Rate of Hydration of Carbon Dioxide and Dehydration of Carbonic Acid at 25°C*

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As a preliminary to kinetic studies of the carbonic anhydrases from human erythrocytes (1, 2), we have measured the rate of the reversible hydration of carbon dioxide in the absence of enzyme by a spectrophotometric indicator technique in a stopped flow apparatus.

We follow Eigen, Kustin, and März (3) (see also the preceding paper by Ho and Sturtevant (4)) in writing the reaction involved as

\[
\begin{align*}
(1) & \quad H^+ + HCO_3^- \rightleftharpoons \text{H}_2CO_3 \quad (2) \\
& \quad k_{12}/k_{13} \quad k_{23} \quad k_{21} \\
& \quad CO_2 + H_2O \rightleftharpoons \text{H}_2CO_3
\end{align*}
\]

Although the full evidence for this scheme is not yet available, it is clearly more general than the "classical" reaction scheme (see e.g. Edsall and Wyman (5)), which assumed that \(k_{12} = k_{13} = 0\). The constants \(k_{12}\) and \(k_{13}\) are very large, \(k_{12}\) probably being of the order of \(5 \times 10^{10}\) M\(^{-1}\) sec\(^{-1}\) and \(k_{13}\) of the order of \(10^8\) sec\(^{-1}\) (3). The absolute values of these velocity constants do not concern us further here; their ratio must be equal to the true ionization constant of \(\text{H}_2CO_3\), which we denote as \(K_{\text{HCO}_3}\). This is reported as \(1.72 \times 10^{-3}\) M at very low ionic strength by Wissbrun et al. (6). The dependence of \(K_{\text{HCO}_3}\) on ionic strength has not been measured; we will assume it to be similar to that of acetic acid.

The ratio \((\text{H}_2\text{CO}_3):(\text{CO}_2)\) at equilibrium is very small (<0.002), and at all pH values in our experiments, the ratio \((\text{H}_2\text{CO}_3):(\text{HCO}_3^-)\) is 0.005 or less. Therefore, \(\text{H}_2\text{CO}_3\) can be neglected in the over-all reaction, which we can write as

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3
\]

and the over-all equilibrium constant can be defined as

\[
K' = \frac{k_{21}}{k_{12}} = \frac{(\text{H}^+)(\text{HCO}_3^-)}{(\text{CO}_2)}
\]

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* For comparison with earlier discussions, we note that the hydration rate constant \(k_{12}\) is experimentally identical with the constant \(k_{\text{HCO}_3}\) of Edsall and Wyman (5) and with \(k_v\) of Pinsent et al. (9). The dehydration constant \(k_{\text{HCO}_3}\) of Edsall and Wyman is identical with \(k_{12} K_{\text{HCO}_3}\) in the notation of the present paper.
The process described by Equation 6 is approximately pseudo first order. It is useful to describe the over-all hydration-dehydration reaction by a constant which we shall call the "apparent first order rate constant," $k_{apparent}$. If we let $x$ and $z$, denote the concentration of the product of the reaction (HCO$_3^-$ for hydration, CO$_2$ for dehydration), at time $t$ and at equilibrium, respectively, then for the initial part of the reaction,

$$\frac{dx}{dt} = k_{apparent}(x - z)$$

(10)

Clearly, if the reaction were strictly pseudo first order in both directions, then $k_{apparent}$ would be a true constant, equal to $k$ the actual rate constant of the reaction, and $a$ is the initial concentration of the reactant. Of course it is not, since (H$^+$) does change to some extent, and this change is indeed the basis for following the reaction by the indicator method. However, it will be seen that in the buffer systems used here, Equation 10 is valid to a high degree of approximation. Therefore, it is useful in calculating initial rates, from which are obtained the true velocity constants, $k_{11}$ and $k_{12}$.

MATERIALS

Carbon dioxide solutions were prepared by bubbling known mixtures of CO$_2$ and N$_2$ from gas cylinders (Medical Technical Gases, Medford, Massachusetts) into deionized H$_2$O in a gas absorption bottle thermostated at 25°. The use of a fritted disk in the bottle greatly speeded the rate of attainment of equilibrium, which, as determined from titration experiments, is achieved in about 15 minutes. Usually, the gas mixture was bubbled through for at least one hour before the experiment began, and the flow of gas was continued as long as the solution was in use.

