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THE ACTIVITY OF CONCENTRATED SOLUTIONS OF CARBONIC ANHYDRASE

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SUMMARY

A rapidly recording stopped-flow apparatus has been used to measure the rate of reactions catalysed by carbonic anhydrase. The method allows high concentrations of enzyme to be used so that the contributions to the observed rate by the non-catalysed and buffer-catalysed rates become unimportant. The rates observed were proportional to the enzyme concentration up to the highest concentrations studied, about one tenth of that found in the red blood cell.

It was shown that under physiological conditions the enzyme is largely in the form uncombined with substrate. The activity of human carbonic anhydrase at physiological temperature, pH, ionic strength and substrate concentration was measured directly. From this it can be estimated that the enzyme in the red cell increases the rate of CO_2 output from bicarbonate by about 13 000 fold. This figure may be compared with the 700-fold increase necessary for CO_2 evolution during the transit of the blood through the lung capillaries.

INTRODUCTION

Many attempts have been made to estimate the catalytic activity of carbonic anhydrase in the human red cell under physiological conditions[1-3]. Most of these estimates have involved extrapolation from the results of measurements made in very dilute enzyme solutions at temperatures below 20°. By using a rapidly recording stopped-flow apparatus it has been possible to make measurements on the catalytic activity of human carbonic anhydrase over a wide range of enzyme concentration up to one tenth of that found in the red cell at physiological pH, temperature, ionic strength and substrate concentration. A new estimate for the catalytic activity in the red cell is thence derivable.

The methods used in the past to study the kinetics of the reactions catalysed by carbonic anhydrase, the hydration of CO_2, and the dehydration of carbonic acid, fall into two main classes: manometric techniques[4] and less accurate changing-pH methods[5]. Both types of measurement are seriously limited by their slowness so that

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they can be employed only with very low enzyme concentrations. The reactions catalysed by the enzyme proceed fairly rapidly in the absence of enzyme and their rates are increased very markedly by rise of temperature. They are also catalysed by non-enzymic solutes particularly the more electronegative constituent of many buffers[6,7]. Under these conditions of low enzyme concentration the observed reaction velocity is thus the sum of the uncatalysed reaction plus contributions due to catalysis by the enzyme and the buffer system. A further complication is that in dilute solution carbonic anhydrase is very susceptible to inactivation by traces of impurities, and possibly also by adsorption at interfaces. In much of the earlier work correct allowance has not been made for these factors, so that a great deal of the published data is contradictory (see discussion by CLARK AND PERRIN[8]).

CLARK AND PERRIN used a combination of the changing-pH method with a rapid reaction technique in studying the effect of activators of carbonic anhydrase. This procedure allowed them to work with much higher concentrations of the enzyme than had been possible previously. This was a great advantage since the enzymically catalysed reaction could be made the only significant contributor to the observed kinetics and also because the enzyme is more stable at higher concentrations[9].

EXPERIMENTAL

The technique employed in the present study was similar to that of CLARK AND PERRIN[8]. All kinetic measurements reported here were made with a rapidly recording stopped-flow apparatus of the type described by GIBSON AND ROUGHTON[10]. The changes in pH were followed by the indicator *p*-nitrophenol, which is particularly suitable for this work. It has a low protein error and has been shown to be virtually non inhibitory at the concentrations used[11]. The absorption maximum of the basic form near 4000 Å is close to the peak sensitivity of the photomultiplier used in the stopped-flow apparatus.

Reactions were initiated by mixing a buffer solution containing enzyme and indicator with a solution of carbon dioxide or sodium bicarbonate. Changes in pH with time were followed photometrically and recorded by an oscilloscope. Calibration of the apparatus was performed by measuring the displacement of the oscilloscope beam when a buffer solution containing indicator in the observation tube was replaced by a similar solution to which small amounts of HCl or NaOH had been added. By limiting the total pH change to 0.1–0.2 unit it was possible to work under conditions where the displacement was proportional to the amount of HCl or NaOH added. This calibration was performed for each buffer concentration and pH used. It was found to be more convenient than the calibration method described by CLARK AND PERRIN[8]. The response time and stability of the recording system employed were such that reactions with half times varying from about 5 msec up to several seconds could be followed readily. The calculation of the reaction rate is described in a later section.

