Chromatographic Method for the Determination of Conditional Equilibrium Constants for the Carbamate Formation Reaction from Amino Acids and Peptides in Aqueous Solution

Jian-Ge Chen,† Mats Sandberg,*,‡ and Stephen G. Weber*,†

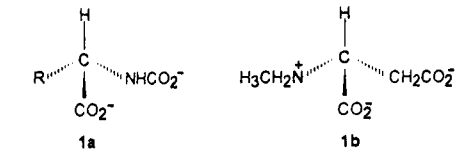
Contribution from the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and Institute of Neurobiology, Department of Histology, Göteborgs Universitet, Medicinaregatan 5, Göteborg, Sweden

Received February 26, 1993

Abstract: A novel and sensitive method has been developed and evaluated for the study of carbamate formation equilibria of amino acids and peptides in aqueous solution. The method is based on reversed-phase liquid chromatography with cetyltrimethylammonium bromide. The reliability of the method was established by comparing the results determined from the present study with the few data in the literature. The relaxation rate of the carbamate reaction was shown to be faster than the chromatographic distribution relaxation rate (seconds). As a result, the retention time of amine solutes is increased in the presence of CO_2. Carbamate formation constants and mole fractions of carbamates at physiological pH of eleven L-α-amino acids and peptides were determined. No correlation between the formation constant and the ammonium pK_a was found. There is significant dependence of the amount of a particular amino acid or peptide that exists as carbamate at pH 7.4 on the pK_a of the ammonium group, however. This is due to mass action rather than reflecting the influence of pK_a on the propensity of the amine to react with CO_2. It is suggested that amino acids and peptides with ammonium pK_a greater than 9.5 do not form significant amounts of carbamates in aqueous solution near neutral pH.

Introduction

Recently, the neurotoxicity of carbamates formed from the reaction of CO_2 with certain L-α-amino acids has been proposed.[1-7] There is physiological evidence that CO_2/HCO_3^- increases the neurotoxicity of L-cysteine[4] and β-(*N*-methylamino)-L-alanine (L-BMAA).[5-6] These results have been rationalized on the basis of the remarkable homology between the CO_2 adducts (carbamates) (**1a**) of the compounds mentioned and the known excitatory amino acid receptor agonist, *N*-methyl-D-aspartate (NMDA) (**1b**). However, because it has not been possible to determine quantitively the carbamates *in vivo*, and such determinations are difficult to imagine, these hypotheses lack direct experimental proof.

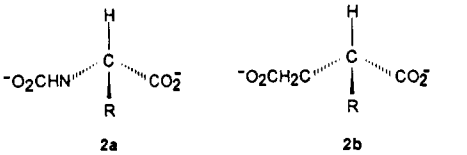


1a

R = CH_2SH (L-cys)

R = $CH_2\overset{+}{N}H_3$ (L-BMAA)

1b



2a

2b

R = $CH(OH)(CO_2^-)$

Other potential physiological effects of carbamates have been proposed.[8-11] Carbamates of ethylenediamine and piperazine were shown to activate mammalian γ-aminobutyric acid (GABA) receptors.[8,9] Mroz[10] recently proposed that CO_2 may theoretically react with inactive amines in the synaptic vesicles to produce active carbamates, i.e. transmitters. After release and interaction with postsynaptic receptors, the carbamate may, if the extracellular environment is less favorable for carbamate formation than the intravesicular environment, spontaneously decompose. This would thus entail an inherent molecular mechanism for transmitter inactivation. Carbamate mimics of carboxylates were shown to react with enzymes.[11] In particular, aconitase, which catalyzes the dehydration of isocitrate, reacts readily with the carbamate of β-hydroxyaspartate (**2a**), which is analogous to isocitrate (**2b**). It is interesting to note that the same workers found that aconitase and glucose 6-phosphate dehydrogenase both actually catalyze the formation of the carbamate which is the substrate mimic.

The implications of these studies, though they are few, are vast because the fundamental biochemical functions of organisms are influenced: the substrate specificity of enzymes, the receptor selectivity for agonists, and chemical signaling.

Given the importance of these compounds, it is surprising that the literature holds very few measurements of carbamate formation constants from amino acids and peptides in aqueous solution. In fact, though glycine has been studied several times,[12,13,14b,15,16a] the only other amino acids and peptides for which formation constants have been measured are alanine,[14a]

* To whom correspondence should be addressed.
† University of Pittsburgh.
‡ Göteborgs Universitet.

(1) Max, B. *Trends Pharmacol. Sci.* **1991**, *12*, 408.
(2) Nunn, P. B.; Davis, A. J.; O'Brien, P. *Science* **1991**, *251*, 1619.
(3) Davis, A. J.; Hawkes, G. E.; O'Brien, P.; Wang, G.; Nunn, P. B. *J. Chem. Res., Synop.* **1991**, 84.
(4) Olney, J. W.; Zorumski, C.; Price, M. T.; Labruyere, J. *Science* **1990**, *248*, 596.
(5) Weiss, J. H.; Christine, C. W.; Choi, D. W. *Neuron* **1989**, *3*, 321.
(6) Weiss, J. H.; Choi, D. W. *Science* **1988**, *241*, 973.
(7) Nunn, P. B.; O'Brien, P. *FEBS Lett.* **1989**, *251*, 31.
(8) Hill, D. R. *Br. J. Pharmacol.* **1983**, *79*, 268P.
(9) Hill, D. R. *Neuropharmacology* **1985**, *24* (2), 147.
(10) Mroz, E. A. *Science* **1989**, *243*, 1615.
(11) Porter, D. J. T.; Alston, T. A.; Bright, H. J. *J. Biol. Chem.* **1987**, *262*, 6552.
(12) Stadie, W. C.; O'Brien, H. *J. Biol. Chem.* **1936**, *112*, 723.
(13) Caplow, M. *J. Am. Chem. Soc.* **1968**, *90*, 6795.

0002-7863/93/1515-7343$04.00/0

β-hydroxyaspartate,[11] glycylglycine,[14l] glycylglycylglycine,[14l] and hemoglobin.[16b] To be sure, there is detailed knowledge of hemoglobin's ability to bind CO_2.[16-19] Pioneering work by Divers[20] led to studies of physiological transport of CO_2 by hemoglobin. This hypothesis was first developed by Henriques[21] and was established later by Roughton *et al.*[17] Insights into the kinetics and mechanism of carbamate formation came later in work by Caplow,[13] and Faurholt *et al.*[14] There is also a general appreciation, from the perspective of CO_2 distribution, that carbamates make up about 2% of the total carbonate in blood,[17b,c] but the literature on quantitative determinations of the formation of a carbamate from an amino acid or peptide is sparse.

The formation constants cannot be predicted. The most complete study of the carbamate reaction is a study by Caplow[13] in which the rate constants of the forward and reverse reactions corresponding to eq 2 were determined for a wide variety of amines, including glycine.

From the rate constants, conditional equilibrium constants (concentrations, not activities) were determined. No correlation between pK_c and pK_a was found.

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+ \qquad K_a = \frac{[H^+][RNH_2]}{[RNH_3^+]} \qquad (1)$$

$$RNH_2 + CO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RNHCOO^- + H^+$$

$$K_c = \frac{[H^+][RNHCOO^-]}{[RNH_2][CO_2]} \qquad (2)$$

The most extensive study of amino acids is that by Armarego and Milloy.[22] They determined the extent of the optical rotation shift and the relative integrations of the α-H peaks of the amine and its carbamate in the ^1H NMR spectra in solutions containing 0.5–1 M amino acid in the presence of 2–3 times that concentration of potassium carbonate. The solution pH was around 10. Most of the common amino acids were studied, and they all showed significant interaction with CO_2.

