CANADIAN THESES ON MICROFICHE

THÈSES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE

Canada

NL 339 (r. 86/08)

THE UNIVERSITY OF ALBERTA

Studies of Hydrophobicity Parameters in Peptides and Proteins

by

© Da-cheng Guo

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL 1986

ISBN 0-315-32555-0

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Da-cheng Guo

TITLE OF THESIS Studies of Hydrophobicity Parameters in Peptides and Proteins

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

YEAR THIS DEGREE GRANTED FALL 1986

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) Dacheng Guo

PERMANENT ADDRESS:

Department of Plant Science
the University of Alberta
Edmonton, Canada T6G 2P5

DATED September 4th 1986

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Studies of Hydrophobicity Parameters in Peptides and Proteins submitted by Da-cheng Guo in partial fulfilment of the requirements for the degree of Master of Science.

.....

Supervisor

.....

.....

.....

.....

Date.....August 25th, 1986.....

for my parents

ABSTRACT

Hydrocarbonaceous bonded stationary phases have been found to be useful physico-chemical tools in assessing the hydrophobicity of peptides. Reversed-phase chromatography at pH 2.0 is the most widely-used HPLC technique for separation of peptides. The ability to predict retention time and relative elution order of peptides in complex mixtures would be of great value. A set of amino acid residue retention coefficients was determined by examining the retention times of synthetic model peptides in reversed-phase high-performance liquid chromatography. Twenty synthetic octapeptides were prepared with the following sequence: Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted with the 20 amino acids found in proteins. The coefficients directly reflected the contribution of side chains of individual amino acid residues to the net hydrophobicity of a peptide. Thus, the retention parameters were used to predict the elution profiles of peptides of known composition during reversed-phase chromatography. A high degree of correlation (0.98) between predicted and observed retention times of 58 peptides not only indicated good predictive accuracy for the coefficients, but also proved that composition is generally the major factor affecting peptide retention time. A number of chromatographic factors were shown to influence peptide retention in reversed-phase

chromatography. These included column length, diameter, *n*-alkyl chain length and ligand density, gradient steepness, flow-rate, temperature, and pH, organic solvent, and ion-pairing reagents used for the mobile phase. Rules for retention time prediction were presented which enabled the experimenter to correct for many of these factors. The results obtained from the chromatograms of peptides and alkylphenones suggested that peptides interact with a reversed-phase support mainly by an adsorption-desorption mechanism and confirmed the importance of using peptide standards to monitor column performance when separating peptides. A set of hydrophilicity HPLC parameters, determined from the retention times of twenty model synthetic peptides at pH 7.0, was applied to predict which amino acid residues were on the surface of a protein and, thus, potentially antigenic. It was recognized that the HPLC hydrophobicity-hydrophilicity parameters obtained in this study correlated best with antigenicity in comparison with other sets of parameters. Furthermore, the predicted surface sites derived from a combination of the three parameters (hydrophilicity, accessibility and mobility) were correlated with the known antigenic sites from immunological studies and surface exposed residues determined by X-ray crystallographic data for several proteins.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. R.S. Hodges for suggesting this project to me, for always being fair and patient, and for his wonderful guidance in the course of my studies.

I would also like to acknowledge the help from Drs. Colin Mant, Bob Parker and Ashok Taneja for contributing to this project, and the help from Ms. Jennifer Van Eyk for preparing the figures. In particular, I would like to acknowledge Dr. Colin Mant for the initial work indicating the feasibility of this project and contributions in the ion-pairing research; Dr. Ashok Taneja for the synthesis of the peptide standards (S1-S5) and Dr. Bob Parker who carried out the computing aspect of the project for obtaining surface profiles for proteins. I also thank all the members of the lab for their help, encouragement, and friendship. It has been enjoyable to study in a lab with such a harmonious working atmosphere.

Special thanks go to the people in Dr.Smillie's lab for showing me the operation of their facilities, and especially to Mike Nattriss for doing the amino acid analyses. I would like to express my appreciation to my supervisory committee: Drs. Smillie, Kay and Vederas for volunteering their time and service. Finally, the financial assistance provided by the University of Alberta Biochemistry Department in the

form of a Graduate Research Assistantship is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Common Amino Acids

	3 letter	1 letter		3 letter	1 letter
Alanine	Ala	A	Lysine	Lys	K
Arginine	Arg	R	Methionine	Met	M
Asparagine	Asn	N	Phenylalanine	Phe	F
Aspartic Acid	Asp	D	Proline	Pro	P
Cysteine	Cys	C	Pyroglutamyl	<Glu	E
Glutamic Acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V
Leucine	Leu	L			

AA	amino acid
Ac	acetyl
aq.	aqueous
Boc	tert-Butyloxycarbonyl
Bzl	benzyl
CH_2Cl_2	dichloromethane
DCC	dicyclohexylcarbodiimide
DIEA	diisopropylethyl amine
DMF	dimethylformamide
For	formyl
g	gram
HI	hydrophobic interaction
HF	hydrogen fluoride
HFBA	*n*-heptafluorobutyric acid
h	hour
HPLC	high-performance liquid chromatography
I.D.	internal diameter

Mbh	4',4'-dimethoxybenzhydryl
MeOH	methanol
N^{im}	imidazole nitrogen
NH_2-terminal	amino terminal
OEt	ethyl ester
OBzl	benzyl ester
RP	reversed-phase
RPC	reversed-phase chromatography
R.T.	room temperature
TLC	thin layer chromatography
tg	gradient elapsed time
TFA	trifluoroacetic acid
Tos	4-toluenesulfonyl (tosyl)
SPPS	solid-phase peptide synthesis

I. INTRODUCTION

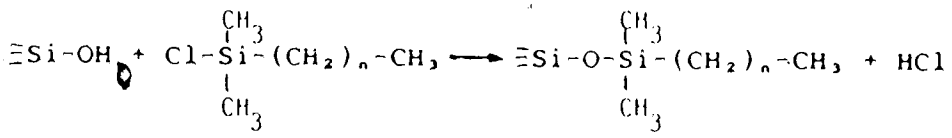
A. Reversed-Phase Chromatography

In modern reversed-phase chromatography, organic functional groups (ligates) are chemically bonded to the silica stationary phase. A characteristic of this technique is the use of bonded stationary phases having nonpolar hydrocarbonaceous ligates such as octyl- or octadecyl moieties with hydro-organic mobile phases. The term "reversed-phase" is used to distinguish this hydrophobic chromatography from the older technique which used polar stationary phases with less polar eluents and had been used almost exclusively in liquid chromatography. It is apparent that the new technique is viewed as a reversal of the relative polarities of the two chromatographic phases. Several comprehensive reviews of reversed-phase chromatography have been written by Regnier and Gooding (1980), Regnier (1983) and Horvath (1983). The best article about laboratory techniques was written by Wilson (1985).

Stationary Phase

Reversed-phase columns are generally composed of uniform porous silica microparticles to which non-polar ligates (e.g. alkyl chains) have been covalently linked via the silanol groups. In general, four basic methods have been used to chemically bond the stationary phase to the silica support. The most popular method is based on a silylation

reaction to form a siloxane (≡si-o-si≡) bond which is very stable in common chromatographic solvents (Colin and Guiochon, 1977; Johnson and Stevenson, 1978; Cooke and Olsen, 1980). Equation 1 represents the silylation reaction between a silica surface silanol and an alkyldimethylchlorosilane. The alkyl chain length may vary from C-1 to C-22. Octyl- (C-8) and octadecyl- (C-18) bonded phases are most often employed for reversed-phase separations (Monch and Dehnen, 1977).



(equation 1)

Because of steric hindrance, the bonding reaction is always incomplete. For example, coverage of nonpolar ligates on the silica support is generally only about 60%-70% of the silica surface area (Lochmuller and Wilder, 1979). The remaining area is occupied by underivatized silanol groups (Majors and Hopper, 1974) (see Fig. 1). Both nonpolar ligates and polar silanol functional groups may serve as binding sites at the stationary phase (Bij *et al.*, 1981). In terms of the alkyl chains as reversed-phase packing materials, the ligates are only 7 Å apart which allows multi-site adsorption of a peptide to the stationary phase (Geng and Regnier, 1984b). This phenomenon is of importance

in the peptide retention mechanism.

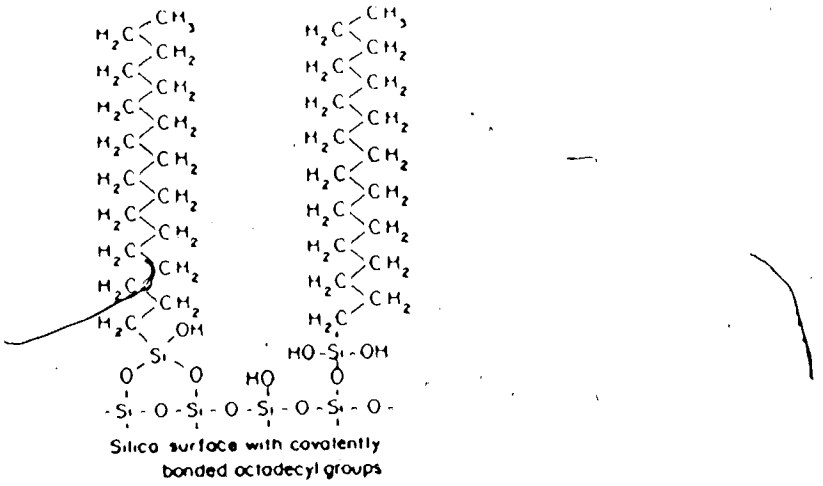


Fig. 1. Silica surface covered with covalently bonded octadecyl groups.

Use of 10-μm , or smaller, silica particles as the base materials gives the best chromatographic efficiency. The small size packings not only provide high performance, which allows better resolution, faster separation and higher detection sensitivity, but also have sufficient bonded phase coverage for high sample capacity (Majors and Hopper, 1974). The mean pore dimension of the most commonly used supports is in the range of 100-300$A°$. The 300$A°$ support is the most useful since it can be used for both peptides and most proteins (Lewis *et al.*, 1980; Wilson *et al.*, 1982; Pearson *et al.*, 1983).

Mobile Phase

Elution of solute molecules from RPC columns often requires an organic solvent which breaks down the intense hydrophobic interactions in the column. The most commonly used solvents of the hydro-organic mobile phase are methanol, acetonitrile, and isopropanol. The eluent strength increases with the concentration of the organic modifier and also depends on the polarity of the solvent.

"Ion-pair" chromatography has become a widely used branch of HPLC. The mechanism of this technique has been described in numerous papers (Hearn *et al.*, 1979a; Heukeshoven and Dernick, 1982; Hearn and Grego, 1984; Hancock and Harding, 1984). The chromatographic process includes the use of ion-pairing reagents which are added to the eluent for selective retention of oppositely charged sample components. When the solute contains potentially ionizable species, the ion-pairing component of the mobile phase may contribute to retention by (i) influencing the ionic state of the solute, (ii) mediating ionization of surface silanol groups on the support, and (iii) forming ion-pairs with the oppositely charged components in a sample. Finally, the resulting effects on chromatographic behaviour are dependent upon the nature and the concentration of the ion-pair reagents.

Column Characteristics

There are some expressions which will be useful in understanding specifications of a column. The most important ones are **RETENTION, RESOLUTION, COLUMN EFFICIENCY** and **COLUMN SELECTIVITY.**

Retention: The retention time (t_R) is the time required for a substance to go from the injector through the column to the detector. The retention of the substance depends on its retardation by the stationary phase.

Resolution: The resolution (R_s) between two peaks is described quantitatively by the expression in equation 2 (see Fig. 2):

$$R_S = \frac{t_{R2} - t_{R1}}{\frac{W_2 + W_1}{2}} = \frac{2\Delta t}{W_2 + W_1}$$

(equation 2)

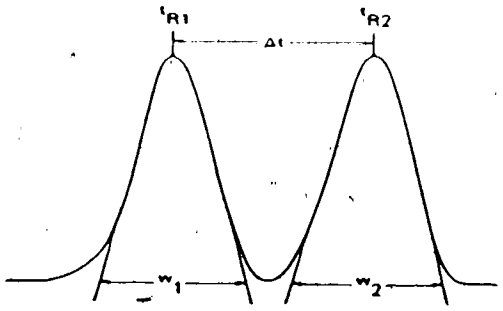


Fig. 2. Definition of resolution.

where t_{R2} and t_{R1}, are retention times of the retained components measured at the peak maximum and Δt is the difference between t_{R2} and t_{R1}. W2 and W1 are the baseline peak widths (min). A resolution value close to 1.0 or

slightly greater than 1.0 is required to resolve two components reasonably (see Fig. 3).

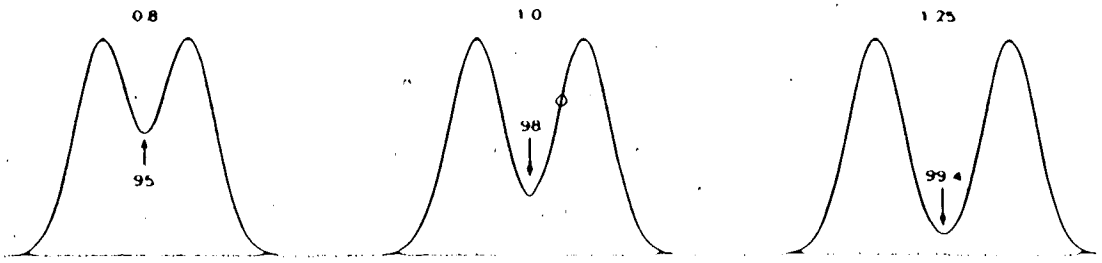


Fig. 3. Standard resolution curves for various R_s values (0.8-1.25). Band size ratio - 1/1. The figure was taken from L.R. Snyder, J. Chromatogr. Sci. 10, 202 (1972).

Column Efficiency: Column efficiency can be determined as shown in equation 3:

$$N = 16 \left(\frac{t_R}{W}\right)^2 = 5.5 \left(\frac{t_R}{W_{1/2}}\right)^2 \qquad \text{(equation 3)}$$

where N is called the number of theoretical plates which describes band broadening compared to the retention time of the component. W is the peak width at the baseline, $W_{1/2}$ the width at 1/2 peak height. An efficient column is able to give very narrow peaks.

Column Selectivity: Column selectivity, α, is measured by equation 4:

$$\alpha = \frac{t_{R2} - t_o}{t_{R1} - t_o} \qquad \text{(equation 4)}$$

By subtracting the time (t_o) for a nonretained peak from the retention time of retained peaks, column selectivity describes the relative separation of two peaks. A comparison of resolution and efficiency versus selectivity is shown in Figure 4:

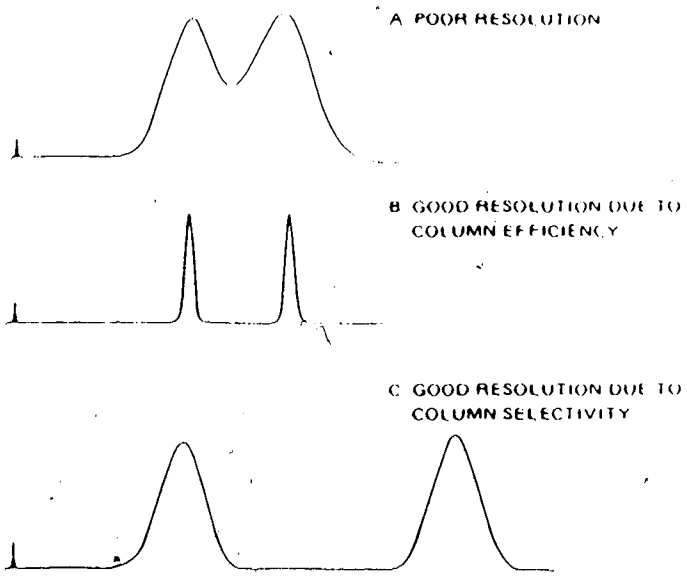


Fig. 4. Resolution and efficiency versus selectivity.

B. Reversed-Phase Chromatography of Peptides

Advantages of RPC

In recent years, RPC has proven to be an extremely useful and powerful technique for the separation of a variety of peptide molecules and has been widely accepted by numerous laboratories for routine peptide isolation. The technique offers a number of practical advantages over the conventional techniques such as paper chromatography and

electrophoresis. These include short analysis time, high resolution and sensitivity, easy sample recovery and suitable methodology for most peptides. The major disadvantages of RPC are poor column-to-column reproducibility and high equipment costs (Wilson, 1985).

It is often necessary to know the primary structure of a peptide or protein from natural sources for the study of structure and function of peptides and proteins. RP-HPLC confers a rapid, sensitive method for the separation of complex peptide mixtures generated by enzymatic and/or chemical digestion of protein samples (Hughes *et al.*, 1979; Hearn, 1980; Deibler *et al.*, 1985). In addition, it is quite useful for analytical and preparative "mapping" of peptide mixtures, allowing peptides containing sites of chemical modification to be isolated for identification. The chemically modified peptides may be detected by their changes in mobility relative to peaks of an unmodified digest (Hancock *et al.*, 1978a; Honegger *et al.*, 1981; Bishop *et al.*, 1981; Manalan *et al.*, 1985).

In many cases, biologically active peptides, including those with hormonal, antibiotic or toxic properties, are available in only minute amounts. The isolation of these compounds is often a difficult task. Many researchers have successfully isolated a variety of biological samples by using RP-HPLC, a very sensitive method for purification (Rzeszotarski and Mauger, 1973; Rubinstein *et al.*, 1977; Rivier & Burgas, 1977; Terabe *et al.*, 1979; Johnson *et al.*,

1980; Meek, 1983; Janssen *et al.*, 1984; James and Bennett, 1985).

Similarly, the purification of desired peptides synthesized by the solid-phase methodology of Merrifield from various undesirable side-reaction products often requires an efficient technique. Hearn *et al.* has described an application of RP-HPLC to the analysis and purification of synthetic peptides and shown that the approach allowed very rapid recognition of deletion or partial deprotection products which were often generated during the synthesis (1979a). In particular, synthesis of a long peptide by solid-phase peptide synthesis would occasionally give rise to serious difficulty in purification. Open column chromatographic procedures based on gel permeation, ion-exchange, adsorption or partition methods were found to have low efficiency in the purification of long peptides. The employment of RP-HPLC in synthetic peptide chemistry has greatly aided the researchers in the separation of closely related peptide impurities.

Although this technique is particularly useful for the separation of relatively small molecules such as peptides, large polypeptides and small proteins have also been successfully chromatographed (Mahoney and Hermodson, 1980).

Chromatographic Behaviour of Peptides

A characteristic of a peptide is that it has hydrophobic (Trp, Phe, Tyr, Leu, Ile *etc.*) as well as

hydrophilic (e.g. Asp, Glu, Arg, and Lys) amino acid residues. Since amino acid side chains vary from polar to nonpolar and charged to neutral, the total hydrophobicity of a peptide depends on its amino acid composition as well as pH of the environment. Variation in hydrophobicity of peptide molecules allows their separation through hydrophobic surface interactions in the reversed-phase mode.

It is well recognized that the mechanism of the peptide retention in reversed-phase chromatography is mainly attributed to the hydrophobic interaction between peptide molecules and the nonpolar ligates on the stationary phase (Bennett *et al.*, 1977; Molnar and Horvath, 1977; O'Hare and Nice, 1979; Wilson *et al.*, 1981). However, the silanophilic interaction of underivatized surface silanol groups with peptides also has an important effect on peptide retention at certain pH's such as neutral pH (Hancock and Sparrow, 1981; Nice *et al.*, 1981). Furthermore, peptide elution in a reversed-phase system is dependent on the hydrophobicity of the solute relative to the polarity of the mobile phase.

In the reversed-phase mode, the more hydrophobic the peptide, the stronger its retention by the column. For an unfolded peptide, the hydrophobicity is mainly related to the summated relative hydrophobic contribution of each amino acid residue in the peptide sequence. However, if the peptide is not completely denatured, the hydrophobic nature of the peptide is dramatically different from that in its unfolded state. This is due to hydrophobic side chains being

buried during the folding process. Therefore, it is not surprising that partially folded and denatured species may possibly create two or more peaks (Hermodson and Mahoney, 1983). This situation may be avoided by using acid solutions in which many peptides are denatured. Lau *et al.* have observed that both reversed-phase supports and the commonly used organic solvents in RPC can cause denaturation of the peptides (Lau *et al.*, 1984; Ingraham *et al.*, 1985). They also reported that the addition of organic solvent during RPC stabilized the secondary structure (α-helix) of the peptide since a representive peptide TM-15 increased its molar ellipticity substantially on the addition of organic solvents.

Peptide retention in reversed-phase systems does not always exhibit the "regular" behavior expected on the basis of the hydrophobic effect. As stressed above, silanophilic interaction contributes to the most irregular retention phenomena. This is due to the underivatized surface silanols on the silica-based supports which are ionized above pH 3.5-4.0. The ionized silanol groups may interact with the basic residues of peptides and cause an anomalous retention behavior within certain pH ranges (Bij *et al.*, 1981).

Peptide positional isomers and diastereoisomers can be separated in RP columns (Terabe *et al.*, 1979; Lundanes and Greibrokk, 1978). The selectivity deviations from elution of isomers can not be explained in terms of difference in summated retention contribution of each amino acid residue.

The separation mechanism of the peptide isomers in RPC has not been satisfactorily explained. However, Terabe *et al.* considered that the separation was based on the difference in molecular hydrophobicities of the peptides (1979). A molecular hydrophobicity of a peptide is concerned with a conformational effect on the hydrophobicity of the molecule. Apparently, a pair of peptides with the same amino acid composition, but different sequences, may have different conformations and therefore, have different affinities for the stationary phase.

C. Hydrophobicity Parameters of Amino acid Residues

Nature of Hydrophobic Interactions

The hydrophobic (fear-of-water) interaction is important to biochemical processes. HI is referred to as "a major driving force in folding of macromolecules, binding of substrate to an enzyme, association of subunits to form a multisubunit enzyme, and the processes involving high levels of aggregation such as the formation of biological membranes" (Tanford, 1973 and 1978).

When a polypeptide chain folds spontaneously in aqueous solution, its hydrophobic side chains will be buried and its polar charged chains tend to be on the surface of the molecule. It means that the hydrophobic side chains are thermodynamically more stable when clustered in the interior of the molecule than when extended into the aqueous

surroundings.

Water molecules have a strong attraction for each other and form a mobile network through hydrogen bonds in a liquid state. When a peptide is introduced into a water network, a hole is created around the hydrophobic side chains of the peptide. Consequently, some hydrogen bonds in the original network are broken. Since the hydrophobic side chains do not interact with water as strongly as water molecules attract each other, the water molecules must orient themselves in a way which reforms the hydrogen bonds that were disrupted by the hydrophobic side chains. In this process, the enthalpy change is small due to a small change in the number of hydrogen bonds. However, structuring of water molecules is associated with a large negative entropy change. The resulting overall positive free energy indicates that the interaction of the nonpolar groups with water will be thermodynamically unfavored. Apparently, the solvation process is governed by a substantial negative change in entropy (Hatefi and Hanstein, 1969; Sturtevant, 1977; Cantor and Schimmel, 1980; Edsall and Mckenzie, 1983).

In contrast, spontaneous association of two nonpolar groups is thermodynamically favored due to a less disruptive effect on the solvent network than the combined effects of two separate groups. The clustering of the hydrophobic groups is not because they like each other, but because they are both disliked by water. Furthermore, HI is a term used to describe the combined effects of London force, hydrogen

bonding, and van der Waals interaction in aqueous solutions (Tinoco *et al.*, 1978).

Determination of Hydrophobicity Parameters

The hydrophobicity parameters for amino acid residues can be determined from several model systems. In 1971, Nozaki and Tanford estimated the hydrophobicity of 12 amino acids, based on the free energies of transfer of hydrophobic side chains to 100% ethanol or dioxane from water (1971). They demonstrated that the difference in free energy between a hydrophobic moiety in 100% ethanol and in water was a measure of the unfavorable free energy of interaction with water and could be used to establish a hydrophobicity scale for hydrophobic side chains.

Similar experiments were carried out by Rekker who found that the partition coefficients in the octanol/water system were somehow linearly related to the hydrophobic fragmental constants (1977). A set of relative hydrophobicity values for 21 amino acids was calculated on the basis of the value of a glycine residue.

Since 1976, a new model system has been used to understand the hydrophobicity properties of the amino acid side chains as part of a peptide. Reversed-phase chromatography provides a physico-chemical method to quantify the hydrophobicity of the amino acid residues. As first noted by Horvath *et al.*, retention times for members of a homologous series, such as oligomers of phenylalanine,

were linearly dependent on the number of residues (1976). This is reasonable because the hydrophobicity should roughly be an additive function of the nonpolar fragments of the phenylalanine polymer. They also proposed that stationary phases with hydrophobic surfaces could be used as probes to obtain information about the hydrophobicity of amino acid residues.

