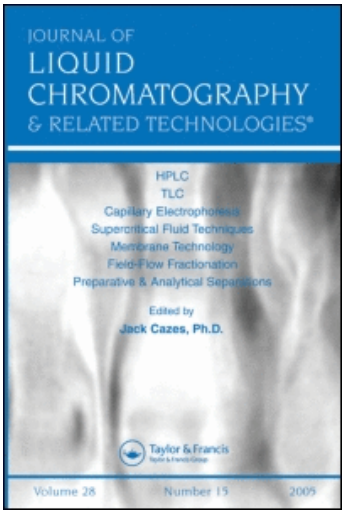

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THE INFLUENCE OF PEPTIDE STRUCTURE ON THE RETENTION OF SMALL CHAIN PEPTIDES ON REVERSE STATIONARY PHASES

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ABSTRACT

Several characteristic structural features of peptides were considered in a study on the retention of small chain peptides on C_8 and polystyrene-divinylbenzene reverse stationary phases. These include the effects of subunit nonpolar, polar, acidic, and basic side chains, the influence of the location (terminal or interior) of a nonpolar subunit in the peptide chain, effects of two or more nonpolar subunits and their location (terminal or interior) in the peptide chain, and the effect of two chiral centers and their location (terminal and/or interior) in the peptide chain. LC data, which were collected in an acidic, zwitterion pH, and basic mobile phase where possible, indicated that location of the side chains in the peptide relative to the terminal charge sites is also an important factor in determining peptide retention. Peptides with side chains containing acidic or basic groups were studied as a function of mobile phase pH. Whether these groups are ionized or not and their location relative to the terminal charged sites strongly influences peptide retention.

INTRODUCTION

Amino acids (AA) and peptides in the past have often been separated by ion exchanger procedures. Recently, modern reversed

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phase liquid chromatography (RPLC), which offers improved versatility, efficiency, and shorter analysis times has been widely used for these separations. Although most investigations have utilized alkyl-modified silica as the stationary phase (1-4), separations by RPLC are also possible on polystyrene-divinylbenzene (PSDV) copolymers which act as nonpolar adsorbents (5,6). With both stationary phases key mobile phase variables are pH, ionic strength, water-organic solvent ratio, and the addition of counterions. Since the PSDV stationary phases are stable throughout the entire pH range they permit a basic mobile phase condition to be used where the AA and peptides can be separated in their anionic form. In contrast alkyl-modified silicas are stable only up to pH 8.

Similarities exist between the two types of stationary phases when applied to AA and peptide separations. For example, manipulation of the mobile phase variables to improve resolution or to alter selectivity is similar; that is, retention decreases as organic modifier concentration increases, eluting strength is in the order CH_3CN > EtOH > MeOH, and the analyte retention is lowest when the mobile phase is at the zwitterion pH. For both stationary phases retention from a buffered mobile phase free of hydrophobic counterions is dominated by interactions between hydrophobic centers within the AA and peptides and the hydrophobic surface of the stationary phase (1-6). If hydrophobic counterions are present, then a double layer type interaction, which is strongly influenced by an ion exchange selectivity prevails under defined experimental conditions (7).

Control of the elutropic properties of the mobile phase and the hydrophobic nature of the stationary phase focuses attention on how intrinsic hydrophobicities exhibited by AA and peptides influences their retention. Success in this goal has already been achieved. For example, AA retention on alkyl-modified silica (2) and PSDV (5,6) can be correlated to carbon number in the AA side chain or to hydrophobicity (8) for the side chain. Retention also correlates to AA partition coefficients in octanol-water distribution (2). For dipeptides, where one AA subunit varies, retention can be correlated to the hydrophobicity of the side chain for the variable AA unit (2,3,5,6,9) while for peptides with repeating AA units, log retention increases linearly as the repeating AA units increase (2,5,6). The effects of mobile phase pH on dipeptide retention appears to be systematic (5,6,10) while di-and tripeptide diastereomer separations follow a structure related elution order (4).

Retention coefficients for individual AA subunits have been estimated via numerical analysis procedures from retention data obtained for a series of long chain peptides of known structure using a known, fixed mobile phase gradient (11-15). These constants are then used to predict the retention time for other peptides providing the same gradient is used and the AA composition of the peptide is known. In general, the correlation between actual and predicted retention times have been favorable (11-15), although there is some disagreement about the coefficient values for certain subunits (15).

The goal of this investigation is to evaluate how individual AA subunits within the peptide chain influence retention of the peptide. Since the AA side chain can be polar, nonpolar, acidic, or basic its influence on retention can be large. The approach taken was to study the retention of a series of peptides whose structures were systematically modified with respect to chirality, type, and location of individual AA subunits (terminal or peptide interior) in the peptide chain. Both alkyl-modified silica and PSDV reverse stationary phases were used with acidic, zwitterion pH, and basic (only with the PSDV stationary phase) mobile phases where the solvent is a water-organic modifier mixture. Since the terminal and side chain acidic or basic groups can be ionized, depending on mobile phase pH, the combined effect of these charge sites and the peptide structure can be evaluated.

