The Carbamate Reaction of Glycylglycine, Plasma, and Tissue Extracts Evaluated by a pH Stopped Flow Apparatus*

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We have used a stopped flow rapid reaction pH apparatus to investigate the carbamate equilibrium in glycylglycine solutions and in three biological tissues, human plasma, sheep muscle, and sheep brain, as well as to investigate the kinetics of carbamate formation in glycylglycine solution and in human plasma.

The rapid reaction apparatus was equipped with a pH sensitive glass electrode in order to follow the time course of pH from 0.005 to 100 s after rapid mixing of a solution of amine or protein and CO₂. Two phases of the pH curve were observed: a fast phase representing carbamate formation, and a slow phase due to the hydration of CO₂ which was uncatalyzed since a carbonic anhydrase inhibitor was added to the biological solutions. From the time course of pH change during the fast phase, \( K_a \), the R-NH₃ ionization constant, and \( K_c \), the carbamate equilibrium constant as well as the velocity constant for the formation of carbamate, \( k_a \), could be calculated from data at different pH and pCO₂. The carbamate formed in glycylglycine solutions over a wide range of pH and pCO₂ was found consistent with the theory of carbamate formation and with published data. At ionic strength 0.16 and 37°, \( pK_a \) is 7.67, \( pK_c \) 4.58. The heat of the carbamate reaction (\( \Delta H \)) was calculated to be -3.2 kcal/mol between 20° and 37°. \( K_a \) of glycylglycine depends quantitatively on ionic strength as predicted by the Debye-Hückel theory. With ionic strength 0.16 \( k_a \) was found to be 2.500 M⁻¹ s⁻¹ at 37°. The activation energy of carbamate formation is 6.7 kcal/mol.

Carbamate measurements in human plasma at pCO₂ from 38 to 359 Torr, pH from 6.9 to 8.3, temperature 37°, and ionic strength 0.15 provided evidence that two kinds of amino groups participate in carbamate formation. From the equilibrium constants computed for the two species they could be identified as \( \alpha \)- and \( \epsilon \)-amino groups. On the basis of a protein molecular weight of 69,000, 0.6 \( \alpha \)-amino groups/molecule with \( pK_a \) = 7.0 and \( pK_c \) = 4.9, and 5.9 \( \epsilon \)-amino groups/molecule with \( pK_a \) = 9.0 and \( pK_c \) = 4.3 contribute to carbamate formation. The velocity constant \( k_a \) was estimated to be 4.950 M⁻¹ s⁻¹ for the \( \alpha \)-amino groups and 13.800 M⁻¹ s⁻¹ for the \( \epsilon \)-amino groups. Under physiological conditions (pCO₂ = 40 Torr, pH = 7.4), the concentration of carbamate in plasma is 0.6 mM and the half-time of carbamate formation is 0.05 s. In extracts prepared from sheep brain at 37°, pH = 7 and pCO₂ = 35 Torr, the carbamate formation was estimated to be 0.06 mM. With pCO₂ = 70 Torr and the same pH and temperature the carbamate concentration in muscle approximates 0.3 mM and increases to 1 mM as pH rises to 8. It is concluded that, as in plasma, a considerable number of \( \epsilon \)-amino groups appear to be available for carbamate formation in these tissues.

Carbamate together with bicarbonate and dissolved CO₂ are the three forms in which CO₂ is known to occur in the body. Although the carbamate reaction has recently again become of considerable interest (1-7), its investigation in biological systems has been largely confined to hemoglobin and red cells, and no studies on other biological tissues have been reported. This is probably due to practical limitations set by previously described methods of carbamate measurement, which are very time consuming and/or require large amounts of solution. We have therefore developed a new method using a pH stopped flow apparatus, which is comparatively quick and convenient, and allows us to study the kinetics as well as the equilibrium of the carbamate reaction (8).

**METHODS**

**Principle**

When a CO₂-free solution containing amino groups is mixed with a buffer-free CO₂ solution of pH ≈ 4, two reactions occur: (a) carbamate
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formation and (b) CO₂ hydration. The former is about 160 times faster than the latter in the absence of carbonic anhydrase (EC4.2.1.1) so that carbamate equilibrium is reached before any substantial amount of CO₂ hydration has taken place. From this fast phase of CO₂ uptake, accordingly, the amount of carbamate formed can be obtained. This technique, called the noncarbonate equilibrium method by Stadie and O'Brien (9), has been utilized by several authors (9-11) who employed gasometric measurements. The method was greatly improved by using a continuous flow rapid reaction apparatus equipped with a CO₂ electrode as described by Constantin et al. (12) to follow the time course of PCO₂ in the mixture (2, 5, 13).

The new method of carbamate determination presented here is based on the same principle but utilizes the measurement of the time course of pH rather than that of pCO₂. According to Equations 1 and 2 (see below) the formation of carbamate leads to a release of protons which in turn is accompanied by a pH change. The time course of pH was followed by a pH electrode which has a response time fast enough to allow the use of a stopped flow instead of a continuous flow technique. Thus, a complete reaction course can be obtained from a single experiment using only 2 ml of protein solution.

Materials and Analytical Methods

Glycylglycine solutions had the following composition: 4 mM glycylglycine (Sigma), 10 mM Veronal (Sigma), and 0.15 M NaCl (tonic strength 0.16). Human plasma from outdated human blood from the blood bank or from fresh blood was diluted 1:1 with 0.15 M NaCl and homogenized for 5 min in a Potter glass homogenizer. This was all done at 0°C. The homogenate was centrifuged at 4000 × g for 30 min, and the clear supernatant, containing the water-soluble proteins, was used for the rapid reaction experiments. Knowing the weight of the original piece of tissue, and the volume of the protein solution prepared from it, the concentration of carbamate measured in the extract could be converted to the amount of carbamate per tissue, wet weight.