It is well known that retention of peptides on reversed-phase columns depends upon the contribution of several interactions, including hydrophobic effects, hydrogen bonding with silica or polar bonded phase moieties and interactions of charged side chain groups with underivatized silanols. Of these, hydrophobic interaction plays the predominant role in effecting solute retention (Molnar and Horvath, 1977; Wehr *et al.*, 1982). To date, most workers are agreed that separation of peptides on reversed-phase supports is dictated primarily by peptide hydrophobicity, which can be approximated by the summation of the hydrophobicity of the individual amino acid side chains. By comparing the retention times and the hydrophobicities of thirty-two peptides and polypeptides, O'Hare and Nice noticed that retention orders of small peptides generally correlated with the sum of the Rekker fragmental constants of their hydrophobic residues (1979). Later on, several sets of hydrophobicity parameters (retention coefficients) were determined for 20 amino acids (Meek, 1980; Wilson *et al.*, 1981; Meek and Rossetti, 1981;

Su *et al.*, 1981; and Sasagawa *et al.*, 1982, 1984a and 1984b). They chromatographed natural peptides using various reversed-phase conditions and recorded retention times for each peptide. The retention coefficient for an amino acid residue was calculated by using two methods of numerical analysis, namely, (1)a multiple regression procedure and (2)a mathematical routine for solving linear equations (Su *et al.* 1981). The calculated retention coefficients not only provide information about the hydrophobicity properties of amino acid side chains, but also can be used to predict the elution profiles of peptides of known composition in RPC. The large differences between the results of these researchers and the results shown in this study will be discussed later in this thesis.

D. Solid-Phase Peptide Synthesis

Solid-phase peptide synthesis developed by Merrifield has brought about a revolution in peptide and protein chemistry. During the last ten years, people have discovered many peptides which possess profound biological activities. Many of these peptides are available naturally in only minute quantities, making isolation and purification extremely difficult. The potential of providing large quantities of these molecules, as well as structural variants by peptide synthesis, has allowed researchers to investigate biological characteristics of peptides, including structure-activity relationships.

In principle, this technique involves the coupling of activated amino acids or peptides to a resin support. Having the peptide attached to the solid support eliminates the laborious purification at intermediate steps that occurs in solution synthesis. Excess reagents, by-products and solvents can be easily removed from the resin by filtration. In addition, the method can be easily automated. The most common strategy used in SPPS is to attach the first amino acid of the peptide chain to the resin support through its carboxylic group and the subsequent amino acids are coupled in the C-to-N direction. This C-to-N assembly offers some advantages over the N-to-C mode of synthesis: (i)several satisfactory Nα-protecting groups are available to prevent racemization of the activated amino acid during coupling; (ii) the activated amino acids can be used at a high enough concentration to drive the coupling reaction to completion; and (iii) decomposition products of the activated amino acid can be removed from the support-bound peptide by filtration during the washing steps. The major disadvantage of SPPS is that the by-products differing from the desired peptide by the deletion, modification or addition of residues remain bound to the resin until the fully assembled peptide is cleaved from the support. Thus, problems in purification can be expected in the synthesis of long peptides. Since the peptides designed for this project are small, ranging from 8 to 10 residues, it should not be difficult to purify the desired peptides.

The basic steps of SPPS are outlined in Figure 5. The solid support is a synthetic polymer, such as copolymer of styrene and divinylbenzene. The carboxyl group of the first amino acid, containing a protected α-amino group, is bound covalently to the polymer. The standard protecting group for an α-amino function is the tert-butyloxycarbonyl (Boc) group which is selectively removed by trifluoroacetic acid (TFA). The newly exposed amino group is present as the acid salt. The TFA salt is neutralized with a base, diisopropylethyl amine (DIEA), leaving the amino terminal uncharged and free to couple. The next Boc-amino acid is then coupled to the aminoacyl resin, usually by the use of dicyclohexylcarbodiimide (DCC). In DCC-mediated coupling reactions, a high degree of activation is afforded by the formation of O-acyl isourea. By repeating the cycle of deprotection, neutralization and coupling, the peptide of desired sequence is assembled on the polymer support.

Following completion of assembly, the protected peptide resin is treated with anhydrous hydrogen fluoride (HF) to cleave the peptide-resin linkage and remove all the side chain protecting groups. In SPPS the functional groups on side chains must be protected by stable blocking groups which will remain completely intact throughout the synthesis, but which can be removed finally to yield the free peptide. The free peptide is extracted from the resin with a suitable solvent (*e.g.* TFA), purified and characterized.

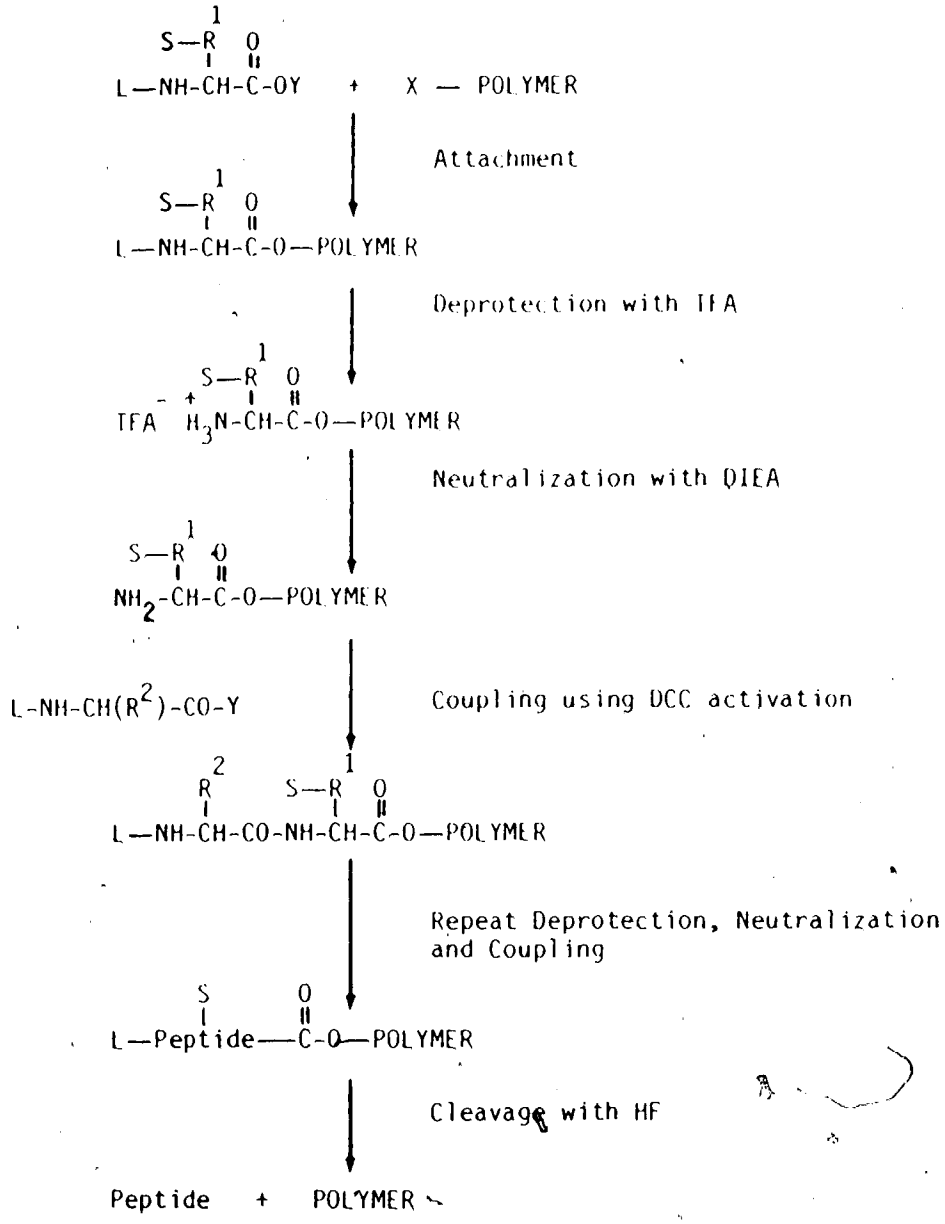


Fig. 3. Steps in solid phase peptide synthesis.
L represents N-terminal blocking group;
S side-chain protecting group; Y activated carboxyl group of protected AA;
X active site on the polymer support;
R^1 amino acid side chain with a functional group requiring protection;
R^2 amino acid side chain requiring no protection;

E. Purpose of This Study

The object of this study has been to develop and evaluate precise hydrophobicity-hydrophilicity parameters for amino acid residues in peptides and proteins from the interaction of peptides with a reversed-phase support. We feel that the use of model synthetic peptides can be a powerful tool in examining the relationship of peptide composition to its chromatographic behaviour in reversed-phase HPLC. A precise method for determining the hydrophobicity parameters of individual amino acid residues is to measure their contribution on retention of a model synthetic peptide, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where position X is substituted by the 20 amino acids found in proteins. For this purpose, we have synthesized a series of short peptides that allow us to determine an empirical set of hydrophobic parameters in reversed-phase chromatography.

Although we have established a set of precise parameters for predicting the retention of any peptide up to 20 residues with known amino acid composition in RPC, the coefficients are valid only for a specific chromatographic system. To extend the application of our retention coefficients, we have examined the effects of some factors which might affect the peptide retention from one system to another. These factors include the choice of the stationary phase, the choice of the buffer system, the pH of the mobile phase, the nature of the ion-pairing agent, the flow-rate, the column temperature and gradient slope. By comparing the

chromatograms at different conditions, we have successfully introduced an internal peptide standard technique to correct for deviations arising from the factors mentioned above.

The peptide retention mechanism in RPC has been investigated by a number of chromatographers. Several hypotheses have been put forward to explain the retention and elution of peptides. These include solvophobic interaction, silanophilic interaction, specific solvation, and exclusion phenomena (Horvath, 1983). By comparing the chromatographic behaviour of peptides with that of small organic compounds, such as alkylphenones, we have postulated an adsorption-desorption mechanism for peptide molecules by emphasizing multi-site interaction of peptides with the nonpolar stationary phase. Furthermore, we have derived a new set of hydrophilicity HPLC parameters from the retention times of 20 model synthetic peptides at pH 7 and used these parameters to predict possible antigenic sites on the surface of a protein. These constants are the first reported parameters derived from AA residues in synthetic peptides.

II. EXPERIMENTAL PROCEDURES

A. Sources of Chemicals and Solvents

Unless otherwise stated, all chemicals and solvents were reagent grade. t-Butyloxycarbonyl (Boc) amino acids were purchased from Vega Biochemicals (Tucson, AZ, U.S.A.), Bachem Fine Chemicals, Inc. (Torrance, CA., U.S.A.), Beckman Instruments, Chemical Dynamics Corp. (South Plainfield, NJ, U.S.A.) and the Protein Research Foundation (Peptide Institute, Inc., Osaka, Japan). Co-poly(styrene, 1% divinylbenzene) benzhydrylamine-hydrochloric acid resin (0.75 mmol of NH_2/g of resin) and co-poly (styrene, 1% divinylbenzene) chloromethyl resin (ca. 1.0 mmol of Cl per g of resin) were purchased from Beckman Instruments Inc. (Palo Alto, CA, U.S.A.). and Pierce Chemical Co. (Rockford, IL., U.S.A.), respectively.

Diisopropylethyl amine (DIEA), dichloromethane (CH_2Cl_2) and trifluoroacetic acid (TFA) were redistilled prior to use. Picric acid was dissolved in CH_2Cl_2 and dried over magnesium sulphate. HPLC-grade acetonitrile (CH_3CN), methanol (CH_3OH), and 2-propanol ($CH_3CHOHCH_3$) were obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). Sodium perchlorate and 88% (w/w) formic acid were also obtained from Fisher Scientific. Reagent grade orthophosphoric acid was purchased from J.T. Baker Chemical Co. (Phillipsburg, N.J. U.S.A.). Sequence-grade anhydrous n-heptafluorobutyric acid (HFBA) was purchased from Pierce Chemical (Rockford,

IL, U.S.A.). Dicyclohexylcarbodiimide (DCC) was obtained from Aldrich Chemical Company Inc. (Milwaukee, U.S.A.). Double distilled water was purified by passage through a Milli-Q water purification system (Millipore Corp., Bedford, MA, U.S.A.).

B. Peptide Synthesis

Attachment of First Amino Acid to the Resin

The peptides were synthesized by the solid-phase synthesis procedure of Merrifield. In terms of C-to-N assembly, the Boc-amino acid desired as the carboxyl residue in the peptide sequence was attached to the resin support. Two kinds of resins were used. Copoly(styrene 1% divinylbenzene) chloromethyl resin was selected to generate free α-carboxyl groups of the peptides. In the case of the model peptides (No. 21 and 23 in Table 2), the carboxyl terminal lysine as the Boc-lysine cesium salt was esterified to the resin. Usually a 2.5 times excess Boc-amino acid cesium salt was reacted for 24 h at 50°C, giving approximately 0.56 mmol substitution of amino acid/gram resin. Following the reaction, the resin was filtered and washed with dimethylformamide (DMF), DMF:H_2O (9:1, v:v), DMF:MeOH (1:1, v:v) by the standard procedure of Gisin (1973). After the final wash, the resin was dried under vacuum in a dessicator.

When a carboxyl terminal amide was desired on the peptide, the Boc-amino acid was coupled with dicyclohexylcarbodiimide (DCC) to the copoly(styrene-1%-divinylbenzene) benzhydrylamine resin. Desired substitutions of the resin for peptide synthesis range from 0.2 to 0.4 mmol/gram (Stewart and Young, 1984). The resin was neutralized with 5% DIEA in CH_2Cl_2 for 1 h. Boc-amino acid (0.4 mmol/g of resin) and DCC (1.1 equivalents) were added to the resin. The mixture was stirred in CH_2Cl_2 for 1 h. This resulted in a substitution of about 0.35 mmol/g of resin, as determined by picric acid monitoring (Hodges and Merrifield, 1975). The remaining free amino groups on the resin were terminated by treatment (1 h) with acetic anhydride/toluene/pyridine (1:3:3, v:v:v).

Elongation of the Peptide Chain

All α-amino groups of amino acids used were protected with the t-butyloxycarbonyl(Boc) group. The following side-chain blocking groups were used: tosyl(Arg), benzyl(Ser and Thr), benzyl ester(Asp and Glu), 2,6-dichlorobenzyl(Tyr), N'-formyl(Trp), N'^{m}-tosyl(His), 4-methoxybenzyl(Cys), 2-chlorobenzoxycarbonyl(Lys), and 4'4'-dimethoxybenzhydryl(Asn and Gln).

The program used for one synthesis cycle is shown in Table 1. The Boc groups were removed at each cycle by treatment with trifluoroacetic acid (TFA)/CH_2Cl_2 (1:1, v:v) for 20 minutes (Table 1: Operation Number I). After each

TABLE 1 Synthesizer program

Operation No.	Step No.	Operation	No. of Washes	Mix time (min.)
I		Deprotection		
	1	CH_2Cl_2	1	1
	2	Prewash 50% TFA/CH_2Cl_2	1	1
	3	Deprotection 50% TFA/CH_2Cl_2	1	20
	4	CH_2Cl_2	5	1
II		Neutralization		
	1	Neutralization 5% DIEA/CH_2Cl_2	3	2
	2	CH_2Cl_2	6	1
III		Monitoring (deprotection)		
	1	Picric acid/CH_2Cl_2 (0.1M)	2	5
	2	CH_2Cl_2	6	1
	3	Neutralization 5% DIEA/CH_2Cl_2	3	2
	4	CH_2Cl_2	3	1
	5	CH_2Cl_2	3	1
IV		Coupling		
	1	Deliver Boc-amino acid/CH_2Cl_2		5
	2	DCC/CH_2Cl_2 coupling		30
	3	CH_2Cl_2	5	1
	4	Isopropanol	2	1
	5	CH_2Cl_2	2	1
	6	Isopropanol	2	1
	7	CH_2Cl_2	6	1

.....continued

Operation No.	Step No.	Operation	No. of Washes	Mix time (min.)
V		Neutralization		
	1	Neutralization 5% DIEA/CH_2Cl_2	3	2
	2	CH_2Cl_2	6	1
VI		Monitoring (first coupling)		
	1	Picric acid/CH_2Cl_2 (0.1M)	2	5
	2	CH_2Cl_2	6	1
	3	Neutralization 5% DIEA/CH_2Cl_2	3	2
	4	CH_2Cl_2	3	1
	5	CH_2Cl_2	3	1
VII		Coupling		
	1	Deliver Boc-amino acid/CH_2Cl_2		5
	2	DCC/CH_2Cl_2 coupling		30
	3	CH_2Cl_2	5	1
	4	Isopropanol	2	1
	5	CH_2Cl_2	2	1
	6	Isopropanol	2	1
	7	CH_2Cl_2	6	1
VIII		Neutralization		
	1	Neutralization 5% DIEA/CH_2Cl_2	3	2
	2	CH_2Cl_2	6	1
IX		Monitoring (second coupling)		
	1	Picric acid/CH_2Cl_2	2	5
	2	CH_2Cl_2	6	1
	3	Neutralization 5% DIEA/CH_2Cl_2	3	2
	4	CH_2Cl_2	3	1
	5	CH_2Cl_2	3	1

deprotection step, the peptide-resin was neutralized with 5% DIEA in CH_2Cl_2 (v/v). The subsequent Boc-amino acid (3 equivalents) in CH_2Cl_2 was added to the peptide-resin followed by a solution of DCC (3.3 equivalents) in CH_2Cl_2 (4ml). The solution was stirred for 30 minutes. Peptide synthesis was carried out automatically on a Beckman peptide synthesizer, Model 990 using the program shown above for the attachment of all amino acids required for the peptide chain. It is worth mentioning that the deprotection and coupling program used is a modification of that previously described by Hodges *et al* (1981) and Parker and Hodges (1985).

In order to drive the coupling reaction to completion, a double coupling method was employed. To obtain satisfactory levels of coupling, the following amino acids (Nα-Boc- and side-chain-protected) were coupled as symmetrical anhydrides: Arg, Gln, Asn, His, Glu, Asp,and Thr. The advantage of anhydride coupling reaction is dramatically increased reaction rates in difficult SPPS couplings. The Boc-amino acid symmetrical anhydrides were prepared immediately before use by the treatment of 6 equivalents of amino acid with 3 equivalents of DCC at 0°C.

Monitoring SPPS

In general, monitoring of SPPS reactions can be employed at three stages: deprotection, coupling, and chain termination. Monitoring at the deprotection step can be

applied to estimate completion of deprotection. Similarly, monitoring at a coupling step or after acetylation yields information about the completeness of the reaction.

The picric acid method was used to monitor SPPS (Hodges and Merrifield, 1975). The monitoring reaction is presented in Figure 6 and the program used is shown in Table 1.

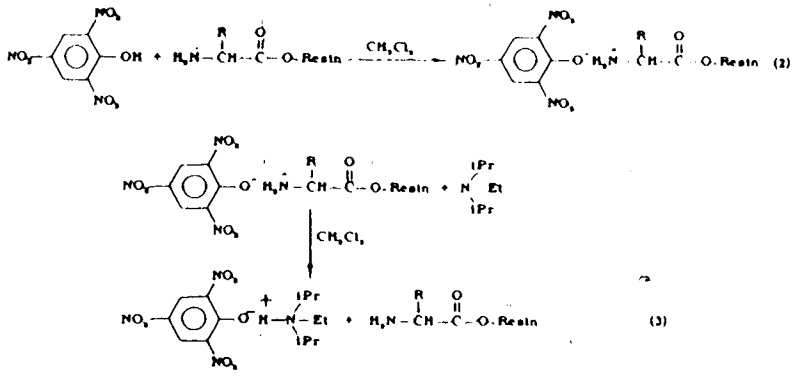


Fig. 6. Solid-phase peptide synthesis monitoring reaction.

The collected DIEA and CH_2Cl_2 washes were diluted to a known volume with CH_2Cl_2 and the absorption of the solution recorded at 362 nm. The concentration of amino groups on the resin (μmoles) was then calculated using a DIEA-picrate molar extinction of 15,000.

Cleavage of Peptides from the Resin

Following deprotection of the N-terminal amino acid, the α-amino group was acetylated by treatment for 30 minutes with acetic anhydride/toluene/pyridine (1:3:3, v:v:v), with

the exception of the peptides in which a free N-terminal α-amino group was required. The completed peptides were cleaved from the resin and simultaneously deprotected by reacting with hydrofluoric acid (15 ml HF/gm peptide-resin), containing 10% anisole at 0°C for 45 min. Anhydrous HF, a strongly protonating acid, is highly volatile, and an excellent solvent for peptides. The cleavage mixture for the methionine-containing peptide-resin also contained free methionine as a scavenger (Tam *et al.*, 1983).

After the reaction was completed, the solvents were removed under reduced pressure at 0°C. The resin was then washed with ether, and the peptides were extracted with TFA (3X10ml). The TFA was removed by rotary evaporation, resulting in light yellow oily materials. The crude peptide was dissolved in water and lyophilized. The N^{im} protecting group of tryptophan, stable to HF cleavage, was removed by treatment with 1M NH_4HCO_3 (pH 9.0) for 24 hours. Removal of the formyl group was monitored by disappearance of the strong 300 nm absorption (Yamashiro and Li, 1973).

C. Purification of Peptides

Apparatus: The HPLC instrument consisted of a Spectra-Physics (San Jose, CA, U.S.A.) SP8700 solvent delivery system and SP8750 organizer module, combined with a Hewlett-Packard (Avondale, PA, U.S.A.) HP1040A diode array

detection system, HP3390A integrator, HP85 computer, HP9121 disc drive and HP7470A plotter. Samples were injected with a 500-μl injection loop (model 7125, Rheodyne Inc., Cotati, CA, U.S.A.). **Columns:** Crude peptides were purified and analyzed on one of the eight reversed-phase columns. The physical characteristics of the packing materials in these reversed-phase columns are shown in Table 8, page 65.

The crude peptides were dissolved in 0.1% TFA/H_2O to a concentration of about 1mg/1ml and centrifuged to pellet any undissolved impurities. The samples were then subjected to reversed-phase HPLC with a linear AB gradient. Solvent A was 0.1% aq.TFA and solvent B was 0.1% TFA in acetonitrile. The crude samples were purified on preparative columns. The solvents of collected fractions were removed by rotary evaporation and the peptides were freeze-dried. To characterize the purified peptides, 10 μg of each purified sample was reinjected onto analytical columns to ensure that the contaminating peptides had been removed.

D. Amino Acid Analysis

Aliquots (50-100μmole /each peptide) of stock solutions of the individual, purified peptides were hydrolyzed in 100-200μl of 6N HCl in evacuated, sealed tubes. Care was taken to remove all oxygen from the hydrolysis tubes. If the peptide contained tyrosine residue(s), 0.1% of phenol(v/v) was added to the 6N HCl. Tubes were incubated at 110°C for 24 h. The hydrolysates were taken to dryness to remove the

HCl, redissolved in a known amount of pH 2.2 buffer and subsequently analyzed on a Durrum 500 amino acid analyzer to confirm peptide composition. The destruction of tryptophan by 6N HCl necessitated the use of 4M aq. methanesulphonic acid, containing 0.2% 3-(2-aminoethyl) indole, as hydrolyzing agent for the tryptophan-containing peptides. The hydrolysate was partially neutralized with an equal volume of 3.5M NaOH prior to analysis (Glazer *et al.*, 1975). Considerable losses of cysteine are found after acid hydrolysis. Thus, cysteine-containing peptides were determined as cysteic acid after performic acid oxidation (Moore, 1963). The performic acid was prepared by mixing 1.0 ml of 30% H_2O_2 and 9.0 ml of 88% formic acid for 1 hour at R.T. and the mixture was cooled to 0°C. About 50 *u*mole of cysteine-containing peptides were dissolved in 2 ml of the reagent. The mixture was kept at 0°C for 4 hours. After the oxidation, 0.3 ml of 48% HBr was added to destroy the remaining performic acid and the solution was removed by rotary evaporation under high vacuum at 40°C with NaOH pellets in the condenser trap. The oxidized product was hydrolyzed with 3 ml of 6N HCl. Analysis of the resulting hydrolysates was performed in the usual manner.

E. Other Methods

Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a relatively simple technique for detecting purity of peptides and amino acids. Pre-coated TLC plates (silica gel 60F-254) were used. The solvents used were either 1-butanol: NH_4OH:H_2O (8:1:1, v:v:v) or 1-butanol:acetic acid:H_2O (4:1:1, v:v:v). The plates were dried for 10 minutes and then sprayed with ninhydrin. Upon heating the ninhydrin chromatoplates at 105°C in the oven, ninhydrin produced purple spots with the peptides or amino acids containing free amino groups.

Thin Layer Electrophoresis

Peptide analogues containing Asn, Asp, Gln, Glu, Arg, Lys and His residues were checked for purity and net charge by thin layer electrophoresis. About 20 umole of each peptide was loaded on the chromagram sheet (Eastman) which was wetted with the pH 6.5 buffer (acetic acid : pyridine : water [3:100:897, v:v:v]). The electrophoresis was performed on Camag equipment (Switzerland) for 1 hour. Peptides were visualized by using the ninhydrin reagent as discussed above.