EXPERIMENTAL PROCEDURE

The reaction was initiated by rapid mixing, in a stopped flow apparatus, of an aqueous solution of CO$_2$ or NaHCO$_3$, and a buffer solution containing an indicator. The apparatus is a slight modification of that described by Spencer and Sturtevant (14). The temperature of the reaction mixture (25.0 ± 0.1°) could be monitored with a thermocouple and swinging mirror galvanometer circuit sensitive to ±0.02°. The indicators chosen for the initial part of the reaction, in the cylinders supplied by the gas company.

The concentration of the CO$_2$ solutions was established by addition of an aliquot to a measured excess of 0.04 M Ba(OH)$_2$, and back titration with 0.01 N HCl, with phenolphthalein as indicator. All of the values for the CO$_2$ concentrations agreed within 2% with the values to be expected from the Henry's law constant (7) and with analyses of the percentages of CO$_2$ in the cylinders supplied by the gas company.

NaHCO$_3$ solutions were prepared freshly from reagent grade crystals. As the solutions are unstable, their pH was monitored before use as recommended by Brinkman, Margaria, and Roughton (13).

All buffer salts were reagent grade. Primary and secondary sodium phosphate, used to prepare the phosphate buffers, were Mallinckrodt products. Parainitrophenol, indicator grade, obtained from Matheson, Coleman and Bell, was recrystallized four times. Metacresol purple from Distillation Products Industries was used without purification.

Buffer compositions are given in Table I. All concentrations and pH values mentioned in this paper refer to the solutions immediately after the reactants have been mixed in a 1:1 ratio in the stopped flow apparatus.

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**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>Total phosphate concentration</th>
<th>(HPO$_4^{2-}$) concentration</th>
<th>Buffer factor Qs in (M/A) X 10$^3$</th>
<th>Ionic strength</th>
<th>Indicator concentration</th>
<th>$k_{4s}$ + k$\text{OH}^{-}$(OH$^{-}$)</th>
<th>$k_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.14</td>
<td>0.075</td>
<td>0.015</td>
<td>4.00</td>
<td>4.66</td>
<td>0.105</td>
<td>1.5 x 10$^{-4}$</td>
<td>0.0435</td>
</tr>
<tr>
<td>6.30</td>
<td>0.025</td>
<td>0.005</td>
<td>1.67</td>
<td>1.80</td>
<td>0.003</td>
<td>1.0 x 10$^{-4}$</td>
<td>0.0404</td>
</tr>
<tr>
<td>6.66</td>
<td>0.026</td>
<td>0.015</td>
<td>2.88</td>
<td>2.83</td>
<td>0.155</td>
<td>5.0 x 10$^{-4}$</td>
<td>0.0451</td>
</tr>
<tr>
<td>6.99</td>
<td>0.025</td>
<td>0.0125</td>
<td>5.59</td>
<td>5.82</td>
<td>0.060</td>
<td>2.5 x 10$^{-4}$</td>
<td>0.0429</td>
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<td>6.90</td>
<td>0.025</td>
<td>0.005</td>
<td>5.52</td>
<td>5.45</td>
<td>0.110</td>
<td>5.0 x 10$^{-4}$</td>
<td>0.0480</td>
</tr>
<tr>
<td>7.05</td>
<td>0.025</td>
<td>0.010</td>
<td>2.75</td>
<td>2.69</td>
<td>0.055</td>
<td>5.0 x 10$^{-4}$</td>
<td>0.0433</td>
</tr>
<tr>
<td>7.55</td>
<td>0.025</td>
<td>0.0207</td>
<td>4.75</td>
<td>4.20</td>
<td>0.069</td>
<td>2.5 x 10$^{-4}$</td>
<td>0.0471</td>
</tr>
</tbody>
</table>

* These figures also include a small correction for the effect of the back reaction due to HCO$_3^-$ ion present in the initial CO$_2$ solution.