All solutions were degassed by exposure to a vacuum of ~0.5 mm Hg (+ water vapor pressure) for 2 hours while being stirred. Aliquots were then transferred into ionometers, pH adjusted to predetermined values by addition of 0.1 N NaOH or HCl, and the solution freed from O₂ and CO₂ for a second time by being flushed with nitrogen and shaken for a total of 30 min. Residual CO₂ was <5 × 10⁻⁴ ml/ml and additional shaking did not lead to a further decrease. Acetazolamide (Lederle) was added to the plasma and tissue extracts in a concentration of 0.01 g/100 ml to ensure inhibition of any carbonic anhydrase possibly present.

Solutions of CO₂ were prepared by equilibrating 0.15 M NaCl solution with CO₂/N₂ mixtures at the temperature of the experiment. The pH of all solutions used for the rapid reaction experiments was measured anaerobically in a thermostatted capillary glass electrode. In the course of preparing solutions with a range of pHi, a titration curve (see Fig. 1) was obtained from which the buffer factor in the pH range of interest was read and used to calculate the amount of H⁺ released by carbamate formation from the pHi record of the rapid reaction experiment (see "Calculation of H⁺ Release Due to Carbamate Formation"). Plasma protein concentrations were determined spectrophoto metrically with the biuret method (19). For calibration bovine plasma albumin (Sigma) was used. Hemoglobin concentrations were determined using the cyanmethemoglobin method as described by Drabkin and Austen (16).

Rapid Reaction Apparatus

The stopped flow apparatus used in this study (Fig. 2) was similar to that of Crandall et al. (17). The output from the glass and reference electrodes (Leeds and Northrup No. 117,145 and 117,147) was amplified (MPA-6/MPA-15 differential preamplifier, Transidyne General, Ann Arbor, Mich.) and could then be read to ±0.002 pH unit on a storage oscilloscope (Tektronix type 564). On a second channel of the oscilloscope a flow signal proportional to the linear speed of the drive was recorded.

Log Time—Owing to the volume between mixing chamber and glass electrode there was a delay (lag time) between mixing and actual pH measurement which was inversely proportional to the velocity of the drive. From the pH measured during flow the extent to which a known reaction (CO₂ hydration in the presence of imidazole) had proceeded during lag time and hence the lag time itself could be calculated. Doing so for different driving speeds an empirical relation between lag time (ranging from 5 to 40 ms for driving pressures of 4.0 to 0.1 atm, respectively) and flow signal was established.

Response Characteristics—Crandall et al. (17) showed that a similar glass electrode instrument could respond to a step change in pH in less than 0.005 s after allowance for the lag time. Recent work by Chow¹ indicates that when the buffer power of the reacting mixture becomes too small, the ability of the electrode to follow rapid changes in pH becomes compromised. Therefore, we tested the apparatus empirically by measuring ΔpH/Δt at a buffer concentration whose buffer power was close to that of the protein solutions used in this study. Mixing 2 mM imidazole buffer with a CO₂ solution at various carbonic anhydrase concentrations a linear relationship between the initial slope of the pH record, ΔpH/Δt, and the carbonic anhydrase concentration is to be expected. Fig. 3 shows that the relation is indeed linear up to ΔpH/Δt = 6 pH/s. Since the electrode thus appears to respond properly up to this velocity, the conditions of the rapid reaction experiments reported in this paper were chosen so that ΔpH/Δt was always <6 pH/s.

Flow Artifact—pH measured during flow may be lower than that measured in the stationary fluid. The size of this artifact depends strongly on the ionic strength of the solution. At μ = 0.003 the flow artifact became as high as 1 pH unit, at μ = 0.15 it was <0.002 pH unit, i.e., negligible. Generally μ was 0.15 so that no correction for the flow artifact was necessary but corrections were applied to pH values measured during flow in solutions with μ = 0.013.

Calculations

It is generally agreed that the carbamate formation of proteins and peptides follows the same reaction scheme as was first established for ammonia by Fausthoit (18). Accordingly, CO₂ reacts with unionized amino groups to form carbamate, i.e., the following equilibria are involved in carbamate formation:

\[ R\cdot NH₂ + CO₂ ⇌ R\cdot NHCO₂⁻ + H⁺ \]

\[ K_1 \]

\[ R\cdot NH₂ + CO₂ ⇌ R\cdot NHCO₂⁻ + H⁺ \]

\[ K_2 \]

where \( K_1 \) is the carbamate equilibrium constant, and \( K_2 \) is the ionization constant of the amino group. The reaction scheme implies that \( R\cdot NHCO₂⁻ \), the carbamic acid, is completely dissociated. This has been shown for glycylglycine at 5°C and pH > 7 (19). We assume this to hold true for the carbamic acid of glycylglycine and proteins at higher temperatures too.

It follows from these reaction equations that the formation of one carbamate group is accompanied by the dissociation of between 1 and 2 protons. It will be shown below (a) how the amount of protons released by carbamate formation was calculated from the pH record and (b) how the carbamate concentration was obtained from this quantity.