The temperature of the reacting solution in the observation tube was measured to an accuracy of $\pm$ 0.1° by means of a copper–constantan thermojunction placed in the tube a few millimetres from the observation point. Experiments at body temperature were carried out in a constant-temperature room.

Materials

The precautions in the manipulation of enzyme solutions described by ROUGHTON AND BOOTH[4] were observed. Water used was distilled once from a metal still and then from glass. In the early part of this work de-ionised water from "Biodeminrolit" mixed-bed resin was used. No effects attributable to this change were observed. Glassware was cleaned in chromic acid and rinsed well with glass-distilled water. Analar chemicals where available were used in making up buffer solutions. Potassium chloride was recrystallised twice from glass-distilled water. *p*-Nitrophenol indicator was recrystallised twice from de-ionized water. The human enzyme used was a crude chloroform preparation made by the method described by ROUGHTON AND BOOTH[4] from outdated transfusion blood. It was stored in small batches at −20° and unfrozen just prior to use. It was found to be stable under these conditions of storage. Enzymes prepared in the same way from sheep and bullock blood obtained at a slaughter-house were also used.

Carbon dioxide solutions were made up in tonometers by equilibrating water with carbon dioxide gas at the required pressure. If used at 38° they were transferred anaerobically to syringes before being warmed. Carbon dioxide concentration was estimated by the Van Slyke manometric apparatus. Bicarbonate solutions were made up shortly before use and were stored in syringes. The pH of the bicarbonate solution was determined on several occasions and was always close to pH 8, showing that negligible amounts of carbonate were present.

For CO_2-uptake experiments 12.5 67 m*M* phosphate buffers were used as in previous work. For CO_2 output however, 20 m*M* imidazole imidazole hydrochloride buffer solutions were preferred for the following reasons: (a) Since only univalent ions are involved the activity corrections are much simpler; (b) Other complications also occur in phosphate buffers which do not seem to arise in imidazole solution; (c) The catalytic activity of the imidazole is even lower than that of phosphate buffers of the same strength; (d) It is true that the chloride content of the imidazole buffers would cause some slight inhibition of the enzyme; this did not prove to be a disadvantage in this work since further chloride in the form of KCl was always added to bring the chloride concentration up to 80 m*M* in order to simulate conditions in the red cell, (e) The ionizing group in this buffer is similar to the group in haemoglobin which is predominantly responsible for blood buffering under physiological conditions.

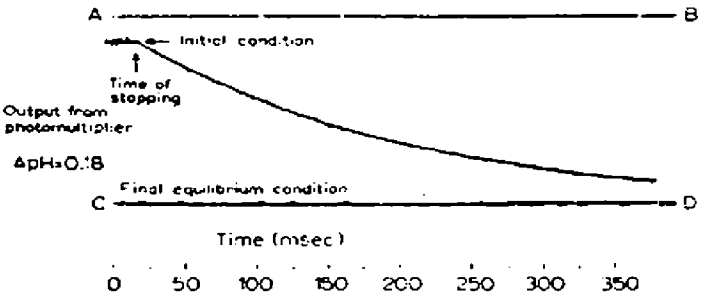

Fig. 1. Typical reaction trace.

CALCULATION

The reaction traces were enlarged on to squared paper and drawn carefully. A typical trace is shown in Fig. 1. The change in pH between the initial and final condition in this case was 0.18 unit. The line AB is produced by the second beam of the oscilloscope and serves only to define the direction of the time axis. The line CD represents the final equilibrium conditions and was recorded on each reaction trace by retriggering the oscilloscope scan at a time greater than ten times the half time of the reaction observed. Since the calibration showed that the deflection of the oscilloscope trace was proportional to the amount of H^+ added to or removed from the buffer the displacement of the trace will also be linearly related to the amount of CO_2 liberated or bound by the reactions.

(a) $$CO_2 + H_2O \rightarrow H^+ + HCO_3^-$$

(b) $$H^+ + HCO_3^- \rightarrow H_2O + CO_2$$

The calculation of the results depends on the fundamental principles underlying the kinetics of reversible enzymic reactions. HALDANE[12] showed that for a reversible enzyme reaction represented by

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P \tag{1}$$

the rate of approach to equilibrium is given by

$$-\frac{ds}{dt} = \frac{dp}{dt} = \frac{s\frac{V_s}{K_{ms}} - p\frac{V_p}{K_{mp}}}{1 + \frac{s}{K_{ms}} + \frac{p}{K_{mp}}} \tag{2}$$

where K_{ms} and K_{mp} are the respective Michaelis constants of the enzyme for substrate and product, s and p are instantaneous values of substrate and product concentration, and V_s and V_p are the respective maximum rates of enzymic catalysis of substrate and of product.