Spectrophotometric Scanning of Peptides

The phenylalanine-, tyrosine-, and tryptophan-containing peptides absorb light significantly in

the ultraviolet. Measurement of light absorption ranging from 240 nm to 300 nm in a spectrophotometer is an extremely rapid and convenient means of examining the peptides which contain the above three amino acids, while all the peptides absorb in the far ultraviolet due to the ultraviolet absorption for peptide bonds. Figure 7 shows the ultraviolet absorption spectra of the tyrosine-containing peptide Ac-G-Y_2-L_3-K_2-amide (Fig. 7A) compared to that of a peptide standard which does not contain any functional groups absorbing light from 240 nm to 300 nm (Fig. 7B).

Measurement of Gradient Elapsed Time

Gradient elapsed time (tg) is defined in this work as the time for the gradient to reach the detector from the proportioning valve via pump, injection loop, and column (Quarry *et al.*, 1984). The value was obtained by adding 20% acetone to solvent B (0.1% TFA in acetonitrile) of an AB gradient (see Fig. 8). The HPLC system was equilibrated with solvent A (0.1% aq. TFA) and the time was measured from the start of the gradient to the observed offscale change in absorbance at 270nm (see arrow point in Figure 8). This was due to a strong absorption of acetone at this wavelength.

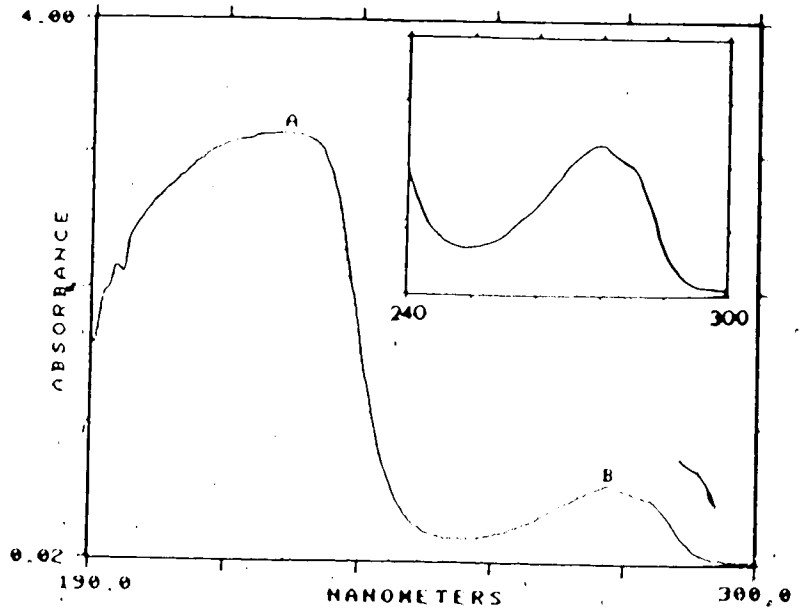


Fig. 7A The ultraviolet absorption spectra of tyrosine-containing peptide: Ac-G-Y-Y-L-L-L-K-K-amide.

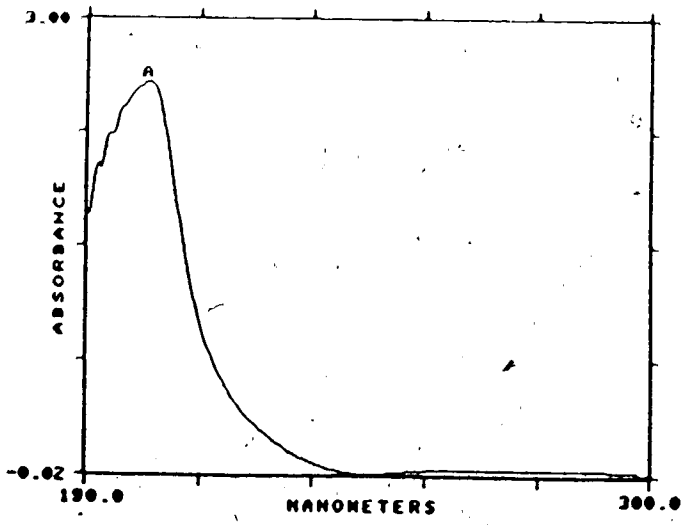


Fig. 7B. The ultraviolet absorption spectra of peptide standard 2: (see Chapter III): Ac-R-G-G-G-G-L-G-L-G-K-amide.

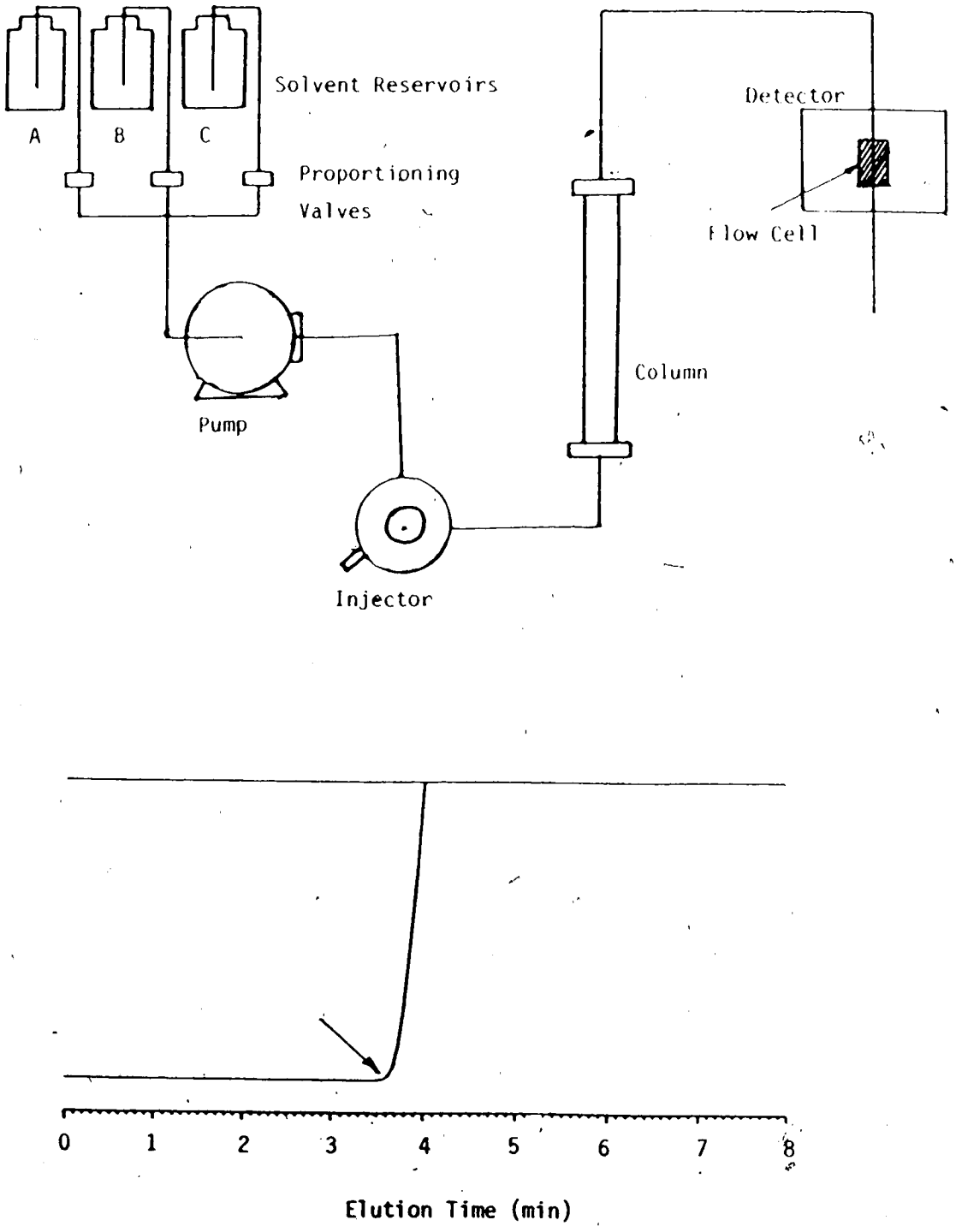


Fig. 8. Schematic diagram of liquid chromatography system and elution profile of acetone, showing the offscale change in absorbance at 270 nm.

III. RETENTION COEFFICIENTS OF AMINO ACID RESIDUES AND PREDICTION OF PEPTIDE RETENTION IN RPC

A. Designing & Characterizing Peptides

The 23 model peptides synthesized are shown in Table 2. All peptides were designed to have eight residues for ease of synthesis with appropriate hydrophobicity such that all 23 peptides would be retained on the reversed-phase columns and eluted with a reasonable acetonitrile concentration. The solubility of the peptides in aqueous solution was ensured by the incorporation of the two lysine residues. Finally, glycine was used to end the peptide chain so that the substituted amino acids (X) would have at least the one peptide bond on the N-terminus in addition to the acetyl group. Two residues were substituted each time for two purposes, namely: (i)amplifying the effect of the substituted amino acid on the peptide retention time; (ii)evaluating those residues with small effects more accurately. Furthermore, all peptides have a common portion of sequence to make the synthesis of the analogs easier. A large stock of Boc-$(Leu)_3$-$(Lys)_2$-benzhydrylamine resin was prepared. Subsequent amino acids were then coupled to 300 mg portions of this common Boc-peptide-resin. The 23 model peptides were used to determine the retention coefficients of amino acid residues in RPC.

Peptide standards have been suggested for accurate monitoring of peptide separation and reversed-phase column

TABLE 2

Sequences and Amino Acid Analysis of Synthetic Model Peptides

	Peptide	Amino Acid			
	Ac-G-X-X-L-L-L-K-K-amide	Gly	X	Leu	Lys
1	-D-D-	1.02	2.02	2.99	2.00
2	-A-A-	0.98	2.01	3.01	2.00
3	-V-V-	1.06	2.08	2.96	1.89
4	-N-N-	0.98	2.00	3.00	2.02
5	-R-R-	0.98	1.95	3.02	2.03
6	-E-E-	0.99	1.99	3.05	1.96
7	-H-H-	0.90	2.07	3.03	2.07
8	-P-P-	1.00	2.04	2.95	1.99
9	-F-F-	0.91	2.03	3.01	2.06
10	-Q-Q-	1.00	1.94	3.03	2.00
11	-G-G-	3.04	——	3.03	1.93
12	-L-L-	0.94	——	4.94	2.11
13	-K-K-	1.10	——	3.00	3.90
14	-M-M-	0.98	1.91	3.00	2.04
15	-Y-Y-	0.93	1.91	3.33	1.82
16	-S-S-	0.99	1.86	3.17	2.01
17	-T-T-	1.10	1.89	3.18	1.83
18	-I-I-	1.03	1.82	3.36	1.80
19	-W-W-	0.95	1.77	3.26	2.06
20	-C-C-	1.09	1.84	2.98	2.09

....continued

Peptide -G-X-X-L-L-L-K-K-	Amino Acid Gly	X	Leu	Lys
21 Ac-G-L-L-L-L-L-K-K-COOH	1.00	———	4.98	2.02
22 NH_2-G-L-L-L-L-L-K-K-amide	1.04	———	4.87	2.08
23 NH_2-G-L-L-L-L-L-K-K-COOH	0.99	———	4.97	2.03

All values were obtained by using the ratio of the total amount of the amino acids to number of the amino acids present in the peptide.

performance (Mant and Hodges, 1986). In particular, they are best suited for monitoring peptide retention and resolution in RPC. Synthetic decapeptide standards were designed with the composition varying as follows: peptide standard 2 (S2), -Gly^1-Gly^4-; S3, -Ala^1-Gly^4-; S4, -Val^1-Gly^4-; S5, -Val^1-Val^4- (Table 3). All peptides contained an Nα-acetylated N-terminal and α-C-terminal amide except S1, which was identical to S3 with the exception of a free α-amino group. The length of the standards (10 residues) was designed to reflect the average size of cleavage fragments from proteolytic digests of proteins. In order to examine the resolving power of a reversed-phase support precisely, the hydrophobicity of the standards only increased slightly between S2 and S5, in particular between S2 and S3 (an increase of one methyl group), S3 and S4 (an increase of two methyl groups) and S4 and S5 (an increase of an isopropyl group). In addition, the presence of positively charged groups in the peptide standards (+2, S2-S5; +3, S1, over the acidic to neutral pH range) allows a good solubility of the peptides in aqueous solvents and sensitivity of the peptides to any ionic, as opposed to hydrophobic, interaction with a reversed-phase support.

An example of the HPLC purification of a model peptide, Ac-G-Y-Y-L-L-L-K-K-amide, is seen in Figure 9. The crude peptide was purified through three chromatographic steps. Figures 9A and 9B show the first analytical chromatograms using a SynChropak RP-P C-18 column and a linear AB gradient

TABLE

Sequences and Amino Acid Analysis of Synthetic Peptide Standards

Peptide Standard	Sequence	Amino Acid					
		Gly	Ala	Leu	Lys	Arg	Val
S1	NH_2-R-G-A-G-G-L-G-L-G-K-amide	4.94	0.98	2.10	1.05	0.95	N.P.
S2	AC--R-G-G-G-G-L-G-L-G-K-amide	5.86	N.P.	2.10	1.08	0.95	N.P.
S3	Ac--R-G-A-G-G-L-G-L-G-K-amide	4.95	0.94	2.07	1.09	1.05	N.P.
S4	Ac--R-G-V-G-G-L-G-L-G-K-amide	4.95	N.P.	1.95	1.10	1.09	0.92
S5	Ac--R-G-V-V-G-L-G-L-G-K-amide	3.94	N.P.	2.10	1.04	0.94	1.98

N.P. denotes that the amino acid residue(s) is not present.

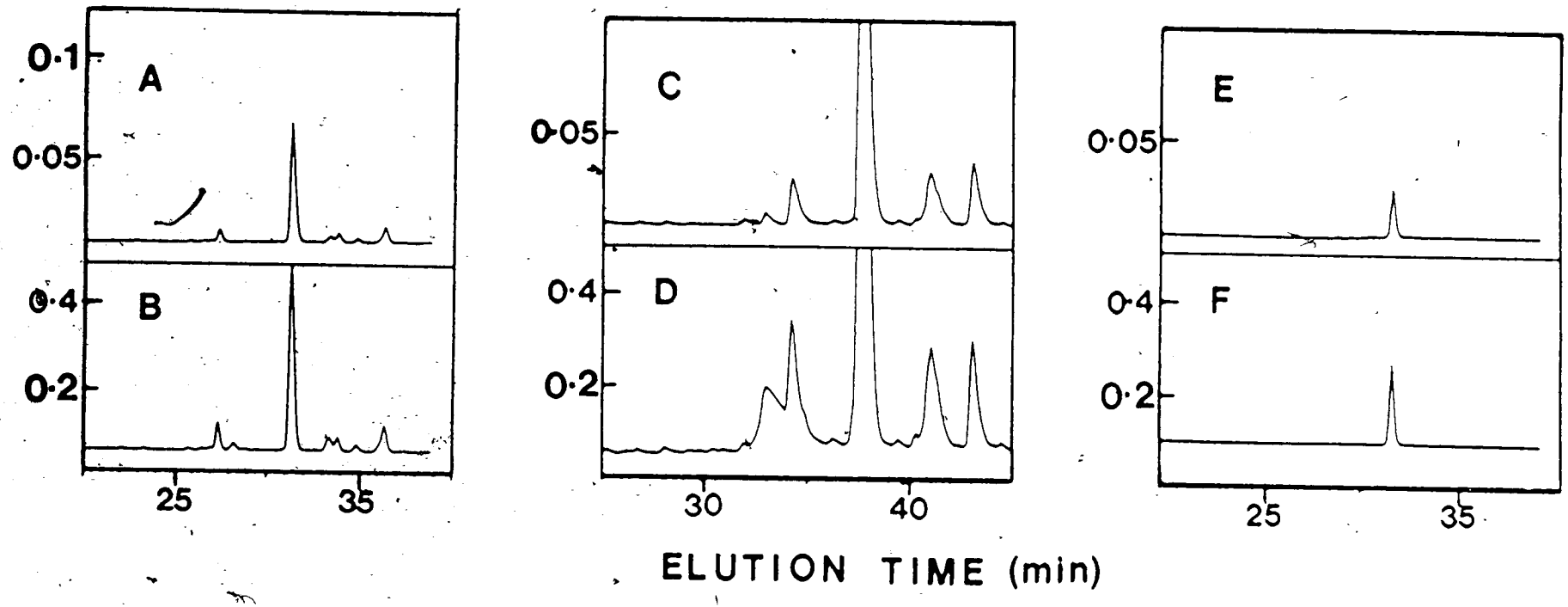


Fig. 9. Chromatograms of the synthetic model peptide with the sequence Ac-G-Y-Y-L-L-L-K-K-amide. UV detection: A,C and E at 280 nm and B,D and F at 210 nm. The left panel, separation of the crude peptide on an analytical kP column (250x4.1 mm I.D.); the middle panel, separation of the crude peptide on a RP preparative column (250x10 mm I.D.); and the right panel, separation of the purified peptide after preparative column on an analytical RP column (250x4.1 mm I.D.). Conditions: linear AB gradient (1% B/min) where A=0.1%aq.TFA and B=0.1%TFA in acetonitrile (pH2.0); 26°C.

for elution, where solvent A is 0.1% TFA/H_2O and solvent B is 0.1% TFA/acetonitrile, at a gradient rate of 1% B/min, a flow-rate of 1 ml/min, and column temperature of 26°C. The use of this chromatographic system yielded completely resolved peaks and appeared well suited for the separation of the synthetic peptides. The sample (25μl of 1mg/1ml) was injected into the analytical column in order to examine the purity of the crude peptide. Figure 9B shows detection of the peptide at 210 nm where peptide bonds absorb and figure 9A at 280 nm where the side chains of the tyrosine residues absorb. In HPLC of peptides, multi-wavelength absorbance is extremely useful in the identification of the peptides containing tyrosine, phenylalanine, and tryptophan residues (Li and Cotter, 1986). An absorption at 210 nanometers was selected for three reasons: (i)considerably high absorption for the peptide bonds and, consequently, high sensitivity of detection; (ii)relative freedom from side chain contribution and (iii)less interference by the buffer systems used for the chromatography (Woods and O'Bar, 1970; Berry, 1982). It is apparent that satisfactory peptide synthesis was obtained. Preparative RP columns were used to purify a large amount of the sample. Figures 9C and 9D show the purification profiles. The crude peptides (200 μl of 1mg/1ml) were run into the preparative column and the main peak was collected for further characterization. At this step, a flow-rate of 2 ml/min was used to speed up the purification. Figures 9C and 9D show the monitoring of the

peptide at 280 nm and 210 nm, respectively. Finally, 10 ul of the purified sample obtained from the second step was reinjected into the analytical column under identical conditions used for the first step to ensure that the contaminating peptides had been removed. As can be seen in Figures 9E(280 nm) and 9F(210 nm), all the impurities surrounding the main peak had been removed and a homogeneous peptide was obtained by this procedure.

The peptides were further characterized by amino acid analysis on a Durrum 500 amino acid analyser to confirm peptide composition. The results are tabulated in Table 2 and Table 3. No corrections were made for hydrolysis loss of sensitive amino acids. Serine and threonine amino acids are partially destroyed by acid hydrolysis, with the amount of destruction dependent on the time of hydrolysis. Isoleucine residues have the β-branched side chains, making hydrolysis slower when two such amino acids are side by side (Glazer *et al.*, 1975). The sulfur-containing amino acid is very susceptible to oxidation under acidic conditions. Therefore, Moore's method for oxidizing the cysteine-containing peptide with performic acid was used to quantitate the amount of cysteic acid (Moore, 1963).

B. Effect of Sequence Specificity

Before synthesizing the 23 peptides used to determine the retention coefficients for the 20 amino acid residues found in proteins and end groups, it was essential to verify

the assumption that each amino acid side chain in a peptide contributes to the the peptide retention independently. In other words, sequence specific effects are minimal and the composition is the major factor affecting peptide retention in reversed-phase chromatography. To verify this concept for the model peptide Ac-G-X-X-L_3-K_2-amide, two peptides of the same composition but differing in sequence were prepared (Peptides 1A and 1B, Table 4). Peptide 1A contained the sequence GLLLG, while Peptide 1B contained the sequence LGLGL, where the leucine residues were inter-spaced by glycine residues. In addition, a number of other homologous peptide pairs were chromatographed on SynChropak C-8 or C-18 columns to examine any sequence specific effect on peptide retention. Analysis was performed under the following chromatographic conditions: a linear AB gradient, where buffer A=0.1% aq.TFA and buffer B=0.1% TFA in acetonitrile, (pH 2.0); flow-rate=1 ml/min; gradient slope=1% B/min; column temperature=26°C.

Table 4 shows that all peptide pairs were eluted as single peaks. It is apparent that the changes in sequence in these peptides of the same composition did not influence peptide retention times in RPC. In other words, composition is generally the major factor affecting peptide elution position. The subtle effects of sequence can be seen with the homologous quartet peptides 5A,5B,5C and 5D (Table 4). These peptides, when chromatographed together, resolved to form a small doublet. It is worth noting that sequence

TABLE 4 Effect of sequence specificity on peptide retention times

Peptide	Sequence	R_t (min)
1A	AcKGLLLGK amide	21.97
1B	AcKLGLGLK amide	
2A	AcAAAKF*AA amide	14.5
2B	AcAAKF*AAA amide	
3A	AcGGFKRPPLRRVR amide	16.0
3B	AcGKFGRPPLRRVR amide	
4A	AcGKFKRGPLRRVR amide	14.6
4B	AcGKFKRPGLRRVR amide	
5A	AcGKFKGPPLRRVR amide	21.24
5B	AcGKFKRPPLRGVR amide	
5C	AcGKFKRPPLGRVR amide	20.97
5D	AcGKFKRPPLRRVG amide	

F* denotes nitrophenylalanine.

variations in small, highly ionized or unprotected terminal peptides can significantly affect peptide retention on RP columns (Pietrzyk *et al.*, 1983, Lundanes and Greibrokk, 1984). However, our results support the view that, in general, retention coefficients for amino acid residues could be used to predict peptide retention.

C. Retention Coefficients of Amino Acid Residues

The types of interactions that could occur between the peptides and the hydrocarbonaceous silica-base support in the reversed-phase mode are ionic, hydrogen bonding and hydrophobic. The first two interactions are mainly due to the surface silanols on the silica gel support, which act as weak acids and are ionized above pH 3.5-4. These weak acids may interact with the functional groups of the peptides chromatographed in RP columns through either ionic or hydrogen bonding interactions or both. Under given mobile phase conditions, it is possible to completely suppress these interactions of the surface silanol groups with solutes. When these interactions are minimal, the hydrophobicity of amino acids in a peptide can be determined on the basis of hydrophobic interactions.

Addition of a low concentration of trifluoroacetic acid to both water and acetonitrile has been found useful in the separation of peptides. The use of TFA enables detection at low UV wavelengths where the peptide bonds absorb strongly. TFA not only provides an acidic medium where ionization of

the surface silanols is suppressed, but also optimizes the resolving power of reversed-phase columns in peptide purification. In addition, TFA is an excellent peptide solubilizing agent and completely volatile so that the separated peptides can be easily recovered from the fractions by lyophilization.

Of the three commonly used solvents in reversed-phase chromatography of peptides, acetonitrile exhibits much superior resolution and selectivity to 2-propanol and methanol and is considered to be the best organic eluent for most practical purposes. Therefore, a water-acetonitrile mobile phase was selected as a suitable elution system in the study of retention behaviour of amino acid residues in the peptides. The chromatographic conditions used for eluting model peptides are as follows: linear AB gradient, where A=0.1% aq.TFA and B=0.1% TFA in acetonitrile, pH 2.0; flow-rate=1 ml/min; gradient slope=1% B/min; column temperature=26°C; detection at 210 nm for monitoring peptide bonds and 280nm for the peptides containing tyrosine-,phenylalanine-, and tryptophan residues. These conditions are routinely used by many chromatographers. The retention behaviour of peptides in modified chromatographic conditions will be discussed in chapter IV.

The excellent elution profiles of representative samples of model peptides are presented in Figures 10A and 10B. The peptides were chromatographed at pH 2.0 on two SynChropak columns: C-18 (Figure 10A) and C-8 (Figure 10B).