Calculation of H⁺ Release Due to Carbamate Formation—Fig. 4 shows an original oscilloscope tracing of a pH stopped flow experiment in which glycylglycine was mixed with CO₂ solution. Three reactions occur upon mixing. In Reaction 1, H⁺ of the acid CO₂ solution is neutralized by the buffer solution. This reaction is practically instantaneous and completed within the lag time of the instrument. In Reaction 2 amino groups and CO₂ react to form carbamate. This reaction has, in this example, a half-time of ~0.03 s. It is completed ~0.12 s after mixing (marked by pHₛᵤⁱᵗ in Fig. 4). Reaction 3 is hydration of CO₂. It has a half-time of ~7 s. At the point where the carbamate equilibrium has been reached no more than 5% of the total reaction has occurred. Therefore, the slope of the pH record becomes approximately linear after the carbamate reaction is completed.

During the constant flow of the solutions (indicated by the level flow signal) a pH plateau, pHₑ, is maintained which represents the pH of the mixture after the lag time has elapsed. After stopping the flow of the solutions (indicated by the abrupt disappearance of the flow signal) a clearly biphasic time course of pH is recorded. The fast phase was

¹We wish to express our appreciation to Dr. Esther Chow, Philadelphia, for suggesting and helping us perform the experiments concerning the response characteristics of the electrode.

²Dr. Esther Chow, personal communication.
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**Fig. 1.** Titration curves used to calculate amounts of $H^+$ liberated by carbamate formation from the pH stopped flow experiments. Obtained in the absence of CO$_2$. a, 4 mM glycylglycine in 10 mM Veronal. Temperature = 37°, ionic strength = 0.16. b, diluted human plasma (protein concentration 3.29 g/100 ml). Temperature = 37°, ionic strength = 0.15. The slope in the pH range 7-8, i.e., the buffer capacity of human plasma, is 0.109 mmol/g of protein/Δ pH and agrees well with value of Van Slyke et al. (14) of 0.104 mmol/g/Δ pH for horse serum. c, extract prepared from sheep skeletal muscle. A 1-liter extract was obtained from 0.19 kg of muscle tissue. The slope of the titration curve at pH = 7 corresponds to a buffer factor of ~15 mmol/kg of muscle/Δ pH. Temperature = 37°, μ = 0.15.

**Fig. 2.** Schematic diagram of the rapid reaction apparatus. When the pneumatic drive plunger (represented by the arrow) pushes the driving block, the reacting solutions (R-NH$_2$ and CO$_2$, respectively) in the pair of 2.5-ml syringes are forced into a four-tangential jet mixing chamber (0.004 ml) out of which the mixture flows against the pH-sensitive glass electrode in the 0.05-ml measuring chamber and then out into a collecting vessel. The flow is stopped when the driving block hits the stopping block. The channel between glass and reference electrode is closed by a Teflon plug with a KCl-saturated cotton thread lying across. This bridge has a negligible resistance of ~20 kohm. The flow signal is generated by a magnetic bar (not shown) attached to the driving block and moving through a copper coil.

**Fig. 3.** Response characteristics of the glass electrode used in the stopped flow apparatus. The initial slope of CO$_2$ hydration as recorded from the glass electrode, $\Delta$PH/Δt, was measured at different concentrations of carbonic anhydrase. The 2 mM imidazole solutions were mixed with 4.43 mM CO$_2$ solutions, temperature = 37°, μ = 0.15. $\Delta$PH/Δt is linearly related to carbonic anhydrase concentration as expected from the kinetics of the enzyme, indicating an adequate response of the electrode up to at least 0.1 pH/s.

When $\Delta$PH$_{carb}$ is multiplied by the buffer factor (BF) as read from the experimental titration curve in the pertinent pH range, the approximate amount of $H^+$ produced by carbamate/liter of solution, $Q_{H^+}$, is obtained:

$$Q_{H^+} = \Delta$PH$_{carb} \cdot BF$$

This value of $Q_{H^+}$ refers to the pH at which carbamate equilibrium is reached, pH$_{equil}$, and to the CO$_2$ concentration established at this point.

It should be noted that $Q_{H^+}$ thus calculated may be up to 90% higher than the actual amount of $H^+$ produced by carbamate formation. This is due to an overestimation of buffer factor, which is lower in the presence than in the absence (in which the titration curves were obtained) of carbamate. However, in calculating the carbamate concentration from $Q_{H^+}$ in the manner described below this error cancels out.

**Calculation of Carbamate Concentration**—Glycylglycine-Veronal mixtures as well as protein solutions may be considered as mixtures of buffering groups not participating and amino groups participating in carbamate formation. If such a solution reacts with CO$_2$ to form only carbamate, an increase in total concentration of protonated groups ($H^+$), i.e., total concentration of buffered protons, occurs which is due to the protons dissociated from carboxylic acid. Protons, which are originally bound by amino groups (R—NH$_2^+$) and then displaced as a consequence of carbamate reaction, are taken up by other buffering
groups and thus do not affect the total concentration of protonated groups in the solution. The increase in $[H^+]$ (plus a negligible increase in the concentration of free $H^+$) is therefore identical with the carbamate concentration:

$$[R-\text{NHCOO}^-] + [H^+] = [H^+]_0$$

(5)

where the indices refer to the situation before (pH$_a$) and after (pH$_b$) carbamate formation, and $[R-\text{NHCOO}^-]$ is the carbamate concentration when carbamate equilibrium is reached. If, simplifying, all buffering groups except the amino groups are described as $H^-$ (unprotonated) and $BH^+$ (protonated), the total concentration of protonated groups in the solution is:

$$[H^+] = [BH^+] + [R-NH_3^+]$$

(6)

