CCLXVI. THE CATALYTIC EFFECT OF BUFFERS ON THE REACTION $CO_2+H_2O \rightleftharpoons H_2CO_3$

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FAURHOLT [1925, 1] has shown that in aqueous solution CO_2 takes part in two independent reactions

$$CO_2+H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-, \quad \text{.....(1)}$$

$$CO_2+OH^- \rightleftharpoons HCO_3^-. \quad \text{.....(2)}$$

The velocity constant of the reaction $CO_2+OH^- \rightarrow HCO_3^-$ is more than a million times greater than the velocity constant of the reaction $CO_2+H_2O \rightarrow H_2CO_3$, the loss of the proton from the H_2O molecule apparently causing, as Faurholt has emphasized, an enormous increase in the affinity for CO_2. In consequence of this, it is only below *p*H 8 that the rate of (2) becomes negligible in comparison with the rate of (1): in the range *p*H 9–10 the two rates are of the same order, whilst above *p*H 10 the rate of (2) becomes predominant.

Similar studies on the rate of combination of CO_2 with very weakly acidic organic hydroxides, such as methyl alcohol, ethyl alcohol and glucose, have shown [Faurholt, 1927] that CO_2 reacts slowly and in an analogous way with the organic hydroxide molecule:

$$CO_2+HOX \rightleftharpoons HCO_3X. \quad \text{.....(3)}$$

The inhibitory effect of the proton is again shown by the fact that the reaction with the organic anion—

$$CO_2+OX^- \rightleftharpoons CO_3X^- \quad \text{.....(4)}$$

—is also very much more rapid and complete than the reaction with the molecule.

In the case of stronger oxy-acids the affinity of the anion for H^+ is correspondingly lower, and Faurholt tacitly assumed that the affinity of the anion for CO_2 would fall *pari passu* and hence that acids with *pK* of the order of 7 (e.g. cacodylic acid) could be safely used as buffers in the measurements of the velocity constants of the reactions $CO_2+H_2O \rightleftharpoons H_2CO_3$ and $CO_2+OH^- \rightleftharpoons HCO_3^-$, without fear of the buffer taking any part other than the "instantaneous" supply or removal of the H^+ ions involved in the ionization of carbonic acid, i.e. $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$. The possibility of any direct reaction of the buffer with CO_2, H_2CO_3 or HCO_3^- (except in the case of the borate ion, which Faurholt suggested might form some special complex with CO_2) was thus implicitly excluded, not only by Faurholt but by all others who have worked in this field.

Preliminary experiments on the activity of carbonic anhydrase in phosphate solutions of varying concentration and *p*H have, however, prompted us to examine more critically the role of the buffer in reactions (1) and (2). To this end we have measured manometrically both the rate of CO_2 uptake by buffer solutions shaken violently with CO_2, and the rate of CO_2 output from bicarbonate buffer mixtures, in the presence of a much wider range of buffer concentration

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and species than heretofore. Our results, especially those in which the buffer concentration is varied 10-fold or more, lead us to conclude that almost all common oxy-acid-salt mixtures which buffer above *p*H 6, not only promote the CO_2 reactions by supplying or removing H^+ ions, but also through their more negative constituents catalyse directly both phases of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$. A preliminary account of these results has already appeared [Booth & Roughton, 1938]. Faurholt's discovery of the activity of the borate ion is thus only a special, though pronounced, example of a general principle.

It was not until nearly the end of the research that we investigated the effect of nitrogen bases as buffers. We were deterred from doing so by the fact that straight-chain nitrogenous bases, such as NH_3 or $CH_2NH_2COO^-$, combine rapidly and reversibly with CO_2 to form carbamino compounds, according to the following scheme [Faurholt, 1925, 2]:

$$R\mathrm{NH}_2 + \mathrm{CO}_2 \rightleftharpoons R\mathrm{NH.COOH}, \quad \text{.....(5)}$$

$$R\mathrm{NH.COOH} \rightleftharpoons \mathrm{H}^+ + R\mathrm{NH.COO}^-, \quad \text{.....(6)}$$

$$\mathrm{H}^+ + R\mathrm{NH}_2 \rightleftharpoons R\mathrm{NH}_3^+. \quad \text{.....(7)}$$

(The inhibitory effect of the proton is shown in this case also by the absence of reaction between CO_2 and $R\mathrm{NH}_3^+$.) The simultaneous occurrence of this carbamino reaction might make it difficult to detect and measure accurately any catalytic effect on the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction.

Cyclic nitrogenous bases have not, so far as we know, been investigated as regards their carbamino-forming power, and it was therefore with special interest that we tested some members of the new range of glyoxaline buffers, recently described by Kirby & Neuberger [1938], to whom we are greatly indebted for samples. Both the cations and the molecules of these substances were found to be incapable of any carbamino reaction with CO_2, but on the other hand the molecules proved to be markedly catalytic towards the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ especially if their pK were greater than 7·0. ($pK = -\log_{10} K$ where $K = [\mathrm{H}^+] \times$ [molecule of base]/[cation of base].) This led us to test such other cyclic nitrogenous bases as were both readily available and sufficiently soluble in water. Similar catalytic activity was always found if the pK were greater than 7, whereas tests of a more extended range of straight-chain N bases showed that these were without catalytic effect, their only reaction with CO_2, if any, being the carbamino formation. These rather remarkable results are summarized in Section II, together with some preliminary data on sulphhydryl and other compounds.

A possible mechanism of these catalyses is given in the discussion, together with some further points of interest which arise in regard to the enzyme carbonic anhydrase.

Methods

Rates of CO_2 uptake were determined by an improvement of Meldrum & Roughton's [1933, 2] manometric boat method. The apparatus (Fig. 1) consists of a lagged tank, a stationary manometer containing toluene coloured with sudan III as gauge liquid, a compensating bottle *E* (about 200 ml.), a CO_2 reservoir *D* (about 200 ml.) which can be connected through fine thermometer tubing *R* to a 60 ml. long-necked glass "boat" and a mechanism, not shown, for shaking the boat about *AR* as axis at an adjustable speed. The shaking must be rapid enough not to limit the rate of CO_2 uptake, but not so violent as to splash solution up into the tube connecting with the manometer. At 315 oscillations per min. the limiting speed is exceeded without the readings becoming erratic.

This speed is kept constant by a synchronous motor and is indicated by a neon lamp stroboscope.

The procedure in an experiment is as follows. The tank is emptied, and dried air is drawn through the connecting tubes to remove condensed moisture. In the meantime the test solution is measured into the "boat", which is stoppered and pushed into a holder in the shaker which holds it firmly by means of two rubber sleeves encircling the boat, all clips being open except C_4. The tank is then filled with water and the stirrer turned on. D is repeatedly evacuated and filled with CO_2 and left with a CO_2 pressure of 30–40 cm. Hg, the clips being closed and water-sealed. The boat, compensating bottle and manometer are evacuated to $\frac{1}{7}$ atm. through C_6 which is closed and placed below the water surface. After 9 min. the boat is shaken for 2 min. to bring the dissolved gases

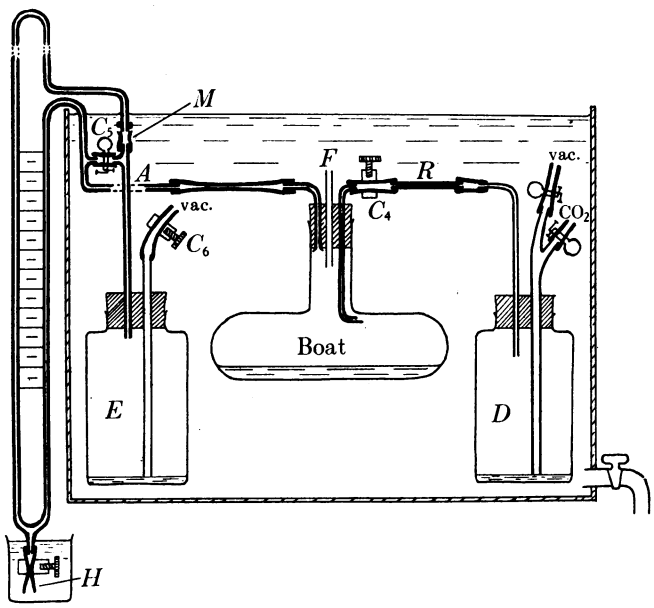


Fig. 1. Simplified diagram of apparatus arranged for CO_2 uptake. Not to scale. The boat is oscillated through 50° at right angles to the plane of the paper. Tube F is only used for anaerobic experiments.

in the test solution into equilibrium with the gas phase, and 1 min. is allowed for the solution to drain to the bottom of the boat. The short circuit C_5 between the two limbs of the manometer is closed and the zero reading taken. CO_2 is slowly let into the boat through the resistance R, by manipulation of C_4, up to the desired pressure. The time taken by this should be about 30 sec. A further period is allowed for the disturbance caused by the admission of the CO_2 to settle down, and then, exactly 2 min. after CO_2 begins to enter, shaking is started and manometer readings taken at 0, 5, 10, 15 sec. etc., up to 5 min. or more. The tank temperature is read. At the end of the experiment the clip C_5 is opened, and air allowed to enter slowly through a capillary inserted at C_6 until all parts of the apparatus are at atmospheric pressure. (If air enters suddenly into the apparatus, when at low pressure, the gauge liquid will shoot over.) Most of the water is meanwhile emptied out of the tank, and the boat then removed, washed and dried.