At equilibrium $\frac{ds}{dt} = 0$, and so

$$\frac{V_s}{K_{ms}} \cdot \frac{K_{mp}}{V_p} \cdot \frac{p_\infty}{s_\infty} = K^* \tag{3}$$

where K^* is the equilibrium constant of the reaction $S \rightleftharpoons P$, and s_∞ and p_∞ are the respective concentrations of substrate and product at equilibrium.

By the principle of conservation

$$s + p = s_\infty + p_\infty \tag{4}$$

Whence by Eqns. 2, 3 and 4

$$-\frac{ds}{dt} = \frac{V_s}{K_{ms}} \times \frac{(s - s_\infty)(1 + 1/K^*)}{(1 + s/K_{ms} + p/K_{mp})} \tag{5}$$

Provided therefore that s/K_{ms} and P/K_{mp} are both small the rate of the enzymic reaction is given by

$$-\frac{ds}{dt} = \frac{V_s}{K_{ms}} \times (s - s_\infty)(1 + 1/K^*)$$

$$= k(s - s_\infty) \text{ where } k = \frac{V_s}{K_{ms}}(1 + 1/K^*) \tag{6}$$

In conditions *in vivo*, in the case of the catalysis of CO_2 uptake and CO_2 output it can be shown that s/K_{ms} and P/K_{mp} are both less than 0.06, and in the experiments reported in this paper in which mammalian physiological conditions were simulated, this limitation was observed.

Since $(s - s_\infty)$ is proportional to the displacement Y of the oscilloscope trace from the line CD, it follows from Eqn. 6 that a plot of log. Y against time should give a straight line of slope equal to $k/2.303$. Such plots were made over the first 30–40% of the reaction and were found to be linear. A typical log. plot is shown in Fig. 2.

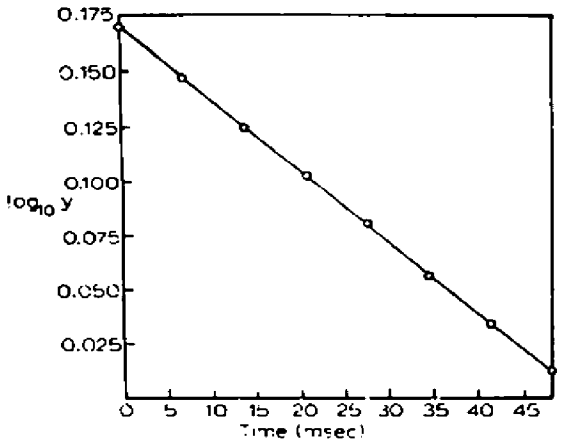

Fig. 2. Log. plot dehydration reaction; pH 7.1, 20°, 12.5 m*M* phosphate buffer, 25 m*M* $NaHCO_3$. Human enzyme preparation 1 in 80.

The value of $[CO_2]_{t=0}$ can be calculated from the composition of the solutions to be mixed, as can also the value of $[CO_2]_{t=\infty}$, provided that the pK's of the buffer system and of the first apparent dissociation of CO_2 are also known under the conditions of ionic strength and temperature employed.

Strictly speaking the above treatment is only valid if the pH remains constant throughout the process. With the small changes of pH in our actual experiments, the errors from neglecting them in the application of Eqn. 5 are very slight.

RESULTS AND DISCUSSION

Enzyme concentration

The catalytic effect of the enzyme has been found to be proportional to its concentration up to the highest concentration employed under almost all the conditions studied. This proportionality holds for both the hydration and dehydration reactions. The effect noticed previously by one of us[13] of a limiting catalytic activity at high enzyme concentration was not confirmed. This effect was indeed found to be due to the influence of the finite response time of the 1954 recording apparatus on the reaction traces. CHANCE[14] states that if the half time of response of the measuring apparatus is somewhat less than the half time of the reaction to be followed then the apparatus may be safely used to follow the reaction. This may be the case for the

main portion of the reaction trace but SIRS[16] has shown that unless the half time of response of the apparatus is less than $^1/_{10}$th to $^1/_{20}$th of the half time of the reaction the initial part of the reaction trace will be greatly distorted. It is the initial part of the trace which is of interest in this case.