Methods that have been used most commonly for the study of carbamate formation include barium precipitation,[13,14] manometry,[12,17] and NMR.[3,22-25] The advantage of NMR is that the chemical shifts observed give credence to the assertion that the carbamate has the structure envisioned (**1a**, **2a**). However, all of these studies were carried out under exceptionally nonphysiological conditions. The experiment that is closest to physiological conditions, and it is exceptional, is a recent NMR study of the formation of the carbamate of L-BMAA.[7] In this experiment the amino acid concentration was 50 mM in a carbonate/bicarbonate solution in D_2O, the pD of which was altered with DCl or NaOD. The bicarbonate concentration ranged from 5 to 250 mM. Higher concentrations are typical.

There is no doubt that amines form carbamates, and there is no doubt that amino acids act like amines in basic conditions. But what is the situation under near-physiological conditions? Can other L-α-amino acids, especially those with lower pK_a values, form a significant amount of carbamate under near-physiological conditions, and can their carbamates have the potential to mimic one of the neurotransmitters? The quantitative data required to predict the extent of the formation of carbamates *in vivo* simply do not exist. Furthermore, the methods to study these reactions under conditions resembling physiological do not exist.

The addition of a carboxylate group will place an additional negative charge on an amine as shown in eq 2. Therefore, reverse-phase liquid chromatography (RPLC) with a cationic surfactant is a natural choice of technique to distinguish carbamates from the free amines. This separation technique is sensitive to molecular charge.[26-35]

This report describes and critically assesses the application of RPLC in the presence of cetyltrimethylammonium bromide to the determination of conditional K_c values of some amino acids and peptides. The particular goals of the current work are to develop a sensitive method to determine carbamate formation quantitatively, to determine the kinetic stability of the carbamates on the chromatographic time scale, and to measure conditional formation constants of representative amino acids and small peptides. After the method has been established we will seek to find a relationship between the formation constant and chemical properties of the amine.

Experimental Section

Reagents. The following reagents were used without further purification: all amino acids and peptides (Sigma, St. Louis, MO), sodium phosphate monobasic (EM Science, Cherry Hill, NJ), sodium phosphate dibasic (Fisher Scientific, Pittsburgh, PA), sodium tetraborate (J.T. Baker, Phillipsburgh, NJ), sodium bicarbonate (Mallinckrodt, Paris, KY), sodium bromide (Fisher), hydrochloric acid and sodium hydroxide (Fisher), *N*-acetylglycine (Sigma), glacial acetic acid and malonic acid (J.T. Baker). Cetyltrimethylammonium bromide (CTAB, Fisher) was recrystallized three times from 5% methanol (EM Science, HPLC grade) in acetone (Fisher) and was washed twice with diethyl ether (Fisher, purified grade) before use.[36]

Instrumentation. An LDC analytical ConstaMetric III pump operated at a flow rate of 1.00 mL/min was used to pump the mobile phase unless otherwise noted. A Rheodyne Model 7125 stainless steel injector with a sensing switch and a 20-μL injection loop was used along with 100-μL

(14) (a) Jensen, A.; Faurholt, C. *Acta Chem. Scand.* **1952**, *6*, 385. (b) Jensen, A.; Jensen, B.; Faurholt, C. *Acta Chem. Scand.* **1952**, *6*, 395. (c) Jorgen, O.; Vejlry, K.; Faurholt, C. *Acta Chem. Scand.* **1952**, *6*, 398. (d) Jensen, A.; Jensen, M. B.; Faurholt, C. *Acta Chem. Scand.* **1952**, *6*, 1073. (e) Jensen, A.; Christensen, R.; Faurholt, C. *Acta Chem. Scand.* **1952**, *6*, 1086. (f) Jensen, A.; Jensen, M. B.; Faurholt, C. *Acta Chem. Scand.* **1954**, *8*, 1129. (g) Jensen, A.; Christensen, R. *Acta Chem. Scand.* **1956**, *9*, 486. (h) Jensen, M. B. *Acta Chem. Scand.* **1957**, *11*, 499. (i) Gronvald, M.; Faurholt, C. *Acta Chem. Scand.* **1960**, *14*, 1374. (j) Johansen, E.; Faurholt, C. *Acta Chem. Scand.* **1960**, *14*, 2240. (k) Jensen, M. B.; Faurholt, C. *Acta Chem. Scand.* **1964**, *18*, 377. (l) Henriksen, O.; Jensen, M. B. *Acta Chem. Scand.* **1967**, *21*, 2819.

(15) Morrow, J. S.; Keim, P. *J. Biol. Chem.* **1974**, *249*, 7484.

(16) (a) Roughton, F. J. W.; Rossi-Bernardi, L. *CO_2: Chemical, Biochemical, and Physiological Aspects*; NASA SP-188; Washington, DC, 1969; p 41–45. (b) Forster, R. E. *CO_2: Chemical, Biochemical, and Physiological Aspects*; NASA SP-188; Washington, DC, 1969; p 55–9. (c) Roughton, F. J. W.; Rossi-Bernardi, L. *CO_2: Chemical, Biochemical, and Physiological Aspects*; NASA SP-188; Washington, DC, 1969; p 61–4.

(17) (a) Roughton, F. J. W. *Physiol. Rev.* **1935**, *15*, 241. (b) Meldrum, N. U.; Roughton, F. J. W. *J. Physiol.* **1933**, *80*, 143. (c) Ferguson, J. K. W.; Roughton, F. J. W. *J. Physiol.* **1934**, *83*, 68. (d) Ferguson, J. K. W.; Roughton, F. J. W. *J. Physiol.* **1934**, *83*, 87. (e) Roughton, F. J. W. *J. Physiol.* **1935**, *85*, 17.

(18) (a) Milla, E.; Geiustina, G.; Margaria, R. *G. Biochim.* **1953**, *2*, 153. (b) Milla, E.; Geiustina, G.; Margaria, R. *G. Biochim.* **1953**, *2*, 434.

(19) Kilmartin, J. V.; Rossi-Bernardi, L. *Nature* **1969**, *222*, 1243.

(20) Divers, E. *J. Chem. Soc.* **1970**, *23*, 171.

(21) (a) Henriques, O. M. *Biochem. Z.* **1928**, *200*, 124. (b) Henriques, O. M. *Biochem. Z.* **1931**, *243*, 241. (c) Henriques, O. M. *Biochem. Z.* **1933**, *260*, 58.

(22) Armarego, W. L. F.; Milloy, B. A. *J. Chem. Soc., Perkin Trans. 1* **1974**, 1905.

(23) Sherry, A. D.; Malloy, C. R.; Jeffrey, F. M. H.; Chavez, F.; Scere, H. K. *J. Magn. Reson.* **1990**, *89*, 391.

(24) Martino, R.; Malet-Martino, M. C.; Vialaneix, C.; Lopez, A.; Bon, M. *Drug Metab. Dispos.* **1987**, *5*, 897.

(25) Lemieux, R. U.; Barton, M. A. *Can. J. Chem.* **1970**, *49*, 767.

(26) Melander, W. R.; Horváth, C.; Tomlinson, E.; Riley, C. M. *Ion-Pair Chromatography*; Chromatographic Science Series; Marcel Dekker, Inc.: New York, 1985, Chapters 2 and 3.

(27) Karger, B. L.; LePage, J. N.; Tanaka, N. *High Performance Liquid Chromatography, Advances and Perspectives*; Academic Press: New York, 1980; Vol. 1, pp 113–206.

(28) Deming, S. N. *CRC Handbook of HPLC for separation of amino acids, peptides and proteins*; CRC Press Inc.: Boca Raton, FL, 1984; Vol. 1, pp 141–152.

(29) Knox, J. H.; Laird, G. *J. Chromatogr.* **1976**, *122*, 17.

(30) Knox J. H.; Hartwick, R. A. *J. Chromatogr.* **1981**, *204*, 3.

(31) Bidlingmeyer, B. A.; Deming, S. N.; Price, W. P.; Sachok, B.; Petrusek, M. *J. Chromatogr.* **1979**, *186*, 419.

(32) Stranahan, J. J.; Deming, S. N. *Anal. Chem.* **1982**, *54*, 2251.

(33) Cantwell, F. F.; Puon, S. *Anal. Chem.* **1979**, *51*, 623.