The preliminary equilibration period is 12 min.; if duplicate boats are used and one is prepared during the equilibration of the other, an observer and an assistant can perform serial experiments at the rate of one every 25 min.

With axial shaking, only a slight vibration is transmitted to the manometer and volume variations from distortion of the thick rubber tubing are negligible. Furthermore all the rubber joints can be water-sealed, the dead space between the boat and the liquid meniscus in the manometer cut down to 1 ml., and the volume of the system exposed to room temperature reduced to about 0·2 ml. These improvements, together with the substitution of toluene for water in the manometer gauge, the modified boat shape and the constant speed motor, are responsible for the increase in accuracy and reliability of the method. When the apparatus is working properly, readings should duplicate to 0·2 mm. At temperatures other than that of the room stirring of the water in the tank must be vigorously maintained during equilibration; otherwise the temperature, and therefore the pressure, in the compensating bottle or boat may vary from one another.

From time to time the whole apparatus must be washed out with water and acetone and dried by flushing with dried air. The manometer is then refilled with de-aerated coloured toluene through the rubber tube *H*. This tube is then filled with de-aerated water to form a seal and immersed in water. A little water is always left in *E* to saturate the gas with vapour.

When autoxidation, leaks or other complications which would give spurious results by manometric methods were suspected, readings were taken in absence of CO_2 during a prolonged first shaking period. For autoxidizable substances experiments were done under anaerobic conditions, by applying a slight modification of the method used by Meldrum & Roughton [1933, 2] for studying the rate of uptake of CO_2 by reduced blood to the present technique. If necessary one component of the required solution could be weighed solid directly into a dry boat, the oxygen removed by washing out with nitrogen and the remaining de-aerated solution added from a tonometer through *F*. In this case the substance makes no contact with oxygen whilst in solution.

Unless otherwise stated all experiments by the CO_2 uptake method were done at 0° with 4·2 ml. solution and an initial CO_2 pressure of 12·8 mm. Hg in a total gas space of 60 ml.

In order to avoid the effect of complications due to the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction, most experiments were done below *p*H 8·0.

Rates of CO_2 output were determined in the same apparatus but with the double compartment boat method of Meldrum & Roughton [1933, 1]. After equilibration of the boat in the tank the solutions were suddenly mixed (by starting the shaker) and the CO_2 evolution followed manometrically. Most of the experiments were done at 0° and at a total gas pressure of $\frac{1}{5}$ atm.

The majority of the experiments were done with the illustrated apparatus which has however now been simplified. The outlet clip, water-seal etc., at *H* have been eliminated and the gauge fluid is introduced into the manometer tube through *M*.

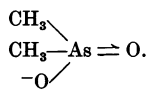
Notes on solutions. Carbonic anhydrase was prepared in semi-purified state by Meldrum & Roughton's [1933, 1] chloroform method.

Buffer solutions were made up with a known acid/ion ratio by adding the calculated quantity of HCl or alkali, and checking the *p*H or titrating to an unbuffered end-point. The *p*H of solutions was determined in most cases colorimetrically but for special cases it was measured by the hydrogen electrode.

Na and K salts were used indiscriminately, except when high concentrations were required and one salt (usually K) is markedly more soluble than the other.

I. Oxy-acid buffers

The two buffers which have been most used hitherto in work on CO_2 kinetics are phosphate ($H_2PO_4^- + HPO_4^=$) and cacodylate ($HCac + Cac^-$), where Cac^- is



We have accordingly investigated these with special care, and since the results and inferences therefrom seem qualitatively the same as for the general class of oxy-acid buffers which function above *p*H 6, we shall first describe the results with phosphate and cacodylate in detail.

CO_2 uptake experiments

Curve *A*, Fig. 2, shows the rate of CO_2 uptake by water, recorded manometrically. The gaseous CO_2 pressure drops to a steady value in about 20 sec.: this rapid uptake is simply due to physical solution of CO_2, the subsequent H_2CO_3 and HCO_3^- formation being negligible.

Curves *B*, *C*, *D*, Fig. 2, show the effect of increasing concentration of total phosphate, the ratio of $[HPO_4^=]$ to $[H_2PO_4^-]$ being kept constant. In each case there is a similar rapid drop of CO_2 pressure in the first 20 sec. (though the drop may be less owing to the solubility of CO_2 in the buffer being lower than in water), and then a prolonged slow phase in which the dissolved CO_2 enters into chemical combination. The slope of this slow phase is seen to be steeper

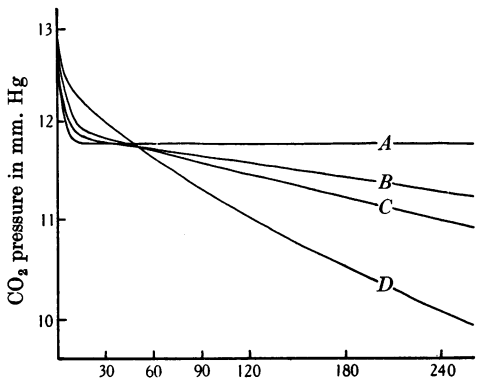


Fig. 2.

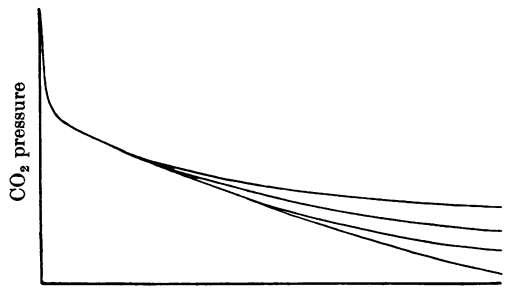


Fig. 3.

Fig. 2. Curves showing the observed course of CO_2 uptake by water and by phosphate at 0°. *A*, water; *B*, 0·0095 *M* phosphate; *C*, 0·14 *M*; *D*, 0·76 *M*. The *p*H was 7·4 at 0·2 *M*.

Fig. 3. Curves showing the expected course of CO_2 uptake by various concentrations of a given buffer, if the buffer itself has no direct effect on the CO_2 reaction. Corrected for effect of changing buffer concentration on physical solubility of CO_2.

throughout its *whole* course, as the phosphate concentration is increased; whereas if the phosphate did no more than just "instantaneously" remove the H^+ ions formed by the reaction $H_2CO_3 \rightarrow H^+ + HCO_3^-$, the slopes of curves *B*, *C*, *D* should be the *same* in their early stages, and the increased buffer concentration should exercise no effect until the latter part of the uptake, when the

velocity of the back reactions becomes significant, i.e. a family of curves like those shown in Fig. 3 should have been obtained.

Clearly there is, in addition, some direct effect of the phosphate upon the reaction, as is indeed shown quantitatively by equation (8) which fits the curves plotted in Fig. 2, i.e.

$$-\frac{d[CO_2]}{dt} = v_u [CO_2] = k_u [CO_2] \{1 + l_u [HPO_4^=]\}, \qquad \ldots\ldots(8)$$

where v_u is the apparent overall velocity constant for CO_2 uptake,
k_u is the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$,
l_u is a catalytic coefficient.

The numerical values of v_u in Tables I and III have been calculated by the method of Brinkman *et al.* [1933], but a correction has also been inserted for the rate of the back reaction, which in the case of phosphate amounts to 2–20 % and in the case of cacodylate to even higher figures. The allowance for the back reaction was made on usual principles and since it requires a knowledge of the end point of the reaction, this was determined either by continuing the experiment for 30 min. or preferably by a separate experiment in which a suitable amount of carbonic anhydrase was added to the mixture, so that the end point was reached in 5 min.