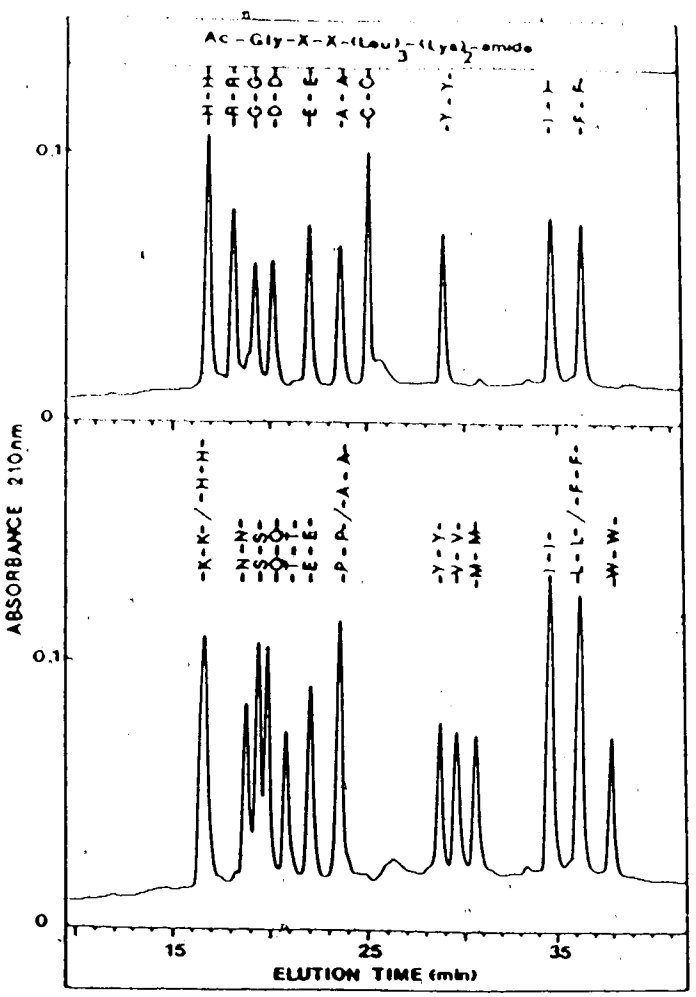


Fig. 10A. Chromatograms of the synthetic model peptides, having the sequence Ac-G-X-X-L-L-L-K-K-amide, where position X is substituted by the 20 amino acids found in proteins. Conditions: column, SynChropak C-18 (250 x 4.1 mm I.D.); linear AB gradient (1% B/min) where A=0.1% aq. TFA and B=0.1% TFA in acetonitrile (pH 2.0); flow-rate= 1ml/min; 26°C; absorbance at 210 nm.

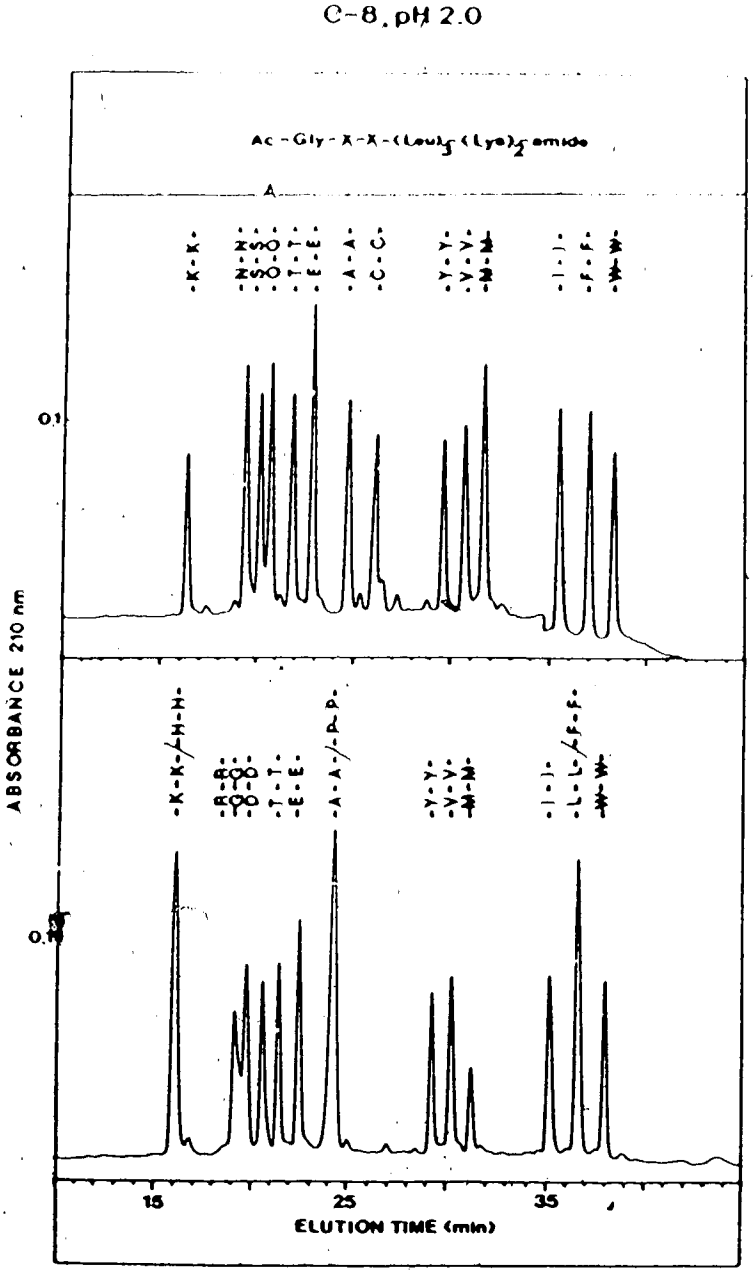


Fig. 10B. Chromatograms of the synthetic model peptides, having the sequence Ac-G-X-X-L-L-L-K-K-amide, where position X is substituted by the 20 amino acids found in proteins. Column, SynChropak C-8 (250 x 4.1 mm I.D.). Same chromatographic conditions as Fig. 10A.

The identification of the peptides on the chromatograms was made by repeating many similar runs. By changing the injection amounts of some peptides while others were kept constant, peptide assignments were made. Consistently similar resolution of the model peptides on several RP columns enabled the determination of an empirical set of retention coefficients, describing the hydrophobicity of amino acid residues at pH 2.0.

A multiple linear equation approach has often been employed to calculate amino acid retention coefficients from RP-HPLC data (Su *et al.*, 1981). This approach is described as follows:

$$a_{1,1}X_1+a_{1,2}X_2+\ldots+a_{1,n}X_n = b_1$$
$$a_{2,1}X_1+a_{2,2}X_2+\ldots+a_{2,n}X_n = b_2$$
$$\vdots$$
$$a_{m,1}X_1+a_{m,2}X_2+\ldots+a_{m,n}X_n = b_n$$

In these equations, $b_1, b_2 \ldots b_n$ are the actual retention times of the peptides; $X_1, X_2 \ldots X_n$ are defined as the retention coefficients of amino acid residues; and $a_1, a_2 \ldots a_{mn}$ describe amino acid composition of the peptides. The retention time of each peptide was assigned by recording the retention time of the pure peptide and the retention of

the same peptide in the peptide mixture. The peptide mixtures were always spiked with a retention-stable peptide such as model peptide Ac-G-L_5-K_2-amide to correct for run-to-run chromatographic differences. Two methods were employed to calculate the retention coefficients for amino acid residues. The first method was a simple multiple linear equation approach. Three linear equations were first solved for the retention coefficients for Lys, Leu and Gly residues by using the retention data from three of the eight residue peptide sequences where -X-X- was Lys, Leu or Gly. The retention parameters for other amino acid residues were then calculated by assigning the three known coefficients into each equation. For the second method, the retention coefficient of each amino acide residue (X) in the eight residue sequence Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide was determined by subtracting the retention time of the synthetic core peptide Ac-Gly-$(Leu)_3$-$(Lys)_2$-amide from the retention time of the eight residue sequence and dividing the result by two. The results of these two methods were very similar. The average values from the two different mathematical methods were adopted for the retention coefficients of amino acid residues. The results are shown in Table 5.

As can be seen, the retention data show that aromatic side chains are the most hydrophobic among 20 amino acid side chains, due to the large size of the aromatic groups. Amino acids with aliphatic side chains have a noticeable

positive contribution to retention, while amino acids with basic side chains have a negative contribution to retention. It is noteworthy that acidic side chains have little or a slightly positive contribution. This is understandable since the side chain carboxyl groups are protonated at pH 2.0. The relative elution order of the peptides is as expected. For example, the -E-E- peptide is more hydrophobic than the -D-D- peptide. Similarly, -A-A- > -G-G-; -T-T- > -S-S-; -I-I- > -V-V-; -Q-Q- > -N-N-. All these peptides differ by two methylene groups. Interestingly, the retention coefficients for Ala compared to Gly and Ile compared to Val are 2.2 and 2.4 minutes, respectively. In both cases we are adding a methyl group to the side chain of Gly or Val and these groups are accessible to interact with the reversed-phase support. However, if we compare the retention coefficients for Glu and Asp; Gln and Asn; Thr and Ser, the increase in the coefficients on adding the extra $-CH_2-$ group is 0.9, 0.6 and 0.8 minutes, respectively. This can be explained by the fact that the $-CH_2-$ group is not as accessible when added to the side chain between the peptide backbone and the hydrophilic functional groups. Moreover, the -L-L- peptide is more hydrophobic than the -I-I- peptide although these side chains contain the same number of carbon atoms. Since isoleucine is β-branched, the β-carbon is close to the peptide backbone and not as available to interact with the reversed-phase support compared to the conformation of the leucine side chain. These results show the excellent

selectivity of the reversed-phase supports used.

The retention coefficients for N- and C- terminal groups (see Table 5) were determined from the sequence Y-Gly-$(Leu)_5$-$(Lys)_2$-Z, where Y=Nα-acetyl (A) or α-amino (B) and Z=Cα-amide (C) or α-carboxyl (D). Figure 11 shows the RP-HPLC profile of mixtures of the four peptides at pH 2.

Table 6 compares our retention coefficients (pH 2.0), obtained from model synthetic peptides, with those reported previously by other research groups using computer-assisted regression analysis of the retention times of a wide range of peptides with varied composition (Meek and Rossetti, 1981; Su *et al.*, 1981; Browne *et al.*, 1982; Sasagawa *et al.*, 1982 and 1984). Retention coefficients were normalized relative to leucine (assigned a value of 100) to allow a direct comparison. As can be seen, there are large discrepancies between the two different approaches. In the studies of these workers, certain amino acid residues did not occur in the composition of the samples as frequently as other amino acid residues. The retention parameters for these amino acid residues would be underestimated as observed by Meek and Rossetti (1981). The other explanation is the conformational effect of some peptides which they used to determine amino acid retention coefficients. This would result in an inaccurate determination of the retention data. It is not quite clear whether there exists any nearest-neighbour or chain-length-dependence effect. The effect of molecular weight on retention is relatively

TABLE 5. Retention coefficients of amino acid residues at pH 2.0.

Amino acid residue	Retention coefficients pH 2.0 (min)
Trp	8.8
Phe	8.1
Leu	8.1
Ile	7.4
Met	5.5
Val	5.0
Tyr	4.5
Cys	2.6
Pro	2.0
Ala	2.0
Glu	1.1
Thr	0.6
Asp	0.2
Gln	0.0
Ser	-0.2
Gly	-0.2
Arg	-0.6
Asn	-0.6
His	-2.1
Lys	-2.1
α-amino	-6.9,-3.0[a]
α-COOH	-0.8

The retention coefficients (in min) were determined from retention times in RPC as shown in Fig. 10.

[a]The charged α-amino group had a smaller effect in an N-terminal Arg residue than an N-terminal residue with an uncharged side chain.

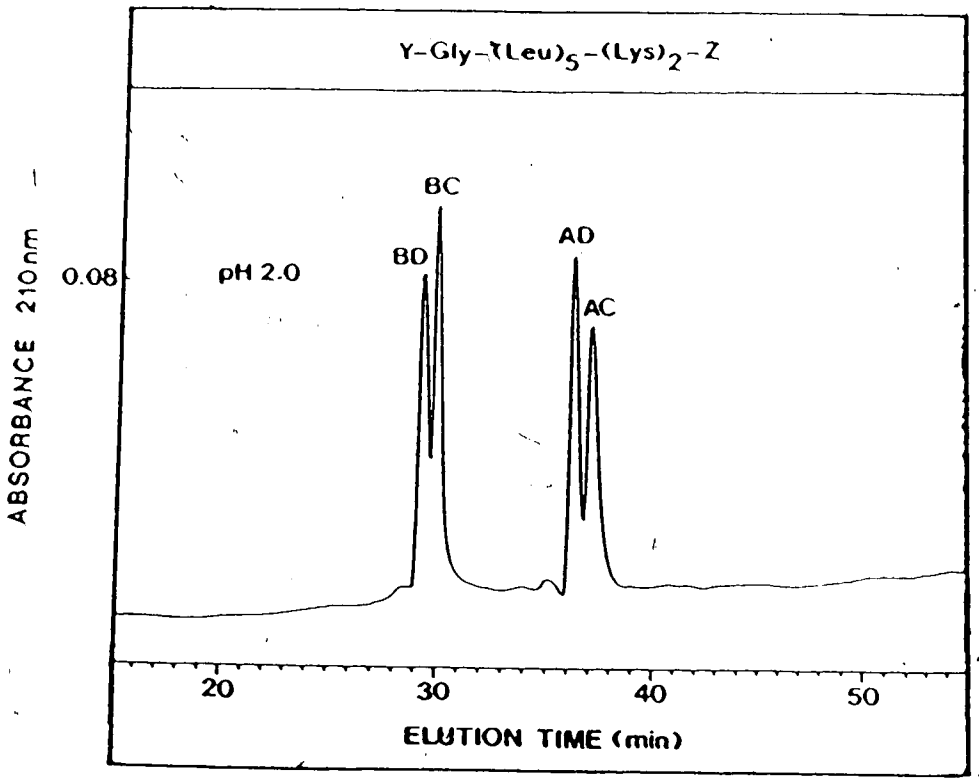


Fig. 11. Chromatogram of four synthetic peptides with the sequence Y-G-L-L-L-L-L-K-K-Z,
where Y = N^{α}-acetyl (A) or α-amino (B)
and Z = C^{α}-amide (C) or α-carboxyl (D).

Conditions: column, SynChropak RP-P C-18 (250 x 4.1 mm I.D.), linear AB gradient; Solvent A consisted of 0.1% aq. TFA and solvent B of 0.1% TFA in acetonitrile, (pH 2.0); 1% B/min; flow-rate, 1 ml/min; 26°C.

TABLE 6. Comparison of predicted retention coefficients of amino acid residues from literature and those obtained from synthetic peptides in this study

Amino acid	Retention coefficients at pH 2 relative to Leucine, taken as 100				
	I (this study)	II	III	IV	V
Trp	109	136	82	- 9	157
Leu	100	100	100	100	100
Phe	100	119	96	80	131
Ile	91	104	33	184	73
Met	68	55	28	113	42
Val	62	28	18	34	48
Tyr	56	80	30	28	70
Cys	32	-	-46	-40	48
Pro	25	30	26	-13	32
Ala	25	9	37	- 4	10
Glu	14	10	-36	-24	11
Thr	7	28	4	-25	- 6
Asp	2	0	-15	61	- 5
Gln	0	12	- 2	14	-21
Gly	- 2	15	- 6	-26	2
Ser	- 2	4	-21	21	-29
Arg	- 7	0	-18	-43	-20
Asn	- 7	-43	-29	-56	-31
Lys	-26	-12	-19	-17	-30
His	-26	33	-11	-85	-23
α-amino	-85,-37[a]	-	21	49	9
α-COOH	-10	-	12	49	17

II: Sasagawa et al., 1982. III: Browne et al., 1982.
IV: Su et al., 1981. V: Meek and Rossetti, 1981.

unimportant in small peptides and is completely eliminated in the model peptides used in this study where single amino acid substitutions were made in an eight-residue peptide. Our approach ensures that 20 amino acids occur in the model peptides with the same frequencies in the substituted positions(-X-X-) and offers the most accurate method for determining retention coefficients.

D. Prediction of Peptide Retention Time

Isolation or quantitation of a specified peptide in a complex mixture by reversed-phase HPLC can be simplified if the elution position of the peptide can be predicted. The amino acid residue retention coefficients, determined in the above section, were applied to predict the elution positions of 58 peptides whose sequences are shown in Table 7. The peptides were chromatographed under the system used to determine the retention coefficients, and peptide standard S4 was always run with the tested samples to correct for deviations arising from the use of different columns or from column aging (see Chapter IV). The predicted retention time (τ) for a peptide equals the sum of the retention coefficients (see Table 5)($\sum R_c$) for the amino acid residues and end groups plus the time for elution of unretained compounds (t_o) and the time correction from the peptide standard (t_s),

$$\tau = \sum R_c + t_o + t_s$$

TABLE 7. Comparison of predicted and observed peptide retention times

Peptide	Sequence	No. of Residues	R_t^{pred} (min)	R_t^{obs} (min)	Error (min)
1	AcKF* amide	2	3.10	3.35	-0.25
2	AcAKF* amide	3	5.03	4.45	0.58
3	AcKF*A amide	3	5.03	3.55	1.48
4	NH_2F*AA amide	3	2.13	2.92	-0.79
5	AcKF*AA amide	4	15.18	16.65	-1.47
6	AcAAKF* amide	4	6.89	6.49	0.40
7	AcAKF*A amide	4	7.07	6.04	1.03
8	AcAKF*AA amide	5	10.87	10.58	0.29
9	AcAAKF*A amide	5	8.71	8.61	0.10
10	NH_2FFGLM amide	5	19.75	20.28	-0.53
11	AcKGLGLK amide	6	11.48	14.64	-3.16
12	AcAAKF*AA amide	6	12.84	13.03	-0.19
13	NH_2AAKF*AA amide	6	11.06	12.56	-1.50
14	AcAAKF*AA OH	6	11.97	13.91	-1.94
15	NH_2AAL**F*AA amide	6	16.08	14.31	1.77
16	NH_2LSL**F*AL amide	6	25.40	26.87	-1.47
17	NH_2LSF*L**ALOEt	6	25.94	26.89	-0.95
18	AcAAAKF*AA amide	7	14.84	14.63	0.21
19	AcAAKF*AAA amide	7	14.79	14.43	0.36
20	AcKGLLLGK amide	7	24.52	23.93	0.59
21	AcKKLLLKK amide	7	21.00	20.73	0.27
22	AcKLGLGLK amide	7	23.57	21.67	1.90
23	NH_2DYMGWMDF OH	8	23.85	26.78	-2.93
24	AcTDLLAGGK amide	8	12.06	13.99	-1.93
25	NH_2TDLLAGGK amide	8	6.23	4.68	1.55
26	AcTDGLAGGK amide	8	4.11	4.57	-0.46
27	AcGAKLEAKG amide	8	6.51	6.08	0.43
28	AcTDLLGGGK amide	8	11.72	12.87	-1.15
29	pEHWSYGLRPG amide	10	19.60	19.88	-0.28
30	NH_2HKTDSFVGLM amide	10	14.47	14.91	-0.44

....continued

Peptide	Sequence	No. of Residues	R_t^{pred} (min)	R_t^{obs} (min)	Error (min)
31	NH_2DMHDFFVGLM amide	10	30.13	29.75	0.38
32	NH_2VQAAIDYING OH	10	16.89	17.06	-0.17
33	NH_2RPKPQQFFGLM amide	11	26.57	26.03	0.54
34	NH_2DVPKSDQFVGLM amide	12	23.52	22.36	1.16
35	AcGKFKGPPLRRVR amide	12	17.03	16.16	0.87
36	NH_2GGFKRPPLRRVR amide	12	11.26	14.18	-2.92
37	AcGKFKRPPLRRVR amide	12	16.84	15.94	0.90
38	AcGKGKRPPLRRVR amide	12	8.04	10.03	-1.99
39	AcGKFGRPPLRRVR amide	12	18.39	16.02	2.37
40	AcGKFKRGPLRRVR amide	12	14.02	14.63	-0.61
41	AcGKFKRPGLRRVR amide	12	14.38	14.55	-0.17
42	AcGGFKRPPLRRVR amide	12	18.19	16.01	2.18
43	AcGKFKRPPGRRVR amide	12	7.70	11.12	-3.42
44	AcGKFKRPPLRGVR amide	12	16.28	15.73	0.55
45	AcGKFKRPPLGRVR amide	12	16.44	16.01	0.43
46	AcGKFKRPPLRRVG amide	12	16.70	15.59	1.11
47	AcGKFKRPPLRRGR amide	12	10.64	12.89	-2.25
48	AcVSKTQTSQVAPA amide	12	12.26	12.76	-0.50
49	AcVSKTETSQVAPA amide	12	12.99	13.07	-0.08
50	AcVSKTATSQVAPA amide	12	17.10	15.51	1.59
51	AcASKTETSQVAPA amide	12	9.81	10.72	-0.91
52	AcDRNAEGYIDAEEL amide	13	30.11	25.67	4.44
53	AcNRNANGYIDAEEL amide	13	26.85	23.85	3.00
54	AcNRDADGYIDAEEL amide	13	28.76	24.72	4.04
55	AcDRDADGYIDAEEL amide	13	29.57	24.98	4.59
56	AcSDQEKRKQISVRGL amide	14	14.17	15.47	-1.30
57a	NH_2AGCKNFFWKTFTSCOH	14	26.57	26.03	0.54
57b	NH_2AGCKNFFWKTFTSCOH	14	26.57	25.92	0.65
58	Ac(GAKLEAKG)$_2$ amide	16	14.97	14.40	0.57

F* denotes nitrophenylalanine; L** denotes norleucine. The contributions of the ethyl ester (Peptide 17) and the pyroglutamic acid (Peptide 29) were considered identical to the amide and acetyl-glutamic acid, respectively.

The accuracy of the predictive method was shown by the correlation between the observed and predicted retention times in RPC. The small average deviation of predicted retention times from observed values (1.3 min) and the high degree of correlation (Figure 12; correlation=0.98, calculated by linear least squares fitting) indicate that our retention coefficients provided excellent predictive accuracy for the peptides studied (ranging from 2-16 residues). By comparison, there were four-minute average error between the predicted and observed retention times for 18 small peptides up to 20 residues by using Meek's retention coefficients. It is noteworthy that the only peptide, 14 residues in length (Table 7), which contained two cysteine residues showed little difference in retention time between its oxidized(57a) and reduced(57b) forms. The same phenomenon was observed in two other peptides containing two cysteine residues each, 21 and 24 residues in length (not listed in Table 7). The results from the limited range of peptides studied seem to suggest that disulphide bridge formation of small peptides has little influence on the interaction of peptide molecules with the hydrophobic stationary phase compared to the same molecules with free cysteine residues. Several peptides predicted to have negative retention times were, as expected, not retained by the reversed-phase columns.

Predicted retention of larger peptides was shown to deviate from actual retention due to conformational effects

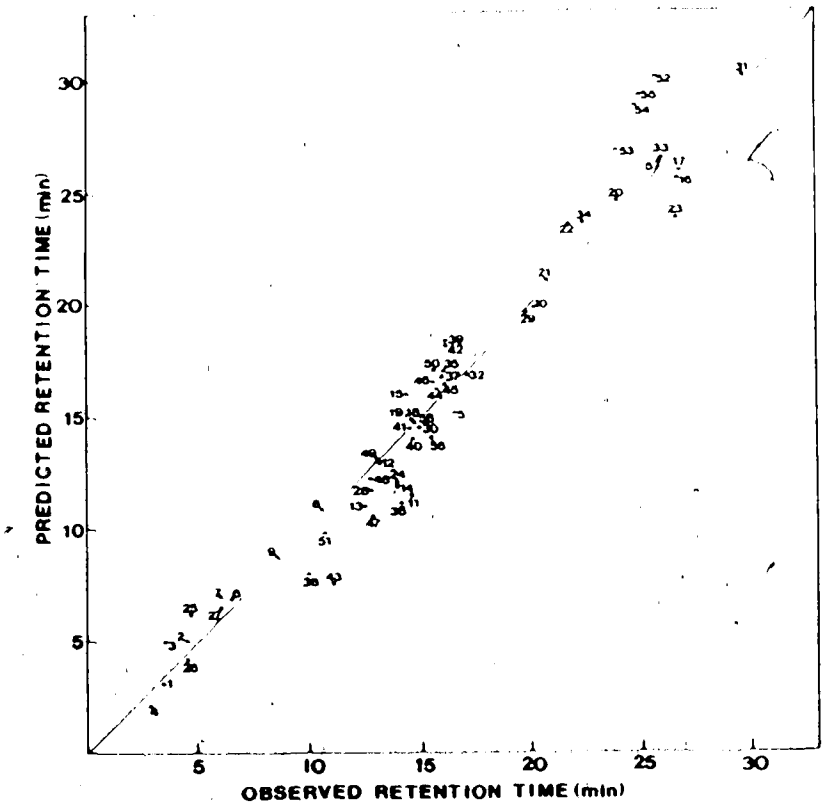


Fig. 12. Correlation of predicted and actual peptide retention times in RP-HPLC. Numbers adjacent to the data points indicate the peptides listed in Table 7. The peptides were chromatographed on SynChropak C-8 or C-18 columns (250 x 4.1 mm I.D.) under the conditions used to determine the amino acid residue retention coefficients: linear gradient (1% B/min) where A= 0.1% aq. TFA and B= 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 1ml/min; 26°C; absorbance at 210 nm.

in solution which reduce the number of exposed hydrophobic residues (Hearn, 1980). The accuracy of peptide retention time prediction decreases significantly for peptides with more than 20 residues. It is believed that the retention time of a large peptide is partially dependent on its molecular weight and introduction of a molecular weight correction may help to increase the predictive accuracy (Monch and Dehnen, 1978; Van Der Zee and Welling, 1982).