Introducing $[\text{Ammtot}]$, the total concentration of amino groups, we then obtain from the definition of $K_2$ in Equation 2 and Equations 5 and 6:

$$[R-\text{NHCOO}^-] = [\text{Ammtot}] - [BH^+]_0 - [R-NH_3^+]_0 - [H^+]_0$$

which rearranges to give:

$$[R-\text{NHCOO}^-] = [\text{Ammtot}] - [BH^+]_0 - [R-NH_3^+]_0 - [H^+]_0$$

(7)

The right-hand side of Equation 7 expresses the increase in the concentration of protonated groups in the solution, when, in the absence of CO$_2$, the pH is lowered from pH$_a$ to pH$_b$, i.e. it is equal to $\Delta$H$_{ac,b}$. BF, where BF is the buffer factor of the solution in absence of CO$_2$. From this consideration and Equations 4 and 7 we arrive at the relation:

$$[R-\text{NHCOO}^-] = \frac{[H^+]_0}{K_2 + 2[H^+]_0} + \frac{[H^+]_0}{K_2 + [H^+]_0}$$

(8)

which allows us to calculate the concentration of carbamate from $Q_{\text{carb}}$. It is possible to simplify the further theoretical treatment by substituting $[H^+]_0$ for $[H^+]_0$ in Equation 8. Usually the difference between pH$_a$ and pH$_b$ is less than 0.05 unit and represents an error in $[R-\text{NHCOO}^-]$ of less than 2%. Therefore, the factor relating $Q_{\text{carb}}$, the amount of $H^+$ dissociated, to the carbamate formed, becomes:

$$\frac{Q_{\text{carb}}}{[R-\text{NHCOO}^-]} = \frac{K_2 + 2[H^+]_0}{K_2 + [H^+]_0}$$

(9)

**RESULTS AND DISCUSSION**

**Carbamate Formation of Glycylglycine**

**Carbamate Equilibrium**

In Fig. 5a $Q_{\text{carb}}$/Glyc, i.e. the approximate number of moles of $H^+$ produced by carbamate formation per number of moles of glycylglycine, is plotted versus the pH at carbamate equilibrium, pH$_{\text{equl}}$, for four CO$_2$ partial pressures. Each curve represents one set of experiments in which glycylglycine solutions of different pH values were mixed with the same CO$_2$ solution (the given values of pCO$_2$ refer to the pCO$_2$ values after physical mixing, before any CO$_2$ has been consumed by the reactions). Fig. 5b shows $Q_{\text{carb}}$/Glyc as a function of pCO$_2$ at constant pH values, one curve for pH 8.0, one for pH 7.5. The points were obtained from the curves of Fig. 5a. Each set of data at constant pH can be used to calculate $K_2$ and $K_z$. Suitable algebraic transformation of Equations 1, 2, and 9 leads to an equation convenient for this purpose:

$$\frac{1}{Q_{\text{carb}}/\text{Glyc}} = \frac{1}{n} \left( \frac{[R-\text{NHCOO}^-]}{[R-\text{NHCOO}^-]} + \frac{[H^+]_0}{K_2 + 2[H^+]_0} \right)$$

(10)

$$= \frac{1}{[R-\text{NHCOO}^-]} + \frac{[H^+]_0}{K_2 + 2[H^+]_0}$$

where $n$ is the number of carbamate binding sites per molecule (1 in the case of glycylglycine) and [CO$_2$] and [H$^+$] refer to the point of carbamate equilibrium. If $1/Q_{\text{carb}}$/Glyc from a set of data of identical pH values is plotted versus $1/[CO_2]$, a straight line should be obtained according to Equation 10. The intercept of this line is $1/n [1 - (K_z/H^+_0)^2 + (K_z/K_z + 2[H^+]_0)^2]$, from which $K_z$ can be calculated if $n$ is known. The slope is $[H^+]_0 (K_z + [H^+]_0)/nK_z(K_z + 2[H^+]_0)$, from which $K_z$ can be obtained. In order to apply the plot according to Equation 10, [CO$_2$] has to be calculated by subtracting $[R-\text{NHCOO}^-]$ from [CO$_2$]$_{\text{mix}}$. 

**FIG. 4.** Original oscilloscope tracing of a pH stopped flow experiment mixing 23.6 mm CO$_2$ solution (pH 3.9) and 4 mm glycylglycine in 10 mm Verona ($\mu = 0.16$, temperature $= 37^\circ$). pH$_a$ ($= 7.23$), the original pH of the glycylglycine solution, is obtained by flowing buffer solution through the measuring chamber. pH$_b$ is the pH established during the flow of the solutions. After stopping pH follows a biphasic time course; the fast phase represents carbamate formation, the slow phase CO$_2$ hydration. At pH$_{\text{equl}}$ carbamate equilibrium is assumed to have been reached. Extrapolating the slow phase (dashed line), pH$_c$ - pH$_b$, the pH change due to carbamate formation (plus neutralisation) is obtained. The upper upper part of the figure shows the flow signal with its base line. The reference point of the time scale is the time of mixing of the solutions, i.e. the time of stopping minus lag time (the lag time was 0.012 s in this experiment).
which presupposes the knowledge of \( K \) (see Equation 8). This problem was solved by a trial and error procedure. In Fig. 6 the plot obtained from the data in Fig. 5b is shown. As predicted by Equation 10, for the data both at pH 8.0 and pH 7.5, straight lines are obtained (correlation coefficients around 0.99). The pK\(_a\) and pK\(_b\) values for 37\(^\circ\) calculated from the plots in Fig. 6 are shown in the first two lines of Table I and are almost identical for the two different pH values. We conclude that these data obtained over a wide range of pH and pCO\(_2\) are entirely consistent with the described theory of carbamate formation, especially with regard to the complete dissociation of carbamic acid.