The results for the hydration reaction using sheep carbonic anhydrase are shown in Fig. 3 under conditions identical to those used by FORREST[13].

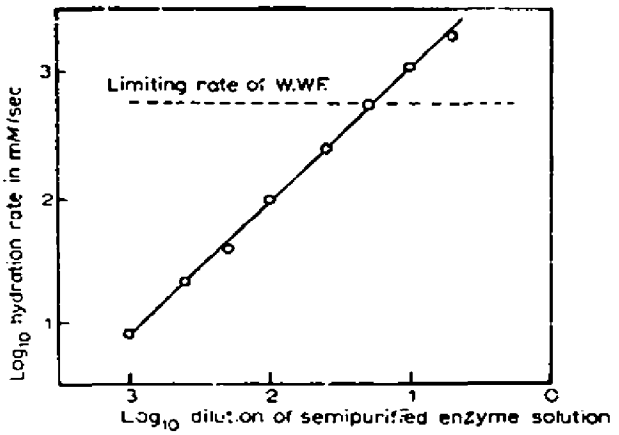

Fig. 3. Catalysis by sheep carbonic anhydrase. Hydration reaction, pH 7.7, 20°, 67 m*M* phosphate buffer, 9.3 m*M* CO_2.

The plot of the logarithm of initial rate of CO_2 uptake against logarithm of enzyme concentration gives a straight line of slope practically equal to unity, thus indicating proportionality between catalytic activity and enzyme concentration over a 200-fold range. The limiting velocity previously reported by FORREST has been exceeded four times without any sign of a plateau appearing at the highest rates measurable by the stopped-flow technique. We hope at an early date to measure rates 10 or so times faster by optical and thermal continuous-flow methods. The

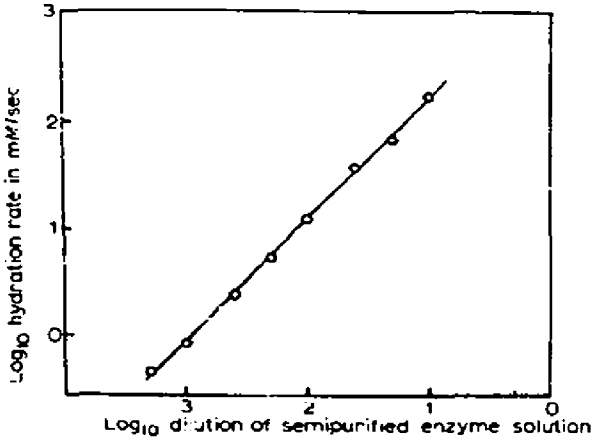

Fig. 4. Catalysis by human carbonic anhydrase. Hydration reaction, pH 7.8, 38°, 12.5 m*M* phosphate buffer, 1.35 m*M* CO_2, 80 m*M* KCl.

latter method has the possible advantage of being applicable even in solution containing haemoglobin of the order of concentration found in the red cells. The results of a CO_2 hydration experiment with human carbonic anhydrase at pH 7.8 and 37° are shown in Fig. 4, which again indicates proportionality between enzymic activity and concentration.

In stronger phosphate buffers it was found that the human enzyme, if present in concentrated solution, has a higher specific activity than in dilute solution. The specific activity of the dilute enzyme solutions could be increased to that found in more concentrated enzyme solutions by adding 10^{-4} *M* EDTA, bovine serum albumin (0.05%), or 10^{-3} *M* reduced glutathione. The inhibition was therefore probably due to impurities in the phosphate buffer used. No such inhibition at low enzyme concentration in these buffers was noted with the bovine and sheep enzyme preparation. The results of a dehydration experiment at pH 7.2 and 38° are shown in Fig. 5.

