systems mostly by addition of diethylbarbituric acid to the sodium salt rather than by the usual practice of adding HCl.

We have no data yet on the action of Cl^- below pH 7.5, but in the case of acetate, the inhibitory effect of which at pH 7.5 is about the same as that of Cl^- , we have some preliminary comparisons of the activity of the enzyme at pH 5.6 with that in cacodylate at the same pH. In 0.06M-acetate the activity is about 0.4 of that in cacodylate, i.e. a degree of inhibition agreeing roughly with that found in 0.06M-acetate at pH 7.5. There is obvious scope for much fuller study of the effects of salts along the quantitative lines worked out by Myrbäck (1926) for amylase and by Mann & Woolf (1930) for fumarase.

The salts of other carboxylic acids would presumably inhibit like acetate, but data are lacking. In early tests on two dicarboxylic acid buffers at pH 5.6, namely, 0.06M-maleate and 0.036M-phthalate, we found activities of only 0.04 and 0.09 respectively of that of cacodylate at this pH.

The fact that 0.048M-Na cacodylate (see bottom row of Table 2) has no appreciable effect gives support to the not quite proven conclusion that moderate concentrations of cacodylate are neither inhibitory nor activating.

In carbonate solutions, the activity of the enzyme is less than in other solutions of the same pH according both to Kiese (1941) and to our own independent results, in which quite large inhibition was observed. This has a bearing on the method of Philpot for estimating anhydrase activity, in which the time is taken for the pH of a solution containing 0.025M- Na_2CO_3 in 0.017M- NaHCO_3 to drop from 10.5 to 7.0 at 0° when CO_2 is bubbled through the solution $\pm$ enzyme. It would be expected, therefore, that the enzyme would be inhibited under these conditions. From the comparisons with the boat method given in the original paper (Philpot & Philpot, 1936), together with the present data on the effect of substrate concentration, etc., we calculate that the degree of inhibition is of the order of 90% as compared with the activities found in the dilute buffer solutions used in Fig. 3. The importance of being sure that so large a degree of inhibition remains constant when the effect of activators and other factors is studied, is even more obvious

than in the case of the standard boat method (see above).

In Brinkman's (1933) method, CO_2 solution is added to a bicarbonate-carbonate solution of pH 9.1, and the time taken for the pH to drop to the turning point of phenol red is measured at 0° . We have no quantitative data as to the degree of inhibition in this case, but there is reason to believe it still to be large, though less than in the Philpot technique.

In view of these drawbacks to the existing CO_2 -indicator methods of measuring anhydrase activity, we have carried out some preliminary experiments with veronal buffer, which is non-inhibitory, in place of carbonate.

SOME RESULTS WITH A CO_2 VERONAL-INDICATOR METHOD

Veronal buffer (3 ml. of 0.022M-veronal in 0.22M-Na salt, pH 7.95), three drops of bromothymol blue, and 2.3 ml. of distilled water (or 0.3 ml. enzyme solution + 2.0 ml. distilled water) are mixed in a 15 ml. weighing bottle which is stoppered and placed in ice for at least 15 min. Ice cold water (5 ml.) saturated with CO_2 (=0.071M- CO_2 as determined by Van Slyke analysis) is then added anaerobically from an all-glass syringe with a long nozzle and the time is observed for the pH to drop to pH 6.3, with the aid of a matching standard of bromothymol blue in phosphate buffer at pH 6.3. By placing the nozzle below the buffer solution the CO_2 can be injected and the solutions all mixed in less than a second without bubbling or loss of CO_2 . The end-point is chosen at a pH where there is a sharp change in the colour of the indicator and the buffer power of the solution is very low, so that the pH is changing rapidly.

In absence of enzyme the following times were noted: 89, 88, 93; mean 90 sec. In presence of enzyme 64, 63, 68, 62; mean 64 sec. The same enzyme solution was used as in Fig. 3 and its final concentration was identical, namely, 14.3 p.p.m.