**Effect of Temperature on \( K \).**—Lines 3 and 4 of Table I represent data obtained at 20°, Line 3 for \( \mu = 0.16 \), Line 4 for \( \mu = 0.013 \). From the pK\(_a\) values of Lines 1 and 3 the reaction heat of the carbamate reaction (Equation 1) is calculated to be \( \Delta H = -3.2 \text{ kcal/mol} \). This relatively low value of \( \Delta H \) for \( K \) implies that, between pH 7 and 8, the overall temperature dependence of carbamate formation of glycylglycine is governed by the high positive reaction heat of the ionization reaction (+10.6 kcal/mol according to Brunetti et al. (21)).

**Effect of Ionic Strength on \( K \).**—The thermodynamic carbamate equilibrium constant, \( K_{eqn} \), is obtained by inserting the appropriate activity coefficients into Equation 1:

\[
K_{eqn} = \frac{f_2 [\text{R-NHC\(\text{O}_2\text{H})]} - 0.5f_1 [\text{R-NH}] [\text{CO}_2]}{f_1 [\text{R-NH}] [\text{CO}_2]} \tag{11}
\]

where \( a_\mu \) is assumed to be given by the pH measured with the glass electrode. Since the carboxylic group of glycylglycine is completely dissociated in the present pH range (13), \( f_1 \) is the activity coefficient of a monovalent, \( f_2 \) the activity coefficient of a divalent anion. The Debye-Hückel theory for more concentrated solutions predicts that

\[
f_1 = 10^{-0.85 \sqrt{\mu}} (1.6) \tag{12}
\]

& 4

\begin{center}
\begin{tabular}{|c|c|c|c|}
\hline
\text{Temperature} & \text{Ionic strength} & \text{pK\(_a\)} & \text{pK\(_b\)} \\
\hline
37° & 77 Torr & 4.60 & 7.87 \\
37° & 145 Torr & 4.56 & 7.87 \\
20° & 40 Torr & 4.45 & 8.29 \\
20° & 77 Torr & 4.74 & 8.32 \\
\hline
\end{tabular}
\end{center}

*Derived from pK\(_a\) = 8.32 (Line 4) correcting for \( \mu = 0.16 \) according to Neuberger (20).
\[
[R-NHCOOH] = \frac{[R-NHCOO^-][H^+]}{K_x}
\]  
(18)

where \( K_x \) is the ionization constant of carbamic acid. Setting \( d[R-NHCOO^-]/dt = 0 \) we obtain from Equations 1, 15, and 16:

\[
k_d = k_a \cdot \frac{K_x}{K_c}
\]  
(17)

Combining Equations 15, 16, and 17 we arrive at:

\[
d[R-NHCOO^-]/dt = k_a \left[ \frac{[CO_2][R-NH_2]}{K_c} - \frac{[R-NHCOO^-][H^+]}{K_c} \right]
\]  
(16)

All dependent variables in this equation can be expressed in terms of pH, the actual pH value during the fast phase of the reaction by means of Equations 19 to 21. The concentration of carbamate at a given time may be expressed applying Equations 4 and 9 analogously:

\[
[R-NHCOO^-] = \frac{[R-NHCOOH] + [R-NH_2] + [R-NH_2COO^-] - [H^+] [R-NHCOO^-]}{K_c} + \frac{[R-NHCOOH]}{K_x} \cdot [H^+]^{1.5}
\]  
(19)

where BF is the average buffer factor of the solution valid for the pH range \( \text{pH}_0 - \text{pH} \). The concentration of carbamate immediately after mixing, \([CO_2_{max}]\), minus the carbamate concentration:

\[
[CO_2] = [CO_2_{max}] - [R-NHCOO^-]
\]  
(20)

where the bicarbonate formation is neglected since it affects the value of \( [CO_2] \) in the studied phase of the reaction by less than 1%. Similarly, the concentration of un-ionized amino groups is calculated from:

\[
[R-NH_2] = \frac{[Am_{tot}] - [R-NHCOO^-]}{K_z} \cdot [H^+]^{1.5}
\]  
(21)

If Equations 19 to 21 are substituted in Equation 18, \( d\text{pH}/dt \) as a function of \( \text{pH} \) and \( t \) can be obtained, but is of little use as it cannot be integrated analytically. Fortunately \( (\text{pH} - \text{pH}_t - \text{s},t) \) is an exponential function of time in all experiments so that a simple relationship of the form

\[
\log(\text{pH} - \text{pH}_t - \text{S},t) = a + b
\]  
(22)

is obtained (correlation coefficients > 0.999, see example in Fig. 7a). Differentiating Equations 22 and 19 allows one to calculate values of \( d\text{pH}/dt \) and \( d[R-NHCOO^-]/dt \). Inserting the latter value together with values of \([R-NH_2], [R-NHCOO^-], \) and \([CO_2]\) (from Equations 19 to 21) into Equation 18 yields \( k_a \). The linearity of the plot of Equation 18 in Fig. 7b shows that the measured kinetic curves are consistent with the theory of carbamate formation. Table III shows the velocity constants of carbamate formation obtained in this way for 37°C and 20°C. It can be seen that the velocity constants, and the activation energy calculated from them, agree reasonably with data obtained by other authors.