EXPERIMENTAL

Chemicals and Instrumentation

Amino acids and many peptides were obtained from Sigma Chemical Co., Chemalog, Vega Biochemicals, and Research Plus. Several peptides were synthesized by reaction of a specific AA or peptide of known structure with (tert-butoxycarbonyl)-L-amino acid N-hydroxysuccinimide ester (BOC-AA). This reaction adds the AA from the BOC-AA to the N-terminus of the starting AA or peptide through the formation of a peptide bond (16,17). Organic solvents and water were LC quality while all inorganic salts were analytical reagent grade.

The PRP-1 (4.1 mm x 150 mm) column, which is a spherical, 10 μm, PSDV copolymer of large surface area and porosity, was obtained from Hamilton Co. The alkyl-modified silica columns were 3.2 and 4.6 mm x 250 mm, 10 μm, Lichrosorb C_8 (Altex and E/M) and 4.6 mm x 250 mm, 10 μm μ-Bondapak C_{18} (Waters). Both Altex Model 332 and Waters Model 202 LC instruments equipped with fixed wavelength detectors or with a Tracor Model 970 or Spectra Physics 770 variable wavelength detector were used.

Procedures

Sample solutions (about 1 mg/ml) were prepared by dissolving mg quantities of the AA or peptide in H_2O, EtOH, or their combination in 6 ml Hypovials fitted with Hycar Septa (Pierce Chemical). Samples were refrigerated when not in use. Operating conditions generally involved sample aliquots of 10 μl, 1 ml/min flow rate, inlet pressures of about 500 to 1400 psi depending on column and eluting condition, detection at either 254 or 208 nm, and controlled temperature at 25°C. Mixed solvents are expressed as per cent by volume. The mobile phase pH was maintained with HCl, NaOH, and phosphate buffer while ionic strength was controlled with added NaCl. Details of these basic LC procedures and calculation of column void volume and capacity factors are provided elsewhere (4-6,7).

RESULTS AND DISCUSSION

Amino acids and peptides change from cations (pH < 2), to zwitterions, and finally to anions (pH > 10) in solution as the pH is increased from an acidic to a basic condition. The charge

form of the AA or peptide strongly influences their retention on reverse stationary phases; in general, a well-defined retention minimum is found when the mobile phase is at the zwitterion pH (5,6,10). If the side chains contain acidic or basic groups, ionization at these sites will contribute to the overall charge on the AA or peptide depending on the pH and ionization constants of the sites. When the mobile phase pH is adjusted so that these groups are also ionized retention is greatly reduced. If all contributing ionization sites are considered the influence of pH on retention can be quantitatively described and predicted (5,10).

Initial LC experiments focused on the retention of AA and a series of dipeptides that differed in only one of the AA subunits. These data were consistent with previous results (5,6) in that retention was found to increase as the hydrophobic property of the side chain increased. The effect was the largest from an acidic or basic mobile phase where the charge resides at either the AA or dipeptide terminal $-NH_2$ or $-CO_2H$ group, respectively, and the smallest from a mobile phase at the zwitterion pH where both groups are charged. These trends were observed for both the PSDV and alkyl-modified silica stationary phases. It should be noted that in these and subsequent studies, LC experiments on the latter stationary phase where restricted to a mobile phase pH of 2 to 8. Thus, the full effect of the presence of anionic sites within the AA and peptides on their retention is only realized with the PSDV stationary phase.

If the side chains also contain acidic or basic sites retention is significantly reduced at mobile phase pH conditions

TABLE I

Retention of Acidic and Basic Dipeptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'			
	pH			
Dipeptide	2.0	5.8	8.6	11.6
L-Tyr-L-Tyr	29.3	6.35	35	0.04
L-Tyr-L-Glu	3.38	0.20	0.48	0
L-Glu-L-Tyr	7.88	0.31	0.24	0
L-Tyr-L-Val	15.4	2.03	19.3	0.48
L-Val-L-Tyr	15.4	1.65	16.5	0.43
L-Tyr-L-Arg	1.67	0.91	6.18	0.31
L-Arg-L-Tyr	4.31	0.82	2.54	0.19

A 10 μm, 150 x 4.1 mm, PRP-1 column using a 1:99 CH_3CN:H_2O, phosphate buffer, μ=0.10M mobile phase at 1.0 ml/min.

where these sites are also ionized. Data illustrating this are shown in Table I (only results for a PSDV stationary phase are shown) where retention of dipeptides with two acidic side chains (Tyr-Glu and Tyr-Tyr), one acidic and one hydrophobic (Tyr-Val), and one acidic and one basic (Tyr-Arg) can be compared.