(34) Staahlberg, J. *Chromatographia* **1987**, *24*, 783 and 820.

(35) Weber, S. G. *Talanta* **1989**, *36*, 99.

(36) Dearden, L. V.; Wooley, E. M. *J. Phys. Chem.* **1987**, *91*, 2404.

Table I. Phosphate–Borate Buffer System[37] Used in the Experiment

pH	$[NaH_2PO_4 \cdot H_2O]$, mM	$[Na_2B_4O_7 \cdot 10H_2O]$, mM
6.52	167.0	33.0
7.17	122.0	48.0
7.35	114.5	50.5
7.55	106.0	53.0
7.84	96.5	56.5
7.98	89.0	59.0
8.31	71.0	65.0
8.60	51.5	71.5
8.82	32.0	78.0
9.49[a]	25.0	70.0
10.31[b]	20.0	65.0

[a] Plus 64.5 mM NaOH. [b] Plus 113.0 mM NaOH.

Hamilton syringes for injections. Waters Nova-Pak C18 analytical columns (3.9 × 150 mm long, 4-μm spherical particle with 60-Å pore size) were purchased from Waters Associates, Millipore Corporation, 34 Maple Street, Milford, MA. A 4.1 × 150 mm Hamilton PRP-3 analytical column with 10-μm spherical poly(styrene–divinylbenzene) 300-Å pore size particles was purchased from Phenomenex, Torrance, CA. Each column was used with a guard column of the same material from the same supplier. Analytes were detected by either a DuPont 852 UV–vis spectrophotometer or a Gilson Model HM UV–vis dual beam spectrophotometer (10-mm lightpath and 70-μL cell), operated at 230 and 210 nm, respectively. In some cases both detectors were used in series.

The pH was measured with an Orion Research pH meter and Fisher Scientific glass electrode.

Chromatograms were collected at 3 points/s by EZChrom (Scientific Software, San Ramon, CA), version 4.5 neg DT, coupled with a DT2802 chromatography interface (Data Translation, Marlboro, MA) on a 25-MHz DKT-386 IBM-compatible PC.

Mobile Phase Preparation. All the buffers contained phosphate and borate[37] and, for pH greater than 9, NaOH. Table I lists the analytical concentrations of sodium phosphate and sodium borate used in the buffers. There are three classes of mobile phases: class A, B, and C, which have the same ratio of [phosphate]/[borate] at a certain pH, as shown in Table I. Mobile phase A also contains 34.0 mM NaBr, B contains 12.5 mM $NaHCO_3$ and 21.5 mM NaBr, and C has 25.0 mM $NaHCO_3$ and 9.0 mM NaBr. The total calculated ionic strength of each mobile phase is 0.30 M. All mobile phases contain 0.70 mM CTAB, which is below its critical micelle concentration.[38] All mobile phases were prepared the night before the experiment with doubly deionized water and were filtered through Millipore aqueous solvent filter paper, type AH (0.45 μm). The containers of mobile phases B and C were sealed after preparation and during the experiment.

Column Preparation. The first coating of CTAB onto the stationary phase of a new column was achieved by passing 150 mL of mobile phase A at 1.00 mL/min through the column. Following this, the solvent was pumped and recycled at 1.00 mL/min (overnight) until a total of 1.35 L of mobile phase had passed through the column. Equilibration of mobile phase and stationary phase was checked by injection of malonic acid at the beginning and the end of the run. Equilibration was reached when the retention times of malonic acid were reproducible.

Chromatographic Procedure. When a new mobile phase with a different total CO_2 content but with the same pH was used, the passage of 100 mL of solvent at 1.00 mL/min was needed before the first injection. When a new mobile phase with a different pH and total CO_2 contentwas used, the passage of 300 mL of solvent at 0.4 mL/min (overnight) was required before the first injection.

All solutions of amino acids and peptides were prepared in their corresponding mobile phases at least 1 h prior to injection unless noted. Injection of water was used to determine the thermodynamic dead volume[39] of the column.[40,41] Temperature and pH were recorded before and after each mobile phase was used. The system temperature was not independently controlled. Experience had demonstrated to us that environmental control was adequate. The ambient temperature was measured six times daily. For the analysis shown in the figures (there are many data not shown in this paper), there were 66 temperature measurements made. The temperature ranged from 25.0 to 26.4 °C, with a mean of 26.0 and a standard deviation of 0.18 °C. pH deviation was within ±0.01 pH unit.

Table II. k'_{RH}/k'_{RCOO^-} Values from Experiments and Regressions

RH	$RCOO^-$	conditions[a]	n[b]	k' ratio	SD
acetate	malonate	A	22	0.391	0.035
acetate	malonate	B	20	0.365	0.047
acetate	malonate	C	22	0.345	0.049

[a] A: No carbon dioxide. B: 12.5 mM total carbonate. C: 25 mM total carbonate. [b] Number of experiments.

Table III. Retention Time of Met, min

	mobile phases					
	pH 6.5		pH 7.5		pH 8.1	
injection	CO_2	no CO_2	CO_2	no CO_2	CO_2	no CO_2
CO_2	1.46	1.47	1.79	1.50	2.58	1.62
no CO_2	1.46	1.48	1.79	1.50	2.45	1.61

The reversibility of the reaction on the chromatographic time scale was checked by using phosphate buffer with and without bicarbonate as mobile phases along with the Nova-Pak C18 column at pH values of 6.5, 7.5, and 8.1. For all other studies, the PRP column was used.

Nonlinear Regression. Estimates of K_c were obtained from nonlinear regression applied to retention time–pH data. The program used the modified Gauss–Newton algorithm. The modification had been done in the program before it was purchased. The program was originally developed by Danuso.[42] It has been automated and installed under the Stata version 3.0 environment by Royston.[43]

Tactics used in the nonlinear regression procedure were to vary the starting values of the parameters and the form of the function fit. Fitting of the curve was done using initial values that generated initial estimates of capacity factor ratio versus pH curves which will approach the actual curves from four different directions: left–down, right–down, left–up, and right–up. Along each direction, different initial values were also used. Goodness of fit was judged by the outcome of the curve, the R^2 value, and the standard error of the parameters.

All amino acids and peptides used are in their L-α-configurations. Standard three-letter abbreviations will be used for amino acid solutes. One letter abbreviations will be used for amino acids in peptides.

All errors reported are 1 standard deviation.

Results

Sensitivity of the Retention to Addition of COO^-. Table II shows the ratio of k' values for the solutes acetate and malonate, which differ by the substitution of a COO^- group for H. This is the same substitution that occurs on conversion of an amine to a carbamate.

Reversibility of Carbamate Formation and Breakdown. The reversibility of the reaction on the chromatographic time scale was checked by a two-factor–two-level experiment; solutes were prepared in solvent with and without carbon dioxide. These solutions equilibrated for less than 5 min before they were injected individually into mobile phases with and without bicarbonate. Injections were made as a function of time. The first injection was 5 min after preparation, and the last one was performed >2 h later. Only one peak was observed during the whole experiment (>2 h) for each of the compounds injected. Table III gives the retention times for a typical amino acid, Met.

(37) Dean, J. A. *Lange's Handbook of Chemistry*, 13th ed.; McGraw-Hill, Inc.: New York, 1985; Section 5, Tables 5-23 and 5-24.
(38) Pramauro, E.; Pelizzeti, E. *Trends Anal. Chem.* **1988**, *7*, 260.
(39) Knox, J. H.; Kaliszan, R. *J. Chromatogr.* **1985**, *349*, 211.
(40) Vit, M. P.; Fahnrich, J. *J. Chromatogr.* **1983**, *281*, 293.
(41) McCormick, R. M.; Karger, B. L. *Anal. Chem.* **1980**, *52*, 2249.
(42) Danuso, F. sgl: Nonlinear regression command. State Technical Bulletin 1; 1991; p 17.
(43) Royston, P. sgl.2: Nonlinear regression command. State Technical Bulletin 7; 1992; p 11.
(44) King, E. J. *J. Am. Chem. Soc.* **1951**, *73*, 1515.
(45) Batchelder, A. C.; Schmidt, C. L. A. *J. Phys. Chem.* **1940**, *44*, 880.
(46) Rey, F.; Varela, A.; Antelo, J. M.; Arce, F. *J. Chem. Eng. Data* **1989**, *34*, 35.
(47) Batchelder, A. C.; Schmidt, C. L. A. *J. Phys. Chem.* **1940**, *44*, 893.
(48) Saxena, R. S.; Gupta, A. *Acta Chim. Hung.* **1984**, *116*, 327.
(49) Smith, M. *Critical Stability Constants*; Plenum Press: New York and London, 1974; Vols. 1 and 2.