**Carbamate Formation in Plasma**

**Carbamate Equilibrium**

Fig. 8 shows results obtained with plasma solutions of ionic strength 0.15 at 37°C. \( Q_{H+/Prot} \), the approximate amount of \( H^+ \) per protein produced by carbamate formation, is plotted versus \( \text{pH}_{\text{eq}} \), the \( \text{pH} \) at carbamate equilibrium (see Fig. 4). \( Q_{H+/Prot} \) was calculated from Equation 4 using the buffer factor of the plasma solution as shown in Fig. 1b. The protein concentration in grams/100 ml, as obtained with the biuret method, was converted to moles/liter using the molecular weight of albumin, 69,000. Four sets of data at four different CO2 partial pressures \( (pCO_2_{mix} = 359, 140, 79, 38 \text{ Torr}) \) were obtained, each set with four to seven different \( \text{pH} \) values between 6.9 and 8.3.

Each point in Fig. 8 represents the average of about five measurements performed at the same \( \text{pH} \) and \( pCO_2 \). The relative standard deviations (variation coefficients) of the values of \( Q_{H+/Prot} \) obtained under identical conditions averaged to 3.5%. They did not vary significantly with the size of the value of \( Q_{H+/Prot} \) for \( 0.5 < Q_{H+/Prot} < 10 \). Values of \( Q_{H+/Prot} \) obtained with plasma from fresh blood did not differ significantly from values obtained at the same \( \text{pH} \) and \( pCO_2 \) with plasma from outdated blood.

**Groups Responsible for Plasma Carbamate**—In the case of hemoglobin, the protein whose carbamate reaction has been studied most extensively, it has been assumed that only the \( \alpha \)-amino groups of the NH4 terminal contribute significantly to carbamate formation at \( \text{pH} < 8 \) (1, 5, 28). How many \( \alpha \)-amino groups per protein are to be expected in plasma, if an arbitrary plasma protein molecular weight of 69,000 is chosen? The number of end groups of some of the plasma proteins is known. Albumin (60% of the plasma protein) and transferrin are single polypeptide chains of 69,000 and 89,000 molecular weight, respectively. Most \( \gamma \)-globulins and fibrinogen have two free \( \alpha \)-amino-terminal amino groups/molecule at molecular weights of 160,000 and 311,000, respectively (29, 30). On the basis of a molecular weight of 69,000, this leads to less than 1 \( \alpha \)-amino group/protein molecule. Therefore, if the assumption that only \( \alpha \)-amino groups form carbamate were true for plasma protein, we would expect \( Q_{H+/Prot} \) to level off at a value < 1. Fig. 8 shows that carbamate/plasma protein does not reach a maximum around 1, but increases steeply with increasing \( \text{pH} \).

At \( \text{pH} 8 \) and \( pCO_2_{mix} = 359 \text{ Torr} \), \( Q_{H+/Prot} \) reaches a value of < 10. This is clearly incompatible with the conception that only \( \alpha \)-amino groups form carbamate: groups in addition to the \( \alpha \)-amino groups must participate in the carbamate formation.

The groups most likely to do this are the \( \epsilon \)-amino groups of the lysine side chains.
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The conclusion that at least two types of groups must be involved in the carbamate formation of plasma is also reached, when a plot of \(1/(Q_{H^+} \text{Prot})\) versus \(1/([CO_2])\) is made of the data obtained in plasma. For sets of data at pH 7.9, 7.7, 7.5, and 7.4 (obtained by interpolation from Fig. 8) we find that, in contrast to the case of glycylglycine, there is no value of \(n\), which yields roughly the same values of \(K_1\) and \(K_2\), or positive values for both \(K_1\) and \(K_2\), at all these pH values. This indicates that the present data are not compatible with the assumption of only one species of binding sites. We therefore made an attempt to fit constants representing two types of binding sites to the data.

Equilibrium Constants \(K_1\) and \(K_2\) and \(n\)—If two types of groups contribute to carbamate formation, \(Q_{H^+} \text{Prot}\), as can be derived from Equations 1, 2, and 9, is given by the following expression:

\[
\frac{Q_{H^+}}{\text{Prot}} = \frac{n_1 K_{21} [R^+]}{K_{21} + [R^+]} \frac{[CO_2]}{K_{12}} \times \frac{n_2 K_{22} [R^+]}{K_{22} + [R^+]} \frac{[CO_2]}{K_{12}}
\]  

(23)

where the indices 1 and 2 designate the two types of groups. Accordingly, the carbamate formation is described by six constants: \(n_1, K_{11}, K_{12}, n_2, K_{21}, K_{22}\). Since the plot according to Equation 10 is not applicable to the case of two types of binding sites, the following procedure was used to find the best values for these constants. Numerical values for the constants were chosen arbitrarily. For all data points shown in Fig. 8 values of \(Q_{H^+} \text{Prot}\) were calculated by inserting these constants and the pertinent values of \([H^+]\) and \([CO_2]\) into Equation 23.

The straight line is the rate constant of carbamate formation \(k_w\). The rate of carbamate formation is calculated from the differentiated regression equation of \(a\) yielding \(dpH/dt\), and the differentiated form of Equation 19. Inserted are the linear regression equation and the correlation coefficient.