The first case provides the greatest change in retention since the dipeptides in basic solution (pH = 11.6) are multivalent anions; that is, both side chain acidic sites as well as the terminal carboxyl groups are ionized extensively. In acidic

solution the hydrophobic effect of the side chain, where the order is Tyr > Glu, increases retention since only the terminal amine group is ionized. Furthermore, the hydrophobic effect of Tyr is greater when it is in position 2 in the dipeptide or when it is furthest from the terminal charged site. Introducing Val into a Tyr dipeptide eliminates one potential ionization site and thus, the retention increases in a basic mobile phase due to the hydrophobic influence of the Val side chain. In an acidic mobile phase the hydrophobicity of the Val side chain and its effect on retention are intermediate relative to the Tyr and Glu side chains.

For the dipeptides in Table I that contain both an acidic and a basic side chain a suppressed retention due to additional ionization at these sites is seen in both an acidic and basic (pH = 11.6) mobile phase. The higher retention of L-Arg-L-Tyr over L-Tyr-L-Arg from an acidic mobile phase and the opposite for a basic mobile phase is consistent with the fact that the hydrophobic side chain is the furthest from the charged site in this order.

The Glu acidic side chain (pK_a = 4.36) ionizes at a lower pH than the Tyr side chain (pK_a = 10.47). This difference has a large effect on retention at mobile phase pHs between the zwitterion pH and a very basic pH. In this region (see data at pH = 8.6 in Table I) the Glu side chain is readily ionized and retention is suppressed. In contrast a higher mobile phase pH is required to bring about appreciable ionization of the Tyr side chain. Thus, retention of the Tyr containing dipeptides increases as the mobile phase pH is increased from the zwitterion pH to a pH where ionization is

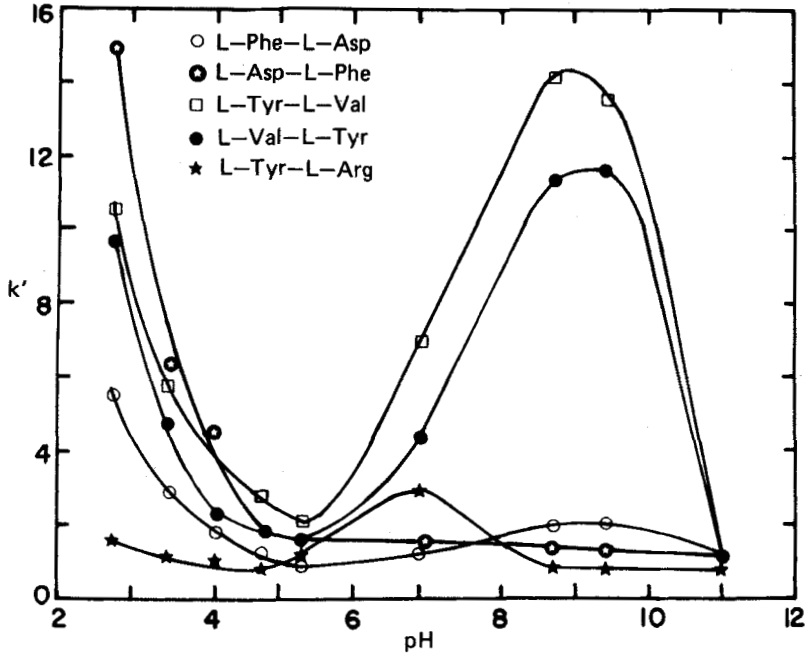


FIGURE 1

Retention of Acidic and Basic Dipeptides on PRP-1 as a Function of pH

A 4.1 x 150 mm, 10 μm, PRP-1 column and a 2:98 $CH_3CN:H_2O$, 1.0 x 10^{-2}M buffer (phosphate), μ = 0.10M (NaCl) mobile phase at a flow rate of 1.10 ml/min.

appreciable. At this point retention decreases sharply. Several examples illustrating this sharp change in retention with mobile phase pH are shown in Figure 1. These data clearly point out the need to carefully control mobile phase pH in order to maintain the expected selectivity when separating peptides with acidic and basic side chains. They also indicate that pH is a powerful tool for changing selectivity when separating peptides of this type from each other or from those without acidic or basic side chains.

No attempt was made to investigate retention of long chain peptides containing several acidic or basic side chains under pH conditions where these groups are and are not ionized. However, the data in Table I (see also Table VI and VII), as well as other data not reported here, indicate that retention would sharply decrease as the number of ionized sites increase, while under mobile phase pH conditions where the sites are not ionized, retention would increase according to the number and hydrophobicity of the side chains present in the peptide.