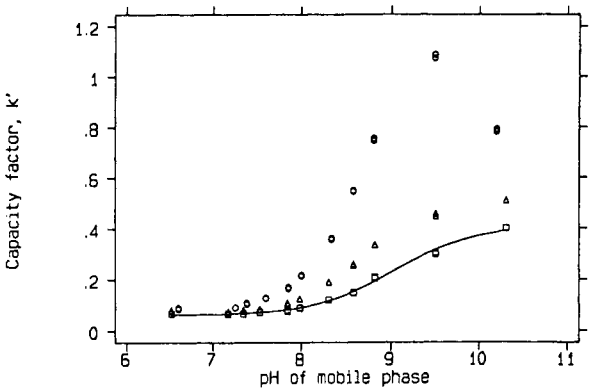


Figure 1. Capacity factor of Met in different mobile phases: (□) no carbonate; (△) 12.5 mM total carbonate; (○) 25.0 mM total carbonate.

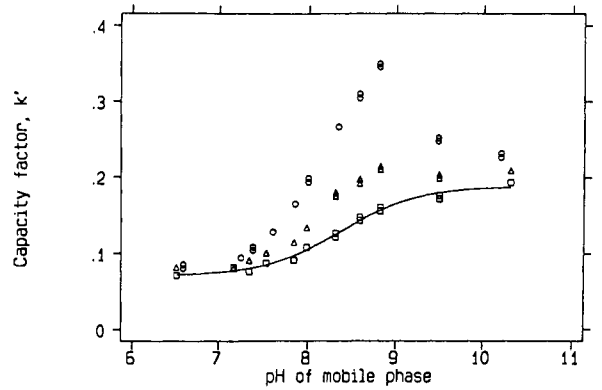


Figure 2. Capacity factor of AG in different mobile phases: (□) no carbonate; (△) 12.5 mM total carbonate; (○) 25.0 mM total carbonate.

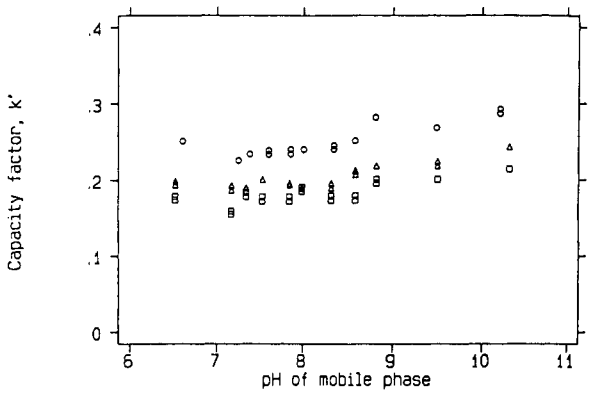


Figure 3. Capacity factor of NAcG in different mobile phases: (□) no carbonate; (△) 12.5 mM total carbonate; (○) 25.0 mM total carbonate.

Chromatographic Titration Curves. Four representative k'_A (without bicarbonate), k'_B (with 12.5 mM total carbonate), and k'_C (with 25.0 mM total carbonate) versus pH curves are shown in Figures 1–4 for the solutes Met, AG, *N*-acetyl-Gly (NAcG), and acetic acid (AcOH), respectively. The solid curves of Met and AG result from the nonlinear regression results with pK_a values of 9.04 and 8.38, respectively.

$k'_{amine,c}/k'_{amine}$ vs pH. Five representative k'_B/k'_A and k'_C/k'_A versus pH curves are shown in Figures 5–9 for Pro, Met, AG, NAcG, and AcOH. The solid curves for Pro, Met, and AG are the results from nonlinear regression, and the solid curves of NAcG and AcOH are the average values.

Carbamate Formation Constants. Carbamate formation constants along with percentage carbamate formed at pH 7.4, total carbonate (TC) = 25.0 mM, I = 0.30 M, and T = 26.0 °C, obtained from nonlinear regression are given in Table IV. R^2 values are greater than 0.99. The correlation coefficient between K_a and K_c is always less than 0.5. The model was relatively insensitive to K_a changes but sensitive to K_c changes.

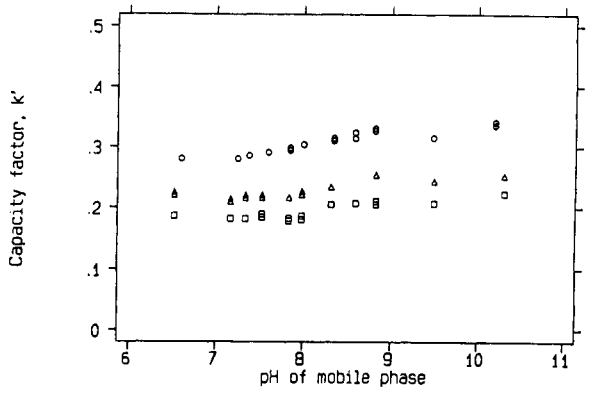


Figure 4. Capacity factor of AcOH in different mobile phases: (□) no carbonate; (△) 12.5 mM total carbonate; (○) 25.0 mM total carbonate.

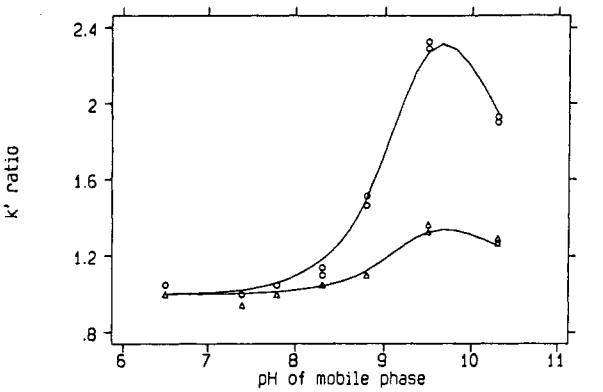


Figure 5. k' ratio plot of Pro: (△) k'_B/k'_A; (○) k'_C/k'_A.

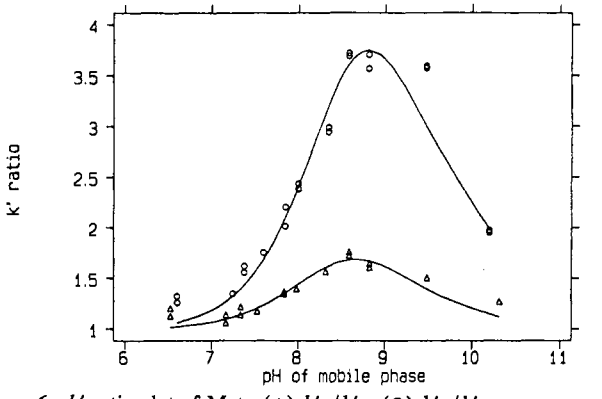


Figure 6. k' ratio plot of Met: (△) k'_B/k'_A; (○) k'_C/k'_A.