† This solution contained 0.10 M NaCl in addition to the phosphate buffer.
for these measurements were \( p \)-nitrophenol, \( pK = 7.149 \) at 25\(^\circ\) (15), and metacresol purple, which was used in one experiment with Veronal buffer at \( pH \) 7.85. Most of the work was performed in phosphate buffers, and a few measurements were made in imidazole and in Veronal buffers.

A pen oscillograph recorded on one channel the per cent transmittance at either 400 m\( \mu \) (\( p \)-nitrophenol) or 578 m\( \mu \) (metacresol purple) as a function of time, and on the other channel the time at which the flow of reactants was abruptly halted.

For calculation of the initial rate of the reaction, it is necessary to know the effect of the addition of \( H^+ \) ions on the absorbance (\( A \)) of the buffer-indicator systems. The nearly linear relation between these two quantities was determined for each system by titrating an aliquot with \( \text{HCl} \) or \( \text{NaOH} \) and following the absorbance change at either 400 m\( \mu \) or 578 m\( \mu \) in a Zeiss spectrophotometer at 25\(^\circ\). The reciprocal of the slope of these titration curves we term the "buffer factor," its units are (moles \( H^+ \) added per liter)/(absorbance change) = \( \Delta \) (acid)/\( \Delta A \). For small amounts of acid added, this may be taken as a constant \( d(\text{acid})/dA = Q_0 \).

The value of the buffer factor for a given system may also be calculated, as follows. Let \( C_B = [(HB) + (B)] \) be the total buffer system concentration, and \( \alpha_B = (B)/C_B \). Similarly, for the indicator system, \( C_I = (HI) + (I) \) represents the total concentration of indicator in both acid and basic forms, and \( \alpha_I = (I)/C_I \). We assume that \( C_B \gg C_I \), so that virtually all buffering is due to \( B \) and \( \text{buffer system concentration} \), \( H^+ = (B)/C_e \). Similarly, for the indicator, \( I = (I)/C_I \).

The calculation of \( Q_0 \) is as follows.

\[
Q_0 = \frac{d(\text{acid})}{dA} = \frac{d\text{acid}}{d\text{pH}} \left( \frac{d\text{pH}}{d\alpha_B} \right) \left( \frac{d\alpha_B}{dA} \right)
\]

Then the value of \( Q_0 \) in Equation 12 is

\[
Q_0 = \frac{C_B[(1 - \alpha_B)]}{C_I[1 - \alpha_I, L \cdot \Delta e]} = \beta_B/\beta_I L \cdot \Delta e
\]

in which \( L \) is the length of the optical path, \( \Delta e \) is the difference between the molar extinction coefficients of both acid and \( HI \) at the wave length of measurement, and \( \beta_B \) and \( \beta_I \) are the buffer and indicator.

For \( p \)-nitrophenol at 400 m\( \mu \), we find \( \epsilon_{B,400} = 200 \) and \( \epsilon_{I,400} = 18,100 \).

Equation 13 is useful for choosing desired values of the buffer factor \( Q_0 \) and for checking the accuracy of experimental values determined by titration. To obtain high sensitivities of response to release of acid, it is obviously desirable to keep \( Q_0 \) low, by using a fairly high concentration of indicator and working at a \( \text{pH} \) fairly close to the \( \text{pK}^+ \) value of the indicator. On the other hand, to maintain nearly first order kinetics as the reaction proceeds, it is important to maintain a high buffer value for the system \( B + HB \), and this of course requires a fairly high value of \( Q_0 \). The choice of the best experimental conditions must be made with both considerations in mind. Calculated and experimentally determined values of \( Q_0 \) are given in Table I.

The over-all hydration-dehydration reaction may be expected to be approximately pseudo first order, an assumption which is justified by the fact that plots of \( \log (A - A_o) \) against time are nearly straight lines. A useful expedient for calculating initial rates is the fitting of an exponential to the experimental points to evaluate \( (dA/dt)_{t=0} \), which is related to the initial rate, \((dx/dt)_{t=0} \), by the buffer factor, \( Q_0 \).