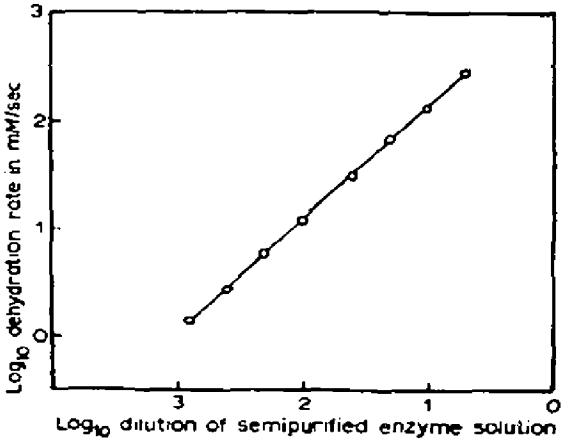

Fig. 5. Catalysis by human carbonic anhydrase. Dehydration reaction, pH 7.2, 38°, 20 m*M* imidazole buffer, 25 m*M* $NaHCO_3$, 80 m*M* total chloride.

Temperature

Experiments on the influence of temperature on the rate of reaction gave a temperature coefficient of 1.45 per 10° for the hydration reaction and 1.4 for the dehydration reaction. The latter figure is in good agreement with the revised value given by ROUGHTON[2]. These experiments were carried out at low substrate concentration so the change in catalytic activity might be due to the influence of temperature on both K_m and V_{max}. Under physiological conditions the substrate concentration is also low so the same temperature coefficients would apply.

Influence of anions

The human red cell contains about 80 mg ions of chloride per litre of water. This concentration of chloride was found to reduce the activity of the enzyme in the hydration reaction (1.2 m*M* CO_2, pH 7.3, $^1/_{80}$ *M* phosphate buffer) to 52% of its

activity in the absence of chloride. This is a slightly smaller effect than would be expected from the figures given by ROUGHTON AND BOOTH[16] on chloride inhibition.

Substrate concentration

DEVOE AND KISTIAKOWSKY[17] found that human carbonic anhydrase did not obey Michaelis kinetics. This finding may be related to the demonstration by NYMAN[18] that the enzyme exists in different forms not all having the same specific activity. A mixture of different forms of an enzyme need not obey Michaelis kinetics although such kinetics could apply to each form individually. It is clear that any further kinetic studies should be made on the separated forms.

It is possible to show experimentally that under physiological conditions the enzyme is only slightly combined with substrate or product and consequently that the hydration and dehydration reactions are approximately first order with respect to CO_2 and HCO_3^- concentrations respectively. If the enzyme was saturated or almost saturated with either substance the reaction would be zero order or intermediate between zero and first order. This point is important when the overall process of CO_2 loading and unloading by the red cell is being considered. The demonstration assumes that only one form of carbonic anhydrase is present but should not be seriously upset if two or more forms are present. HALDANE's expression Eqn. 2 may be rewritten

$$\frac{ds}{dt} = \frac{K' \frac{V_p}{K_{mp}} s - \frac{V_p}{K_{mp}} p}{1 + \frac{s}{K_{ms}} + \frac{p}{K_{mp}}} \tag{7}$$

The initial reaction rate with substrate concentration s is given by

$$-\frac{ds}{dt} = \frac{K' \frac{V_p}{K_{mp}} s}{1 + \frac{s}{K_{ms}}} \tag{8}$$

and the rate of the back reaction with substrate concentration p

$$\frac{ds}{dt} = \frac{\frac{V_p}{K_{mp}} p}{1 + \frac{p}{K_{mp}}} \tag{9}$$

If s is identified with CO_2 and p with bicarbonate

$$K' = \frac{[HCO_3^-]}{[CO_2]} = \frac{[H^+][HCO_3^-]}{[H^+][CO_2]} = \frac{K_1'}{[H^+]}$$

where K_1' is the apparent first ionisation constant of carbonic acid.

The results of activity measurements on human carbonic anhydrase under conditions close to physiological are shown in Table I.

TABLE I

INITIAL RATES OF CO_2 HYDRATION BY 1/100 DILUTION OF HUMAN ENZYME PREPARATION

pH 7.11, 80 mM KCl, 38°, K^* = 11.2.