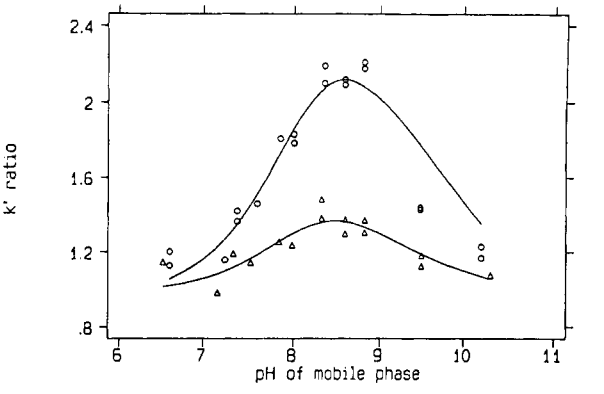


Figure 7. k' ratio plot of AG: (△) k'_B/k'_A; (○) k'_C/k'_A.

Discussion

Analysis of the Method. It is first important to establish that the substitution of a CO_2 group for a proton results in an increase in retention in the chromatographic system that contains CTAB.

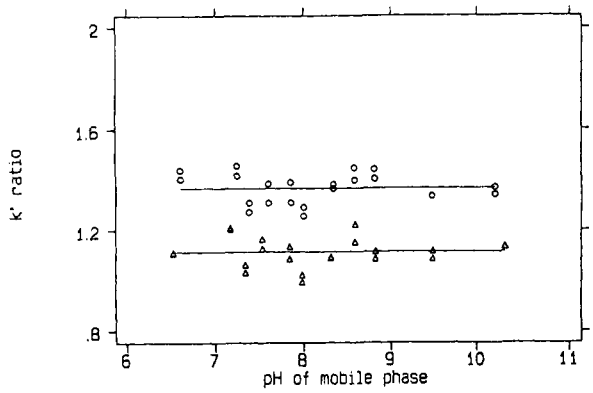


Figure 8. k' ratio plot of NAcG: (△) k'_B/k'_A; (○) k'_C/k'_A.

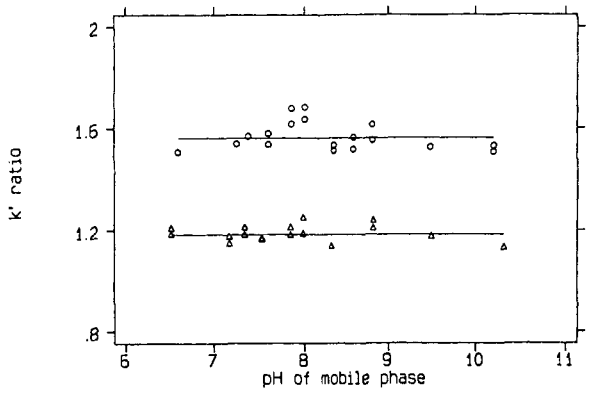


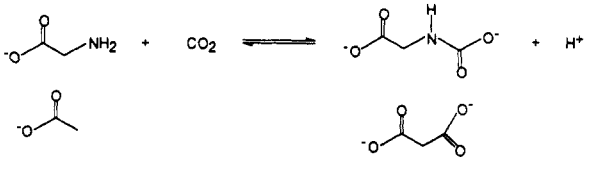
Figure 9. k' ratio plot of AcOH: (△) k'_B/k'_A; (○) k'_C/k'_A.

Table IV. Conditional Carbamate Formation Constants and Percentage Carbamate at pH = 7.4

solute	pK_a[a]	$K_c \pm s$[c]	$pK_c \pm s$[c]	100Z
Gly	9.58[44]	1.58×10^{-5}	4.80	0.26
GP	8.60[b]	$5.22 \times 10^{-5} \pm 6.75 \times 10^{-7}$	4.28 ± 0.01	7.37
GG	8.38[b]	$5.44 \times 10^{-5} \pm 7.13 \times 10^{-6}$	4.26 ± 0.13	12.2
GGG	8.26[b]	$5.78 \times 10^{-5} \pm 6.75 \times 10^{-6}$	4.24 ± 0.12	14.9
Ala	9.68[45]	$1.27 \times 10^{-5} \pm 1.30 \times 10^{-6}$	4.90 ± 0.10	0.16
AG	8.38[b]	$2.48 \times 10^{-5} \pm 1.50 \times 10^{-6}$	4.61 ± 0.06	5.62
AAA	8.37[b]	$2.11 \times 10^{-5} \pm 8.00 \times 10^{-7}$	4.68 ± 0.04	4.98
Asn	8.68[b]	$1.15 \times 10^{-5} \pm 7.73 \times 10^{-7}$	4.94 ± 0.07	1.39
Met	9.04[b]	$6.87 \times 10^{-5} \pm 5.53 \times 10^{-6}$	4.16 ● 0.08	3.72
Ser	9.24[46]	$1.87 \times 10^{-5} \pm 2.69 \times 10^{-6}$	4.73 ± 0.14	0.67
Asp	9.52[47]	$2.52 \times 10^{-5} \pm 2.50 \times 10^{-8}$	4.60 ± 0.00	0.48
Pro	10.4[48]	$6.28 \times 10^{-5} \pm 2.80 \times 10^{-6}$	4.20 ± 0.04	0.16

[a] pK_a of ammonium form of the amino group under our experimental conditions. [b] Determined by this experiment. [c] Run-to-run standard deviation.

Scheme I



The acetate/malonate pair is analogous to the monoanionic Gly and its carbamate (Scheme I). The comparison of malonate to acetate shows that a proton has been lost and a carboxylate has been added, just as in the carbamate formation.

Table II shows that the ratio of k' values (acetate/malonate) is about 0.37 for the whole pH range being studied (6.5–10.5). The standard deviations are large, about 12% of the mean, but acceptable for data taken over a period of weeks. There is a small shift in the ratio as the CO_2 content in the mobile phase changes, resulting from the change in the ionic content of the eluent.[40] We can conclude that the chromatographic system is sensitive to the substitution of COO^- for H in a molecule over the pH range of interest.

The kinetics of the carbamate formation play a crucial role in the separation. There are three kinetic regimes. The chemical relaxation time, τ_c, can be much shorter than the separations relaxation time, t; then only one peak will be observed. Second, τ_c can be commensurate with t; then there will be two overlapping peaks in the chromatogram. Finally, if τ_c is much larger than t, there will be two separate peaks for the carbamate and the corresponding amine if the capacity factors of the two are different.

Table III shows the retention times of Met in six chromatographic systems; with and without CO_2 at three pH values. The two rows correspond to the injections of Met that had been equilibrated with CO_2 (+) and without CO_2 (–). The main result is that the influence of CO_2 on retention does not require that CO_2 be injected with the analyte. If it is the carbamate formation that shifts the retention, then the formation occurs on the column. Another observation, consistent with the latter, is that there is a single peak from the injections of Met. A test for the relative rates of the chemical and the separation relaxations can be performed by calculating the Damkoehler number, Da,[50] which is given by the ratio of residence time in the mobile phase to the relaxation time of the reaction. It has been used for theoretical studies of the influence of chemical kinetics on chromatographic peak shape and position.[51,52]

$$\mathrm{Da} = \frac{L}{\mu_0} k_1 \left([\mathrm{CO_2}] + \frac{[\mathrm{H^+}]}{K_c} \right) \tag{3}$$

where L = length of the column, μ_0 = velocity of an unretained solute, and k_1 = the forward rate constant in reaction 2. Values of Da generated from eq 3 using the forward rate constant and the equilibrium constant for Gly ($k_1 = 1.26 \times 10^4$ M^{-1} s^{-1}) from Caplow's[13] paper range from 1470 (pH 6.5) to 0.2 (pH 10.3) at TC = 12.5 mM. Values generated using Faurholt's data[14b] are higher, ranging from 8700 to 1.2 under the same conditions. When Da is much less than unity, two peaks would be expected. When Da is around unity, a single broadened peak is expected. Indeed, somewhat broadened peaks were observed for all the amino acids and peptides tested at pH values greater than 8.8. We may expect that this is a general result for amino acids and peptides because Faurholt has found that, for 21 amines with pK_a values ranging from 8 to 12, the value of k_1 fell in the narrow range $1.11 \times 10^5 \pm 7.6 \times 10^3$ M^{-1} s^{-1}.