The time intervals over which v_u has been calculated have usually been 2 min. each, and in any given experiment at least four such intervals have been chosen between 1·5 and 8·0 min., v_u as given in Tables I and III thus being the average of four or more values. The individual figures in any one experiment usually agree with the mean to within 10 %.

A rougher, but less laborious, mode of calculation was used for the other data, which are mostly of a preliminary nature. This consisted in measuring the time t for the CO_2 pressure to drop through one or more small specified ranges in the early part of the slow phase of CO_2 uptake. Clearly $v_u = 1/t \times$ a constant, which is obtained from the curve of a solution of known v_u (determined as in Table I).

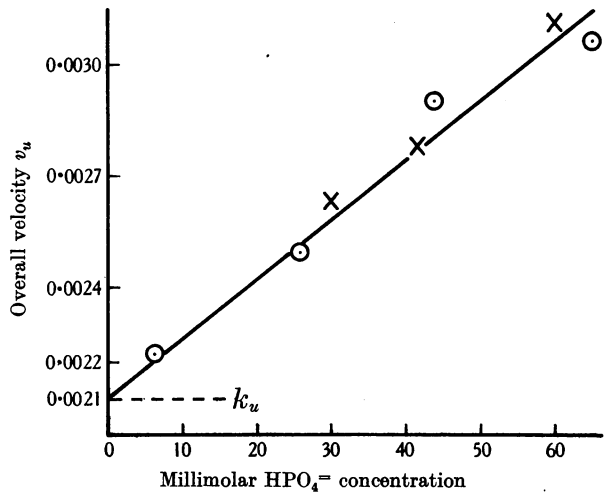


Fig. 4. Effect of $[HPO_4^=]$ on velocity of CO_2 uptake. ⊙ our experiments; × calculated from data of Brinkman *et al.* [1933].

Fig. 4 shows that there is a linear relation between v_u and $[HPO_4^=]$, at a ratio of $[HPO_4^=] : [H_2PO_4^-]$ of 2 : 1, as equation (8) requires. The corresponding

value of l_u, namely 8, together with l_u values for ratios ranging from 8 : 1 to 1 : 1 are given in Table I. The l_u values agree with one another within experimental error, thus demonstrating the validity of equation (8).

Table I. *Effects of phosphate and cacodylate on CO_2 uptake rate*

$[HPO_4^=] : [H_2PO_4^-]$	Conc. range (total phosphate, *M*)	l_u
8·0	0·01–0·20	7·7
3·2	0·01–0·20	8·0
2·0	0·01–0·10	7·7
1·0	0·01–0·10	8·5
	Mean	8·0

$[Cac^-] : [HCac]$	Conc. range (total cacodylate, *M*)	l_u
7·0	0·02–0·20	8·5
4·0	0·02–0·20	9·4
	Mean	9·0

A $[HPO_4^=] : [H_2PO_4^-]$ ratio of 1 : 1 was the lowest that could be used, for below this the CO_2 taken up becomes so small and the back reaction increases so fast that accurate determinations are not feasible. For the same reasons it was impossible to reduce the $[Cac^-] : [HCac]$ ratio below 4 : 1, for the pK of cacodylic acid is about 0·8 pH below the pK_2 of phosphoric acid, and even at 4 : 1 the back reaction corrections amount to as much as 30 %, though in spite of this the v_u values over different intervals in any one experiment tally satisfactorily.

Above a total buffer concentration of 0·2 *M*, the physical solubility of CO_2 is appreciably depressed and the interpretation of the results thus becomes uncertain: in rough experiments, however, up to *M* it was found that v_u continued to increase nearly linearly with $[HPO_4^=]$. In the case of cacodylate v_u increased rather faster than $[Cac^-]$: this may be just an ordinary deviation from the law of mass action in strong solution, but it might also be due to some change in the state of the cacodylate at higher concentrations, such as the formation of a polymeride which is catalytically more active. A similar deviation occurs with chromate buffer solutions, in which there is known to be an equilibrium between the hydrochromate ion, $HCrO_4^-$, and the dichromate ion, $Cr_2O_7^=$, the amount of the latter increasing rapidly as the total chromate concentration is raised.

It was of interest to see whether equation (8) could also be applied to results published by earlier authors before the effect of the buffer was suspected. Adequate data are available in two such papers, and l_u values calculated therefrom are given in Table II. The excellent agreement with the present findings is a strong confirmation of the validity of our conclusions. Table II further shows that equation (8) is valid over a wide range of CO_2 concentration, namely 0·001 *M* (present paper) to 0·03 *M* (Faurholt).

Table II. *Values of l_u calculated from data in the literature*

Source	Buffer	CO_2, *M*	l_u	l_u from Table I
Faurholt [1925, 1, Table IV]	Cacodylate	0·03	9·5	9·0
Faurholt [1925, 1, Table VI]	Phosphate	0·018	8·4	8·0
Brinkman *et al.* [1933, Table III]	Phosphate	0·002	8·3	8·0

Extrapolation of the data, summarized in Table I (or in Table III [Brinkman *et al.* 1933]) to zero $[HPO_4^=]$ gives us for the first time an accurate value for k_u,

the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. The mean value of k_u is 0·0021, i.e. about 25 % lower than the hitherto accepted value of 0·0027 [Table I, Roughton, 1935, 1], which is, of course, wrong, for in the calculations on which it was based no account was taken of the direct catalytic effect of the buffer.

Arguments against the effect being due to neutral salt action. It will be convenient now to exclude the possibility that the effect of the buffer may be accounted for by some kind of "neutral salt action" or ionic activity factor. This we do on the following grounds.

(*a*) The observed effects are larger than expected, and indeed are appreciable at concentrations, e.g. 0·01 *M*, much lower than those at which typical neutral salt action manifests itself. This holds not only for phosphate and cacodylate, but more so for the very active substances, such as selenite, tellurate and sulphite, to be dealt with later.

(*b*) Table III shows that addition of high concentrations of typical neutral salts, such as KCl and $NaNO_3$, to 0·04 *M* phosphate buffer (1 : 1), only increases the CO_2 uptake rate to a relatively slight extent, the l_u values for these salts being at most only one-sixteenth that of phosphate.

Table III. *Effect of neutral salts on the rate of CO_2 uptake by 0·04 M phosphate*

Salt	l_u
$(NH_4)_2SO_4$	0·2
$NaNO_3$	<0·05
$NaNO_2$	0
KCl	0·3
NH_4Cl	<0·05
KI	0·07
KCNS	0·5
$K_3Fe(CN)_6$	0·4

CO_2 output experiments

If the action of the buffer (or some accompanying impurity) is purely catalytic, then the reverse reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ should be equally affected. On the face of it, this should be easy to test by measuring the rate of CO_2 output from mixtures of bicarbonate with varying strengths of buffer. Unfortunately, however, variation of the buffer strength will also affect the activities of the ions in solution, and may thus independently alter the rate of CO_2 output, which is directly proportional to the product of the H^+ and HCO_3^- activities. The exact nature of the possible "activity effects" is shown by the following treatment.

$$\text{The rate of } CO_2 \text{ output} = \frac{d[CO_2]}{dt} = v_o\,[H_2CO_3] = \frac{v_o a_H f_{HCO_3}[HCO_3^-]}{K_1}, \qquad \text{.....(9)}$$

where v_o = overall output velocity constant,

a_H = hydrogen ion activity,

f_{HCO_3} = activity coefficient of HCO_3^-,

K_1 = true first ionization constant of H_2CO_3.

Now
$$a_H = \frac{K_2 f_{H_2PO_4}[H_2PO_4^-]}{f_{HPO_4}[HPO_4^=]}, \qquad \text{.....(10)}$$

where $f_{H_2PO_4}$, f_{HPO_4} = activity coefficients of $H_2PO_4^-$ and $HPO_4^=$ respectively,

K_2 = second ionization constant of H_3PO_4.