That is, Equation 10 leads to the relation

\[
\left( \frac{dx}{dt} \right)_{t=0} = -Q_0 \left( \frac{dA}{dt} \right)_{t=0} = -Q_0(A_o - A_o) \frac{d \ln (A - A_o)}{dt} \quad \text{at} \quad t = 0
\]

The value of \( k_{\text{apparent}} \) is obtained from the limiting slope of plots of \( \log (A - A_o) \) against time since

\[
\log (A - A_o) = \log (A_o - A_o) - \frac{k_{\text{apparent}} t}{23}
\]

in which \( (CO_2) \) stands for the initial \( (CO_2) \).

An Alternative Calculation

We note here briefly another method of calculating \( k_{\text{apparent}} \). The value of \( x_o \) in Equation 10 is given by

\[
x_o = K'_I (CO_2)/[K'_I + h_o]
\]

in which \( h_o \) is the hydrogen ion activity at equilibrium. If one makes a series of measurements of \( k_{\text{apparent}} \) in a given buffer, at different values of \( (CO_2)_o \), and extrapolates to \( (CO_2)_o = 0 \), it is obvious that in this limiting case \( h_o \) becomes equal to \( h_e \), the initial \( H^+ \) ion activity of the buffer. In the limiting case \( (CO_2)_o = 0 \), therefore, the entire course of the reaction follows a first order equation, and from Equations 10 and 17

\[
\frac{dx}{dt} = k_{\text{apparent}}(x_o - x) = k_{11} \left[ 1 + \frac{h_o}{K'_I} \right] (x_o - x)
\]

The value of \( k_{\text{apparent}} \) is determined by Equation 15 from absorbance measurements as a function of time, at several values of \( (CO_2)_o \). The plot of \( k_{\text{apparent}} \) against \( (CO_2)_o \), which is found to be linear within the limits of error, is extrapolated to \( (CO_2)_o = 0 \). Equation 18 then determines the value of \( k_{11} \); a correction of course is necessary for Reaction 7, as in Equation 16. The values of \( k_{11} \) so determined in the same set of buffers listed in Table I below agree within the limits of error with those obtained by the initial rate method (Equation 14). Full details of the experimental data and the calculation of rate constants by this method, with a more general derivation of Equation 18, are given in the thesis of B. H. Gibbons, cited in the ini-
Results

Hydration Reaction

The initial rates of the hydration reaction in three buffer systems are plotted as a function of initial CO₃ concentration in Fig. 1. The lines in Fig. 1 represent the value of $k_{13} + k_{OH}$ (OH⁻) obtained from the average of the six points for each system. Each point in Fig. 1 represents the average of four separate runs. The data used to calculate $k_{13}$, and the resulting values of this constant, are given in Table I for nine different buffer systems.

The most serious source of error lies in the determination of the buffer factor, because the titration curves are not truly straight lines. We estimate their probable uncertainty as ±3%. Apart from this, the reproducibility of a rate constant determined by the initial rate method was found to be ±1.5%, the relative mean deviation of the rate constants calculated from the individual points at one pH was never greater than ±1.8%. The uncertainty in the concentration of the CO₃ solutions may be ±1.5%.

Small corrections were made to the hydration constant in the two most acid solutions studied (Table I) to allow for the small amount of bicarbonate ion present initially in the COP solutions. A similar but larger correction (+8%) was required for the amount of bicarbonate ion by Reaction 7. The final average value of $k_{13}$, after making these corrections, is $5.5 \times 10^4$ M⁻¹ see⁻¹.

Dehydration Reaction

The first order rate constant for dehydration was measured at pH 7.05, in 0.025 M phosphate buffer. It was found that the initial absorbance on mixing the reactants in the stopped flow apparatus was independent of the concentration of NaHCO₃.

The concentration of (H⁺) ions was calculated as described in the text. The value of $k_{13}$ is derived from the values of: (initial rate)/(H⁺) (HCO₃⁻), listed in the last column, after making two corrections discussed in the text: (a) for the back reaction due to CO₂ initially present in the bicarbonate; and (b) for disappearance of bicarbonate ion by Reaction 7. The final average value of $k_{13}$, after making these corrections, is $5.5 \times 10^4$ M⁻¹ see⁻¹.

Dehydration constant (see below).

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Small corrections were made to the hydration constant in the two most acid solutions studied (Table I) to allow for the small amount of bicarbonate ion present initially in the COP solutions. A similar but larger correction (+8%) was required for the dehydration constant (see below).