Substrate	Rate
3.2 mM CO_2	17.2 mmoles/l/sec
20 mM $NaHCO_3$	9.9 mmoles/l/sec
3.2 mM CO_2 + 20 mM $NaHCO_3$	6.9 mmoles/l/sec

Applying Eqns. 8 and 9 to these data it follows that

$$\frac{\frac{V_p}{K_{mp}}}{1+\frac{s}{K_{ms}}} = 0.48 \text{ and } \frac{\frac{V_p}{K_{mp}}}{1+\frac{p}{K_{mp}}} = 0.495$$

Therefore

$$1+\frac{s}{K_{ms}} \simeq 1+\frac{p}{K_{mp}}$$

Hence

$$\frac{s}{K_{ms}} \simeq \frac{p}{K_{mp}}$$

or both these quantities are very much smaller than unity. In the former case the use of Eqn. 7 above gives

$$\frac{V}{K_{mp}} = \frac{(11.2+3.2+20)}{1+2\frac{s}{K_{ms}}} = 6.9$$

From Eqns. 8 and 9 it follows that

$$\frac{\frac{V_p}{K_{mp}}(35.8+20)}{1+\frac{s}{K_{ms}}} = (17.2-9.9) = 7.3$$

Hence

$$\frac{1+\frac{s}{K_{ms}}}{1+2\frac{s}{K_{ms}}} = \frac{6.9}{7.3}$$

hence

$$\frac{s}{K_{ms}} \simeq \frac{p}{K_{mp}} = 0.06$$

The actual values of K_{ms} and K_{mp} which could be derived in this way are not very reliable as slight inaccuracies in the activity measurements reported in Table I would lead to different values of these constants being derived. It is clear however

that $K_{m_{CO_2}}$ and $K_{m_{HCO_3^-}}$ are both much larger than the concentrations of these substances in the red cell under physiological conditions. Eqns. 8 and 9 can then be simplified to

$$\frac{d[CO_2]}{dt} = K^* \frac{V_p}{K_{mp}} [CO_2] \tag{10}$$

and

$$\frac{d[HCO_3^-]}{dt} = \frac{V_p}{K_{mp}} [HCO_3^-] \tag{11}$$

The rate multiplication by carbonic anhydrase is thus proportional to $K^* V_p/K_{mp}$ in the case of CO_2 uptake and to V_p/K_{mp} in the case of CO_2 output, and so depends upon enzyme concentration but not on substrate or product concentrations.

Enzyme content of the red cell

Various estimates of the catalytic effect of the carbonic anhydrase have been made by previous workers. In these calculations it has been generally assumed that the effect remains proportional to the enzyme concentration over the whole range extending from the dilute (0.1–1.0 mg/l) carbonic anhydrase solutions in which the activity was measured up to the concentrations (2–3 g/l) found in the red cell. This assumption has now been verified for semi-purified carbonic anhydrase up to concentrations of about one tenth of those in the red cell and this provides some support for the basic assumption in the earlier calculations. Unfortunately owing to the optical interference by the haemoglobin it is not possible to apply the present method to red cell haemolysates at higher concentrations than have already been studied by the classical manometric methods, but it is hoped however to get over this difficulty by means of other methods, *e.g.* the rapid thermal technique.

We assume however, that the rates of enzyme concentration in the semi-purified solution to that in the human red cell can be deduced from a comparison of the catalytic activity of diluted semi-purified solution and red cell haemolysates.

In the calculation now to be given the following data were used: (a) The non-enzymic rate of CO_2 output at 37° from 25 m*M* $NaHCO_3$ and 20 m*M* imidazole buffer at pH 7.2 in presence of 80 m*M* chloride, was found to be 0.33 m*M* CO_2/sec; (b) In presence of semi-purified enzyme solution, diluted 1 in 5, the rate of CO_2 output from the mixture used in (a) was 288 m*M*/sec; (c) The enzyme content of the semi-purified enzyme solution was 33.5% of the average present in the human red cell[19].

The magnification of the rate of CO_2 output in the red cell, under physiological conditions, should then be

$$\frac{288}{0.33} \times 5 \times \frac{1}{0.335} = 13\,000$$

This estimate may be compared with previous ones, which have ranged from 1500 to 8500 fold, but it may be pointed out that the conditions in our present calculation, though still not fully physiological, are much nearer this ideal than have obtained

in any work hitherto. The present estimate of 13 600-fold magnification gives about 1800% margin of safety over the minimum figure by which the reaction

$$H^+ + HCO_3^- \rightleftharpoons CO_2 + H_2O$$

needs to be catalysed, if it is not to restrict the rate of CO_2 output in the lung (see Table ix of ROUGHTON[20]).

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