Therefore
$$\frac{d[CO_2]}{dt} = \frac{v_o K_2 f_{HCO_3} f_{H_2PO_4}[H_2PO_4^-]}{K_1 f_{HPO_4}[HPO_4^=]}[HCO_3^-]. \qquad \text{.....(11)}$$

The activity coefficients of divalent ions, such as $HPO_4^=$, are much more affected, and in a more specific manner, than those of univalent ions, by changes in ionic strength, and there is no reason to suppose that the fraction $f_{HCO_3} \times f_{H_2PO_4}/f_{HPO_4}$ should remain constant as the phosphate concentration is reduced by dilution with water. But if the buffer is, instead, diluted with a mixture of K_2SO_4 and KCl, of the same total molarity as the highest concentration of buffer and with the same molar proportions of $SO_4^=$ to Cl^- as of $HPO_4^=$ to $H_2PO_4^-$ in the buffer, this difficulty should be practically overcome: for Landolt & Bornstein's Tables show that the activity coefficients of K_2SO_4 and Na_2SO_4 agree to within 0·01 with the activity coefficient of Na_2HPO_4 in pure solutions of the respective salts at the same concentration (up to 0·1 *M*), and it is therefore reasonable to suppose that in phosphate + sulphate mixtures the activity coefficients would be the same as in pure phosphate buffers of the same total molarity. A similar argument is applied to the Cl^- and $H_2PO_4^-$ ions.

All solutions, except bicarbonate, were freed from CO_2 by repeated evacuation and shaking. To compensate for the carbonate content of the bicarbonate solution, an equivalent amount of HCl was placed in the other solution used in the double compartment boat.

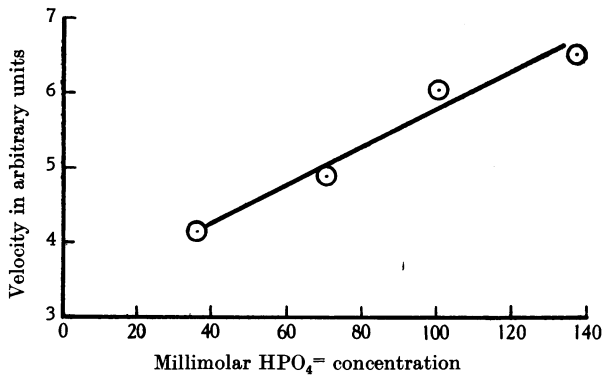


Fig. 5. Effect of $[HPO_4^=]$ on velocity of CO_2 output.

The methods of calculation were similar to those used in the CO_2 uptake work. Fig. 5 shows that there is again a linear relation between $[HPO_4^=]$ and v_o/K_1 and we may therefore write

$$\frac{v_o}{K_1} = \frac{k_o}{K_1}\{1 + l_o\,[HPO_4^=]\}, \qquad \text{.....(12)}$$

where k_o is the true velocity constant of the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$, and l_o is the catalytic coefficient for CO_2 output corresponding to l_u for CO_2 uptake. Table IV gives the values of l_o for phosphate + sulphate + chloride mixtures and also for cacodylate + chloride ± acetate mixtures. It will be noted that in the case of both buffers l_o is independent of wide variations in *p*H and that the l_o values agree, within experimental error, not only with the l_u values of Table I but also with the l_u values given in Table IV, which were specially determined in buffer salt mixtures of the same composition as used in the output experiments. This last result proves that phosphate and cacodylate are both true catalysts of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$.

The assumption that the ionic dissociation and reformation of H_2CO_3 are too fast to limit the rates of uptake or output of CO_2, even when the buffer

Table IV. *Effects of phosphate and cacodylate on CO_2 output rate*

	$[HPO_4^=]:[H_2PO_4^-]$	l_o	l_u
Phosphate + $SO_4^=$ + Cl^-	3 : 1	8·5	8·9
Phosphate + $SO_4^=$ + Cl^-	1 : 3	8·6	—
	$[Cac^-]:[HCac]$	l_o	l_u
Cacodylate + Cl^-	7 : 1	—	8·5
Cacodylate + Cl^-	2 : 1	9·2	—
Cacodylate + Cl^- + acetate	1 : 2	8·9	—

concentration is below $M/100$, perhaps requires some further words. Ionic reactions of this kind are generally assumed to be "instantaneous". A minimum value for their rate is given by rapid reaction velocity experiments such as those of Roughton [1930] which have shown that in presence of $M/10$ buffer the ionization of weak acids similar to H_2CO_3 has a half-period of less than 0·0003 sec. —probably far less. Whether decrease of buffer concentration would increase the half-period appreciably is very doubtful, since the ionization in complete absence of buffer, i.e. $H_2CO_3 + H_2O \rightleftharpoons HCO_3^- + H_3O^+$, is probably *per se* extremely fast. But, even if the rapid ionization of H_2CO_3 in buffer solutions were entirely due to a direct reaction with the buffer anion, i.e. $H_2CO_3 + A^- \rightleftharpoons HCO_3^- + AH$, a decrease of buffer concentration from $M/10$ to $M/100$ could not raise the upper limit for the half-period to more than $10 \times 0{\cdot}0003$ sec. $= 0{\cdot}003$ sec. Now calculation shows that, for the overall rate of CO_2 uptake in $M/100$ buffer solution to be retarded appreciably by the ionization of H_2CO_3, the half-period for the latter must be $> 0{\cdot}01$ sec. The action of the buffers cannot therefore be due to any effect on the ionic reaction $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$, but must be due entirely to their effect on the molecular reactions of CO_2 with water. Of several further points in favour of this we need only mention that the numerical values of k_u and k_o/K_1, as found by extrapolating to zero buffer concentration the respective rates of CO_2 uptake and output, check satisfactorily in two independent ways with the requirements of the law of mass action when applied to the molecular reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$. (Details will be given in a later paper by one of us.) If the rate of ionization of H_2CO_3 were of any limiting influence at low buffer concentrations no such checks would be obtained.

Additive test. An experimental comparison of the rates of CO_2 output from a mixture containing

(*a*) 0·017 *M* $NaHCO_3$ + 0·075 *M* K_2HPO_4 + 0·025 *M* KH_2PO_4 + 0·1 *M* KCac, with

(*b*) 0·017 *M* $NaHCO_3$ + 0·075 *M* K_2HPO_4 + 0·025 *M* KH_2PO_4 + 0·1 *M* Na acetate,

showed that the rate of (*a*) was 1·52 times that of (*b*). This demonstrates that the catalytic effects of the phosphate and cacodylate ions are additive, for on this basis we should have

$$\frac{\text{rate of } (a)}{\text{rate of } (b)} = \frac{1 + (8{\cdot}55)\,(0{\cdot}075) + (9{\cdot}05)\,(0{\cdot}1)}{1 + (8{\cdot}55)\,(0{\cdot}075) + (0{\cdot}6)\,(0{\cdot}1)} = 1{\cdot}49.$$

In this fraction 8·55, 9·05 and 0·6 are the respective l_o values of $HPO_4^=$, Cac^- and CH_3COO^- (Table IV).

The observed and calculated ratios agree much better than might have been expected from the size of the experimental errors.

Evidence that the catalytic action of the buffers is not due to an accompanying impurity. An impurity present in the salts and/or alkalis used in making the buffer solutions might be responsible for the catalytic effects observed.

The alkalis should first be considered, for they are used both by the manufacturer in preparing salts, such as KH_2PO_4, and by ourselves in making up the buffers. Two tests eliminate this possibility. (*a*) The effects are the same whether NaOH or KOH is used. (*b*) No increased effect is found when the buffer is treated with additional alkali if the latter is afterwards neutralized with an equivalent amount of HCl. It will be recalled that Cl^- itself does not inhibit—see Table III.

As regards the phosphate, we find exactly the same amount of catalysis whether all the phosphate solution is made up from KH_2PO_4 (Kahlbaum puriss. or AnalaR) or from Na_2HPO_4, $12H_2O$ (Kahlbaum puriss. or AnalaR). It seems unlikely that all these reagents would contain just the same amount of catalytic impurity. Furthermore since the effect is always proportional to the $[HPO_4^=]$ or $[Cac^-]$, the impurity would have to have the same *p*H-activity curve as both phosphate and cacodylate, the *pK* of which differ by 0·8. This is impossible if the impurities in the two cases are the same, whereas if there are two different impurities it would be a remarkable coincidence that their respective *p*H-activity curves should both happen to be identical with the ionization curves of the corresponding buffers.

Addition of sufficient carbonic anhydrase to double the rate of CO_2 uptake by *M*/50 phosphate, is also found to double the rate of CO_2 uptake by *M*/5 phosphate which, in absence of enzyme, it will be remembered, is nearly twice the rate of uptake by *M*/50 phosphate. The catalytic actions of carbonic anhydrase and phosphate thus appear to multiply one another instead of being merely additive, as would be expected if the phosphate catalysis were due to traces of an impurity. A possible mechanism for the multiplicative effect is put forward later.