Dehydration Reaction

The first order rate constant for dehydration was measured at pH 7.05, in 0.025 M phosphate buffer. It was found that the initial absorbance on mixing the reactants in the stopped flow apparatus was independent of the concentration of NaHCO₃.
In Table 1, it appears that the hydration constant $k_1'$ calculated from initial rates increases with the phosphate concentration. The data strongly support the observation of Roughton and Booth (11) that $\mathrm{HPO}_4^{2-}$ ions catalyze the reaction. To distinguish between phosphate catalysis and a direct effect of ionic strength, 0.1 M NaCl was added to one of the usual buffers; within experimental error, there was no change in the rate.

Fig. 2 shows the relationship between $k_1'$, calculated from initial rates, and the concentration of $\mathrm{HPO}_4^{2-}$, giving an extrapolated value for $k_1'$ of 0.0375 sec$^{-1}$ at zero buffer concentration. This value is within a few per cent of that obtained in 0.025 M imidazole buffer, pH 7.31, and in 0.025 M Veronal buffer, pH 7.85. This would suggest that these buffer ions have little or no catalytic effect on the reaction. Roughton and Booth (11) reported the coefficient $l$ (Equation 9) to be 1.5 for imidazole, and 8 for phosphate and Veronal. For the Veronal system, there seems to be a discrepancy here. The coefficient for phosphate calculated from Fig. 2 is close to 8, in good agreement with the previous work.

Since the early measurements of the rate of hydration of $\mathrm{CO}_2$ by Fauchold (17), there has been substantial agreement on the value of the hydration rate constant at temperatures close to 0°. However, such concordance does not exist with respect to the value at 25°. Presumably, this is because the difficulties of making accurate measurements increase with the rate of the reaction.

Nearly all previously reported values of $k_1'$ at 25°, except for those of Kiese (18), which are not understood, are much lower than that given here. Roughton’s (19) value of 0.037 sec$^{-1}$ at 25° is very close to ours, but it was derived indirectly from the kinetics of the dehydration reaction, combined with equilibrium data for the ionization of carbonic acid. It is difficult to evaluate the sources of error in some of the other methods used to measure $k_1'$ directly. In the manometric technique of Mills and Urey (21) possessed some difficulties mentioned by the authors, and their data for the rate of hydration at 25° show considerable scatter. The method of Maren (22), although very useful for comparative measurements, probably involves considerable uncertainties insofar as absolute values of rate constants are concerned.

Ho and Sturtevant (4) have used an apparatus similar to ours and practically the same methods, including calculation of initial rates and measurement of a “buffer factor.” The chief difference lies in the method of preparing $\mathrm{CO}_2$ solutions. We agree closely with their values for $k_1'$, except that they found no catalysis by $\mathrm{HPO}_4^{2-}$ ions. This point remains unresolved.

We have remarked above that variation of bicarbonate concentration did not seem to alter the initial absorbance in dehydration runs in phosphate buffer. On the other hand, preliminary measurements of the dehydration reaction in imidazole buffer showed a sharp rise in absorbance of the $p$-nitrophenolate indicator on mixing with bicarbonate. Without attempting a detailed analysis, we note that this difference is to be expected. At constant total concentration of indicator, the absorbance is determined by $\alpha_{\text{im}}$, the fraction of indicator in the basic form. By Equation 11, this is determined by pH - $pK_{\text{im}}$. On increase of the ionic strength, in a phosphate buffer, both pH and $pK_{\text{im}}$ decrease, but the decrease in pH of the phosphate buffer will be somewhat greater, because of its higher charge type. This will tend to lower $\alpha_{\text{im}}$ slightly. On the other hand, the small amount of $\mathrm{CO}_3^{2-}$ ion present in the bicarbonate will react with the buffer, thus raising pH and $\alpha_{\text{im}}$. The net result is that $\alpha_{\text{im}}$ remains virtually constant. In imidazole buffers, on the other hand, pH increases with increasing ionic strength, whereas $pK_{\text{im}}$ decreases; hence $\alpha_{\text{im}}$ increases sharply on mixing with bicarbonate.