In order to focus on the effect of individual side chains in longer chain peptides, peptides were obtained or synthesized that have structures which systematically change with respect to side chain hydrophobicity, charge site, and/or chirality. Subsequently, retention data for these peptides were determined on the PSDV and alkyl-modified silica stationary phases as a function of mobile phase pH. Since the former stationary phase permits a wider mobile phase pH only data for this stationary phase are reported here (18).

The effect of a repeating hydrophobic side chain on retention from an acidic, zwitterion pH, and basic mobile phase is shown in Figure 2 where retention of a series of $(L\text{-}Ala)_x$ peptides is correlated to the peptides hydrophobicity. This was determined by a summation of the assigned hydrophobicity (0.53) for each Ala subunit according to Rekker (8). Similar results were obtained on the C_8 column at pH conditions where column stability is maintained. As hydrophobicity increases, retention increases

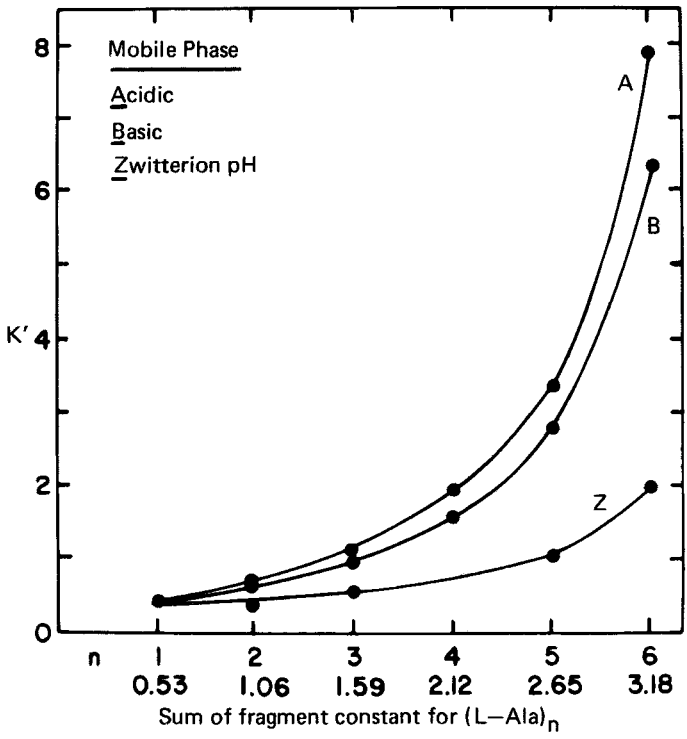


FIGURE 2

Retention of a Series of $(L\text{-}Ala)_x$ Peptides as a Function of Hydrophobicity

Conditions are the same as Figure 1 except the solvent is 100% H_2O and pH = 1.75, 5.20, and 11.00.

systematically whether the analyte is an anion, a cation, or a zwitterion. However, the rate of change is the lowest for the latter condition or where both the terminal $-NH_2$ and $-CO_2H$ groups are ionized.

Table II lists retention data as a function of mobile phase pH for a series of $L\text{-}Phe\text{-}(Gly)_x$ peptides where the Phe unit is systematically shifted through the peptide. Since the charge on

TABLE II

Retention of L-Phe-$(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
Peptide	2.1	5.9	10.0
L-Phe	2.32	0.98	1.58
Gly-L-Phe	6.44	1.05	2.34
L-Phe-Gly	3.00	1.40	3.29
L-Phe-Gly-Gly	2.59	1.33	4.13
Gly-Gly-L-Phe	8.07	1.10	1.78
L-Phe-Gly-Gly-Gly-Gly	2.40	1.63	5.27
Gly-L-Phe-Gly-Gly-Gly	5.03	2.81	5.95
Gly-Gly-L-Phe-Gly-Gly	6.50	3.13	4.18
Gly-Gly-Gly-L-Phe-Gly	6.75	2.60	3.60
Gly-Gly-Gly-Gly-L-Phe	9.27	1.42	1.87

Column-mobile phase conditions are the same as Table I except the mobile phase solvent is 5:95 $CH_3CN:H_2O$.

the peptide can be shifted from one end (terminal $-NH_2$ group) in the peptide to the other (terminal $-CO_2H$ group) or at both ends (at the zwitterion pH), the effect of the position of the hydrophobic side chain relative to the charged site can be ascertained. As the chain length of the L-Phe-$(Gly)_x$ peptides in Table II increases, the trend becomes more obvious and the effect is clearly