That the effect can be due to metallic impurities is very unlikely because:

(*a*) Certain metals, notably Fe, are precipitated by phosphate at *p*H 8·0, yet the supernatant fluid from phosphate solutions which had been kept a long time showed the same effect as freshly prepared solutions.

(*b*) No increased effect was observed on adding to 0·04 *M* phosphate, *p*H 7·1, various possible impurities in traces, including many cations, e.g. Li^+, Cu^+, Cu^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Mg^{++}, Hg^{++}, Pb^{++}, Fe^{++}, Fe^{+++}, Sb, La, U, Sn. The following cations had no (or very slight) additive effect on the rate of CO_2 uptake by cacodylate buffer, *p*H 6·8: Ca^{++} (0·2 *M*), Ba^{++} (0·2 *M*), Sr^{++} (0·2 *M*), Th^{++++} (0·001 *M*), La^{+++} (0·0005 *M*). At 0·0006 *M*, however, La^{+++} had a marked accelerating effect, but at this concentration a precipitate developed in the boat. 0·012 *M* Cu^{++} and 0·1 *M* Rb^+ in β-glycerophosphate, *p*H 6·8, had no effect.

(*c*) The rate of CO_2 uptake by phosphate, *p*H 7·1, is unaffected by adding 0·08 *M* cyanide.

This varied evidence, taken as a whole, makes it safe for the present to attribute the catalytic action in the main, if not entirely, to the more electronegative constituent of the buffer itself.

Further experiments on the nature of the catalysis

The natural question next arises as to whether this effect is limited to the cacodylate, the secondary phosphate and the borate ions (Faurholt's work) or whether it is shown by the more negative constituent of oxy-acid buffers in general. We have tested the matter by CO_2 uptake experiments on a wide range of buffers, either in pure solution or when mixed with phosphate buffer, in which case their additive effect, if any, on the rate of uptake by phosphate has been worked out. At *p*H > 9·0 results become less easy to interpret owing to the

appreciable intervention of the $CO_2 + OH^- \rightleftharpoons HCO_3^-$ reaction, and we have therefore, for the present, restricted ourselves in the main to buffers with acid constituents of pK below 9·0. Our object being to make a broad preliminary survey of the whole field, we have in most cases contented ourselves with the rougher method of calculating l_u described above, and we have neglected any complications which may arise from the possible effects upon the solubility of CO_2 of certain of the substances which were used in high concentration: for these reasons we do not claim the same exactitude for these results as for those given in Tables I and IV, though we do believe that their order of magnitude is correct. The values of l_u, in terms of the more negative constituent of the buffer, are

Table V. *Effect of various oxy-acid buffers on rate of CO_2 uptake*

	Buffer	pK at 0°	[ion] : [acid]	[ion] + [acid],* M	l_u
I.	Phosphate	pK_2 7·1	—	—	8·0
	Phosphite	pK_2 6·7	2·0	0·20	6
	Pyrophosphate	—	—	—	10–50
	α-Glycerophosphate	6·4	10·0	0·40	4·5
	β-Glycerophosphate	6·3	3·0	0·25	3·0
	Phosphoglycerate	6·0	18·0	0·15	2·5
	Hexosediphosphate	6·3	9·0	0·23	8·5
	Cacodylate	6·3	—	—	9·0
	Arsenate	pK_2 6·8	2·0	1·20	6·0
II.	Maleate	pK_2 6·1	8·0	0·95	2·0
				1·00	1·6†
	Citrate	pK_3 5·4	c. 20·0	2·00	1·5†
	Veronal	8·0	3·0	0·18	8·0
III.	Chromate	pK_2 6·4	3·0	0·1	c. 50
	Borate	9·5	0·016	1·45	c. 150†
IV.	Sulphite	pK_2 7·0	3·0	0·1	900
	Selenite	pK_2 8·0	0·3	0·02	1700
				0·0036	> 2000†
	Tellurate	pK_1 7·8	1·0	0·05	600
V.	Formate	3·8	—	1·0	0·5†
	Acetate	4·7	—	1·0	0·6†
	Phthalate	pK_2 5·3	—	0·8	0·8†
	Oxalate	pK_2 4·1	—	0·6	1·4†

* Highest total concentration used. † Measured in 0·04 M phosphate buffer.

given in Table V which shows clearly that the effect sought for is very widespread, being considerable with all oxy-acids of $pK > 6$, and in general increasing with pK. For convenience in discussion Table V has been divided into the following sections.

Group I consists of buffers closely related in structure to phosphate and cacodylate, and with pK in the range 6·0–7·0. Phosphite, with a pK_2 rather lower than the pK_2 of phosphate, has an l_u value also slightly less (i.e. 6·0 compared with 8·0). The organic phosphates with still lower pK have their l_u values correspondingly lower, except hexosediphosphate. But the molecule of this substance has two phosphate groups, each with pK at 6·3. Hence for calculating l_u, a M solution of hexosediphosphate should from the present point of view correspond to a 2·0 M solution of the ion. On this basis, l_u comes to be of the same order as for the other organic P compounds. Pyrophosphate shows considerable catalytic effect, but it is present both in the trebly and quadruply ionized forms under the pH conditions studied, and the respective catalytic contributions of these two forms have not yet been worked out.

Group II. Two organic acids, maleate and citrate, with pK near the lower limit at which activity is shown, have rather small l_u values. Veronal with a pK of 8·0 shows, as would be expected, a higher l_u value.

Group III. The two inorganic buffers chromate and borate have l_u values about 10 times greater than those in group I. In the case of chromate there is, as already mentioned, some uncertainty owing to the presence in solution of appreciable amounts of $Cr_2O_7^=$, besides the $HCrO_4^-$ and $CrO_4^=$ ions.

Faurholt [1925, 1] commented on the anomalous results found by him with borate even at pH 8·0, where complications arising from the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction should only be slight. Inspection of his figures shows that the discrepancies to which he refers could be explained if we assign an l_u value to the borate ion of the order of 100–200—a reasonable figure in view of the pK of boric acid, i.e. 9·5. In borate-phosphate mixture we find an l_u value of about 150, thus confirming this suggestion.

Group IV. Sulphite, selenite and tellurate,[1] showed the greatest effects of any so far tested, l_u being in the neighbourhood of 1000. Careful controls showed that the results were not due to autoxidation. Their activity, being so much greater than that of other substances of similar pK, must be due to their special chemical constitution: in this connexion it is of interest to find this high activity associated with three elements of the same group (VI) of the Periodic Table. A further point of interest about the action of selenite will be discussed later.

The effects of chromate and selenite on CO_2 output were also investigated. The l_0 values in the two cases were found to be of the same order as the l_u values.

Group V contains some typical carboxylic acids with $pK < 5{\cdot}0$. Their l_u values, with the doubtful exception of oxalate, are all distinctly below 1·0. It thus appears that as the strength of the acid rises a range is reached (pK 6·0–5·0) in which the catalytic effect of the acid anion tends to fade out.

The effect of the bicarbonate ion

The l_u value for the HCO_3^- ion is not included in Table V, since it cannot be obtained either by the methods so far used in this paper or from data given in previous papers. It is, however, important to measure it, both from the point of view of the physico-chemical mechanism of the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and also of practical applications to biological problems. Since the true first pK of H_2CO_3 is about 3·7 the l_u value for HCO_3^- would not, from Table V, be expected to exceed 1·0. The following modification of the usual technique shows that, in point of fact, the l_u value must be less than 0·5 and may be zero.

A 0·2 M $NaHCO_3$ in 0·004 M Na_2CO_3 mixture was prepared by dissolving the requisite weights of the two salts in recently boiled distilled water with minimal contact with air so as to reduce CO_2 exchange. 5 ml. of this solution were placed in the boat and shaken at 0° with the standard CO_2 pressure (in the usual way) for 90 min., i.e. until equilibrium was practically reached. The $[CO_3^=]$ was found to have increased to 0·005 M, and the $[HCO_3^-]$ to have dropped correspondingly. The pH of the solution was calculated to be 8·6. After 1 min. pause for drainage, a second vol. of CO_2 equal to the first was introduced into the boat in the usual way, and an ordinary experiment then carried out to completion. Still more $CO_3^=$ was formed and there was a further slight fall in pH. The overall velocity constant of CO_2 uptake was then calculated from the time course of the CO_2 uptake during the second shaking though far larger corrections were necessary than usual for (*a*) the velocity of the back reaction (50–60 %) and (*b*) the velocity

[1] Prepared from B.D.H. telluric acid. Two samples from other sources were unsatisfactory as they gave heavy precipitates when partially neutralized by alkali.

of the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction which at pH 8·6 is about 30 % of the velocity of the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction. Fortunately, these two large corrections are opposite in sign, and in consequence the final value of the overall velocity constant should be accurate to about ± 10 %. The average of four calculated values came out to 0·00208, in very close agreement with the value, 0·0021, given above for k_u, the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. The joint catalytic effects of the HCO_3^- and $CO_3^=$ ions in this experiment thus cannot at most have exceeded 10 %, which means that l_u for HCO_3^- ion alone $> 0{\cdot}5$—a negligible value for practical purposes in work at $[HCO_3^-] < 0{\cdot}05\ M$.