The solid curves in Figures 1 and 2 show the calculated effect of ammonium ion dissociation on retention in the absence of CO_2. As the pH increases, the fraction of amino acid in the zwitterionic form decreases, that in the anionic form increases, and hence retention time increases. The shapes of these curves are analogous to those observed for the carboxylic proton dissociation by Horvath *et al.*[53] using RPLC and by Kong *et al.*,[54] using RPLC with an anionic surfactant and organic modifier. The other two sets of points represent retention in the presence of CO_2/HCO_3^-. These data clearly show the effect of the presence of CO_2/HCO_3^- on the overall capacity factor. Figures 3 and 4 are for two controls, NAcG and AcOH. The curves are flat, which indicates that there is no effect of pH on the retention of these fully ionized carboxylates over the range studied, in the presence or in the absence of CO_2.

We conclude, then, that the shift in the retention of amines that occurs at high pH and in the presence of CO_2 is caused by the reversible formation of the carbamate in the chromatographic system. We will use this shift quantitatively to determine the

(50) Damkoehler, G. Z. *Electrochem.* **1936**, *42*, 846.
(51) Melander, W. R.; Lin, H. J.; Jacobson, J.; Horváth, C. *J. Phys. Chem.* **1984**, *88*, 4527.
(52) Jeng, C. Y.; Langer, S. H. *J. Chromatogr.* **1992**, *589*, 1.
(53) Horváth, C.; Melander, W.; Molnar, I.; Molnar, P. *Anal. Chem.* **1977**, *49*, 2295.
(54) Kong, R. C.; Sachok, S.; Deming, S. N. *J. Chromatogr.* **1980**, *199*, 307.

formation constants of several amino acids and peptides in the following way. The k' for a solute that exists in several forms in chemical equilibrium is the mass-weighted average of the k' values of the individual forms. Thus, for the case in the absence of CO_2, the observed overall capacity factor, k'_{amine}, is

$$k'_{amine} = k'_p f_p + k'_d f_d \quad (4)$$

where f_p and f_d are the mole fractions of the ammonium (protonated) form and the amine (deprotonated) form, respectively, and k'_p and k'_d are the corresponding k' values.

The ratio of the fraction of ammonium ion and amine is a constant, A, for a given pH and ammonium ion K_a, thus

$$f_p = \frac{A}{1+A} \quad (5)$$

$$f_d = \frac{1}{1+A} \quad (6)$$

where $A = [H^+]/K_a$and

$$k'_{amine} = \frac{Ak'_p + k'_d}{A+1} \quad (7)$$

In the presence of CO_2 there are three forms in simultaneous equilibrium

$$f_{p,c} + f_{d,c} + Z = 1 \quad (8)$$

where Z represents the fraction of the amine that is carbamate. Note that the ratio $f_{p,c}/f_{d,c}$ is still A. However, the sum of the two fractions is now

$$f_{p,c} + f_{d,c} = 1 - Z \quad (9)$$

Here we will consider that the fraction of the amine that exists as the carbamic acid is negligible. This is a good assumption for basic solutions. This leads to the following expression for the $k'_{amine,c}$, the observed overall k' value for the amine in a CO_2-containing mobile phase.

$$k'_{amine,c} = (1 - Z)k'_{amine} + Zk'_c \quad (10)$$

where k'_c is the k' value of the carbamate. The ratio of the k' values in the presence of CO_2 to those in its absence is

$$\frac{k'_{amine,c}}{k'_{amine}} = 1 - Z + Z\frac{k'_c}{k'_{amine}} \quad (11)$$

Substitution of eq 5 into eq 12 leads to eq 13, which was fit to the data.

$$\frac{k'_{amine,c}}{k'_{amine}} = \left\{\frac{[H^+] + K_a}{K_a k'_{d,c} + [H^+]k'_{p,c}} - 1\right\}Z + 1 \quad (12)$$

where

$$Z = \left\{\left(\frac{H^+}{K_c(TC)}\right)\left(1 + \frac{K'_1}{[H^+]} + \frac{K'_1K'_2}{[H^+]^2}\right)\left(1 + \frac{[H^+]}{K_a}\right) + 1\right\}^{-1} \quad (13)$$

$$k'_{p,c} = \frac{k'_p}{k'_c}$$

$$k'_{d,c} = \frac{k'_d}{k'_c}$$

$$K'_1 = \frac{[HCO_3^-][H^+]}{[CO_2]}$$

$$K'_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]}$$

Thermodynamic K'_1 and K'_2 values from the literature[55] were corrected by the Davis[56] equation to our experimental conditions, $I = 0.30$ M and $T = 299$ K. The values used in eq 12 were $K'_1 = 10^{-6.05}$ M and $K'_2 = 10^{-9.74}$ M.

The weakness of the approach is that we do not know k'_c. As we must determine the unknown, chemically meaningful parameter K_c by fitting eq 12 to data, it is in principle possible to determine k_c' simultaneously. However, numerical experiments have shown that many of the parameters in the nonlinear regression are correlated. We are loathe to accept the results of nonlinear regression in which there is a significant correlation between pairs of parameters. Because of the correlation, k'_c cannot be determined from the regression. This has led to the following numerical approach.

The value of pK_c for Gly has been determined by many workers.[12,13,14b,15,16a] We have taken that value of 4.80 as a known constant. The pK_a of the ammonium form of Gly is 9.58.[44] By taking these parameters as constants, nonlinear least squares regression using eq 12 and our experimental data can be used to estimate $k'_{p,c}$ and $k'_{d,c}$ at each of the two concentrations of CO_2/HCO_3^-. We then assume that these values hold true for all other amino acids and peptides. This is a central assumption, which is not beyond question. The assumption is consistent with the Martin equation,[57] which simply states that the overall free energy change for a molecule sorbing to the stationary phase is the summation of free energy terms from different fragments of the molecule.

Scheme I shows the analogy between the acetate/malonate pair and the pair consisting of glycine and its carbamate. Values of $k'_{d,c}$ should be comparable to the k' ratios shown in Table II if the assumption is valid. For mobile phase condition B, $k_{d,c}$ is 0.42 ($n = 2$, SD = 0.22), while, for mobile phase condition C, it is 0.31 ($n = 2$, SD = 0.05). The larger error in case B is due to the relatively small retention shift at 12.5 mM TC. The conditions in the chromatography are never such that complete formation of the carbamate from the amino acid occurs, so the k' ratio is only indirectly measured through the regression. Nonetheless, there is acceptable agreement between these values and those in Table II.

We would like to use these values for $k_{d,c}$ for all of the amino acids and peptides. However, there is a small concern that the k' shift due to the formation of a carbamate would be significantly influenced by the amino acid R group. While we cannot resolve this issue directly, there are analogous data which seem to support the presence of a negligible effect of the R group. The influence of dissociation on the retention of a number of phenylacetic acids in reversed-phase liquid chromatograpy has been determined by Horváth *et al.*[58] The ratio k'_{RCOO^-}/k'_{RCOOH} was 0.336 ± 0.048, $n = 6$. The consistency of the ratio is the important point, not the numerical value. The phenylacetic acids studied had a wide range of functional groups and substitution patterns, yet the k' ratio discussed was virtually constant. Our admittedly small data set also shows consistency. These facts along with the Martin equation justify our use of $k'_{d,c}$ and $k'_{p,c}$ as constants for a given TC.

The ratios of the upper curves of k' versus pH, with CO_2, to the lower one, without CO_2, are generated and shown in Figures

(55) Edsall, J. T.; Wyman, J. *Biopysical Chemistry*; Academic Press, Inc.: New York, 1958; Chapter 10.
(56) Butler, J. N. *Ionic Equilibrium, A Mathematical Approach*; Addison-Wesley Publishing Co., Inc.: Reading, MA, 1964; Chapter 12, pp 429–450.
(57) Karger, B. L.; Snyder, L. R.; Horváth, C. *An Introduction to Separation Science*; John Wiley and Sons: New York, 1973; pp 55–61.
(58) Horváth, C.; Melander, W.; Molnar, I. *Anal. Chem.* **1977**, *49 (1)*, 142.