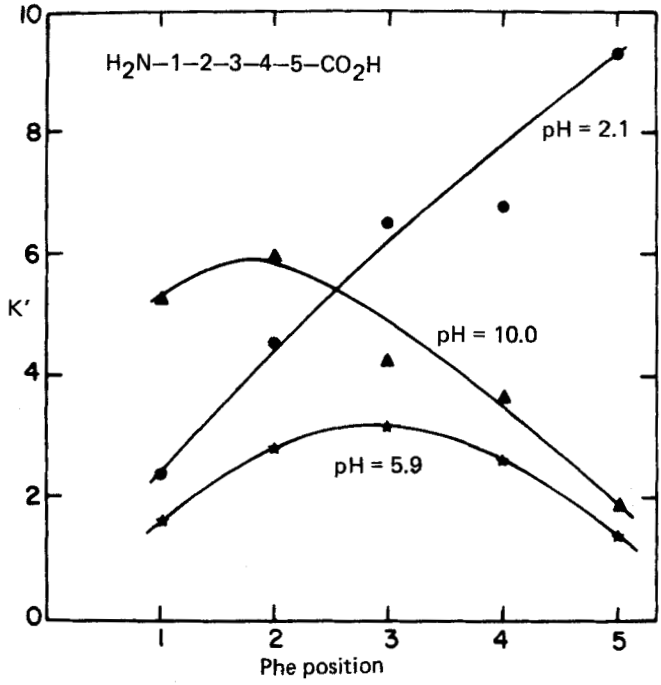


FIGURE 3

Effect of the Location of the Phe Subunit on the Retention of a Series of L-Phe-$(Gly)_4$ Peptides on PRP-1 as a Function of pH

Column conditions are given in Table II.

illustrated in Figure 3 where retention of the 5-unit peptides are plotted for the three mobile phase pH conditions as a function of the position of the L-Phe in the peptide. Whether the mobile phase is basic (terminal $-CO_2^-$ group) or acidic (terminal $-NH_3^+$ group), retention increases as the hydrophobic side chain is moved away from the charge site. In the former case this is when the side chain is at subunit 1 while in the latter this is at subunit 5. For a zwitterion pH, where both terminal groups are charged, the

highest retention is favored when the side chain is at subunit 3 or midway to the two charge centers.

The effects of two and three hydrophobic centers are shown in Table III and Table IV, respectively, where retention data for a series of $(L\text{-}Phe)_2\text{-}(Gly)_x$ and $(L\text{-}Phe)_3\text{-}(Gly)_x$ peptides are shown. Additional hydrophobic centers increase retention and consequently the mobile phase eluting power was increased (% CH_3CN increased) in order to reduce retention times. Although not all peptide combinations were available, the general trends in retention, even though more complex, are consistent with those observed for peptides with only one hydrophobic center (Table II). For example, the highest retention is favored by PPG and PPGG in basic solution where both hydrophobic centers are as far as possible from the $-CO_2^-$ terminal charged site and lowest in acidic solution where they are as close as possible to the $-NH_3^+$ terminal charged site. Similarly,at the zwitterion pH the highest retention is favored by those peptides where the hydrophobic centers are in the interior positions (GPPG, GPPGG) and lowest retention when these centers are at the terminal ends (for example, compare PGP to PGGP).

The correlation between peptide retention and a systematic peptide structure change is also illustrated in Table V. Whether Gly (set 2 peptides),Phe (set 3 peptides),or Phe-Phe (set 4 peptides) is added to the N-terminus of set 1 peptides, the retention order is essentially the same as found in their absence (compare to set 1 peptides). Other similar trends are apparent when comparing the peptides in Table II to IV.

TABLE III

Retention of $(L\text{-Phe})_2(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
Peptide	2.2	6.4	10.05
L-Phe-L-Phe	3.39	1.46	3.95
L-Phe-L-Phe-Gly	1.80	1.56	3.99
Gly-L-Phe-L-Phe	5.49	1.68	2.02
L-Phe-Gly-L-Phe	4.86	1.32	2.11
L-Phe-Gly-L-Phe-Gly	3.09	1.69	2.77
L-Phe-Gly-Gly-L-Phe	3.24	1.08	2.79
Gly-L-Phe-Gly-D,L-Phe[a]	5.02 5.53	1.64	1.90
Gly-L-Phe-L-Phe-Gly	3.69	2.84	2.52
L-Phe-L-Phe-Gly-Gly	1.45	1.57	3.94
Gly-L-Phe-Gly-L-Phe-Gly	3.64	1.87	2.31
Gly-L-Phe-L-Phe-Gly-Gly	3.20	2.34	2.40
Gly-L-Phe-Gly-Gly-L-Phe	4.36	1.31	1.39

Column-mobile phase conditions are the same as Table I except that the mobile phase solvent is 1:4 $CH_3CN:H_2O$.

a. Data are for LL and LD, respectively, where resolved.