The experiment also gives an upper limit of about 25 for the l_u value of the $CO_3^=$ ion. Recent experiments at higher $[CO_3^=]$ suggest that the limit is almost certainly much lower.

II. Basic nitrogenous buffers and other substances

Basic nitrogenous buffers

We have tested three classes of N-containing bases: (*a*) compounds with the basic N in a closed ring, e.g. glyoxaline, nicotine, (*b*) compounds with the basic N in a straight chain, e.g. aniline and (*c*) mixed compounds containing both cyclic basic N and straight chain basic N, e.g. histidine. The results with class (*a*) being the most straightforward will first be described.

Cyclic N bases. Four members of the glyoxaline series and three other compounds were tested as regards their effect on CO_2 uptake by the methods described in Section I (see Table VI). Fig. 6 shows a typical pair of results for

Table VI. *Effect of cyclic nitrogenous bases*

Buffer	pK at 0°	[base] : [ion]	l_u	l_o
2:4(or 5)-Dimethylglyoxaline	8·80	0·33	12·5	—
4(or 5)-Methylgloxaline	7·97	2·0	9·5	—
		1·0	11	—
		0·5	9·5	—
		0·25	10	—
Glyoxaline	7·40	1·0	1·5	—
		2·0	—	*c.* 2
4(or 5)-Hydroxymethylglyoxaline	6·85	4·0	1·6	—
Nicotine	8·5 (8·0 at 25°)	0·5	13	—
Pilocarpine	7·30	1·0	4·6	—
	(7·0 at 15°)	1·0	—	5·0
Pyridine (in phosphate)	5·7 (5·3 at 20°)	50·0	<0·6	—

an equimolar buffer mixture of glyoxaline and glyoxaline hydrochloride at total concentrations of glyoxaline ranging from 0·036 to 0·38 M. It will be noted that the CO_2 uptake during the initial rapid phase is in both cases practically the same and is equal in amount to that expected from the solubility coefficient of CO_2, thus showing that in this case there is no rapid reversible combination of the base with CO_2 to form a compound of a carbamino type. (Contrast the results obtained in Fig. 7 with a typical straight chain N compound.) Nor was there any such indication in the case of any of the other compounds listed in Table VI.

When v_u, the overall velocity constant for CO_2 uptake, is calculated as in Section I and plotted against the concentration of unionized base (i.e. the more negative constituent of the buffer) a straight line is obtained. The slope of the

line shows that the l_u value for glyoxaline is about 1·6. Extrapolation of the line back to zero base concentration gives a value for k_u, the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$, in close agreement with that already found in Section I.

The l_u values given in Table VI have all been calculated in terms of the more negative constituent: the validity of this procedure was tested thoroughly in the case of 4(or 5)-methylglyoxaline by experiments at four different [N base : [N cation]] ratios ranging from 2·0 to 0·25. It is seen that l_u is constant within experimental error. It may be reasonably assumed that the other compounds in the Table would, on test, yield the same result.

Table VI also shows that there is a correlation between l_u and pK similar to that already found for the oxy-acid buffers in Table V (though in the present case the available range is smaller): thus, for example, the two compounds with $pK > 8{\cdot}0$, nicotine and 2:4(or 5)-dimethylglyoxaline, each have an l_u of about 12, whereas pyridine with a $pK < 6$ has a barely detectable l_u value. The compounds of intermediate pK have, broadly speaking, intermediate l_u values.

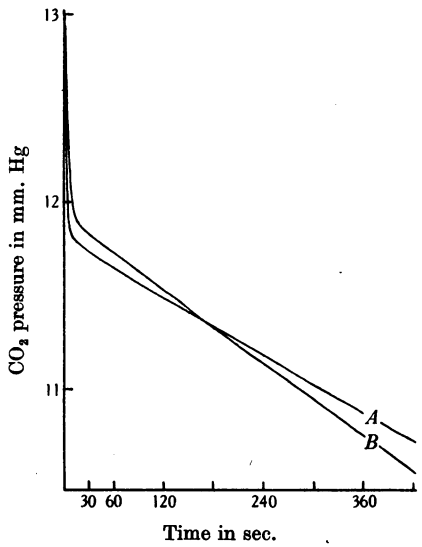


Fig. 6.

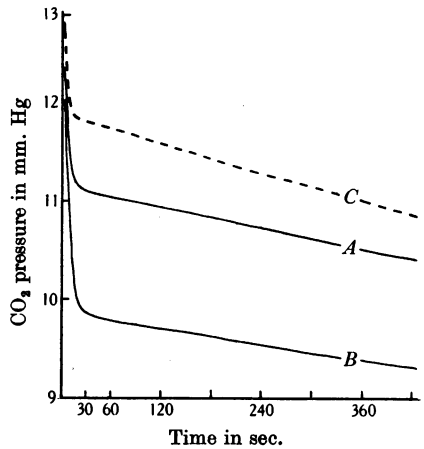


Fig. 7.

Fig. 6. Observed course of CO_2 uptake by glyoxaline. *A*, 0·018 *M* unionized glyoxaline + 0·018 *M* glyoxaline ion; *B*, 0·19 *M* unionized glyoxaline + 0·19 *M* glyoxaline ion.

Fig. 7. Observed course of CO_2 uptake by hydroxylamine buffer solutions. *A*, 0·036 *M* NH_2OH + 0·012 *M* NH_3 + OH; *B*, 0·085 *M* NH_2OH + 0·026 *M* $NH_3{}^+OH/HPO_4{}^=$ + 0·011 *M*; *C*, 0·033 *M* H_2PO_4 for comparison.

Evidence for the catalytic nature of the effect was sought for as before, namely by comparing the l_0 values, obtained from CO_2 output experiments at different buffer concentrations, with the l_u values. Unfortunately, owing to experimental error and theoretical uncertainty, satisfactory accuracy can only be attained, if the l_0 value is > 5 and the $pK < 7$ (as is the case both for phosphate and cacodylate buffers). Of the compounds listed in Table VI pilocarpine approaches this ideal most nearly: the l_0 value from experiments over a range of 0·05–0·20 *M* total pilocarpine concentrations is 5·0, in satisfactory agreement with the l_u value of 4·6. A similar, but much less accurate test showed that the

l_o and l_u values of glyoxaline are of the same order of magnitude. The actual equation for the overall velocity constant of CO_2 output by N base buffers differs slightly from equation (11): it is

$$\frac{d[CO_2]}{dt} = \frac{v_o K_B f_{HCO_3} f_B [B^+]}{[B]} [HCO_3^-], \qquad \text{.....(13)}$$

where $[B^+]$, $[B]$ are the concentrations of N cation and base respectively.

K_B is the ionization constant of the base $= [B][H^+]/[B^+]$.

f_B is the activity coefficient of the N cation.

The calculation of l_o in the case of N base buffers is thus much more sensitive to activity coefficient errors than in the case of the oxy-acid buffers, since a product of activity coefficients is involved instead of a quotient of activity coefficients as in equation (11).

Straight chain N bases. Faurholt [1925, 2] has measured the equilibrium constant K_{diss} for the carbamino reaction between CO_2 and the following straight chain amines: NH_3, CH_3NH_2, $(CH_3)_2NH$ and $CHNH_2COO^-$ (glycinate). As a measure of affinity of the bases for CO_2 we may take

$$1/K_{diss} = \frac{[CO_2][B]^2}{[B^+][carb]}, \qquad \text{.....(14)}$$

where [carb] is the concentration of carbamino compound formed. The values of $1/K_{diss}$ at 0° ranged from 10^3 to 10^6 indicating a very large affinity of these bases for CO_2. This, together with the fact that the pK value at 0° in all four cases is >10, makes it very difficult by the present methods to determine whether these bases have an appreciable l_u value, though Faurholt's work indirectly speaks against such a possibility.