5–9 for the solutes Pro, Met, and AG and for the controls NAcG and AcOH, respectively. A few qualitative conclusions can be drawn from the graphs. A comparison of the Pro, Met, and AG curves with the controls shows that the method is sensitive to carbamate formation. The maximum[12,15,23–25] results from the opposing influence of pH on the concentration of the reactants; increasing the pH increases [RNH_2] at the expense of [$R-NH_3^+$], but it decreases [CO_2]. The method yields well-defined peaks, which is important to the success of the regression.

Determinate errors and imprecision in the method can originate from several sources. The low retention time of analytes, especially at low pH values is the largest problem. When t_R approaches t_0, retention is minimal and k' approaches zero. There are two implications of having a low k'. A simple propagation of errors treatment shows that (see eq 14) the relative error in k' is inversely proportional to the difference $t_R - t_0$, if t_0 is known precisely. Thus, the error in measuring k' becomes larger the closer t_R is to t_0.

$$\frac{\sigma_{k'}}{k'} = \frac{\sigma_{t_R}}{t_R - t_0} \tag{14}$$

Second, there is a potential determinate error. The simple quantity t_0 is notoriously difficult to determine accurately,[38] so to guard against an unduly large influence of t_0 on the accuracy of the k' ratio, t_R should be as large as possible, as can be seen in eq 15.

$$k'_x/k'_y = \frac{t_{R,x} - t_0}{t_{R,y} - t_0} \tag{15}$$

Errors in k', of course, lead to errors in the values of K_c determined from the regression. This problem limits the application of the method at present to amines with conditional formation constants $>10^{-6}$. The assumption made in the regression, that $k'_{p,c}$ and $k'_{d,c}$ are constant, is another possible error. The agreement shown in Table II and the results of Horváth *et al.*[58] for phenylacetic acids indicate that the assumption is a good one. Finally, correlations and local minima are possible in all nonlinear regressions. We have made efforts to eliminate the latter source of error by seeking solutions from many starting places. The assumption referred to above has allowed us to do the regression with at most two parameters. In all cases, the correlation coefficient between the parameters determined by nonlinear least squares, K_a and K_c, was less than 0.5, which is quite acceptable.

There are disadvantages of the method. First of all, different ions present in the mobile phase can yield different surface potentials on the stationary phase and therefore affect the retention times for charged species.[59] This problem is reflected by the baseline shift of the k' ratio plots of the controls (Figures 8 and 9) as well as the amines (Figures 5–7). The shift is caused by the substitution of bromide for bicarbonate to keep the ionic strength constant.[59] Because of this ion effect, the k' ratio is not doubled when the total carbonate concentration is doubled. This is also shown by the $k'_{acetate}/k'_{malonate}$ ratio and values in Table II; condition B has larger values than condition C. We have made an effort to eliminate this problem by using the same types of ions in the different mobile phases. Second, the method is more tedious and time consuming than spectroscopic methods.

Conditional K_c for Some Amino Acids and Peptides. Quantitative results from nonlinear regression are shown in Table IV. The Brønsted plot of pK_c versus pK_a determined in this experiment is shown in Figure 10. The graph shows no relationship between pK_c and the pK_a, as was the case shown by Caplow[13] for a wider variety of amines. The poor correlation in the Brønsted plot could be due to the presence of extra stabilization or destabilization energy provided by intramolecular interactions of some carbamates but not others. For example, the large K_c values observed for thiosemicarbazide[13] ($pK_a = 1.75$, $pK_c = 4.21$), semicarbazide[13] ($pK_a = 3.65$, $pK_c = 4.79$), and hydrazine[13] ($pK_a = 8.20$, $pK_c =$ 4.68) could be due to the enhancement of the nucleophilicity of the amines by the unshared pairs of electrons on the neighbor group.[60] The unexpectedly small K_c of aniline ($pK_a = 4.58$, $pK_c = 6.02$) determined by Caplow[13] was explained by amine stabilization from resonance.[13] We speculate that the side chains of some amino acids and peptides can play an important role on the relative carbamate formation constants.

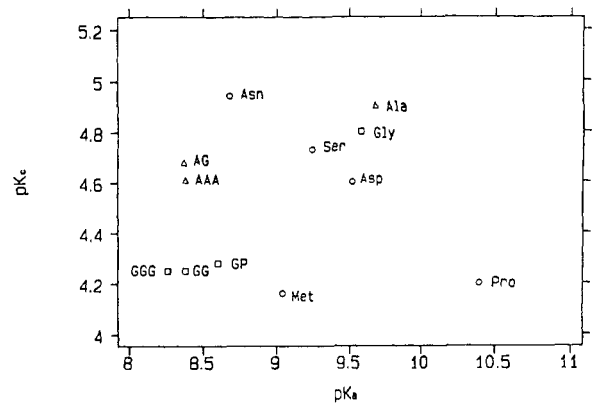


Figure 10. Conditional carbamate formation constant versus ammonium pK_a: (□) Gly family; (△) Ala family; (○) others.

Table V. pK_c Values Determined by Faurholt Using Ba^{2+} Precipitation

compound	pK_a[a,49]	pK_c[b]
Ala	9.88	5.14[14a]
Gly	9.78	4.64[14b]
Gly-Gly	8.25	4.32[14l]
Gly-Gly-Gly	8.09	4.21[14c]
n-propylamine	10.57	4.21[14f]
sec-propylamine	10.67	4.89[14f]
n-butylamine	10.64	4.30[14h]
sec-butylamine	10.56	4.78[14h]
tert-butylamine	10.69	5.20[14h]

[a] Thermodynamic dissociation constants of ammonium; $T = 298$ K and $I = 0$. [b] $pK_c = pK'_1 - pK_{Eq}$; K_{Eq} was defined and used by Faurholt.[14]

Structure and reactivity. Gly, GP, GG, and GGG (□) vs Ala, AG, and AAA (△). The Gly family has higher formation constants than the Ala family, as determined by this experiment and in agreement with the literature.[14a,b] The only difference between these two series is the R group on the α-C, which is H for Gly and CH_3 for Ala. A consideration of the structure[61] shows that the methyl group of the Ala family can interfere sterically with the *N*-carboxylate group. Steric effects also explain Faurholt's pK_c values of *n*-, *sec*-, and *tert*-alkylamines (see Table V). Qualitatively, our data obtained by RPLC (see Table IV) agree with those of Faurholt obtained by precipitation (see Table V); pK_c decreases in the order of Ala > Gly > GG > GGG. Quantitative comparison is complicated because of different conditions and methods used.

The larger K_c for peptides, as compared to amino acids, could be explained by the interaction between the carbamate oxygens and the amide proton[62–67] (in a seven-membered ring) or the electrostatic interaction between the carbamate oxygen and the amide nitrogen.[68–71] Such structures, which certainly are present in nonpolar solvents, are less important in water,[72] so the explanation is not entirely satisfying.

(59) Stahlberg, J.; Hagglund, I. *Anal. Chem.* **1988**, *60*, 1958.
(60) Carey, F. A.; Sundberg, R. J. *Advanced Organic Chemistry*; Plenum Press: New York, 1990; Part A, pp 284–90.
(61) Stryer, L. *Biochemistry*, 3rd ed.; W. H. Freeman and Co.: New York, 1988; pp 18–20.
(62) Novoa, J. J.; Whangbo, M. H. *J. Am. Chem. Soc.* **1991**, *113*, 9017.
(63) Smith, D. A.; Vijayakumar, S. *Tetrahedron Lett.* **1991**, *32*, 3613.
(64) Smith, D. A.; Vijayakumar, S. *Tetrahedron Lett.* **1991**, *32*, 3617.
(65) Gellman, S. H.; Dado, G. P. *Tetrahedron Lett.* **1991**, *32*, 7377.
(66) Dado, G. P.; Gellman, H. *J. Am. Chem. Soc.* **1992**, *114*, 3138.
(67) Guo, H.; Karplus, M. *J. Phys. Chem.* **1992**, *96*, 7273.
(68) Song, S.; Asher, S. A.; Krimm, S.; Shaw, K. D. *J. Am. Chem. Soc.* **1991**, *113*, 6359.
(69) Wang, Y.; Spiro, T. G.; Purrello, R.; Jordan, T.; Spiro, T. G. *J. Am. Chem. Soc.* **1991**, *113*, 6359.