TABLE IV

Retention of $(L\text{-}Phe)_3(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
	2.4	6.4	10.2
L-Phe-L-Phe	0.765	0.333	0.602
L-Phe-L-Phe-L-Phe	2.96	1.63	2.82
L-Phe-L-Phe-L-Phe-Gly	2.91	1.50	2.72
L-Phe-L-Phe-Gly-D,L-Phe	2.24 2.69	1.31 1.82	2.14
L-Phe-L-Phe-L-Phe-Gly-Gly	1.24	1.28	2.43
L-Phe-L-Phe-Gly-Gly-L-Phe	1.59	1.00	1.61
L-Phe-L-Phe-Gly-L-Phe-Gly	2.02	1.76	2.37

Column-mobile phase conditions are the same as Table I except the mobile phase solvent is 3:7 $CH_3CN:H_2O$. a. Data are for LLL and LLD, respectively, where resolved.

Several $(L\text{-}Leu)_y\text{-}(Gly)_x$ peptides were also studied (17). Although this series did not cover as great a range as with the $(L\text{-}Phe)_y\text{-}(Gly)_x$ peptides, the available data indicated similar retention trends that correlated to the number and location of the Leu hydrophobic side chains within the peptide chain.

Table VI lists retention data for several $(L\text{-}Tyr)\text{-}(Gly)_x$ peptides as a function of pH. In this series the single Tyr subunit introduces an acidic side chain which provides a charged site in addition to the terminal $-CO_2^-$ group providing the mobile phase

TABLE V

Correlation of Peptide Structure and Retention on PRP-1 for $(L\text{-Phe})_y(Gly)_x$ Peptides

Set	Acidic Mobile Phase
1	PGG < GGP
2	PPGG < PGGP ≃ PGPG
3	GPPGG < GPGPG < GPGGP
4	PPPGG < PPGGP < PPGPG
	Zwitterion pH Mobile Phase
1	GGP < PGG
2	PGGP < PGPG ≃ PPGG
3	GPGGP < GPGPG < GPPGG
4	PPGGP < PPPGG < PPGPG
	Basic Mobile Phase
1	GGP < PGG
2	PGGP ≃ PGPG < PPGG
3	GPGGP < GPGPG ≃ GPPGG
4	PPGGP < PPGPG < PPPGG

See Tables I to IV; P=L-Phe, G=Gly.

TABLE VI

Retention of $(L\text{-Tyr})(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
Peptide	2.0	5.9	11.1
L-Tyr	4.72	1.14	0
L-Tyr-Gly	5.60	1.94	0.10
Gly-L-Tyr	11.0	1.10	0
Gly-Gly-L-Tyr	15.6	1.46	0
Gly-L-Tyr-Gly	10.5	2.68	0.18
L-Tyr-Gly-Gly	5.34	2.28	0.16
Gly-Gly-Gly-L-Tyr	18.8	1.99	0.11
Gly-Gly-L-Tyr-Gly	12.8	3.83	0.13
Gly-L-Tyr-Gly-Gly	10.9	4.74	0.31
L-Tyr-Gly-Gly-Gly	5.96	2.97	0.24

Column-mobile phase conditions are the same as Table I except that the mobile phase solvent is 100% H_2O.

pH is high enough. Thus, in a strongly basic mobile phase retention is sharply reduced due to the additional charge. At the zwitterion pH and in an acidic mobile phase the Tyr subunit imparts its own hydrophobic contribution to the retention; as expected retention is low from the former and high from the latter mobile phase. Several other retention trends are apparent and are

consistent with those already indicated in the previous discussion when the influence of the additional charged sites are accounted for. For example: 1) In acid solution retention increases as the Tyr subunit is shifted further away from the charged N-terminus: 2) At the zwitterion pH the highest retention is favored when the Tyr subunit is moved to the chain interior. Also, higher retention occurs when the Tyr subunit is at the N-terminus rather than at the terminal $-CO_2H$ group. 3) In basic solution, even though retention is barely detectable, the data indicate that higher retention is favored when the Tyr subunit is located further away from the charged terminal $-CO_2H$ group. 4) Although not shown in Table VI, retention passes through a maximum between the zwitterion and strongly basic pH which correlates to the stepwise ionization of the terminal $-CO_2H$ group and side chain acidic site (see Figure 2) (5,10).