IV. FACTORS AFFECTING PEPTIDE RETENTION IN RPC

As discussed in chapter III, it is possible to predict peptide elution positions under the specific chromatographic conditions used to determine the retention coefficients. Peptide retention is affected by a number of factors, such as the nature of the bonded stationary phase, the buffer system used, the pH of the mobile phase, the organic mobile phase component, the flow-rate, the column temperature and gradient slope. By examining peptide elution profiles under various conditions, information was obtained to expand our predictive method and improve our understanding of the peptide retention mechanism. It was also found that an internal peptide standard was required to make accurate peptide predictions.

A. Effect of Different Reversed-Phase Packings

Peptide separation in RPC is based on the hydrophobic differences between the samples being chromatographed and the bonded phase of the support material. It is clear that peptide retention is affected by the nature of the reversed-phase packing. In other words, columns from different sources may vary depending on the physical characteristics of the packing materials (McNair, 1984). The physical properties of the packing materials used in this study are presented in Table 8. The effects of column configuration (column length and internal diameter), ligand density, particle size and column aging on peptide retention

were investigated.

Chromatography of model peptide mixtures, whose sequence variations have been described in Chapter III, was carried out on three different RP columns (SynChropak C4, SynChropak C8, and Whatman C8) to examine the effects of the alkyl chain length as well as ligand density on peptide retention (Fig. 13). Similar elution profiles were obtained with almost identical elution orders of peptides; the main difference was a shift in peptide retention times (Cooke *et al.*, 1983). The affinity order of RP packings for peptides was found to be Whatman C8 > SynChropak C4 > SynChropak C8. The increased retention on the SynChropak C4 compared to the C8 column is mainly due to the increased n-alkyl chain ligand density (double for the C4 column). This agrees with the results of other studies (Majors and Hopper, 1974; Karch *et al.*, 1976; Henrikson *et al.*, 1978; Lau *et al.*, 1984). With a given mobile phase, increased ligand density generally results in greater peptide retention. For the small peptides used in this study (8 residues, ca, 900 daltons), only a limited part of the long alkyl chains participates in the retention process (Berendsen and de Galan, 1980). Lau *et al.* have observed that the length of the n-alkyl chain has little influence on peptide resolution (1984). Figure 13 shows slightly better overall resolution on the two C-8 columns compared to the C-4 column (Arg/Gly, Leu/Phe/Trp separations).

TABLE 8 - Physical Properties of the Reversed-Phase Packing Materials.

Reversed-Phase	Chain Length of Bonded Material	Column Length (mm)	Column I.D. (mm)	Carbon Loading (% W/W)	Particle size um	Pore size Å
Bechman Ultrapore RPSC C-3	3	75	4.6	2.9	5	300
SynChropak RP-4	4	250	4.1	7.5	6.5	300
SynChropak RP-8	8	250	4.1	7.5	6.5	300
Whatman Partisil 5 C-8	8	250	4.6	9.0	5	60
Ultrapore RP-300 C-8	8	220	4.6	—	10	300
SynChropak RP-P	18	50	4.1	10.0	6.5	300
SynChropak RP-P	18	250	4.1	10.0	6.5	300
SynChropak RP-P	18	250	10.0	10.0	6.5	300

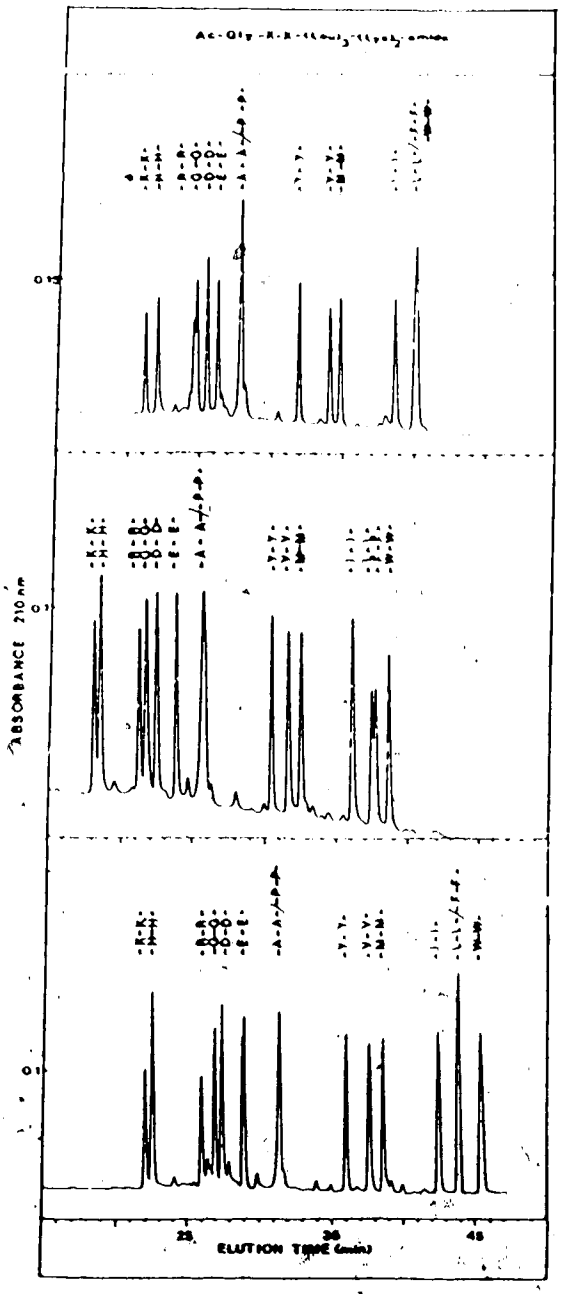


Fig. 13. Chromatograms illustrating the effects of three different reversed-phase columns on the retention of synthetic model peptide mixtures. Top, SynChropak C-4 column (250 x 4.1 mm I.D.); middle, SynChropak C-8 column (250 x 4.1 mm I.D.); bottom, Whatman C-8 column (250 x 4.6 mm I.D.). Conditions: linear gradient (1% B/min) where A=0.1%aq. TFA and B=0.1%TFA in acetonitrile, (pH 2.0); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm.

To investigate further the effects of column configuration and aging on peptide retention, a mixture of five synthetic peptide standards, whose sequence variations have been described in Chapter III, was tested on four SynChropak RP columns. Figure 14 shows the elution profiles of the peptide standard mixtures chromatographed at pH 2.0 under the conditions used to obtain our retention coefficients (see Fig. 10). The elution profiles for these four reversed-phase supports were found to be similar with the acetonitrile solvent system (Wilson *et al.*, 1981; Geng and Regnier, 1984a). The peptide mixture was dissolved in 0.5% aq.TFA (Fig. 14A-C) or 2% aq.TFA (Fig. 14D). Following sample injection, the absorbance peak at 210 nm, produced by the excess of TFA concentrations in the sample, represented the elution time for unretained compounds (t_o). Columns of different sizes have different times for t_o. It is apparent that the peptides bind more tightly to a new column (Fig. 14A) than a similar column extensively used over a period of months (Fig. 14B). The results indicate the influence of column aging on peptide retention. Interestingly, only slightly shorter peptide retention was observed on the 5-cm column (Fig. 14C) compared to the 25-cm (Fig. 14A), demonstrating only a small effect of column length on peptide retention (Grego and Hearn, 1984). Also, similar elution profiles were obtained on a preparative column (Fig. 14D; 10 mm I.D.) and an analytical column (Fig. 14A; 4.1 mm I.D.). This result validates the use of our retention

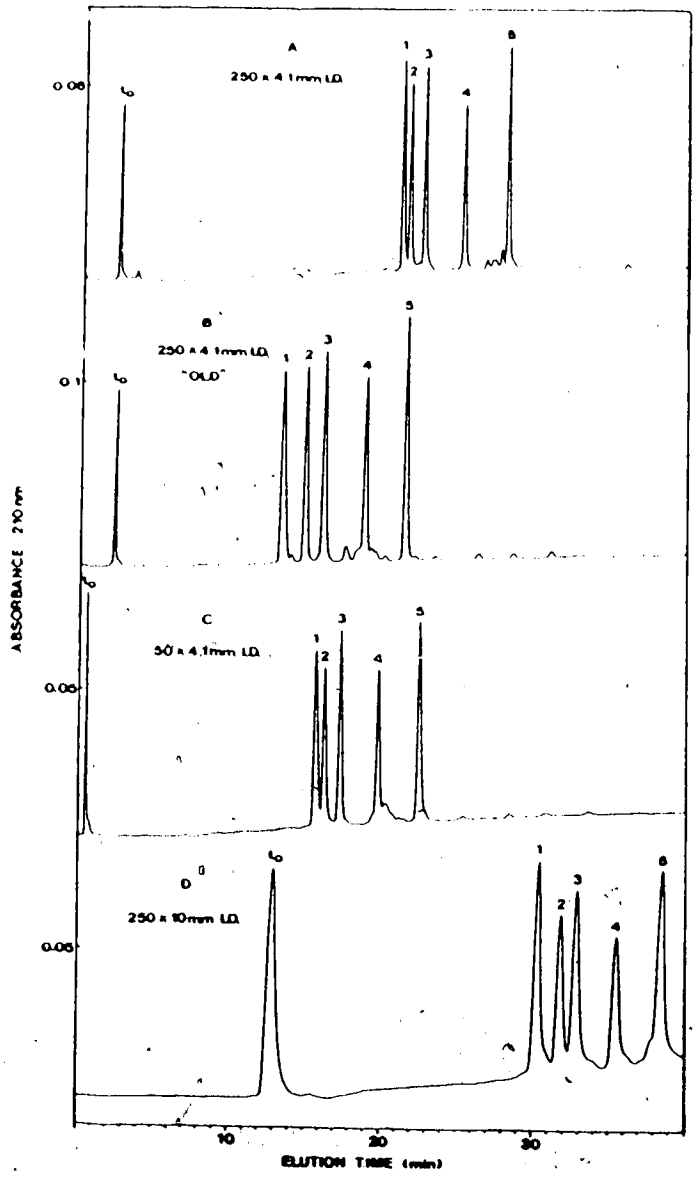


Fig. 14. Chromatograms illustrating the effects of four different RP columns on the retention of the peptide standards. Columns: SynChropak C-18 at pH 2.0, (A) 250 x 4.1 mm I.D.;(B)250 x 4.1 mm, I.D.; (C)50 x 4.1 mm I.D.; (D)250 x 10 mm I.D. Detailed column descriptions are found in Table 8. Conditions:linear gradient (1% B/min) where A=0.1%aq. TFA and B=0.1% TFA in acetonitrile; flow-rate, 1 ml/min; 26^{o}C; absorbance at 210 nm.

coefficients on columns of varying diameter.

For small peptides, pore size and particle size are not the important column parameters affecting retention and resolution in the reversed-phase mode (Hearn, 1980). Sorbents of smaller particle size usually have a higher column efficiency. However, Wilson *et al*. observed almost identical chromatographic behaviors of peptides when 5 *um* or 10 *um* particle-size packings were tested (1981). The particle sizes of all the packings used in this study are similar (5-6.5 *um*) and have little effect on resolution.

Table 9 shows a comparison of the retention times of the standard peptide mixture on the four SynChropak C-18 columns, a SynChropak C-8 and a Whatman C-8 column. The predictive method has been detailed in Chapter III. These results indicate the importance of an internal peptide standard to correct for column and instrumentation variations when the amino acid coefficients from HPLC data of our model synthetic peptides are applied to predict the retention time of any peptide of known composition on different columns.

Effect of Different Organic Solvents

The aqueous buffer often has a profound effect on both peptide retention and resolution. In general, optimization retention and resolution can be achieved by manipulation of mobile phase as well as stationary phase parameters. The most popular eluting solvents are methanol, acetonitrile,

TABLE 9. Predicted and observed retention times for a peptide standard mixture on different RP-columns[a]

	Column (see Experimental)											
	1		1 ("old")[b]		2		3		4		5	
Peptide standard	pred.[c]	obs.[c]	pred.	obs.	pred.	obs.	pred.	obs.	pred.	obs.	pred.	obs.
1	19.0	20.9	12.7	13.2	13.7	15.6	29.4	30.2	14.0	14.0	16.5	16.6
2	19.8	21.4	13.5	14.7	14.5	16.2	30.2	31.7	14.8	15.9	17.3	18.9
3	22.0	22.4	15.7	16.0	16.7	17.3	32.4	32.8	17.0	17.2	19.5	20.1
4	25.0	25.0	18.7	18.7	19.7	19.7	35.4	35.4	20.0	20.0	22.5	22.5
5	30.2	27.9	23.9	21.4	24.9	22.4	40.6	38.3	25.2	22.8	27.7	25.4

Conditions: linear gradient (1% B/min) where A = 0.1% aq. TFA and B = 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 1 ml/min.

a: Detailed column descriptions are found in Table 8. b:This column was extensively used over a period of 4 months. c:The column temperature for all runs was 26 C.

Column 1: SynChropak RP-P (C-18) (250 x 4.1 mm I.D.). Column 2: SynChropak RP-P (C-18)(50 x 4.1 mm I.D.)
Column 3: SynChropak RP-P (C-18) (250 x 10 mm I.D.). Column 4: SynChropak RP-8 (C-8) (250 x 4.1 mm I.D.).
Column 5: Whatman Partisil 5 C-8 (250 x 4.6 mm I.D.).

and isopropanol. Of these three organic solvents, the order of effectiveness in eluting peptides from a reversed-phase column was: isopropanol > acetonitrile > methanol (Wilson *et al.*, 1981; Lau *et al.*, 1984).

Figure 15 shows the elution profiles of the identical model peptide mixtures when chromatographed on a SynChropak C-8 column using a linear AB gradient (1% B/min), where A=0.1% TFA/H_2O and B=0.1% TFA in isopropanol (top), acetonitrile (middle) or methanol (bottom). As noted, retention times of the model peptide mixtures were shifted in accordance with the polarity of the solvents being used. The increasing retention times of the peptides with increasing polarity of organic solvent also confirm the effectiveness order noted above. In general, the TFA-acetonitrile eluent gives good resolution for most peptides and allows absorbance monitoring at 210 nm for high-sensitivity detection. Isopropanol is a stronger eluent for peptides than acetonitrile and may be required for elution of very hydrophobic components. High viscosity of the isopropanol system sometimes causes high column backpressure and low efficiency (Lundblad and Noyes, 1984). Although the resolution of the peptides in the methanol system is not as good as that of the acetonitrile system, methanol can be used for very hydrophilic molecules. All the mobile phase systems mentioned to this point may be used with absorbance detection of peptides at 210 nm. Furthermore, the addition of a chaotropic salt, $NaClO_4$, to

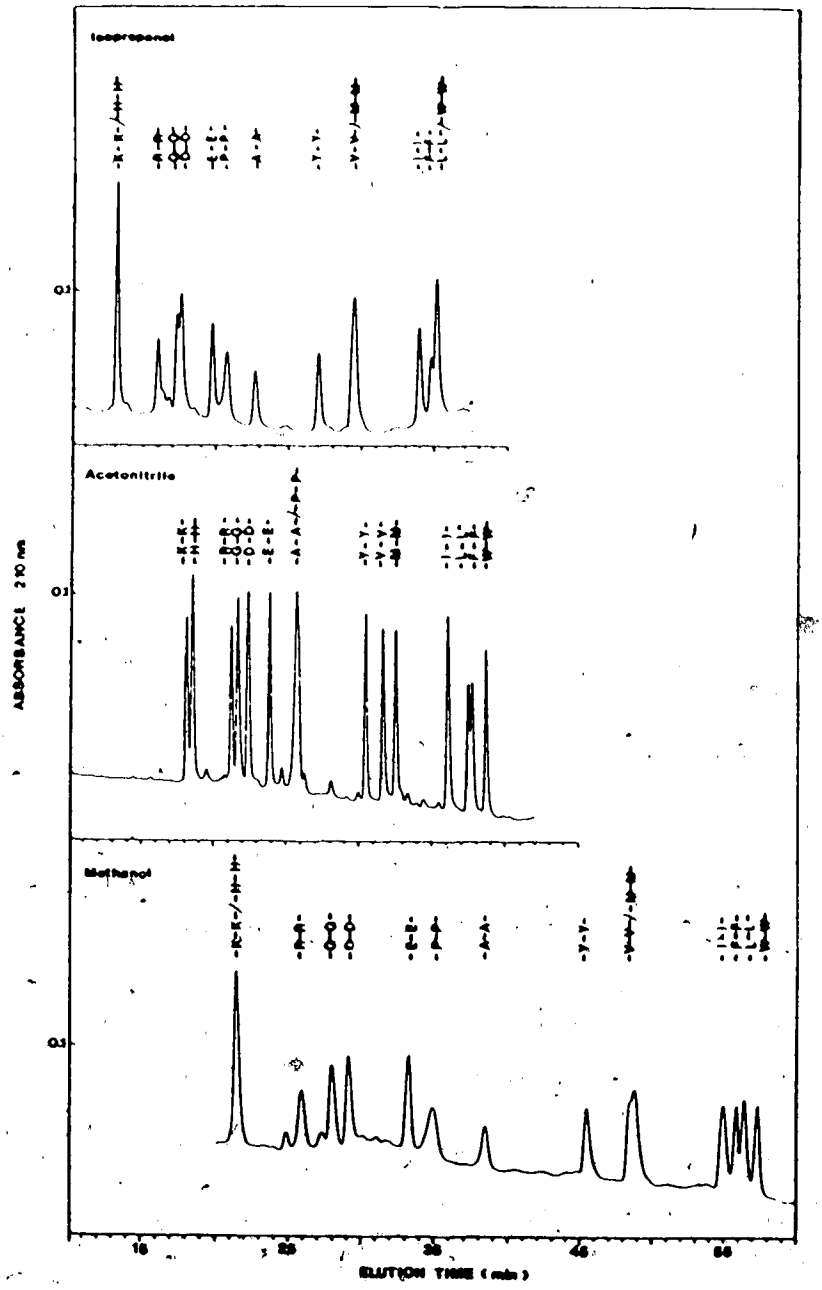


Fig. 15. Chromatograms illustrating the effects of three different organic solvents on the retention of the synthetic model peptide mixtures. Organic solvents: top, isopropanol; middle, acetonitrile; bottom, methanol. Conditions: linear gradient (1% B/min), where A=0.1%aq. TFA and B=0.1% TFA in one of the above three organic solvents, (pH 2.0); flow-rate, 1ml/min; 26^{o}C; absorbance at 210 nm.

the pH 7.0 buffers (e.g. A=aq.$(NH_4)_2HPO_4$, 0.1M $NaClO_4$; B=40% H_2O-60% acetonitrile containing 0.1M $NaClO_4$) was found to be essential for producing good resolution (Hatefi and Hanstein, 1969).

C. Effect of Ion-Pairing Reagents

Ion-pairing reagents are usually employed to improve the resolution of charged peptide molecules (Poll *et al.*, 1982). In general, two principle mechanisms of ion-pairing have been suggested, one involves the formation of ion-pairs in the mobile phase with the sample molecule prior to adsorption to the stationary phase and the other involves covering the stationary phase surface such that ion-pairing reagents act as a "dynamic ion-exchanger" (Schaaper and Teller, 1980; Melander *et al.*, 1980). Whatever the mechanism, the resolving power of ion-pairing reagents is attributed to their interaction with the charged residues of a peptide.

The retention characteristics of peptides on chemically bonded hydrocarbonaceous stationary phases were found to be influenced by the addition of low concentrations of suitable counterions (Hearn and Hancock, 1978; Hancock *et al.*, 1978b). In the case of anionic ion-pairing reagents, all anionic counterions are potentially capable of ion-pairing with the positively charged residues of a peptide, thereby reducing its overall hydrophilicity and increasing peptide retention (Bennett *et al.*, 1979; Vigh *et al.*, 1982). For

example, phosphoric acid (H_3PO_4) and perfluorinated acids (*e.g.* trifluoroacetic acid and heptafluorobutyric acid) are commonly used anionic counterions. They differ in their ability to interact with the stationary phase. The effects of H_3PO_4, TFA and HFBA on retention of a series of model synthetic peptides were compared. The model peptides, described in Chapter III, were chromatographed on a SynChropak C-18 column at a flow-rate of 1 ml/min, a temperature of 26°C and the water-acetonitrile solvent system containing different ion-pairing reagents (A=0.1% aq. H_3PO_4, TFA or HFBA and B=0.1% of the three respective ion-pairing reagents in acetonitrile). A plot of R_t/N (R_t=peptide retention time; N=number of positive charges on the peptide) for each of the three ion-pairing reagents versus the values obtained for the other two reagents is shown in Fig. 16. These results compare the average contribution of each positive charge on the peptides to any change in their retention times in the presence of different counterions. An excellent degree of correlation (correlation coefficient=r; A compares HFBA and TFA, r=0.999; B compares H_3PO_4 and TFA, r=0.998 and C compares H_3PO_4 and HFBA, r=0.997) was obtained between the combinations of R_t/N for the three ion-pairing reagents. The results suggest an essentially equal contribution by each positively charged residue to shifts in peptide retention when changing from one ion-pairing reagent to another. One important point that can be drawn from the result of Figure 16 is that retention

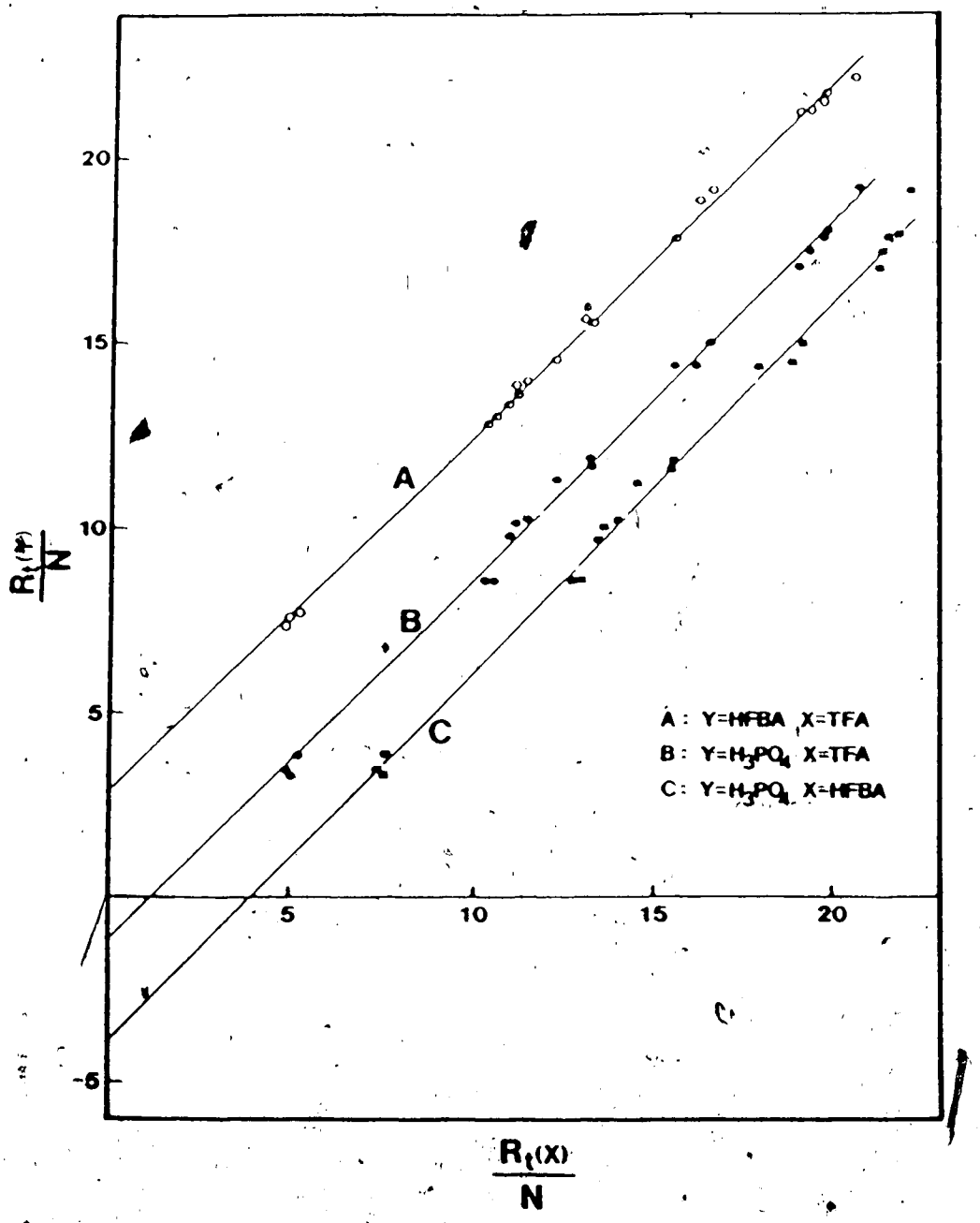


Fig. 16. Correlation of contribution of each positively charged group to changes in peptide retention in RP-HPLC in the presence of different counterions. Conditions: column, SynChropak C-18 (250 x 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% H_3PO_4, TFA or HFBA as ion-pairing reagent; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. R_t denotes retention time; N denotes number of positively charged groups in peptides.

times of the basic peptides increase with increasing hydrophobicity of the counterions. This is in agreement with the studies of several investigators (Hearn *et al.*, 1979b; Benn[redacted] *et al.*, 1980; Schaaper *et al.*, 1980; Bennett, 1983; Hancock and Harding, 1984). The "ion pairs" formed alter the retention behavior of basic peptides substantially, whereas that of neutral peptides is not affected in the presence of different counterions.