The dependence of the dehydration rate constants on ionic strength is not firmly established. However, if the acetic acid analogy is correct for the ionic strength dependence of $K_{\text{HCO}_3}$, then it can be shown that the ratio $k_1'/K_{\text{HCO}_3}$ is a constant for all moderate ionic strengths, and since $k_1'$ does not change, $k_1'$ $K_{\text{HCO}_3}$ cannot do so either. It also follows that the dependence of $k_1'$ on ionic strength must be equal to that of $1/K_1$. Therefore, comparison between different values of $k_1'$ $K_{\text{HCO}_3}$ should be valid providing they have been calculated from direct measurement of $k_1'$, combined with appropriate values of $K_{\text{HCO}_3}$.

Eisen et al. (3), using a temperature jump method, found $k_1' = 5.6 \times 10^4$ M$^{-1}$ sec$^{-1}$ at 25° and 0.1 M ionic strength. From $K_{\text{HCO}_3} = 2.7 \times 10^{-4}$, this gives $k_1'$ $K_{\text{HCO}_3} =$ 15.1 sec$^{-1}$. This is 10% higher than our value measured in the presence of phosphate ions, so that the discrepancy becomes approximately 25% if one assumes phosphate catalysis.

The value of Ho and Sturtevant (4) for $k_1'$ at very low ionic strength is 10.2 $\times 10^4$ M$^{-1}$ sec$^{-1}$; $k_1'$ $K_{\text{HCO}_3}$ from their data is therefore about 17.5 sec$^{-1}$, or approximately 25% higher than our value.

If we compute the energy of activation, $E_a$, of the hydration reaction from $k_1'$ at 25° as reported here and the well established figure of 0.0221 sec$^{-1}$ at 0° (5), both at zero buffer concentration, we obtain 19,200 cal mole$^{-1}$. Pinsent et al. (9) calculated from their data a value of 19,000 at 0°, but concluded that it fell rapidly with increasing temperature to 10,750 at 38°. Our
value of $k_1'$ would indicate that $E_a$ actually is nearly independent of temperature.

Our data provide a test of the relation between rate and equilibrium constants in Equation 3. From the present measurements at pH 7.05 in 0.025 M phosphate buffer, $k_1'/k_2' = 7.4 \times 10^{-7}$. The appropriate value for $K_1'$ interpolated from the work of Harned and Bonner (23) at this ionic strength (0.055) is $6.76 \times 10^{-7}$. In view of the experimental errors involved, the agreement is reasonably satisfactory. Edsall and Wyman (5) had noted earlier that the older rate data did not fit the equation very well. The concordance of rate and equilibrium constants is considerably closer when the new kinetic data are employed.

**REFERENCES**


**SUMMARY**

The rate constants for the hydration of CO2 and dehydration of H2O3 have been measured at 25° in a stopped flow photometric apparatus. The velocity constant for the hydration reaction, after correction for the reaction between CO2 and OH- ions, is 0.0375 ± 0.002 sec⁻¹, both in imidazole buffer and also in phosphate buffers, when the data are extrapolated to zero phosphate concentration. We observe a catalytic effect of secondary phosphate ions on the reaction, in agreement with earlier findings of Roughton and Booth. This appears to be a specific catalysis by the phosphate ions, not an ionic strength effect. Apart from the catalytic effect of phosphate, our results are in excellent agreement with those reported in the accompanying paper by Ho and Sturtevant, but are higher than most of the values reported earlier at 25°. The dehydration velocity constant $k_2'$ was found to be $5.5 \times 10^{-4}$ M⁻¹ sec⁻¹ at 25° and 0.055 ionic strength. When this is multiplied by the ionization constant $K_{H^O_3}$, which is taken as $2.5 \times 10^{-4}$ at this ionic strength, we obtain a value of $k_2'K_{H^O_3} = 13.7$ sec⁻¹.

**Acknowledgments**—We are indebted to Professor Julian M. Sturtevant of Yale University for arranging for the fabrication of the stopped flow unit according to his design, and for advice in setting it up. Our discussions with him, with Dr. I. R. Gibbons, and with Dr. Chien Ho have been most valuable.
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