We have, however, tested several straight chain bases of $pK < 9{\cdot}0$, and, in accordance with Faurholt's views, have found a much lower carbamino affinity for CO_2 so that it has been possible to allow for it fairly well in calculation of l_u. Typical results for hydroxylamine at a $[B] : [B^+]$ ratio of 3 : 1 and total concentrations 0·048 *M* and 0·11 *M*, are shown in Fig. 7.

The CO_2 uptake in the rapid phase is much greater than that taken up in physical solution (see control curve for CO_2 uptake by phosphate, and also Fig. 2) the excess, due to carbamino formation, is, as is to be expected from equation (14), proportional (at constant $[B] : [B^+]$) to the total hydroxylamine concentration. The data of Fig. 7 indicate that $1/K_{diss}$ for hydroxylamine is about 4, i.e. about 1/500 the value for ammonia, thus showing the marked effect of substituting an —OH group both on pK and on the affinity for CO_2.

It will be seen that the rate of CO_2 uptake during the slow phase is less in the 0·048 *M* hydroxylamine solution than in the phosphate control, and in the 0·11 *M* hydroxylamine solution less still. This is partly due to the lower pressure of CO_2 remaining in the gas phase and partly due to the fact that the $[HCO_3^-]$ in the hydroxylamine solution is formed not only from CO_2 coming from the gas phase but also from CO_2 dissociating from the carbamino compound, which is maximal at the end of the rapid phase and decreases as the solution becomes more acid [*v.* Roughton, 1935, 2]. Approximate corrections can be made for both of these effects: the former is obviously proportional to the extent by which p_{CO_2} is lower than the control, whilst the latter can be shown to be roughly equal to the $[carb]/[CO_2]$ in solution at the end of the rapid phase, provided that the [carb] is not too large. The carbamino corrections in the case of Fig. 7 amount to 7 and 20 % respectively and when applied, together with the p_{CO_2} corrections, show that the true rate of HCO_3^- formation in the hydroxylamine

solution is, within experimental error, equal to the basal rate in absence of catalyst, thus showing that hydroxylamine has no appreciable l_u value. Similar results were obtained with the other compounds listed in Table VII.

Table VII. *Straight chain N bases*

Compound	pK	Carbamino formation	l_u
Hydrazine	8·4 at 20°	+ + +	Undetectable
Hydroxylamine	6·2 at 20°	+ +	,,
Aniline	4·5 at 20°	+	,,
Acetamide	*c.* 0	0	,,
Urea	*c.* 0	0	,,

+ + + denotes that $1/K_{diss}$ is of same order as $1/K_{diss}$ for NH_3.
+ + denotes that $1/K_{diss}$ is of the order of 10^{-2} times $1/K_{diss}$ for NH_3.
+ denotes that $1/K_{diss}$ is of the order of 10^{-4} times $1/K_{diss}$ for NH_3.
0 denotes that $1/K_{diss}$ is less than 10^{-6} times $1/K_{diss}$ for NH_3.

Mixed compounds. From the above results we should expect that a compound which possesses both a basic cyclic N and a basic straight chain N should act both as CO_2 carrier and CO_2 catalyst. We have verified this in preliminary experiments on histidine, and two of its derivatives of physiological interest, dissolved in phosphate buffer. The results are shown in Table VIII.

Table VIII. *Effects of histidine and derivatives on CO_2 uptake rate*

Compound	pK* at 22° Glyoxaline N	pK* at 22° Side chain N	l_u	Carbamino formation
Histidine	6·15	9·3	*c.* 1·2	+ + +
Carnosine	6·8	9·5	*c.* 6	+ + +
Anserine	7·0	9·5	*c.* 3	+ + +

* Values and assignments from Deutsch & Eggleton [1938] who kindly supplied samples of anserine and carnosine.

Other buffers

We have also tested the catalytic effects of the anions of three non-oxy-acid weak acids, namely HCN (pK at 0° *c.* 10·0), H_2S (pK at 0° *c.* 7·0) and HF.

Cyanide. The l_u of CN^- was difficult to measure owing to the volatility and high pK of HCN. The following special technique was used. Scheele's HCN solution (4%, B.D.H.) was mixed with an equal volume of water in a burette over mercury, to avoid HCN loss by evaporation. Titration to pH 6·0 gave the mineral acid content of the solution (0·031 N), and further titration to pH *c.* 12 (Tropoeolin O as indicator) gave the HCN content (0·79 N). 4 ml. of this solution + 0·8 ml. of $M/2$ KCN were placed in the boat, the latter stoppered at once and the experiment carried out as usual except that the gas pressure was left at 1 atm. instead of being reduced. The standard amount of CO_2 was introduced from a reservoir at 19 cm. Hg positive pressure. The rate of uptake of CO_2 by this cyanide buffer mixture, which contains 0·66 M HCN and 0·06 M CN^-, was found to be about twice that of the basal rate below pH 7·5 in absence of catalyst. Fourfold dilution of the cyanide buffer in a second experiment showed a CO_2 uptake rate about 1·9 times the basal rate. The increase above the basal rate in the two experiments was mainly due to the rate of the $CO_2 + OH^-$ reaction, which in a 1 : 11 cyanide-HCN buffer mixture (pH *c.* 9·0) should be of the order of 80%[1] of the $CO_2 + H_2O \rightarrow H_2CO_3$ basal rate. If the residue of

[1] Exact allowance is difficult owing to uncertainty as to the pK of HCN at 0° and of the size of the appropriate activity corrections.

the increase is attributed to the catalytic effect of the CN^- ion, its l_u value comes out to be roughly 1·0 or less. CN^- therefore does not appear to belong to the catalytic cyclic N family, which at such a high pK should show an $l_u > 10{\cdot}0$. Nor does it form any carbamino compound, according to the present experiment and the previous ones of Meldrum & Roughton [1933, 2].

Sulphide. The pK_1 of H_2S at 0° is about 7·0 so that a catalytic effect of HS^- might possibly be expected. Unfortunately the high volatility of H_2S leads to appreciable loss of H_2S into the gas phase of the boat from the liquid as the latter becomes more acid during the CO_2 uptake. To minimize this, the $[HS^-]$ was kept down to 0·012 *M* by dissolving the requisite amount of NaHS (obtained by half-neutralizing Na_2S (A.R.) with HCl) in a buffer mixture containing 0·05 *M* 2:4(or 5)-dimethylglyoxaline base + 0·05 *M* dimethylgloxaline cation (*p*H *c.* 9·0). Calculation showed that, even so, a correction of 15% must be inserted. The experiment was done in much the same way as the cyanide experiment and the uptake rate was found to be about 15% slower than the control uptake rate by the same buffer mixture without added NaHS. Thus when the correction for H_2S volatility is applied the two rates agree within experimental error, showing that the catalytic effect of 0·012 *M* HS^- is inappreciable. The l_u value of the HS^- cannot therefore exceed 10·0 and may be zero.

The amount of CO_2 taken up during the rapid phase was found to be the same whether HS^- was present or not, thus showing no evidence of any appreciable rapid reversible combination between CO_2 and HS^- analogous to the carbamino reaction.

We have not yet tested any more complicated sulphhydryl acids, such as thiolacetic acid, owing to the higher pK of their –SH group (*c.* 10·0) and their instability.

Fluoride. The pK of HF is 4·45 at 25°, and the l_u value, calculated from an additive experiment with 0·6 *M* NaF + phosphate buffer, is 1·1. This value agrees closely with that of the oxy-acids of pK *c.* 4·0, and is distinctly higher than the range found for neutral salts of strong acids in Table III.

Discussion

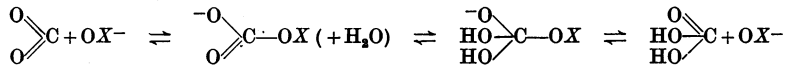
The mechanism of the catalysis

In the CO_2 output experiments the additional rate due to the catalysis is proportional to the product of the $[H_2CO_3]$ and the more negative constituent of the buffer, e.g. $[HPO_4^-]$ or $[Cac^-]$, and is independent of the $[HCO_3^-]$ except in so far as the latter conditions the $[H_2CO_3]$. This means that H_2CO_3, not HCO_3^-, is the substrate acted on by the catalyst, i.e. the reaction catalysed must be $CO_2 + H_2O \rightleftharpoons H_2CO_3$ and not $CO_2 + OH^- \rightleftharpoons HCO_3^-$. Preliminary confirmation of this has been obtained by CO_2 uptake experiments between *p*H 9·0 and 10·0 with two different $[H_2BO_3^-] : [H_3BO_3]$ ratios and varying total borate concentration. It was found that the fraction of the overall uptake rate due to the $CO_2 + OH^- \rightarrow HCO_3^-$ rate was practically unaffected by changes in $[H_2BO_3^-]$ although the fraction due to the $CO_2 + H_2O \rightarrow H_2CO_3$ was greatly affected by $[H_2BO_3^-]$, which showed its usual l_u value > 100.