The validity of the method was first checked by calculating approximately the value of k_u (the velocity constant of the uncatalyzed reaction $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$) from the blank tests without enzyme. Allowing a period of 1 sec. for the time of mixing of the CO_2 and buffer solutions, we have

$$\begin{aligned}\text{At } t = 1 \text{ sec., } [\text{'veronate' ion}] &= 0.022 \times 3/10.5 \\ &= 0.0063 \text{ M,} \\ [\text{HCO}_3^-] &= \text{zero,} \\ [\text{CO}_2] &= 0.071 \times 5/10.5 = 0.0337 \text{ M.}\end{aligned}$$

$$\begin{aligned}\text{At } t = 90 \text{ sec., } [\text{'veronate' ion}] &= 0.00015 \text{ M,} \\ [\text{HCO}_3^-] &= 0.00615 \text{ M,} \\ [\text{CO}_2] &= 0.0276 \text{ M,}\end{aligned}$$

since $[\text{HCO}_3^-] \text{ at } t = 90 - [\text{HCO}_3^-] \text{ at } t = 1$ equals $[\text{veronate}] \text{ at } t = 1 - [\text{veronate}] \text{ at } t = 90$ equals $[\text{CO}_2] \text{ at } t = 1 - [\text{CO}_2] \text{ at } t = 90$.

The average $[\text{CO}_2]$ during the experiment = 0.0307 M.

So k_u is approximately equal to $[\text{HCO}_3^-]$ formed/
 $\{[\text{average CO}_2] \times (90 - 1)\}$

$$= 0.00615 / (0.0307 \times 89) \\ = 0.00225 \text{ mol./l./sec.}$$

This value must, however, be reduced by about 2% to allow for the catalytic effect of the veronate ion on the $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ reaction. The final value, i.e. 0.0022, agrees well with the accepted value for k_u at 0° , namely, 0.0021.

The enzymic activity is next calculated as follows. The overall rate of enzymic and blank reactions together is proportional to $1/(64 - 1)$ sec., and the rate of the blank reaction alone to $1/(90 - 1)$ sec.

The ratio of the intrinsic enzymic rate to the blank rate therefore

$$= (1/63 - 1/89) \div 1/89 = 0.413.$$

So that the rate of the enzymic hydration of CO_2

$$= 0.413 \times 0.00225 \times 0.0307 \\ = 2.85 \times 10^{-5} \text{ mol. CO}_2/\text{l./sec.}$$

From equation (1)

$$\frac{k_{eu} [\text{average CO}_2] E}{[\text{average CO}_2] + K_{mu}} = 2.85 \times 10^{-5}$$

$$\text{or } k_{eu} E = \frac{2.85 \times (0.0307 + 0.009)}{0.0307} \times 10^{-5}$$

$= 3.7 \times 10^{-5}$ mol. $\text{CO}_2/\text{l./sec.}$ over the pH range covered in the experiment, i.e. 7.9–6.3.

According to Fig. 3, the manometric value of $k_{eu}E$ at the beginning of the experiment (pH 7.9) = 8.0 and at the end (pH 6.3) = 3.0, and it can be shown by numerical approximation that the average value during the run should be about equal to the actual value at pH 7.35, i.e. 5.5.

The figure for $k_{eu}E$ obtained by the all-liquid method thus comes out at about 70% of that expected from the manometric data. About one-third of the c. 30% deficit may be accounted for by slight inhibitory action of the indicator (Kiese & Hastings, 1940b) and the remainder (20%) is not large in view of the experimental uncertainties of the two methods ($\pm 10\%$) and the approximate character of the calculations. The degree of agreement in these preliminary tests suggests on the whole that any special effects of adsorption and/or inactivation at the gas/liquid interface in the manometric technique have been mainly controlled by our procedure.

Further tests under a wider variety of conditions are obviously desirable, but it is hoped that the detailed working out as given above may be of use in further research in this field, particularly in the study of those activators which, according to Leiner (1941), are so specially effective in the enzymic hydration of CO_2 , as investigated by the all-liquid method.