As stressed above, peptide retention and resolution are affected by different columns due to column aging and physical properties (see Table 8). A correction value which relates peptide retention times in different ion-pairing systems can be obtained from the chromatographic behavior of synthetic peptide standards for a specific column used. A series of five synthetic peptide HPLC standards (S1-S5) was chromatographed on a SynChropak C-18 column and an Aquapore C-8 column to examine the changes in the resolution and retention under different ion-pairing reagent systems (Fig.17). As seen in Fig. 17, the elution orders of the peptides were identical on both columns but differences in overall resolution were apparent. Thus, the peptide HPLC standards were used to calculate the required retention time correction between different ion-pairing systems for a specific column used. Table 10 indicates the retention times of the peptide standards for all three ion-pairing systems, together with the average contribution of each anionic counterion per positive charge to changes in peptide

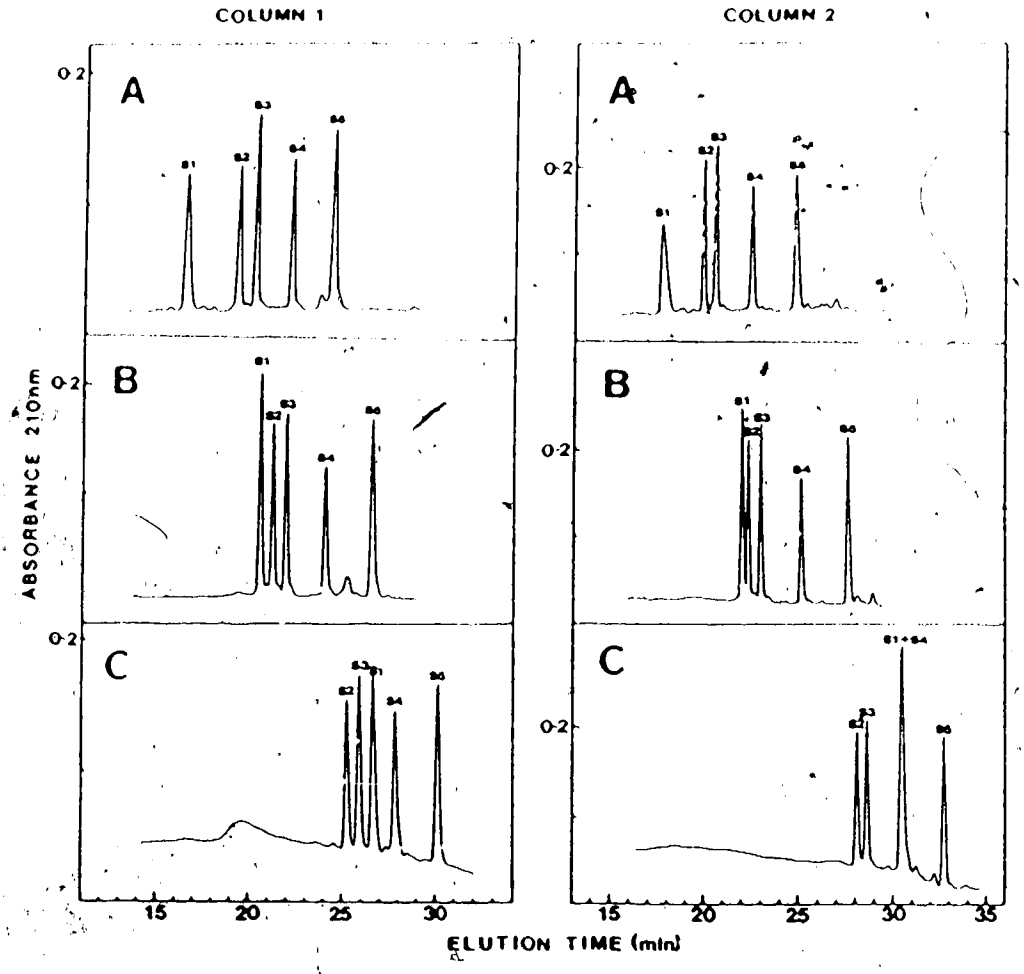


Fig. 17. Chromatograms illustrating the effect of ion-pairing reagents on the separation of a mixture of synthetic peptide standards in RP-HPLC. Conditions: Column 1, SynChropak C-18 (250 x 4.1 mm I.D.); Column 2, Aquapore C-8 (220 x 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% H_3PO_4 (Panel A), TFA (Panel B) or HFBA (Panel C); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1-S5 are described in Chapter III.

TABLE 10. Effect of ion-pairing reagent on retention times of five synthetic peptide HPLC standards in RP-HPLC.

Column 1

Peptide Standard	Retention Time (min) H_3PO_4	TFA	HFBA	Δ/N (HFBA-TFA)	Δ/N (TFA-H_3PO_4)
S1	16.3	20.5	26.7	2.1	1.4
S2	19.2	21.2	25.3	2.1	1.0
S3	20.0	21.9	25.9	2.0	1.0
S4	22.0	24.0	27.8	1.9	1.0
S5	24.2	26.5	30.1	1.8	1.2
				Av. 2.0	Av. 1.1

Column 2

Peptide Standard	Retention Time (min) H_3PO_4	TFA	HFBA	Δ/N (HFBA-TFA)	Δ/N (TFA-H_3PO_4)
S1	17.8	21.9	30.4	2.8	1.4
S2	20.0	22.3	28.1	2.9	1.2
S3	20.7	22.9	28.6	2.9	1.1
S4	22.6	25.0	30.4	2.7	1.2
S5	24.8	27.5	32.7	2.6	1.4
				Av. 2.8	Av. 1.3

Conditions: Column 1, SynChropak C_{18} (250 x 4.1 mm I.D.); Column 2, Aquapore C_8 (220 x 4.6 mm I.D.); linear gradient, where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% H_3PO_4, TFA or HFBA as ion-pairing reagent; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1-S5 are described under experimental.

* Δ denotes difference in retention time of a peptide between two ion-pairing reagent systems; N denotes number of positively charged groups in peptide.

retention between the HFBA and TFA systems and between the TFA and H_3PO_4 systems. The values were obtained by dividing the difference in retention time (ΔR_t) of the peptide standards in two ion-pairing systems by the number of positive charges they possess (N), summating the resulting values:

$$(\Delta^1/N + \Delta^2/N + \ldots\ldots \Delta^5/N)$$

and dividing this summated figure by 5. The final values obtained correspond to the plot intercepts on the ordinate shown in Fig. 16.

Prediction of retention time of a peptide between two counterion systems can be simplified by only considering Δ/N (Δ/N = the average contribution of each positively charged residue to shifts in peptide retention) and n (n= the number of positively charged groups of the peptide). Thus, the counterion correction factor (t_i) for a peptide of interest can be obtained by multiplying n by Δ/N,

$$i.e.\ t_i = n \times (\Delta/N)$$

When the retention time of a peptide of interest is known in the presence of one counterion system, its predicted position in another counterion system is simply described by the expression, $\tau = R_t + t_i$, where τ is the predicted peptide retention time in the desired counterion, R_t is the observed retention time in another counterion system, and t_i is the counterion correction factor. To test the accuracy of retention time prediction between systems containing different ion-pairing reagents, the above

prediction method was applied to the separation of a mixture of basic peptides of varying numbers of positively charged groups (see Table 11) on both SynChropak C-18 and Aquapore C-8 columns. The elution profiles of the peptides in the presence of anionic counterions for the SynChropak C-18 column are presented in Fig. 18. The results clearly show increasing retention times of the peptides with increasing hydrophobicity of the counterion ($HFBA^-$ [C]>TFA^- [B]>$H_2PO_4^-$ [A]). Also, the elution order of the peptides significantly changed from one counterion system to another. This is clearly illustrated in Fig. 18, where the elution order of peptides 1, 3 and 6 (containing 1, 3 and 6 positively charged residues, respectively) was reversed as the counterion changed from $H_2PO_4^-$ (Panel A) to $HFBA^-$ (Panel C)(Grego *et al.*, 1983).

Table 12 compares the predicted and observed retention times for the peptides in the H_3PO_4 ion-pairing system or HFBA system. The prediction was based on the observed retention times of the same peptide mixture in the TFA system. The results presented in Table 12 illustrate the accuracy of the method for the peptides examined. The average deviations of predicted and observed values for the seven peptides on the SynChropak column were 0.8 min (TFA - H_3PO_4) and 1.1 min (TFA - HFBA); for the Aquapore column, the values were 0.7 min (TFA - H_3PO_4) and 1.3 min (TFA - HFBA). The small average deviations prove the value of this predictive method.

TABLE II Sequences of a mixture of peptides of varying numbers of positively charged groups.

PEPTIDE	SEQUENCE	NUMBER OF POSITIVELY CHARGED GROUPS
0	Ac-I-D-L-L-G-AMIDE	0
1	Ac-V-S-K-I-E-I-S-Q-V-A-P-A-AMIDE	1
2A	Ac-R-G-A-G-G-L-G-L-G-K-AMIDE	2
2B	Ac-R-G-V-G-G-L-G-L-G-K-AMIDE	2
3	R-G-A-G-G-L-G-L-G-K-AMIDE	3
4	Ac-S-D-Q-E-K-R-K-Q-I-S-V-R-G-L-AMIDE	4
5	Ac-G-K-F-K-R-P-P-L-R-R-V-G-AMIDE	5
6	Ac-G-K-F-K-R-P-P-L-R-R-V-R-AMIDE	6

Ac = N^{α}-acetyl; amide = C^{α}-amide. Amino acid residues are denoted by the single letter code. Peptide 3 has a free α-NH_2 group.

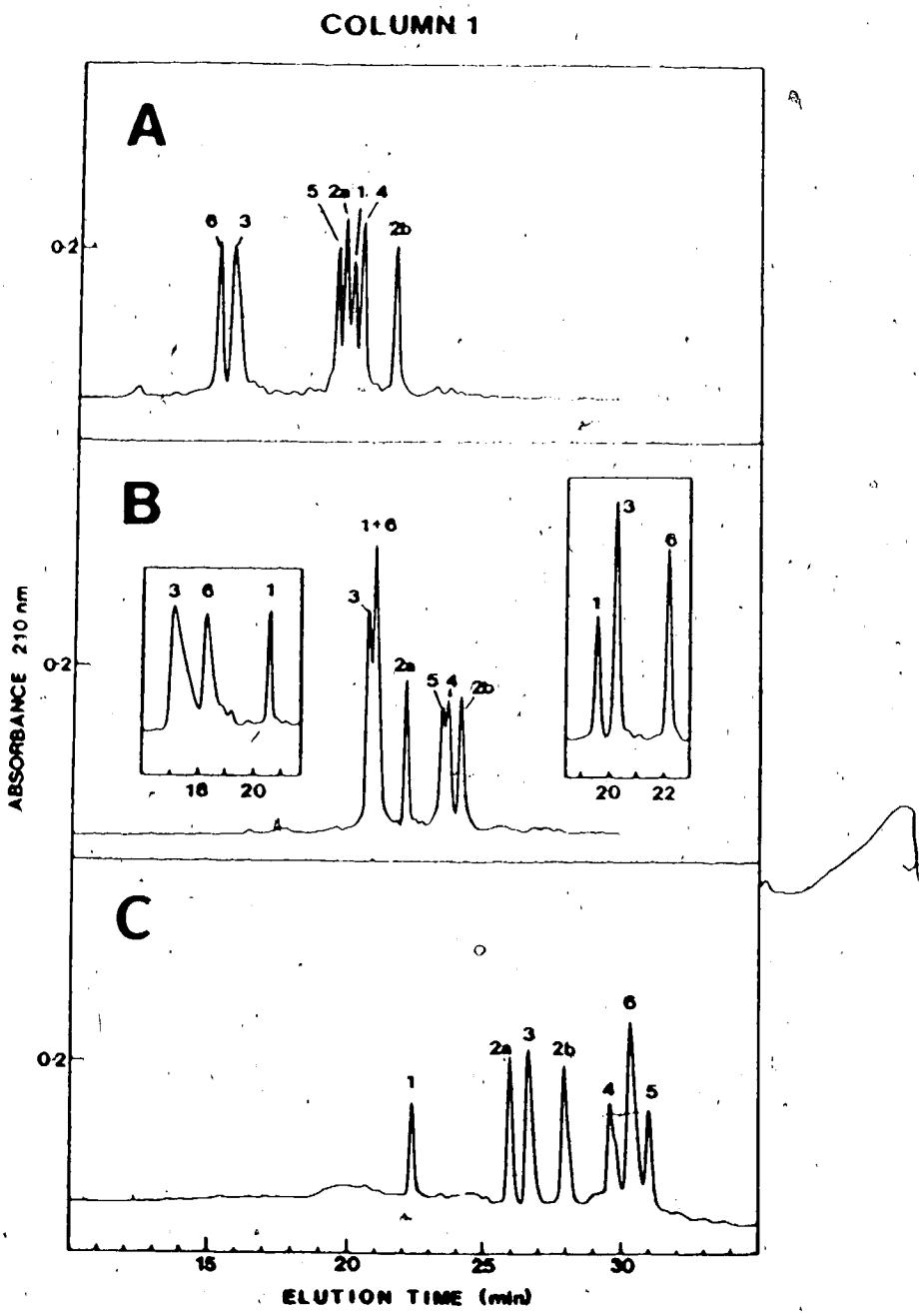


Fig. 18. Effect of ion-pairing reagents on the separation of a mixture of basic peptides in RP-HPLC. Conditions: column, SynChropak C-18 (250 x 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% H_3PO_4 (Panel A), TFA (Panel B) or HFBA (Panel C); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Panel B, insets: left, 0.01% TFA in solvents A and B; right, 0.4% TFA in solvents A and B.

TABLE 12. Comparison of predicted and observed retention times for a mixture of basic peptides of varying numbers of positively charged groups.

Column 1

Peptide	R_t^{obs} * TFA	τ ** H_3PO_4	R_t^{obs} H_3PO_4	τ HFBA	R_t^{obs} HFBA
1	21.0	19.9	20.1	23.0	22.4
2a	22.1	19.9	19.8	26.1	26.0
2b	24.1	21.9	21.7	28.1	28.0
3	20.8	17.5	15.8	26.8	26.7
4	23.6	19.2	20.4	31.6	29.6
5	23.5	18.0	19.5	33.5	30.4
6	21.0	14.4	15.3	33.0	31.0

Column 2

Peptide	R_t^{obs} TFA	τ H_3PO_4	R_t^{obs} H_3PO_4	τ HFBA	R_t^{obs} HFBA
1	20.5	19.2	19.8	23.3	23.7
2a	22.5	19.9	19.3	28.1	28.9
2b	25.6	23.0	21.9	31.2	30.4
3	21.3	17.4	15.6	29.7	29.9
4	24.0	18.8	19.3	35.2	32.2
5	23.7	17.2	17.6	37.7	35.6
6	20.5	12.7	12.6	37.3	35.6

Conditions: Column 1, SynChropak C_{18} (250 x 4.1 mm I.D.); Column 2, Aquapore C_8 (220 x 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% H_3PO_4. TFA or HFBA as ion-pairing reagent; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequences of peptides are shown in Table II.

* R_t^{obs} denotes observed retention time of a peptide.

** τ denotes predicted retention times of peptides, calculated as described in text.

The observed retention times of the seven peptides in H_3PO_4, TFA and HFBA (Table 12) were plotted against the average increase in retention time per positively charged residue (X) (obtained from peptide standards S1-S5, with TFA or HFBA as counterion [Table 10]) compared to the retention times of the peptide standards when H_3PO_4 was used as the mobile phase acid (for TFA, X=1.1; for HFBA, X=1.1+2.0=3.1 [Table 10]). Different slopes of the plots for each peptide were observed (Fig. 19A). With the exception of peptide 3, the plots show a linear relationship between peptide retention time and counterion hydrophobicity. When the number of positively charged residues in the peptides was taken into account, R_t/N was then plotted against X, showing relatively parallel profiles (Fig. 19B). These results further support the assumption that each positive group exerts an essentially equal effect on peptide retention. Thus, retention times of peptides in the presence of ion-pairing reagents not only follow the order of relative hydrophobicities determined by the nature of the amino acid side chains, but also rely on the number of positively charged groups and the polarity of the counterion.

The actual retention of a peptide was also found to be affected by the concentration of counterions (Hearn *et al.*, 1979b; Schaaper and Teller, 1980; Hearn and Grego, 1981). The effect of concentration of ion-pairing reagents on the retention of five synthetic peptide standards was studied with a linear AB gradient, where A= water and

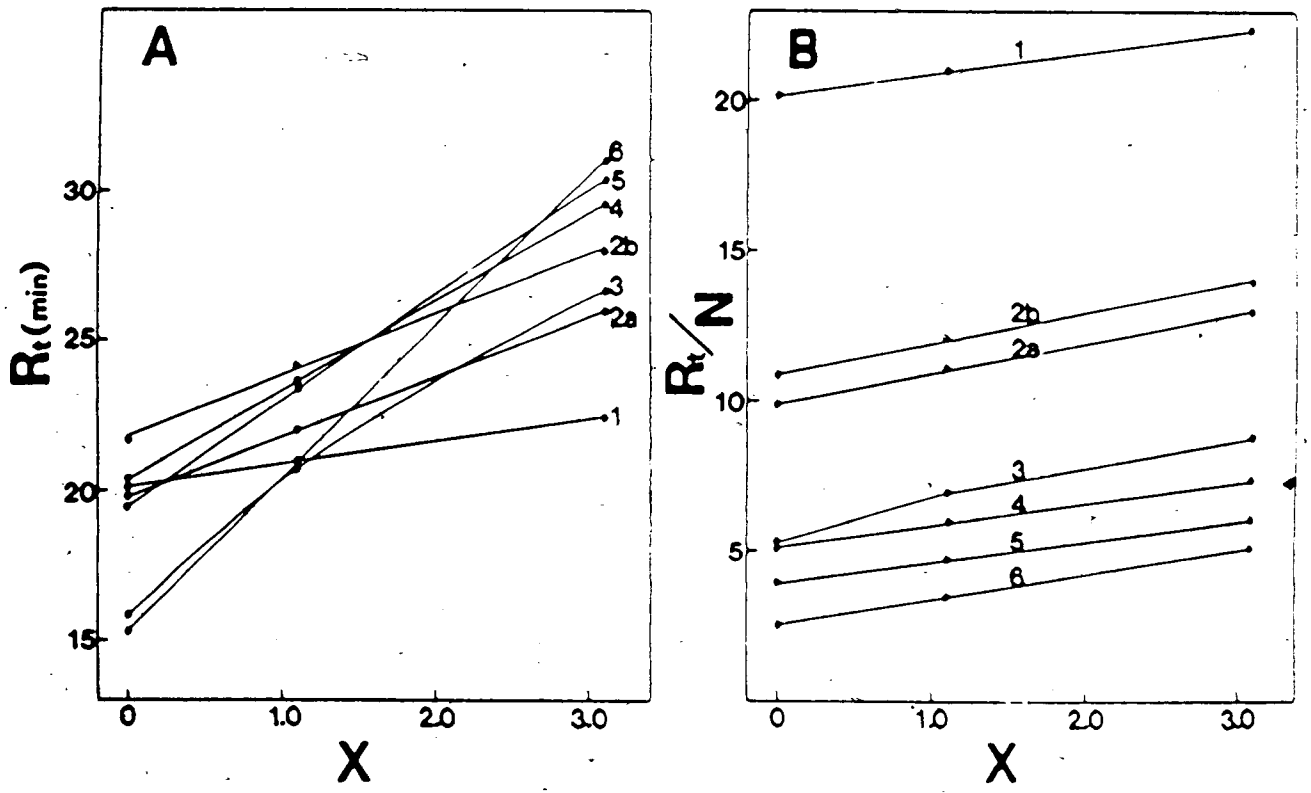


Fig. 19. Effect of hydrophobicity of ion-pairing reagents on peptide retention in RP-HPLC. Panel A: retention time (R_t) versus the average increase in retention time (X) per positively charged group, obtained from the peptide standards S1-S5, when TFA or HFBA was used as the mobile phase acid compared to to the retention times of the peptide standards when H_3PO_4 was used as the mobile phase acid; X= 1.1 (TFA) and 3.1 (HFBA) (Table 10). Panel B: R_t/N versus X, where N denotes number of positively charged groups in peptide.

B=acetonitrile, both solvents containing 0.01%-0.5%(v/v) of H_3PO_4, TFA or HFBA. Fig. 20 shows increasing peptide retention times with increasing concentration of TFA in the mobile phase. Among five peptide standards, S1 (3 positively charged groups) has a greater change in its elution position relative to the other four peptides (all possessing 2 positively charged groups) as the concentration of TFA increased. To consider the average effect per positively charged residue of varying counterion concentration, a plot of R_t/N (R_t= retention times of the peptides in the TFA system; N= number of positively charged residues they possess) versus concentration of TFA in the mobile phase was made (Fig. 21). The similarity of the curves for all five peptide standards suggests an essentially equal effect of TFA concentration on each positively charged residue. This conclusion can also be applied to the other anionic counterion systems (*e.g.* H_3PO_4 or HFBA). The results indicate that the effect of counterion concentration on peptide retention time should be considered in retention time prediction. To simplify the prediction procedure, it is recommended that a suitable concentration of ion-pairing reagent such as 0.1% (v/v) be consistently used.

Column selectivity was found to be significantly changed when different TFA concentrations were used in the mobile phase. Several peptides of similar size but varying in the number of positively charged groups (Table 11) were chromatographed in TFA concentrations varying from 0.01% to

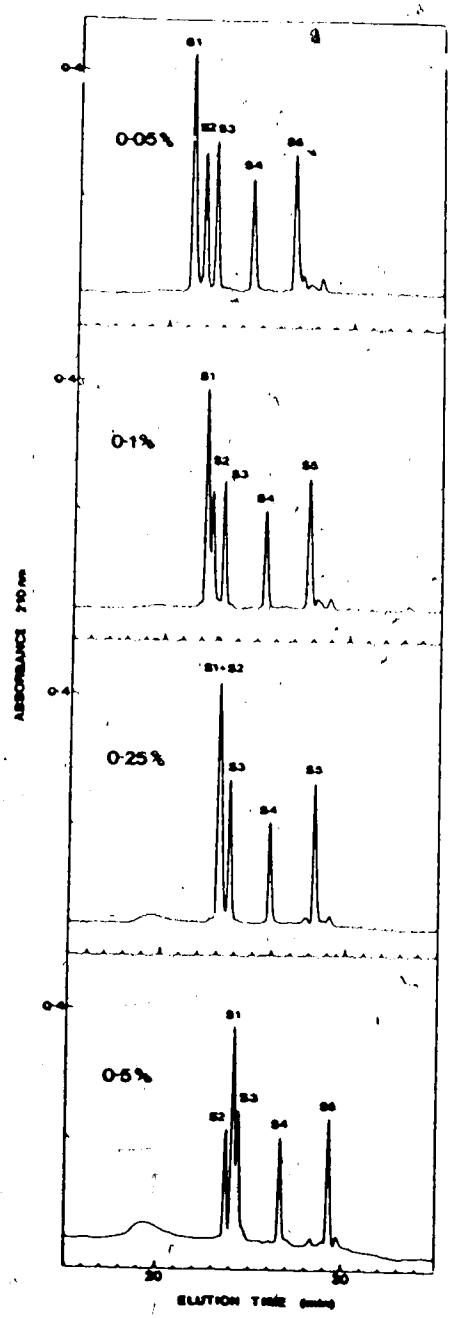


Fig. 20. Effect of concentration of ion-pairing reagent on retention times of five synthetic peptide standards in RR-HPLC. Column, Aquapore C-8 (220 x 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both containing 0.05%, 0.1% 0.25% or 0.5% TFA; flow-rate, 1 ml/min; 26°C.