![Fig. 7. a, linearization of the pH record of an experiment mixing glycylglycine and CO\(_2\) (pH = pH\(_{\text{fast}}\) - \(s_1\) t) represents the distance between the pH curve of the fast phase of the pH record and the extrapolated slow phase (see Fig. 4). Also shown are the linear regression equation and the correlation coefficient. b, plot of the left versus right of Equation 18 for the experiment shown in a. The slope of the straight line is the rate constant of carbamate formation \(k_w\). The rate of carbamate formation is calculated from the differentiated regression equation of \(a\) yielding \(dpH/dt\), and the differentiated form of Equation 19. Inserted are the linear regression equation and the correlation coefficient.](http://www.jbc.org/)}
The calculated values of $Q_{CO_2}$/Prot, $q$, were then compared with the measured values, $q_m$. It has been mentioned that the relative standard deviations were about the same for all numerical values of $Q_{CO_2}$/Prot. Therefore, the differences $q_i - q_m$ were weighted by dividing by $q_i (51)$, and the sum of squares for all data points, $\phi$, was calculated from

$$\phi = \sum_{i=1}^{m} \left( q_i - q_m \right)^2 \quad (24)$$

were $m$ is the number of data points. Using the gradient technique of Fletcher and Powell (32), the values of the six constants were varied until the minimum for $\phi$ was found.

The procedure was started using $[CO_2_{mix}]$ as an estimate of $[CO_2]$, the $CO_2$ concentration at carbamate equilibrium. After obtaining first estimates of the equilibrium constants, carbamate concentrations, $[R-NHCOO^+]$, could be calculated from $Q_{CO_2}$/Prot. This allowed us to get new estimates of $[CO_2]$ via Equation 20. Then the entire procedure was repeated until the differences between the old and the new estimates of $[CO_2]$ were negligible ($<0.1\%$). In order to ensure that the constants obtained in this way reflect the true, not merely a local, minimum of $\phi$, the initial guesses of the constants $K_1$ and $K_2$ were varied within ±1 order of magnitude, those of the $n$ value within ±100%. The calculations were performed on a Siemens 4004/BS 1000 digital computer.

The constants obtained from this fit are compiled in Table IV. They were used to calculate the curves in Fig. 8 for the respective experimental conditions of the four data sets. It can be seen that the calculated curves reasonably fit the measured data points. The overall goodness of the fit can be expressed quantitatively by $R^2$, the fraction of variation in the dependent variable ($Q_{CO_2}$/Prot) which is explained by variations in the independent variables ($[CO_2]$ and $[H^+]$) (33). $R^2$ was found to be 0.97. This high value indicates that, in spite of the diversity of the plasma proteins, plasma carbamate can be adequately described on the basis of two species of participating groups. As a measure of the reliability of the numerical values obtained for the single constants their standard errors may be taken. The latter were calculated following the procedure proposed by Tukey (34) and Dammkoehler (33) and are listed in Table IV.

The values of constants listed in Table IV show that the plasma carbamate concentration is a function of $pH$ and $pCO_2$ plasma. This is in agreement with the results obtained by Stadie and O'Brien's (39) data is possible, since their measurements were done in concentrated plasma protein solutions at pH 0 in plasma at 37°C and at pH 9 only.

It can be seen from Fig. 9 that, in the physiological range of pH and $pCO_2$, plasma carbamate is more sensitive to changes in $pH$ than in $[CO_2]$. This stipulates an extremely small arteriovenous concentration difference of plasma carbamate: increasing the arterial $pCO_2$ of 37 Torr by 14% to its venous value of 42 Torr (40), and shifting pH from 7.46 to 7.43 (which is a 7% increase in $H^+$ concentration), leads to a rise of carbamate concentration from 0.631 mM to 0.633 mM. This is a 7% increase in $H^+$ concentration). Plasma carbamate does not contribute significantly to the $CO_2$ exchange in the respiratory cycle. It may play a role, however, during transient $pCO_2$ changes by virtue of the fact that its formation is 2 orders of magnitude faster than the formation of bicarbonate, which in plasma is not catalyzed by carbonic anhydrase.

Carbamate Kinetics

In order to evaluate the kinetics of carbamate formation in plasma the pH records of seven stopped flow experiments covering the pH range from 7.6 to 9.1 were analyzed. $pCO_2_{mix}$ was 38 Torr in all experiments. Temperature was 37°C, ionic strength 0.15. The first two-thirds of the fast phases of the pH records were linearized as described for glycylglycine: plotting $\log (\Delta pH - \Delta pH_s)$ versus time straight lines with correlation coefficients $>0.998$ were obtained for all experimental records studied. After differentiating the regression equations obtained from these plots, for any value of $pH$ within the linearized part of the experimental pH curve a corresponding value of $dpH/dt$ could be calculated. The experimental pairs of $pH$ and $dpH/dt$ thus obtained were used for the kinetic analysis as shall be shown below.

Different Kinetics of $\alpha$- and $\epsilon$-Amino Groups—An attempt was made to explain the carbamate kinetics in plasma with the assumption of an identical velocity constant $k_d$ for the two types of carbamate binding sites but the values obtained
groups are not only different with respect to their \( pK_a \) values, as an indication that the two kinds of carbamate forming depended strongly on the pH. \( k_{a1} \) dropped from a value of 12,000 \( M^{-1} s^{-1} \) at pH 9 to 7,000 \( M^{-1} s^{-1} \) at pH 7.6. This was taken as an indication that the two kinds of carbamate forming groups are not only different with respect to their \( pK_a \) values, but also show a different kinetic behavior. Indeed, Chipperfield (27) has shown that the carbamate formation velocity constant of amino acids and peptides increases with the \( pK_a \) value of the amino group. Therefore, different velocity constants, \( k_{a1} \) and \( k_{a2} \), were assigned to the two species of groups and their values were fitted to the experimental data.