THE MELDRUM & ROUGHTON ENZYME UNIT

Meldrum & Roughton (1933) defined the enzyme unit provisionally as the amount which, when dissolved in 2 ml. of phosphate solution (M/10 Na_2HPO_4 in M/10 KH_2PO_4) and mixed with 2 ml. of bicarbonate solution (M/5 NaHCO_3 in M/50 NaOH), halves the time for evolution of the second quarter of total CO_2 at 15° . This unit was useful in the early work on the enzyme, but fails to satisfy present-day requirements for the following reasons. (1) No allowance is made for the progressive inactivation of the enzyme during the shaking: even at 0° this is considerable and variable. (2) At the concentrations of phosphate used there is an inhibition of the order of 40%, which may also be variable with different enzyme preparations. (3) No corrections for diffusion are made. At 15° they would be higher than at 0° , and therefore more sensitive to changes in the conditions of shaking, etc. (4) No account is taken of the possibility of a response curve of the type shown in Fig. 1B.

On these grounds it has not seemed feasible to translate the Meldrum & Roughton unit to the absolute units of this paper, and we do not recommend the continued use of the former unit except for relatively crude work. For accurate estimations of carbonic anhydrase activity the most suitable manometric procedure would seem to be the measurement of the rate of CO_2 uptake at 0° by dilute phosphate ($> 0.02\text{M}$) or chloride-free veronal buffer in the range pH 7.5–8.5, with graded amounts of enzyme, by the technique and mode of calculation described in this paper.

DISCUSSION

The question of activators. Most of the data in this paper are on crude and semi-purified enzyme solutions in the presence of one of the limited series of buffers which have little or no effect on the activity of the enzyme, in so far as this can be established by varying the buffer concentration over the range 0.01–0.10M. No studies were made with activators, since our work was done before their discovery. According, however, to Leiner (1941) and Main & Locke (1941) the effect of activators on red cell solutions is small, if appreciable, presumably because the carbonic anhydrase is already fully 'activated' by accompanying substances derived from the haemolyzed cells. Our data on the latter may therefore well be independent of activator effects. With the semi-purified enzyme solutions, the effect of activators is stated to be much more pronounced, but in any detailed study thereof it is clearly necessary to make full allowance for the effect of pH, buffers and the other factors dealt with

above. The methods and results developed in this and the accompanying paper should therefore be of service in, and form a basis for, more exact and comprehensive research on the effects of activators in semi-purified and highly purified enzyme solutions.

The actual existence of activators was for some time vigorously debated in Germany between Leiner and Kiese, the latter of whom was unable to detect any activation in many of the instances brought forward by Leiner. Owing to the war some of the relevant publications may well have escaped our notice but in the most recent work of Kiese (1942) to reach us clear proof is advanced as to the ability of glutathione to activate both the enzymic hydration and dehydration of CO_2 . This led Kiese to withdraw from his earlier denial of the existence of activators, but he concluded, and we believe with reason, that 'obviously the conditions for activation are not yet sufficiently clear'. As pointed out in a previous section, in most of the work which has been done on activators the enzyme preparations are already considerably inhibited by the medium in which the estimation is made. Many so-called 'activations' may indeed be no more than reversible restoration of activity lost through (a) the inhibitory effect of the buffer itself, or of traces of impurities present in it, in the distilled water or on the walls of the glass apparatus; (b) adsorption at interfaces and surface denaturation. These possibilities are obviously of particular importance in the case of highly purified enzyme solutions, wherein enzyme concentrations of the order of $\text{m}/300,000,000$ are dealt with and the protective substances present in the less purified preparations have been removed. In such work it is essential not to omit *any* of the precautions listed by Roughton & Booth (1946), and it is equally important to verify that the relation between enzyme concentration and activity is linear and not of the form shown in Fig. 1*B*, otherwise entirely misleading results would be obtained. In none of the previous work known to us on activators have all these points been covered, and for that reason quantitative knowledge on this subject seems open to question, though it must be admitted that the effects so far described appear too large for the qualitative demonstration of activators to be doubted. As suggested elsewhere (Roughton, 1943) it would be very desirable to adopt the rapid flow methods of Hartridge & Roughton (1923) to the estimation of carbonic anhydrase activity so that far higher concentrations of the purified enzyme could be used, thus reducing the chance of activity loss through the factors mentioned above. With this technique it should also be possible to work at body temperature which is outside the range of the manometric method even with diffusion corrections. Data derived from such estimations are obviously de-

sirable for a more accurate appraisal of the role of the enzyme in physiological processes.