Table VII lists retention data for several $Trp(Gly)_xTyr$ peptides as a function of pH. The low retention at a basic pH and the high retention at a pH intermediate to the zwitterion and very basic pH is consistent with the ionization of the acidic side chain of the Tyr subunit. In basic solution retention increases as the number of Gly units increases but at the intermediate pH retention decreases. Retention is low at the zwitterion pH and high in acidic solution due to the hydrophobic contribution of Trp and Tyr. Unlike the $(Ala)_x$ series in Figure 2, retention passes through a maximum as the number of Gly units increases. The reason for this is not readily apparent since retention (see Table VI) increases in the order G-G-G-Tyr > G-G-Tyr > G-Tyr and the addition of the Trp subunit to the N-terminus of these chains

TABLE VII

Retention of L-Trp$(Gly)_x$ L-Tyr Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'			
	pH			
Peptide	2.0	5.9	9.5	11.1
L-Tyr	0.6	0.2	0.2	0
L-Trp	5.30	2.12	1.85	1.61
L-Trp-L-Tyr	28.1	7.43	15.9	1.31
L-Trp-Gly-L-Tyr	42.8	9.48	13.0	1.38
L-Trp-$(Gly)_2$-L-Tyr	34.8	9.92	11.6	1.41
L-Trp-$(Gly)_3$-L-Tyr	29.6	7.29	11.0	1.56
L-Trp-$(Gly)_4$-L-Tyr	27.1	7.10	10.8	1.77
L-Tyr-$(Gly)_2$-L-Trp	4.62	4.43	4.27	

Column-mobile phase conditions are the same as Table I except the mobile phase solvent is 1:9 $CH_3CN:H_2O$.

results in the retention maximum at Trp-G-G-Tyr. The effect of reversing Trp and Tyr is seen by comparing data for Tyr-G-G-Trp to Trp-G-G-Tyr in Table VII. Since the charged sites in the former are at both ends in a basic mobile phase, retention is much lower. In an acidic and zwitterion pH mobile phase switching the Tyr and Trp to N-terminus and carboxyl-terminus, respectively, reduces retention significantly.

Separation of dipeptide diastereomers on a C_8 (4) and PSDV (6) stationary phase has been reported previously. For dipeptides

with hydrophobic and hydrophilic side chains the data indicate that the L-L and D-D enantiomers always coelute first followed by the coelution of the D-L and L-D enantiomers regardless of mobile phase pH. Table VIII lists retention data for a series of $(Leu)_2(Gly)_x$ peptides where the two chiral centers provided by the two Leu subunits occupy different positions in the peptide chain. The effect of the hydrophobic side chain provided by the Leu subunits is consistent with the trends already described. However, the nature and location of the chiral centers also strongly influence peptide retention. When the Leu chiral centers are adjacent the L-L and D-D enantiomers coelute first as in the case of dipeptide diastereomers (4,6). When the chiral centers are not adjacent a significant change in selectivity is observed. That is, the L-L and D-D enantiomers coelute either second or very close to or with the L-D and D-L enantiomers. This trend is independent of mobile phase pH. However, the selectivity for the separation of the diastereomers, in general, favors the order acidic > basic > zwitterion mobile phase pH (see Table VIII). The trends shown in Table VIII were also observed on the C_8 stationary phase (18) at mobile phase pH conditions where column stability is maintained.

The observed elution order for the diastereomeric dipeptides can be correlated to their preferred molecular conformation (4). Since the two hydrophobic side chain groups and the two terminal groups, which are charged depending on the mobile phase pH, are on the same side in the D-L and L-D dipeptide enantiomers and on opposite sides for the L-L and D-D enantiomers, the retention of

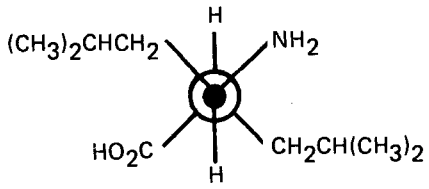
TABLE VIII

Retention of L-Leu-D,L-Leu(Gly)$_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

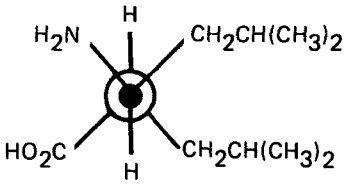
	Capacity Factor, k'					
	pH					
	2.20		6.25		10.1	
Peptide[a]	LL	LD	LL	LD	LL	LD
L-Leu-D,L-Leu	1.87	6.02	0.46	1.52	1.67	2.08
L-Leu-D,L-Leu-Gly	0.93	3.23	0.51	1.83	2.02	2.90
L-Leu-Gly-D,L-Leu	2.89	2.14	0.61	0.48	1.15	1.05
Gly-L-Leu-D,L-Leu	3.23	7.90	0.86	1.67	0.89	1.83
L-Leu-Gly-Gly-L,D-Leu	2.10	1.84	0.56	0.42	1.01	0.94
Gly-Gly-L-Leu-D,L-Leu	3.21	7.32	0.80	1.44	0.67	1.25
L-Leu-D,L-Leu-Gly-Gly	0.85	2.89	0.65	2.08	2.07	2.92
Gly-L-Leu-Gly-D,L-Leu	3.63	3.51	0.82	1.00	0.93	1.04
Gly-L-Leu-Gly-Gly-D,L-Leu	3.19	2.92	0.83	0.67	0.90	0.80
L-Leu-Gly-Gly-Gly-D,L-Leu	1.63	1.69	0.39	0.44	0.71	0.71
L-Leu-D,l-Asp	3.93	9.41	0	0	0	0.73
L-Leu-D,L-Arg	4.29	8.31	1.95	8.27	30	34