Whilst not excluding the possibility of a chain mechanism we think it more likely that the catalysis can be explained by the intermediate compound formation between CO_2 and H_2CO_3 on the one hand, and the more negative constituent of the buffer on the other.

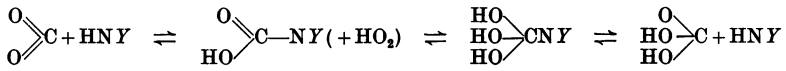
In the case of the oxy-acid buffers the compounds might be of a "carbonato"

type, analogous to the reversible compounds between CO_2 and organic hydroxides studied by Faurholt:



We have to suppose that H_2O can combine with the CO_2 bound to OX^- to form the corresponding $H_2CO_3^-.OX$ intermediate compound which in turn decomposes to $H_2CO_3 + OX^-$. We then have the catalytic scheme formulated in the equation. This scheme explains why (*a*) the reaction $CO_2 + OH^- \rightleftharpoons HCO_3^-$ is not catalysed; probably the electrostatic repulsion between HCO_3^- and OX^- hinders the formation of the necessary $HCO_3^-.OX^-$ intermediate compound, and (*b*) the catalytic activity of the oxy-acid ion tends to disappear when the pK of the acid is $< 6{\cdot}0$. Table VII shows how the affinity of the straight chain N bases for CO_2 declines *pari passu* with the decrease in pK, i.e. decreases in affinity for H^+. Similarly we might suppose that the combination of CO_2 with the $—O^-$ in weak acid anions persists up to a certain point as the strength of the acid increases but finally tends to become negligible. On this view the lack of catalysis is due to failure to form the necessary intermediate compound.

In the case of the cyclic nitrogenous base buffers the intermediate compound with CO_2 might be of a carbamino type:



With straight chain N compounds no reaction occurs between water and the carbamino compound of the N bases, but in the cyclic N compounds the postulated carbamino compound might, for some reason connected with the chemistry of the ring, tend to hydrate, forming the unstable intermediate which breaks down to H_2CO_3 and the free N base. The catalytic scheme is thus complete. Although at present we have no direct evidence in favour of these hypotheses, we have found them very useful working guides, and therefore have felt justified in mentioning them.

Additive effects

We have already alluded to the fact that the catalytic effects of $HPO_4^=$ and Cac^-, when jointly present, are additive and the same is also true of the following pairs—maleate + phosphate, chromate + phosphate and probably also borate + phosphate. Carbonic anhydrase and phosphate, on the other hand, tend to multiply the effects of each other. It was therefore of special interest to see whether our most active inorganic catalyst, selenite, is additive or multiplicative. It is difficult, owing to ionic activity uncertainties, to make such experiments fully satisfactory from a theoretical standpoint, but some preliminary results with selenite + phosphate, and selenite + chromate mixtures do suggest that in both cases selenite multiplies the effect of the weaker catalytic ion. Selenite may thus turn out to be a weak inorganic analogue of carbonic anhydrase, in which case a further detailed study of its catalytic mechanism would be well worth while.

As to the mechanism of the multiplicative effect it is possible that selenite and/or carbonic anhydrase may catalyse the formation of the intermediate compound between CO_2 and the more weakly catalytic anions, which we have supposed to be a preliminary to the catalysis and which may not itself proceed very rapidly. Various other explanations are however possible.

The effect of pH on carbonic anhydrase

As already hinted in the introduction, the original aim of the present research was to determine the effect of *p*H upon the activity of carbonic anhydrase.

Two difficulties were already obvious at the start, namely (*a*) in the *p*H range above 8·0, where CO_2 reacts in two ways (see equations (1) and (2)) there is the problem of sorting out the respective effects of the enzyme on these two different processes, and (*b*) the Michaelis constant of the enzyme is very high, and hence it is impracticable to work at substrate concentrations sufficient to saturate the enzyme—a prerequisite in *p*H-activity work. The results described in this paper, though at first sight adding yet a further complication, may on further investigation prove to simplify the study of the effect of *p*H on carbonic anhydrase. Thus in regard to (*a*) preliminary experiments suggest that carbonic anhydrase, like phosphate and other buffers, only catalyses the $CO_2+H_2O \rightleftharpoons H_2CO_3$, and does not affect the $CO_2+OH^- \rightleftharpoons HCO_3^-$. In regard to (*b*), it is already known from unpublished observations of the writers and others, that the activity of carbonic anhydrase increases markedly over the *p*H range 6·0–8·0. If it turns out that the activity of the enzyme in promoting CO_2 uptake at constant CO_2 pressure when plotted against *p*H gives a curve resembling the ionization curve of a weak acid, a result exactly analogous to the results with phosphate etc. would be obtained, and it might by further analogy be fair to infer therefrom the actual *pK* of the active group of the enzyme, even though the enzyme had not been saturated with substrate in the experiments. All this is perhaps too much to hope for, but at all events we have some valuable new pointers as to the investigation of the mode of action of carbonic anhydrase.

The catalytic effects herein described occur in many physiological buffers containing CO_2, and should therefore be borne in mind as possible factors in diverse physiological and biochemical processes and experiments. In particular, they may be of interest in bone equilibria, wherein carbonato-phosphate reactions of a type somewhat similar to that postulated in our catalytic schemes may take place, especially in regions of high local phosphate concentration.

Summary

1. Detailed improvements have been made in the manometric technique for measuring the rate of CO_2 uptake by solutions.

2. When CO_2 is taken up by buffer solutions the buffer substance itself has some direct effect on the reaction besides "instantaneous" removal of H^+ concerned in the ionization of H_2CO_3 to $H^+ + HCO_3^-$. Experimental proof is offered that the effect cannot be due to impurities in the solutions or to "neutral salt" action, but is proportional to the concentration of the more negative constituent of the buffer. Thus in the case of phosphate buffer, the overall velocity constant, v_u, is given by

$$v_u = 0{\cdot}0021\ [CO_2]\ \{1 + l_u\ [HPO_4^=]\},$$

l_u, the catalytic coefficient, being 8, and for cacodylate 9. The figure 0·0021 represents the true velocity constant of the reaction $CO_2+H_2O \rightarrow H_2CO_3$ and is about 25 % lower than the erroneous value previously accepted.

3. All other oxy-acids, so far tested, which buffer in the *p*H range 6–9 show similar effects, whereas salts of stronger acids have much smaller effects ($l_u < 1{\cdot}5$). The effects with sulphite, selenite and tellurate are much larger (l_u of order of 1000).

4. Straight chain N bases, e.g. NH_2OH, show no appreciable catalytic activity, though they combine readily with CO_2 to form carbamino compounds. Cyclic N bases, e.g. glyoxaline, on the other hand do not form carbamino compounds but do act catalytically. The effect is proportional again to the concentration of the more negative constituent—in this case the unionized N base. Values for l_u of the order of 10 were found for several cyclic bases of $pK > 7{\cdot}0$. With weaker bases the effect tends to disappear. Mixed compounds, e.g. histidine, show both catalytic action and carbamino formation.

5. Similar effects are observed on the rate of *output* of CO_2 from bicarbonate solution suddenly mixed with buffer, l_0 (output) for the oxy-acid buffers phosphate, cacodylate, chromate, selenite, and for the N base buffers pilocarpine and glyoxaline being equal to l_u. The effect must therefore be a catalysis.

6. It is shown that the buffers only catalyse the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and not the $CO_2 + OH^- \rightleftharpoons HCO_3^-$ reaction. A mechanism is suggested in which CO_2 (or H_2CO_3) combines reversibly with the more negative constituent of the buffer.

7. The effects of phosphate and cacodylate are additive, but of the enzyme and phosphate are probably multiplicative.

8. Some biochemical implications of these results are discussed. They must be allowed for in work involving velocities of CO_2 reactions. Selenite is suggested as a possible inorganic model of carbonic anhydrase.

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