The activity of carbonic anhydrase in the intact red cell. In 1931, a year before carbonic anhydrase was discovered, Dirken & Mook showed that, when a suspension of red cells was mixed with serum saturated with CO_2 , equilibrium as regards CO_2 and bicarbonate was reached to within 10% in about 0.2 sec. In such experiments a considerable amount of CO_2 hydration occurs inside the red cell, and since this process, as well as all the others involved, is accomplished in 0.2 sec. there is no escape from the conclusion that the intact red cell has a high anhydrase activity. This result was confirmed by the manometric experiments of Booth (1938) which show clearly the importance also of accessibility factors, namely the chloride-bicarbonate interchange and the rapid supply and removal of H^+ ions by buffers inside the red cell. Further interesting evidence, of a qualitative kind, was available after Mann & Keilin's (1940) discovery of the inhibitory action of sulphanilamide. It was easy to show manometrically that the rates of CO_2 -uptake and output by red cell suspensions are greatly reduced in the presence of sulphanilamide (Roughton, Dill, Darling, Graybiel, Knehr & Talbott, 1941; Davenport, 1941; see also the later experiments of Fegler, 1944). The same result was likewise obtained by Keilin & Mann (1941) and by Jacobs & Stewart (1942) using less direct, though very elegant, methods of observation.

Thus there is no doubt whatever of a high anhydrase activity inside the red cell, though its magnitude is still uncertain. Owing to the limiting effect of diffusion and of the 'accessibility factors' studied by Booth, no direct measurement has yet been made of the activity of the intact red cells, though such estimation is much needed for appraising the exact role of carbonic anhydrase in speeding up the exchange of CO_2 between the blood and the lungs or tissues. In default thereof, Roughton (1935) has tentatively assumed that the activity remains proportional to concentration up to the high values present in the red cell and, from the activities of the red cells when laked, diluted and measured with the standard Meldrum & Roughton method, he estimated that the anhydrase of the red cells was capable of magnifying 1500-fold the rate of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ at 38° . No proper allowance could, however, be made at the time for the effects of pH, and of concentrations of substrate, phosphate and chloride on the activity of the enzyme, and with the new data on these factors given in this paper the figure of 1500-fold needs to be replaced (tentatively) by one of 7500-fold (Roughton *et al.* 1941). A further increase might be necessary for the effect of activating factors in the cell. Roughton (1935) also calculated that the

reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ needs to be magnified 150 times in rate within the blood if the CO_2 in the lung capillaries is to come to within 10% of equilibrium with the CO_2 in the alveolar air in 1 sec., i.e. the average time hitherto assumed to be spent by the blood in the lung capillaries. This calculation, however, requires modification in light of the more recent data on (i) the velocity constants of the $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ reaction and the true first ionization constant of carbonic acid at 38° (Roughton, 1943, Table 1); (ii) the average time spent by the blood in the human lung capillaries, which, according to Roughton's (1945) theoretical estimations, ranges from 0.75 sec. at rest to 0.35 sec. in hard work. On this basis, magnifications ranging from 300- to 650-fold would be required in the circulating blood, and if the calculated magnification of 7500-fold in the intact red cell is a correct figure it follows that in normal blood the enzyme is present in 12- to 25-fold excess of minimum requirements. These revised figures accord rather more closely with the results of Roughton *et al.* (1941) on the effects of sulphanilamide on CO_2 output in man at rest and during exercise than did the previous figures which these authors used.

SUMMARY

1. Manometric determinations are reported on the effect of substrate concentration, pH and neutral salts on the activity of semi-purified and crude carbonic anhydrase at 0°. The activities are calculated in mol. CO_2 /l. absorbed or evolved per second, and allowance is made for (i) the limiting effect of

diffusion, (ii) progressive denaturation caused by the shaking, (iii) inhibitory effects of the buffers and of impurities present in the solution.

2. The Michaelis constant is found to be independent of pH: its value at 0° = $0.009 \text{ M} \cdot \text{CO}_2 \pm 0.001$.

3. In the pH range in which CO_2 uptake and output can both be measured, carbonic anhydrase catalyzes each phase equally.

4. The effect of pH was studied over the range pH 5.6–10.0 with the aid of four main buffer systems, namely, cacodylate, phosphate, veronal and dimethylglyoxaline, the inhibitory effect of these buffers being inappreciable at concentrations below 0.05M. The pH curve shows a minimum between 6.0 and 7.0, and is still rising at pH 10.0. The shape is therefore probably not that of the ionization curve of a univalent weak acid, as had been previously reported.