The fitting procedure was done in the following way. Arbitrary values of \( k_{a1} \) and \( k_{a2} \) were inserted into the differential equations describing the formation of carbamate by the two kinds of groups, which were formulated analogously to the kinetic equations for glycylglycine (Equations 18 to 21). These equations were integrated numerically for the conditions of kinetic experiments using the Runge-Kutta method described by Zurmühl (31). For identical values of pH, theoretical values of \( \frac{dpH}{dt} \) \( (d^t) \) obtained in the course of the integration were compared with experimental values of \( \frac{dpH}{dt} \) \( (d) \) obtained from the linearized experimental record, and the sum of squares, \( F \), was calculated for all kinetic experiments according to:

\[
F = \sum_{i=1}^{m} (d_i - \tilde{d}_i)^2
\]

where \( m \), the number of compared values of \( \frac{dpH}{dt} \), was given by the step size of the integration. This procedure did not require knowledge of the lag time. In a manner similar to that described for the carbamate equilibrium constants, the best values of \( k_{a1} \) and \( k_{a2} \) were determined by searching the minimum of \( F \).

The velocity constants of carbamate formation in plasma obtained in this way are listed in Table IV together with the constants of Table IV.

Forster et al. (2) determined an overall kinetic constant of 11,000 \( M^{-1} s^{-1} \) for human hemoglobin which would compare quite well with an overall \( k_a \) for plasma protein as may be seen from Table IV. The dependence of \( k_a \) on \( pK_a \), expressed as \( \Delta \log k_a/\Delta pK_a \), is calculated from Table IV to be 0.29, a value which agrees well with Chipperfield's (27) value of 0.26 for amino acids and peptides. The half-time of carbamate formation in plasma, estimated from the constants of Table IV, is 0.047 s with \( pCO_2 = 40 \text{Torr} \), pH = 7.4, and a protein concentration of 7 g/100 ml. This is 150 times faster than the half-time of the uncatalyzed hydration of CO.

**Carbamate Formation in Tissues**

We attempted to examine the question of carbamate formation in tissues by virtue of the present method. This question has been extensively discussed (41-47), mainly because of its bearing on the validity of the determination of intracellular pH from intracellular \( pCO_2 \) and total CO2 concentration, but never has been resolved. Fig. 10 shows the results obtained for an extract from skeletal muscle (thigh) from sheep. Measurements at two partial pressures, \( pCO_2 \) of 74 and 140 Torr, in a pH range between 6.4 and 8.3 were performed. The same pattern of pH dependence as in plasma was found: for both \( pCO_2 \) partial pressures, \( Q_{H^+} \) per tissue wet weight, is less than 1 mmol/kg at pH < 7, but shows a steep increase with pH at pH > 7. At pH 8 \( Q_{H^+} \) reaches a value of about 10 mmol/kg. This pronounced increase in \( Q_{H^+} \) in the alkaline pH range suggests that in muscle, as in plasma, a large number of \( \epsilon \)-amino groups are available for carbamate formation. Table V shows data for brain tissue of sheep. For \( pCO_2 = 3.5 \text{Torr} \), measurements at three pH values were done. As in muscle, \( Q_{H^+} \) shows a strong dependence on pH. Assuming a value of 1.5 for \( \gamma \) (see Equation 9), the carbamate concentrations are estimated to be 0.33 mM for muscle at pH 7.0 and \( pCO_2 = 70 \text{Torr} \), and 0.08 mM for brain at 35 Torr and pH 7.0. It should be noted that not all the carbamate-forming material of the tissue may have been contained in the tissue extracts and these concentrations therefore may be somewhat underestimated.

**Note on "Barium-soluble Fraction of CO2" in Muscle and on Effect of Carbamate on Determination of Intracellular pH by CO2 Method—Conway and Fearon (41) concluded that in rat muscle there is a barium-soluble noncarbonate CO2 fraction of 8 mmol/kg out of 14.8 mmol/kg of total CO2. This is more than 10 times the carbamate concentration we expect from Fig. 10 for sheep muscle at an intracellular pH of 7. The data in Fig. 10 do show, however, that a carbamate concentration of 8 mmol/kg can occur in muscle, but at pH values far above the physiological range.

We suggest that the high values of noncarbonate CO2 found by Conway and Fearon (41) and by Butler et al. (46) may be an artifact caused by formation of appreciable amounts of carbamate during the experimental procedure they used. In their analytical procedure, 2-g pieces of freshly excised muscle tissue are introduced into 0.2 N KOH and left there for an hour, during which period the pH in the muscle pieces increases from 7 to about 12. Several minutes are required for the average pH in the muscle to go from pH 8 to pH 9.5. Rough calculations show that, although the pCO2 of the tissue decreases with increasing pH, the intracellular carbamate concentration may easily rise to 8 mmol/kg as pH rises from 8 to 9.5, and that in this pH range only a few seconds are necessary for this amount of carbamate to be formed. Thus, we conclude that carbamate formation in the muscle exposed to KOH (rather than an...
inhibition of carbonate precipitation in the muscle extract as inferred by Butler et al. (46) may have caused erroneously high values of barium-soluble \( \text{CO}_2 \) in these studies.

From the present data, determinations of the intracellular pH, \( \text{pH}_i \), by the \( \text{CO}_2 \) method (see reviews in Refs. 43, 44, and 47) appear to be far less affected by a neglect of intracellular carbamate concentrations than has been postulated by Conway and Fearon (41). In brain with \( \text{pH}_i = 7, \text{pCO}_2 = 35 \) Torr and different pH values using tissue extract. Temperature 37\(^\circ\), ionic strength 0.15.

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