Column-mobile pahse conditions are the same as Table I except the mobile phase solvent is 15:85% CH_3CN:H_2O; for Leu-Asp and Leu-Arg it was 100% H_2O and the pH was 2.1, 5.9, and 9.5. a. Peak assignments were made from chromatographic data obtained for each enantiomer.

the former is greater due to the higher overall hydrophobic concentration of the side chains. This is illustrated below



L–L



L–D

where the conformation for the L-L and L-D dipeptide diastereomers for Leu-Leu are shown looking down the peptide chain. (The D-D and D-L forms would be the mirror images of the conformations shown.) This preferred conformation prevails when the two chiral centers are adjacent to each other in longer chain peptides. If the chiral centers are separated by a nonchiral subunit, such as Gly, the concentration of hydrophobic side chains in the preferred conformation changes. Construction of models using the criteria discussed elsewhere (4) indicates a slightly greater concentration of side chain hydrophobicity for separated chiral centers rather than for adjacent ones. For example, models of Leu-Gly-Leu indicate side chains on adjacent sides for L-G-L and D-G-D enantiomers and opposite sides for L-G-D and D-G-L enantiomers. However, the change is not large enough to produce a retention reversal but rather a similarity in retention (see Table VIII). If additional Gly subunits are inserted between the chiral centers the retention difference is reduced further. This is due to the increased separation of the

chiral centers and to the change in the preferred molecular conformation.

Several workers (19,20) have suggested that elution orders are reversed (D-L and L-D elute first followed by the coelution of the D-D and L-L enantiomers) for the separation of diastereomeric dipeptides containing basic side chains which are in the ionized form. The data in Table VIII do not agree with this and indicate that the elution order for diastereomers containing either ionized acidic or basic side chains is consistent with the retention order observed for other diastereomeric dipeptides (4,6).

The data reported here clearly indicate that modest structural differences in small chain peptides often lead to major changes in peptide selectivity, particularly when coupled with control of mobile phase pH. Often elution orders can be reversed by a suitable change in mobile phase pH. Consider the L-Phe-$(Gly)_4$ pentapeptide series in Figure 3 (see also Table II). In an acidic mobile phase retention with favorable resolution follows the order GGGGP > GGGPG > GGPGG > GPGGG > PGGGG while in a basic one the elution order is essentially reversed. If a zwitterion mobile phase pH is used GGPGG is retained the most, however, resolution of the mixture is poor compared to either an acidic or basic mobile phase.

Prediction of peptide elution time (11-15) is possible by the establishment of a coefficient characteristic of each AA as being either a terminal or an interior subunit. A mixed solvent

mobile phase gradient and a constant pH was usually used in establishing the AA coefficient (11-15). Since the alkyl-modified silica was the stationary phase, the constants were established only at an acidic pH (pH < 3) and/or near zwitterion pH conditions (pH 5 to 7). The data shown here clearly indicate, at least for small chain peptides, that the location of the subunit within the peptide interior significantly influences peptide retention. For example, GPGG, GGPGG, and GGGPG would be predicted to have identical elution times when using the AA coefficients. Figure 3 clearly indicates that this is not the case and the location of the Phe within the peptide significantly influences retention. Other examples demonstrating this are apparent from Tables II to VIII.

Several other factors, in addition to those indicated by Sasagawa et al. (15), may account for the variance in the reported AA coefficients. 1) The mixed solvent gradient usually covers a wide range of added organic modifier (often from 0 to 60%). This can influence the ionization of the weak acids and their salts and subsequently change the apparent pH of the mobile phase; the effect should be more noticeable in the acidic mobile phase. 2) The type of co-anion used for the buffer components can have a strong influence on the retention of the peptides, particularly if the peptides are retained in a cation (or anion) form via a double layer interaction (7,21-22). 3) The data reported here indicate that the retention is not independent of the location of a given AA subunit within the

peptide. However, it is important to note that for short chain peptides like those used in this study, peptide shape is probably not yet a significant factor. For example, approximately 4.3 AA subunits are required for one helix turn. In previous studies (11-15) long chain peptides were used and it is reasonable to suggest that peptide shape factors will tend to reduce and perhaps normalize individual contributions of AA side chains within the peptide.

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