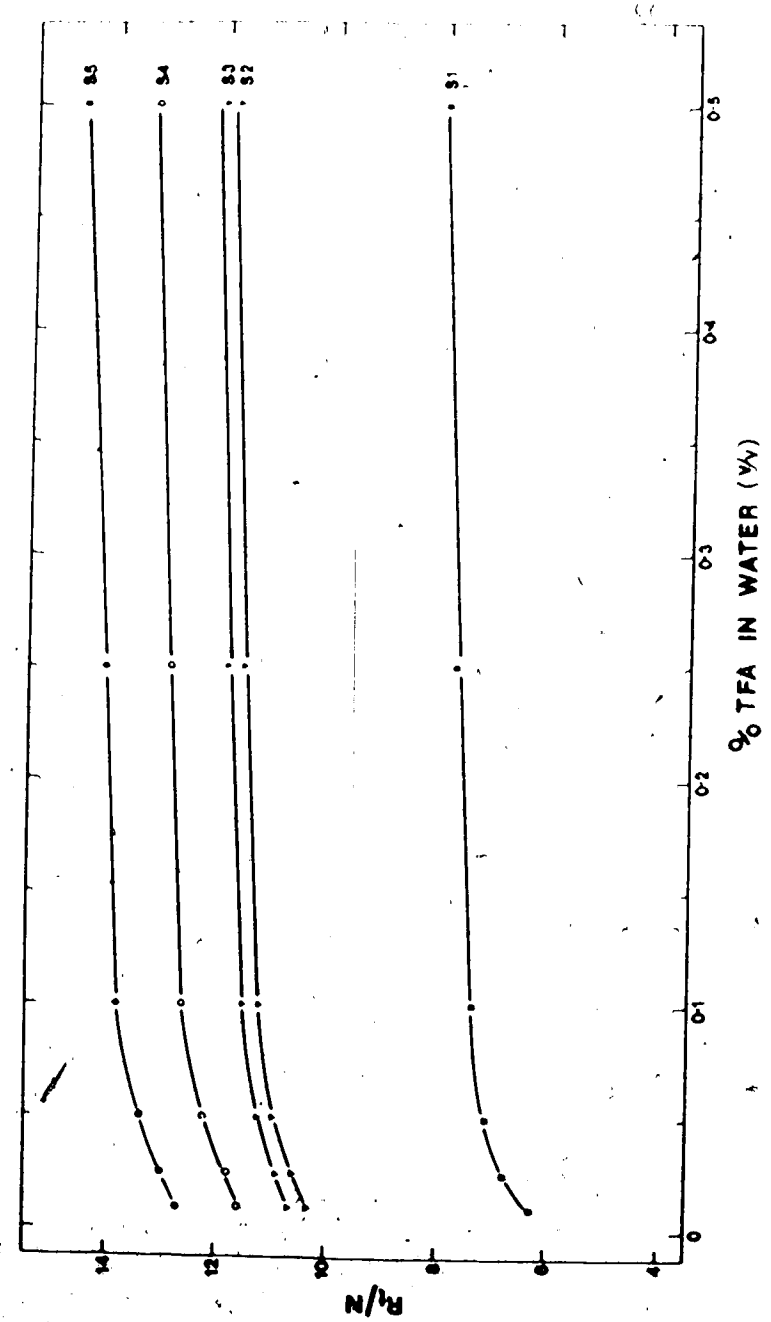


Fig. 21. A plot of the average effect per positively charged group of five peptide standards versus the concentration of ion-pairing reagent in RP-HPLC. Column: Aquapore C-8. Chromatographic conditions as Fig. 20. R_t denotes peptide retention time; N denotes number of positively charged groups in peptides.

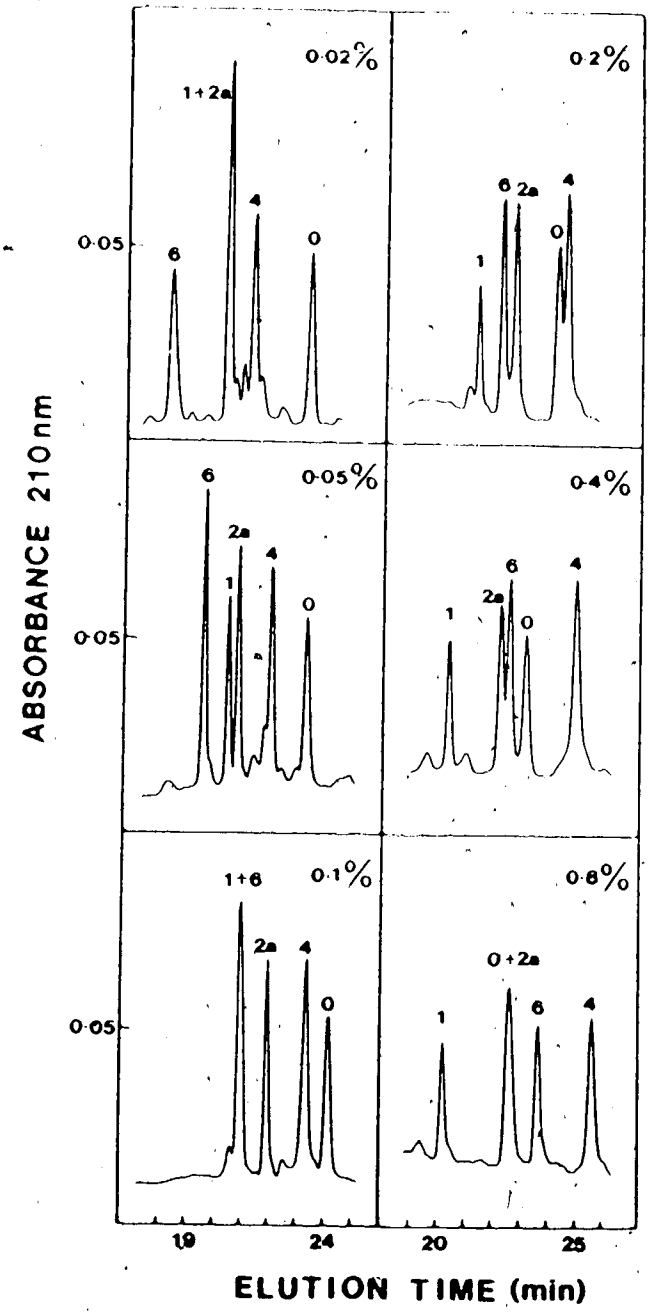


Fig. 22. Effect of concentration of ion-pairing reagent on selectivity of a mixture of peptides in RP-HPLC. Conditions: column, SynChropak C-18 (250 x 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.02%, 0.05%, 0.1%, 0.2%, 0.4%, or 0.8% TFA; flow-rate, 1 ml/min; 26 °C; absorbance at 210 nm. Sequence variations of the peptides are listed in the Table 11.

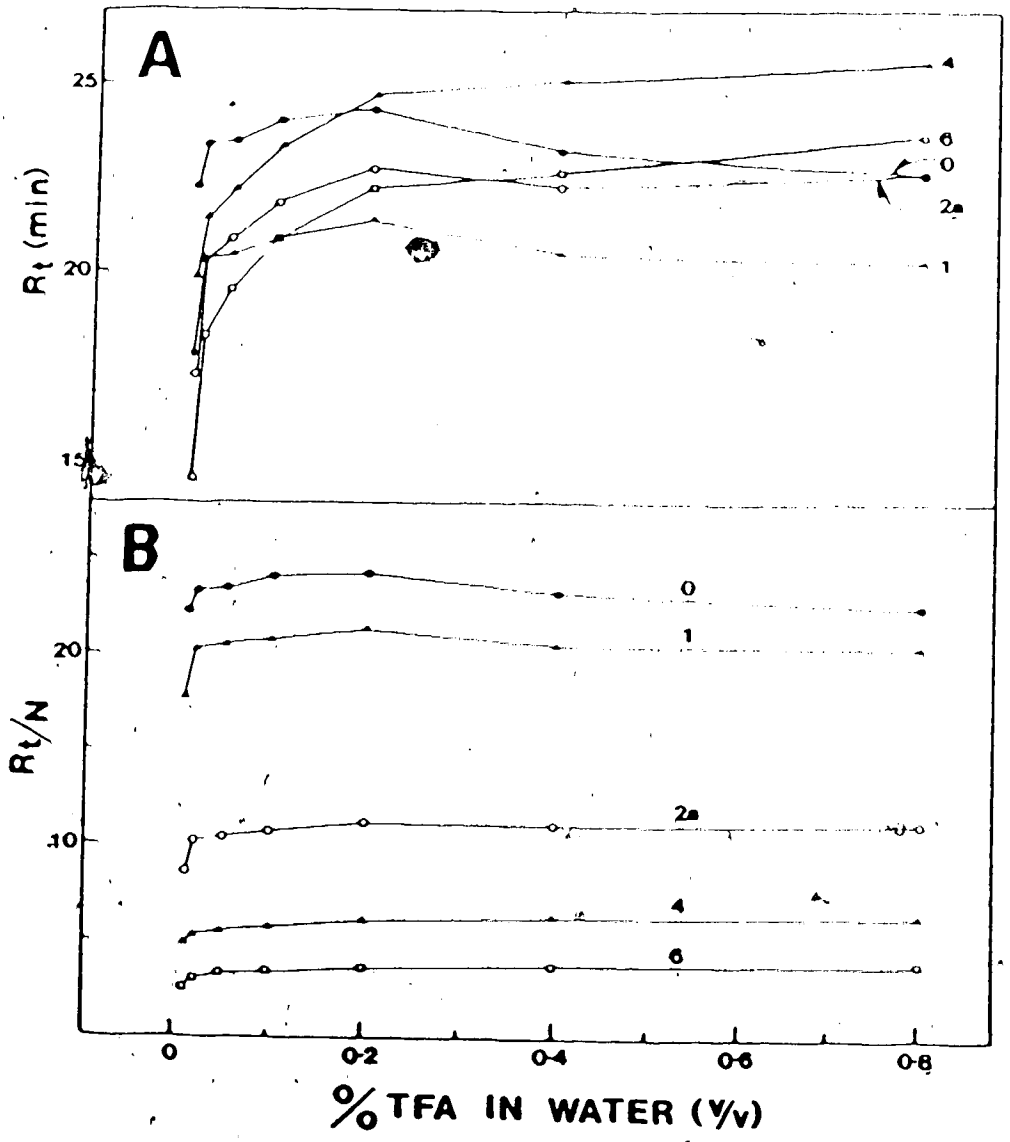


Fig. 23. Effect of concentration of ion-pairing reagent on retention times of a mixture of peptides in RP-HPLC. Chromatographic conditions as Fig. 22. R_t denotes peptide retention time; N denotes number of positively charged groups in peptide.

0.8% (v/v). As shown in Fig. 22, the peptides exhibited a changing selectivity profile as the concentration of TFA in the mobile phase was increased. This was also demonstrated in the insets of Fig. 18. The elution order in the right inset was 1, 3 and 6 at 0.4% TFA , compared to 3, 6 and 1 at 0.01% TFA in the left inset of the same figure. The plots of retention times of the peptides against TFA concentration in the mobile phase (Fig. 23, Panel A) initially increased but eventually became essentially independent of increasing TFA concentration (Hearn *et al.*, 1979). Plotting R_t/N versus TFA concentration (Fig. 23, Panel B) produced similar profiles for the five peptides, suggesting an equal effect of counterion concentration on each basic residue. It was noticed that the retention time of peptide 0 was affected least over the TFA concentration range tested. Peptide 0 contains no basic residues, serving as a marker in the study of the retention behavior of basic peptides under different counterion concentration conditions.

D. Effect of Flow-Rate

A change in mobile phase flow-rate can result in changes in both peak retention and resolution (Glajch, 1986). The left panel in Figure 24 shows that retention times of peptides increase rapidly with decreasing flow-rates. Since the elution profiles of the peptides are found to be very similar at different flow-rates, it seems to suggest that flow-rate has little effect on retention

time of a peptide provided the gradient elapsed time (t_g, see Chapter II: Experimental) is subtracted from the retention time of the peptide. Figure 25 shows that plotting the differences ($R_t - t_g$) against flow-rate results in straight line plots. Apparently, flow-rate has little effect on retention times of the peptide standards, once the value for t at each flow-rate is taken into account. Hence, the predicted retention time of a peptide at different flow-rates may be calculated by simply correcting for the varying gradient elapsed times. Thus, from the retention coefficients determined in Chapter III (chromatographic conditions: linear gradient, where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile; 1% B/min; 1 ml/min; 26°C) the predicted retention time of a peptide at varying flow-rates is obtained by subtracting the gradient elapsed time at 1 ml/min (t_g) from the predicted retention time at 1 ml/min (τ) and adding the result of dividing t_g at 1 ml/min by the desired flow-rate (y, ml/min)

$$\tau(y) = (\tau - t_g) + \left(\frac{t_g}{y}\right)$$

The resolution of peptide standards S4 and S5 on the chromatograms was also calculated under varying flow-rates. Plotting resolution versus flow-rate (Fig. 26) demonstrated increasing resolution of the two peptides with increasing flow-rate and decreasing gradient slope (Jones *et al.*,

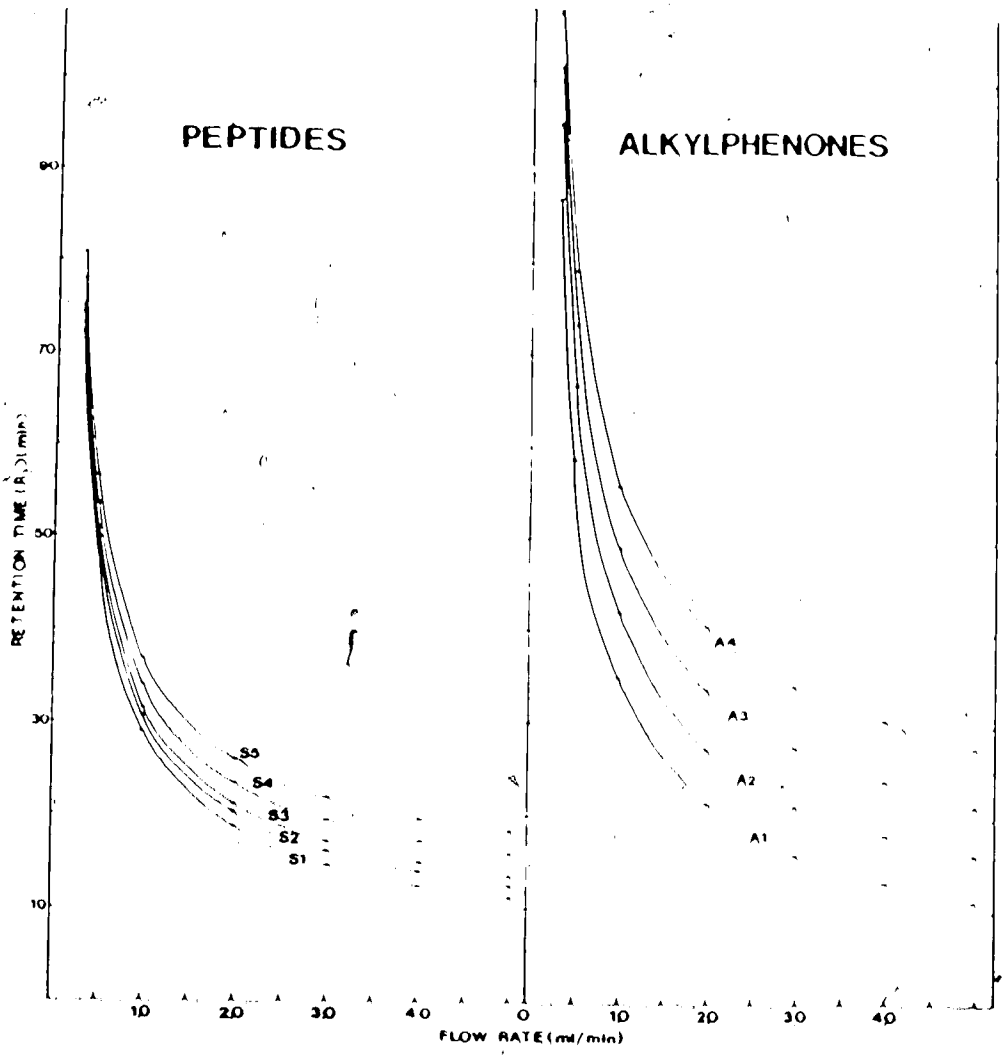


Fig. 24. Effect of flow-rate on the retention times of alkylphenone and synthetic peptide standards in RP-HPLC. Conditions: column, SynChropak C-18(250 x 10 mm I.D.); linear gradient (1% B/min) where A=0.1%aq. TFA and B=0.1 TFA in acetonitrile (pH 2.0); flow-rate, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1-S5 are described in Chapter III. A1-A4 denote acetophenone, propiophenone, n-butyrophenone, and valerophenone, respectively.

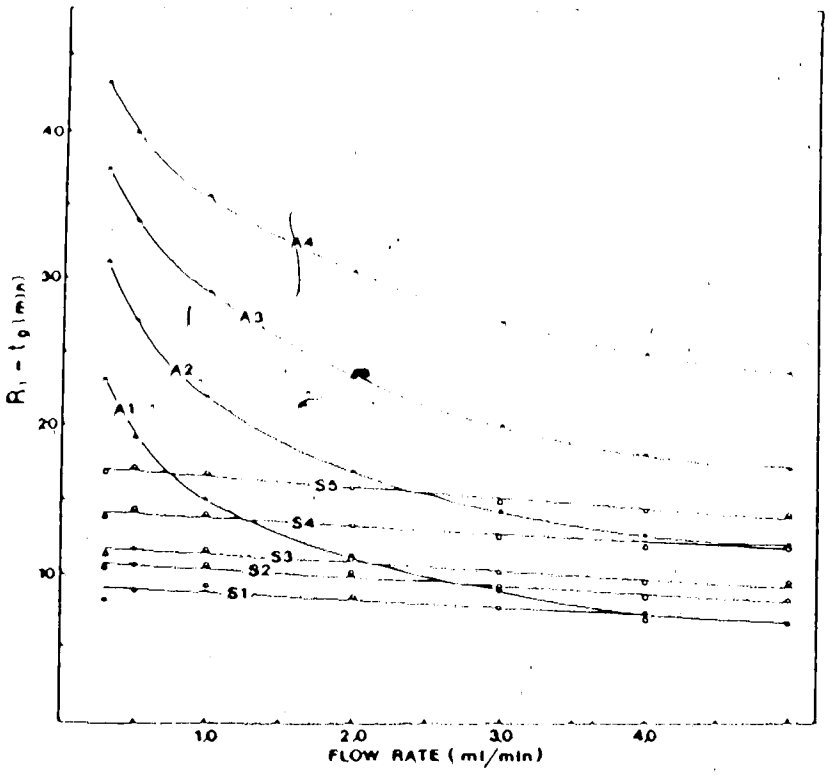


Fig. 25. Effect of subtracting gradient elapsed time from retention times of alkylphenone and synthetic peptide standards in RP-HPLC at different flow-rates. Sequence variations of peptide standards S1-S5 are described in Chapter III and alkylphenones A1-A4 are described in Fig. 24. Chromatographic conditions as Fig. 24.

1980). Peak height was noticed to increase with decreasing flow-rate and increasing gradient slope. As observed above, flow-rate variations have negligible effect on the retention differences ($R_t - t_g$) of the peptides and the distance between the S4 and S5 peaks (Δt) remains essentially the same at all flow-rates. However, the tendency for the peptides to diffuse decreases as the flow-rate increases, producing smaller peak widths (W_1, W_2, see Chapter I) and, hence, improved resolution. In contrast, improved resolution is observed when the gradient slope decreases, since the resulting increase in Δt more than compensates for any concomitant increase in peak widths. Jones *et al.*, also noted that decreased column efficiency was obtained with increasing mobile phase flow-rate (1980). This is partly due to poor penetration of solute molecules into the particle pores of the column support. In terms of this phenomenon, manipulation of mobile phase flow-rates in the separation of peptides should involve comprehensive consideration of peptide retention, resolution and column efficiency. It is important to choose an optimum flow-rate for the separation of a given sample (Glajch, 1986).

Figure 24 also compares the effects of flow-rate on the retention times of synthetic peptide HPLC standards and four alkylphenone HPLC standards. It is seen that the retention times of the alkylphenones increase more rapidly (right) with decreasing flow-rates than those of the peptides (left). The contrast can best be visualized by comparing the

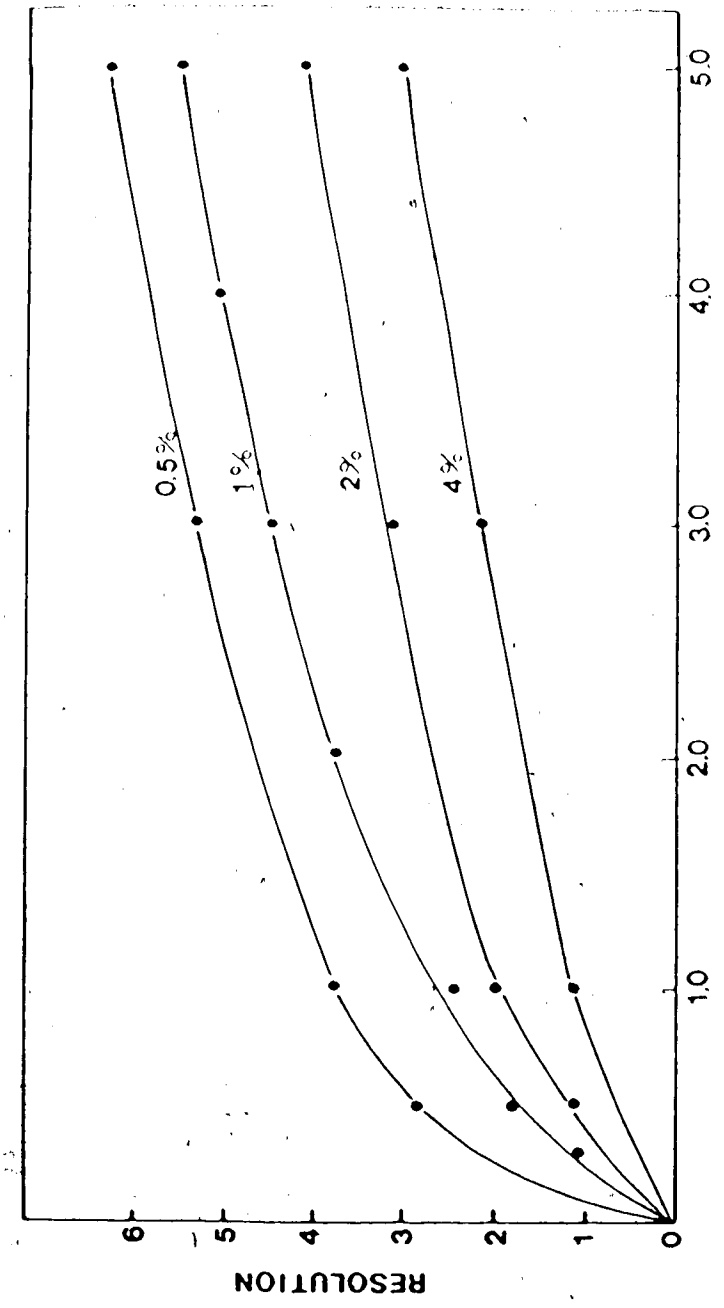


Fig. 26. Effect of flow-rate and gradient slope on resolution of two synthetic peptide standards (S4,S5) in RP-HPLC. Conditions: column, Synchropak C-18 (250 x 10 mm I.D.); linear gradient (0.5%, 1%, 2% or 4%/min where A=0.1%aq. TFA and B=0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 0.3-5.0 ml/min; 26°C; absorbance at 210 nm.

alkylphenone and peptide standards with similar retention times at 5 ml/min (S1 and A1, S4 and A2) and following the increasingly dissimilar profiles of the standard pairs as the flow-rate decreases. The relationship between (R_t-t_g) and flow-rate for the alkylphenone and peptide standards derived from the results shown in Fig. 24, is demonstrated in Fig. 25. The difference between the alkylphenone and peptide profiles is apparent and flow-rate has much greater effect on the retention times of the alkylphenone standards than those of the peptide standards. Since solute partitioning is controlled by mobile phase flow-rate, peptide standards seem to mainly follow an adsorption-desorption mechanism which is not governed by the effect of flow-rate. In terms of this principle, plotting the retention differences (R_t-t_g) of the peptide standards against flow-rate should result in straight-line plots with zero slope, *i.e.* little or no effect of flow-rate on retention time. However, the slightly negative slope of the peptide plots suggests some increase in peptide partitioning as the flow-rate decreases. For practical purposes, this deviation from zero slope is negligible.

E. Effect of Column Temperature

Some researchers found that peptide retention times were minimally influenced by different column temperatures (15°-70°) (Wilson *et al.*, 1981). We did observe a reduction in peptide retention time with increasing temperature.

Figure 27 demonstrates a successive decrease in retention times and slightly improved peptide resolution as the temperature was increased from 26°C (the value used to determine our coefficients) to 66°C in 10°C increments. The average change in peptide retention time with temperature was 0.13 min/°C. This value can be taken into consideration in predicting peptide retention at a temperature other than that used to determine our retention coefficients.