5. The anions of many neutral salts were found to be inhibitory at pH 7.4 when present in concentrations of 0.02–0.1M, the series running $\text{SO}_4^- < \text{Cl}^- \ll \text{Br}^-$, NO_3^- , I^- . The anions of most carboxylic acids are also inhibitory.

6. The enzyme is strongly inhibited in the bicarbonate-carbonate solutions used in the Philpot method of estimation, and an alternative all-liquid method using veronal buffer is therefore proposed.

7. The relation of the present work to that of other investigators on activators of carbonic anhydrase is discussed.

8. The quantitative activity of the enzyme in the red cell *in vivo* is reconsidered in the light of the new data reported in this paper.

REFERENCES

- Booth, V. H. (1938). *J. Physiol.* **93**, 117.
 Brinkman, R. (1933). *J. Physiol.* **80**, 171.
 Davenport, H. W. (1941). *Amer. J. Physiol.* **133**, 257.
 Dirken, M. N. J. & Mook, H. W. (1931). *J. Physiol.* **73**, 349.
 Euler, H., Josephson, K. & Myrbäck, K. (1924). *Hoppe-Seyl. Z.* **134**, 39.
 Fegler, J. (1944). *Nature, Lond.*, **153**, 137.
 Hartridge, H. & Roughton, F. J. W. (1923). *Proc. Roy. Soc. A*, **104**, 376.
 Jacobs, M. H. & Stewart, D. R. (1942). *J. gen. Physiol.* **25**, 539.
 Keilin, D. & Mann, T. (1940). *Biochem. J.* **34**, 1163.
 Keilin, D. & Mann, T. (1941). *Nature, Lond.*, **148**, 493.
 Kiese, M. (1941). *Biochem. Z.* **307**, 400.
 Kiese, M. (1942). *Naturwissenschaften*, **30**, 122.
 Kiese, M. & Hastings, A. B. (1940a). *J. biol. Chem.* **132**, 267.
 Kiese, M. & Hastings, A. B. (1940b). *J. biol. Chem.* **132**, 281.
 Leiner, M. (1940). *Naturwissenschaften*, **28**, 316.
 Leiner, M. (1941). *Biochem. Z.* **311**, 119.
 Leiner, M. (1943). *Biochem. Z.* **315**, 30.
 Main, E. R. & Locke, A. (1941). *J. biol. Chem.* **140**, 909.
 Mann, T. & Keilin, D. (1940). *Nature, Lond.*, **146**, 164.
 Mann, P. J. G. & Woolf, B. (1930). *Biochem. J.* **24**, 427.
 Meldrum, N. U. & Roughton, F. J. W. (1932). *J. Physiol.* **75**, 3P.
 Meldrum, N. U. & Roughton, F. J. W. (1933). *J. Physiol.* **80**, 113.
 Myrbäck, K. (1926). *Hoppe-Seyl. Z.* **159**, 1.
 Philpot, F. J. & Philpot, J. St L. (1936). *Biochem. J.* **30**, 2191.
 Roughton, F. J. W. (1935). *Physiol. Rev.* **15**, 241.
 Roughton, F. J. W. (1941). *J. biol. Chem.* **129**, 141.
 Roughton, F. J. W. (1943). *Harvey Lectures*, **39**, 96.
 Roughton, F. J. W. (1945). *Amer. J. Physiol.* **143**, 621.
 Roughton, F. J. W. & Booth, V. H. (1938). *Biochem. J.* **32**, 2049.
 Roughton, F. J. W. & Booth, V. H. (1946). *Biochem. J.* **40**, 309.
 Roughton, F. J. W., Dill, D. B., Darling, R. C., Graybiel, A., Knehr, C. & Talbott, J. H. (1941). *Amer. J. Physiol.* **135**, 77.
 Scott, D. A. & Fisher, A. M. (1942). *J. biol. Chem.* **144**, 371.
 Scott, D. A. & Mendive, J. R. (1941a). *J. biol. Chem.* **139**, 691.
 Scott, D. A. & Mendive, J. R. (1941b). *J. biol. Chem.* **140**, 445.