Percentage Carbamate vs pK_a of the Amine. Table IV shows the mole fractions of carbamates for the various amino acids and peptides being studied in this report at pH = 7.4 and TC = 25.0 mM.

Equations 1, 2, and 8 can be rearranged to yield

$$\frac{1}{Z} = 1 + \frac{H^+}{K_c[CO_2]}\left(1 + \frac{[H^+]}{K_a}\right) \qquad (16)$$

If $[H^+]$ and K_c were constant, then $1/Z$ would be a linear function of $(1 + [H^+]/K_a)$. Linear regression of data at pH 7.4 for 12 determinations of Z at pH 7.4 shows a significant ($p < 0.005$) correlation, but a rather poor fit ($r^2 = 0.57$), and an intercept near unity (0.91). Thus, the general trend for a group of amines is that the fraction existing as carbamate at pH 7.4 increases, due to mass action, as pK_a decreases. That having been said, it is still necessary to know K_c to predict accurately the quantity of a particular amine that exists as the carbamate.

The reason for the loose correlation observed is the relatively small range of K_c (1 order of magnitude) that exists in the group of amines with K_a values ranging over >2 orders of magnitude. As a consequence of this, the concentration of the amine form of the amino acid varies more than K_c, so its concentration, rather than its thermodynamic tendency to react, dominates. From the admittedly small set of data, one can tentatively conclude that carbamate formation in solutions at neutral or near-neutral pH is probably unimportant for amines with pK_a >9.5.

Comments on Neurobiological Issues. The principle focus of this work has been to demonstrate the need for a flexible method to determine amino acid and peptide carbamate formation constants and to develop and evaluate such a method. Even though we have not yet determined formation constants for all of the amino acids, or even for all of the most important amino acids, we have learned enough to make some speculative comments on certain aspects of the biological implications of carbamates.

It is important to recognize that carbamate formation occurs with CO_2 *per se*. Increased CO_2 in the inspired air causes profound effects on brain function. The actions of CO_2 are not simple, as depression, increased excitability, seizures, and anesthesia are observed depending on the concentration of CO_2.[73] In this context it is interesting to note that Woodbury and Karler in 1960 discussed carbamate formation as one possible cause for their findings.[73] It is obvious from our results that increased carbamate formation from amino acids and peptides occurs as a function of elevated CO_2 and that this potentially may have importance for the adverse effects on brain function. An altered carbamate/native amino acid (or peptide) ratio may, for example, alter the substrate availability for enzymes inside brain cells and change neuroactive substances to inactive substances, and *vice versa*, in the extracellular space (see Introduction).

Examples of situations other than inspiration which may result in increased carbamate formation include seizures in the hippocampus which are accompanied by moderate elevations in CO_2 concentrations,[74] and the dramatic elevation of CO_2 observed during ischemia.[75] In the latter case the parallel fall in pH may counteract carbamate formation. After brain ischemia, intracellular alkalosis has been reported, which would favor carbamate formation.[76] Rapid alkalinization of the brain extracellular space is observed during excitatory synaptic transmission.[77] As carbamate formation also is a fast process, increased extracellular carbamate formation is theoretically possible during such events.

$$HCO_3^- + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CO_2 + H_2O \qquad (17)$$

The presence of carbonic anhydrase (CA), a glial enzyme which promotes the hydration of CO_2 to carbonic acid (reaction 17), assures that the local brain concentration of CO_2 is kept fairly close to its equilibrium value.[78] However, as glial cells develop late during maturation, the CA concentration is extremely low in young compared to adult rats.[78] The spontaneous CO_2 equilibrium with carbonic acid is slow. The rate constants ($k_1 = 5.5 \times 10^4$ M^{-1} s^{-1} and $k_{-1} = 0.0375$ s^{-1})[79] indicate that the time required for a concentration perturbation to relax by 99% is 40 min at pH 7.4 and 150 min at pH 8.0. Thus, carbamate formation in normal situations and during seizures, ischemia, and synaptic transmission may be more pronounced in young individuals compared to adult ones, due to the lack of CA and consequently higher local CO_2 concentrations.

Summary and Conclusions

A new and sensitive method for the study of carbamate formation has been developed and evaluted. The reliability of the method has been confirmed by comparing the results determined in this experiment with the results in the literature. Although this experiment was run under conditions of [total amine] = ≤1 mM, [total carbonate] = 12.5 and 25.0 mM, I = 0.30 M, and T = 299 K, other conditions more nearly physiological are possible. Amine concentrations can be lowered, ionic strength can range from 0.05 to 0.5, and the temperature can cover the range from about 0 to 70 °C. The method can also be used at any pH consistent with chemical stability of the column packing material.

Carbamate formation and breakdown has been shown to be much faster than the chromatographic time scale (seconds) for pH values less than about 9. The separation relaxation time can be decreased if a shorter and a higher efficiency column is employed at a higher flow rate. The chemical relaxation time, on the other hand, can be increased by decreasing the concentrations of the reactants and the temperature of the solution. The combination of these two approaches will lower the Damkoehler number, which might enable the method to obtain kinetic information on the carbamate reaction. Another advantage of working at lower Damkoehler number is in analysis. Under low-Damkoehler-number conditions one can envision the separation of injected carbamates. Therefore the direct determination of them in physiological samples may be possible.

Conditional carbamate formation constants for some amino acids and small peptides were determined for the first time. No relationship between the formation constants and the pK_a of the amines was found, but a general relationship was found between the percentage of carbamate at a certain pH and the amine pK_a for the compounds studied in this report. It is suggested that amino acids with pK_a values greater than 9.5 do not form significant amounts of carbamates under physiological conditions.

Acknowledgment. We are grateful to the Medical Research Council of Sweden (Grants K91-12V-09772-01 and B93-12X-00164-29B) and the National Institutes of Health (Grant GM-44842) for financial support of the research. M.S. is supported by the Swedish National Science Research Council (Grant B-Bu 1905-308).

(70) Wang, Y.; Spiro, T. G.; Purrello, R.; Georgiou, S.; Spiro, T. G. *J. Am. Chem. Soc.* **1991**, *113*, 6368.
(71) Mirkin, N. G.; Krimm, S. *J. Am. Chem. Soc.* **1991**, *113*, 9742.
(72) (a) Portnova, S. L.; Bystrov, V. F.; Tsetlin, V. I.; Ivanov, V. T.; Ovchinnikov, Y. A. *Zh. Obshch. Khim.* **1968**, *38*, 428. (b) Weinstein, B. *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*; Marcel Dekker; New York, 1974; Vol. 3, p 21.
(73) Woodbury, D. M.; Karler, R. *Anesthesiology* **1960**, *21*, 686.
(74) Pinard, E.; Trembley, E.; Ben-Ari, Y.; Sexlaz, J. *Neuroscience (Oxford)* **1984**, *13*, 1039.
(75) Kraig, R. P.; Pulsinelli, W. A.; Plum, P. *Am. J. Physiol.* **1986**, R348.
(76) Chopp, M.; Linde, V.; Chen, H.; Knight, R.; Helpern, J. A.; Welch, K. M. A. *Stroke* **1990**, *21*, 63.
(77) Chen, J. C. T.; Chester, M. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7786.
(78) Woodbury, D. M.; Engstrom, F. I.; White, H. S.; Chen, C. F.; Kemp, J. M.; Chow, S. Y. *Ann. Neurol.* **1984**, suppl. 16, 5135.
(79) Gibbons, B. H.; Edsall, J. T. *J. Biol. Chem.* **1963**, *238* (10), 3502.