Peptide separation in reversed-phase mode is governed by the hydrophobic interactions between the peptide molecules and the non-polar stationary phase supports. As noted by Ben-Naim, hydrophobic interaction should be temperature-dependent (1980). Thus, it is expected that there should be an increase in the strength of the hydrophobic interaction with increasing temperature and this would be due to an increase in the entropy between the non-polar parts of the peptide molecules and the hydrophobic reversed-phase support (Snyder, 1979). This effect favors peptide retention in a reversed-phase column. On the other hand, temperature also affects the mass transfer of solutes between the mobile and the stationary phases and speeds up peptide elution. A higher transfer rate of the solutes between these two phases results from an increase in diffusion rate at elevated temperatures (Rubinstein, 1979). These two effects may govern the solute retention process. However, hydrophobic interaction is probably not the major or the dominant factor involved in the retention process at

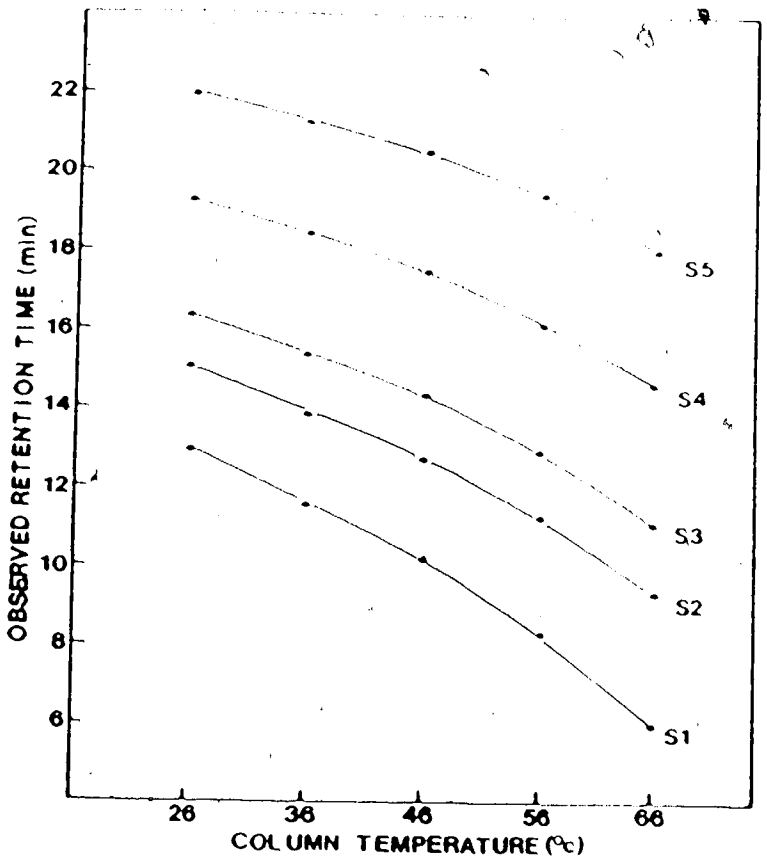


Fig. 27. Effect of temperature on retention time of five synthetic peptide standards in RP-HPLC. Conditions: column, SynChropak C-8 (250 x 4.1 mm I.D.); linear gradient (1% B/min) where A= 0.1% aq. TFA and B= 0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 1 ml/min; temperature, 26°C, 36°C, 46°C, 56°C, or 66°C; absorbance at 210 nm. Sequence variations of peptide standards S1-S5 are described in Chapter III.

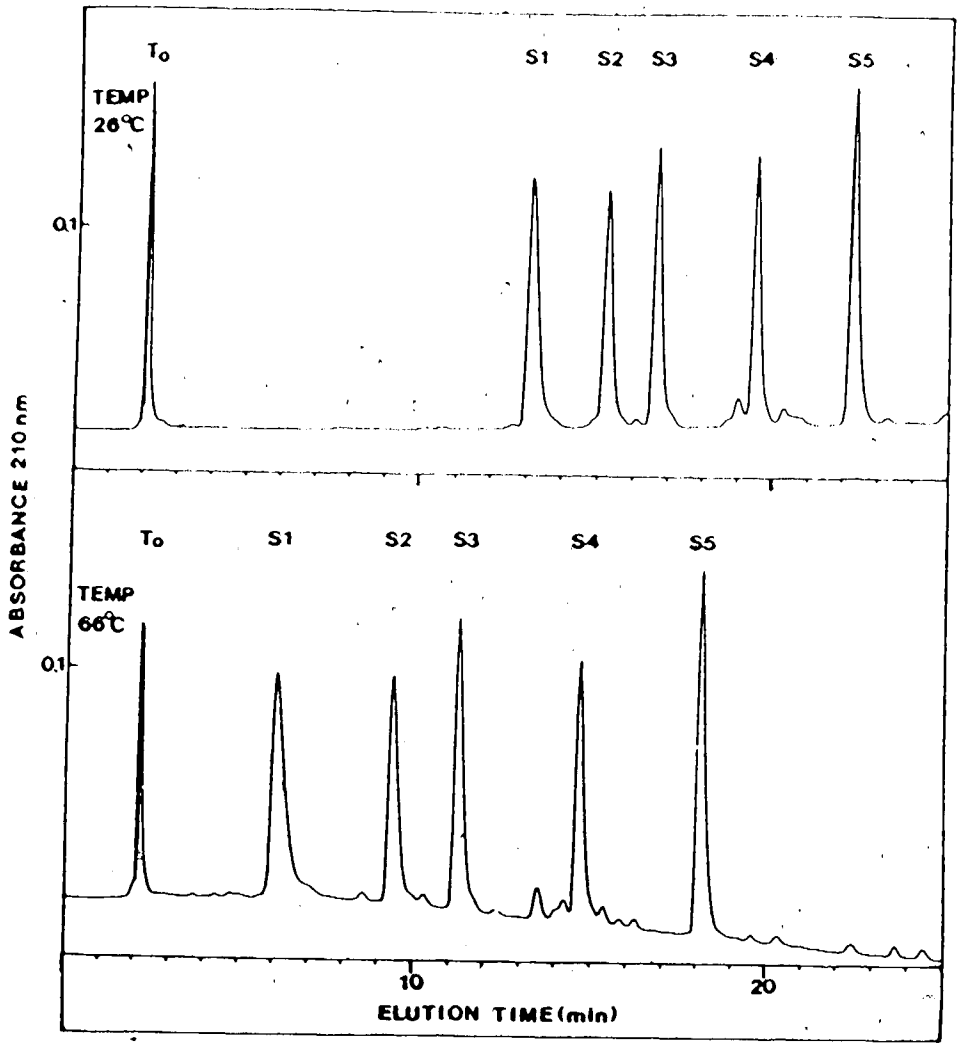


Fig. 28. Chromatograms of five synthetic peptide standards at 26°C and 66°C in RP-HPLC. Column: SynChropak C-8 (250 x 4.1 mm I.D.); linear gradient (1% B/min) where A= 0.1% aq. TFA and B= 0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 1 ml/min; temperature, 26°C or 66°C; absorbance at 210 nm. Sequence variations of peptide standards S1-S5 are described in Chapter III.

elevated column temperatures. Thus, the rate of movement of the peptide samples in the stationary and mobile phase is much faster at a higher column temperature compared to a low column temperature.

The improved peptide resolution at higher temperature (Fig. 28) is probably due to the increasing solubility of the solute in mobile phase and the decrease in solvent viscosity. In reversed-phase chromatography the hydrophobic support and the organic solvent cause denaturation (Lau *et al.*, 1984). Depending on the hydrophobic ligand density of the support and the conditions used for chromatography multiple peaks from a single protein component can often be observed (Ingraham *et al.*, 1985). Increasing temperature can result in denaturation and isolation of a single component instead of multiple peaks. This improvement may be advantageous. However, improved resolution must be balanced against possible peptide or protein degradation, or acceleration of column aging at elevated temperatures.

F. Effect of Gradient Slope

The effect of gradient slope on peptide retention was studied by examining chromatographic behaviour of a mixture of five synthetic peptide HPLC standards under different gradient conditions. The peptides were resolved on a SynChropak C-8 column (250 x 4.1 mm I.D.), with linear AB gradients (solvent A is 0.1% TFA in water; solvent B is 0.1% TFA in acetonitrile, pH=2.0) of 0.5%, 1%, 2%, and 4% B/min

at a flow-rate of 1 ml/min and a temperature of 26°C. Figure 29 shows a plot of retention times of the peptides versus the reciprocal of the gradient slopes. Ideally, a linear relationship should exist between peptide retention and the reciprocal of the gradient slope, with the plots for all five peptides intercepting at the gradient elapsed time, t_g (see Chapter II: Experimental; t_g =7.0 min in this experiment). However, an increase in peptide partitioning as the gradient steepness decreases may be responsible for causing a deviation from strict linearity at 0.5% B/min. In the gradient range used by most investigators (0.5-4% B/min), the relationship between peptide retention time and reciprocal of gradient slope may be considered linear. Thus, one can use retention data from one gradient slope to predict retention times in another. The predicted retention time of a peptide at varying gradient rates [$\tau(x\%)$] may be calculated by subtracting the gradient elapsed time at 1 ml/min (t_g) from the predicted retention time at 1% B/min (τ), multiplying by the reciprocal of the desired gradient slope and again adding t_g,

$$\tau(x\%) = (\tau - t_g)\ (1/x\%) \pm t_g$$

Hence, peptide retention predictions can be made for different gradient slopes, no matter what gradient slope was used to determine a particular set of coefficients.

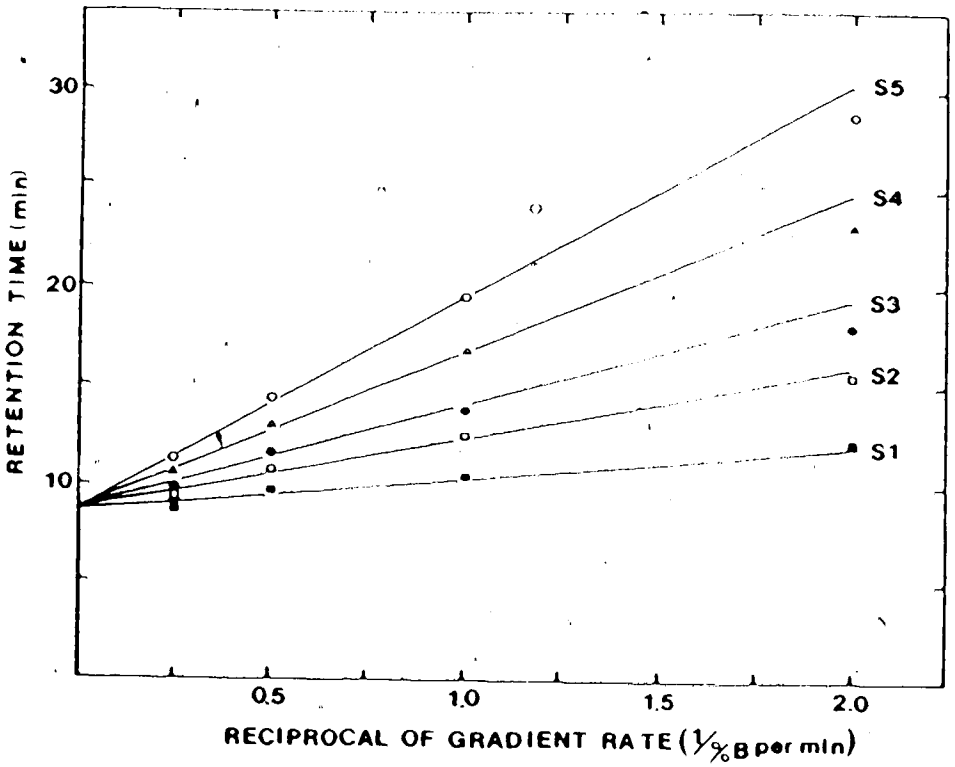


Fig. 29. A plot of retention times of five synthetic peptide standards versus the reciprocal of the gradient slope (1/% B per min). Conditions: column, SynChropak C-8 (250 x 4.1 mm I.D.); linear gradient (0.5%, 1%, 2% or 4% B/min), where A= 0.1% aq. TFA and B= 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1-S5 are described in Table 3.

G. Requirement for Peptide Standards

Reproducibility of peptide retention may depend on a variety of factors, including instrumentation variations, column packing materials, solvents, ion-pairing reagents and operating conditions. The resolving ability of packings of identical chain length may also vary from manufacture to manufacture or from batch to batch of support from the same manufacturer. Appropriate HPLC standards are needed to correct for the retention variations. The internal-standard method--running the HPLC standard with the sample of interest--is particularly useful for monitoring sample retention and resolution in RPC. By using an internal peptide standard, it should be possible to predict retention under most chromatographic conditions. Although neutral compounds such as alkylphenones have been available as HPLC standards, we have shown that peptide standards are necessary for peptide retention prediction. Figure 30 demonstrates the elution profiles of three synthetic peptides and two alkylphenone standards on two SynChropak C18 columns of different lengths, a Whatman C8, and a Beckman Ultrapore C3 column used under identical conditions (linear AB gradient, where A=0.1% aq.TFA and B=0.05% TFA in acetonitrile, pH=2; 2% B/min, 1 ml/min, 26°C). Two points can be drawn from the chromatograms: (1)column length has a greater effect on the retention times of the alkylphenones than on those of the peptides (compare the identical materials in two columns of different length, C-18 5-cm and

C-18 25-cm), and (2)the *n*-alkyl-chain matrices have different effects on the retention of the alkylphenones compared to the peptides. It is apparent that peptide retention times on C-8 and C-18 columns of the same length are very similar, while the alkylphenones are bound more tightly to the C-8 material, which has about double the ligand density of the C-18 material. These results suggest that the alkylphenones are resolved mainly by a partitioning mechanism, while the peptides are separated mainly by an adsorption/desorption mechanism. The small difference in peptide retention as a function of column length and *n*-alkyl chain length is consistent with the peptide separation mechanism involving adsorption/desorption steps instead of the multiple-step partitioning process which is associated with the chromatography of small molecules. These conclusions agree well with the results of the flow-rate experiments (see Figures 24 and 25) and the observations of Colin and Guiochon (1978).

The difference in the separation mechanisms between the alkylphenones and the peptides is probably due to the different molecular sizes and interaction modes with a reversed-phase support. The peptides used in this study are not only larger in size than alkylphenones, but also contain multiple hydrophobic groups on the molecules. Once they are injected into the reversed-phase column, the peptides will be adsorbed onto the hydrophobic surface of a support. It is noteworthy that multiple hydrophobic regions on both the

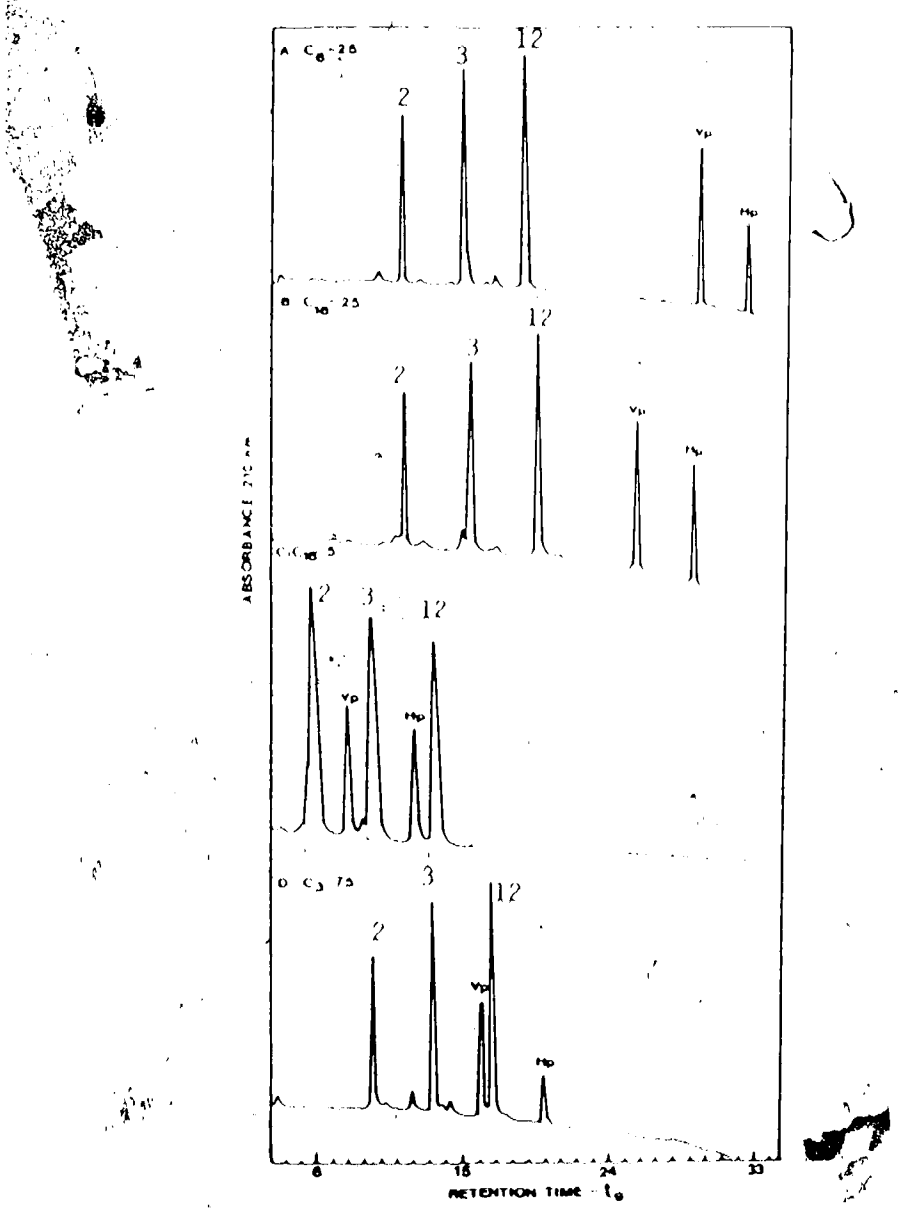


Fig. 30 Chromatograms illustrating effects of different column lengths and supports of different chain lengths on retention of three peptides and two alkylphenones. Panel A, Whatman C-8 (250 x 4.6 mm I.D.); B, SynChropak C-18 (250 x 4.1 mm I.D.); C, SynChropak C-18 (50 x 4.1 mm I.D.); D, Beckman C-3 (75 x 4.6 mm I.D.). Position X in the synthetic model peptide sequence (Table 2)was substituted by Leu (Peptide 12), Val (Peptide 3), or Ala (Peptide 2). VP=valerophenone; HP=hexanophenone. Conditions: linear gradient (2% B/min) where A =0.1% aq. TFA and B=0.05% TFA in acetonitrile(pH 2.0); flow-rate,1 ml/min; 26°C;absorbance at 210 nm.

peptides and the support may participate in the adsorption process (Pearson *et al.*, 1983; Geng and Regnier, 1984a). Desorption is accomplished through the introduction of an organic solvent in the mobile phase. The organic solvent may function in two ways: (1)displace the peptides by interacting with both the hydrophobic matrix and the peptides, and (2)disorder the structure of the water organic solvent system so that the peptides may be eluted off the column (Fausnaugh *et al.*, 1984). On the other hand, the alkylphenones have smaller molecular size and favor separation by a partitioning mechanism (Lochmuller and Wilder, 1979). The alkylphenone molecules partition between the mobile and stationary phases without strong adsorption on the non-polar ligates of a reversed phase support. In practice, it is preferable to use the compounds which are structurally similar to the sample of interest for the accurate retention prediction. Therefore, peptide standards are essential for prediction of peptide retention time and cannot be substituted by non-peptide molecules.

H. Calculation of Peptide Retention Time

In reversed-phase chromatography, the retention time of a peptide can be predicted by summing values that reflect the contribution (in minutes) of each amino acid residue and the peptide terminal groups to peptide retention time (see Table 5). Several predictive methods can be applied for different chromatographic conditions.

(1)The predicted retention time is based on the following operating conditions: a linear AB gradient, *i.e.* starting composition of 100% A, followed by increasing concentration of B at 1% per minute (where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile), a flow-rate of 1 ml/min, and a temperature of 26°C. By introducing an internal peptide HPLC standard, the predicted retention time (τ) for a peptide equals the sum of the retention coefficients (ΣR_c) for the amino acid residues and the end groups plus the time for elution of unretained compounds (t_o) and the time correction for the peptide standard (t_s):

$$\tau = \Sigma R_c + t_o + t_s$$

These corrections (t_s and t_o) allow for the use of (a)any HPLC apparatus, (b)reversed-phase columns of any length or diameter, (c)reversed-phase packings of any *n*-alkyl chain length and ligand density, (d)any temperature, (e)any flow-rate. This predictive method does not apply when using different organic solvents, since the predicted retention time of a peptide is not directly proportional to the solvent polarity. For example, large selectivity differences are observed when comparing the elution profile using a water-acetonitrile mobile phase with the one using a water-methanol mobile phase (see Fig. 15). In general, it is accepted that acetonitrile is the best solvent for most

peptide separations.

(2) If gradient slopes other than 1% B/min are used, the prediction should be based on the predicted value at 1% B/min:

$$\tau(x\%) = (\tau - t_g)(1/x\%) + t_g$$

where t_g is the gradient elapsed time at the desired flow-rate, determined at 1% B/min.

(3)If anionic counterions other than TFA are used in the mobile phase, prediction of peptide retention time in the second counterion system can be made in two ways:

(i)In the first approach, the calculation is based on the predicted retention time in a TFA counterion system plus the correction factors , including t_o, t_* and t_i (t_i is defined as the counterion correction factor and determined by multiplying the number of positively charged groups of the peptide by the average contribution of each positively charged residue to shift in peptide retention [nxΔ/N]):

$$\tau = \Sigma R_c + t_o + t_* + t_i$$

(ii)The second approach, detailed in section C of this Chapter, is based on the observed retention time in a TFA counterion system plus the counterion correction factor

(t_i):

$$r = R_i + t_i$$

Where R_i is the observed retention time in a TFA counterion system.

The second approach is more accurate than the first, since deviations between the predicted and observed retention times of peptides in the counterion system used to determine the retention coefficients are magnified when predicting values in another system.

Retention time is partially dependent on the molecular weight of a peptide. The effect of molecular weight on retention is relatively unimportant in small peptides but becomes significant with larger ones. Thus, the accuracy of peptide retention time prediction profoundly decreases beyond about 20 residues.

Peptide retention prediction can be applied to simplify the identification of specified peptides in RP-HPLC. The major advantage is that the position of a peptide(s) of interest in the elution profile of a peptide mixture will be narrowed down to a small section in the chromatogram, saving much time and effort in subsequent purification. Furthermore, useful information about the relative order of peptide elution of a complex mixture can be obtained by the

use of this predictive method.

V. USE OF PEPTIDE HYDROPHILICITY PARAMETERS IN PREDICTING SURFACE REGIONS ON PROTEINS

Antigenic sites of proteins must be on the surface in order to interact with antibodies. Therefore, these sites are most likely to be hydrophilic. Indeed, Green *et al.* have found that the entire surface of the hemagglutinin molecule was antigenic when the synthetic peptide fragments of the protein surface were used as immunogens (1982). According to this distinction, prediction of most antigenic sites from amino acid sequences can be made on the basis of the hydrophilicity parameters of amino acid residues. The ability to predict possible antigenic sites on the surface of a protein would be useful in immunological studies.

A new set of hydrophilicity HPLC parameters was derived from the retention times of 20 model synthetic peptides, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted with the 20 amino acids found in proteins. It was found that the HPLC parameters obtained in this study at pH 7.0 could be applied to predict the surface sites which were correlated to the known antigenic sites from immunological studies and surface exposed residues determined by X-ray crystallographic data for several proteins.

A. Retention Coefficients and a Hydrophilicity Scale

It has been discussed in Chapter III that the retention time of a peptide from reversed-phase chromatography is related to the summed hydrophobicity and hydrophilicity of

the amino acid residues in that peptide. Retention coefficients at pH 7.0 were determined for 20 amino acids from the HPLC retention data of a model peptide mixture on a SynChropak C-18 column (for calculation methods see Chapter III). It is noteworthy that addition of sodium perchlorate ($NaClO_4$) to the mobile phase (A=aq. 10mM $(NH_4)_2HPO_4$/0.1 M $NaClO_4$; B=0.1 M $NaClO_4$ in 60% aq. acetonitrile) was found to be essential to provide the excellent resolution shown in Fig. 31 (Meek, 1980). In the absence of perchlorate, the peptides exhibited high retention times and peak broadening due to the ionic interaction between ionized surface silanols and basic residues on the peptides. This interaction can be suppressed in the presence of $NaClO_4$ salt. Impurities in the buffer salts were removed by passing the pH 7.0 buffer A and the aqueous component of buffer B through a preparative column.

By comparing the retention parameters for pH 7.0 with those for pH 2.0, significant changes in the retention coefficients are noted in the values for His, Arg , Lys, Asp and Glu (Table 13). A histidine residue is deprotonated above pH 6-6.5 and the effect of losing a positive charge is shown by a large positive shift in retention coefficient from pH 2.0 to pH 7.0. Higher retention coefficients of the basic residues (Arg, Lys) at pH 7.0 are probably due to ionic interaction with the negatively charged silanols above pH 3.5-4 that were not completely suppressed by sodium perchlorate. At pH 7.0, the side chains of the acidic

C-18, pH 7.0

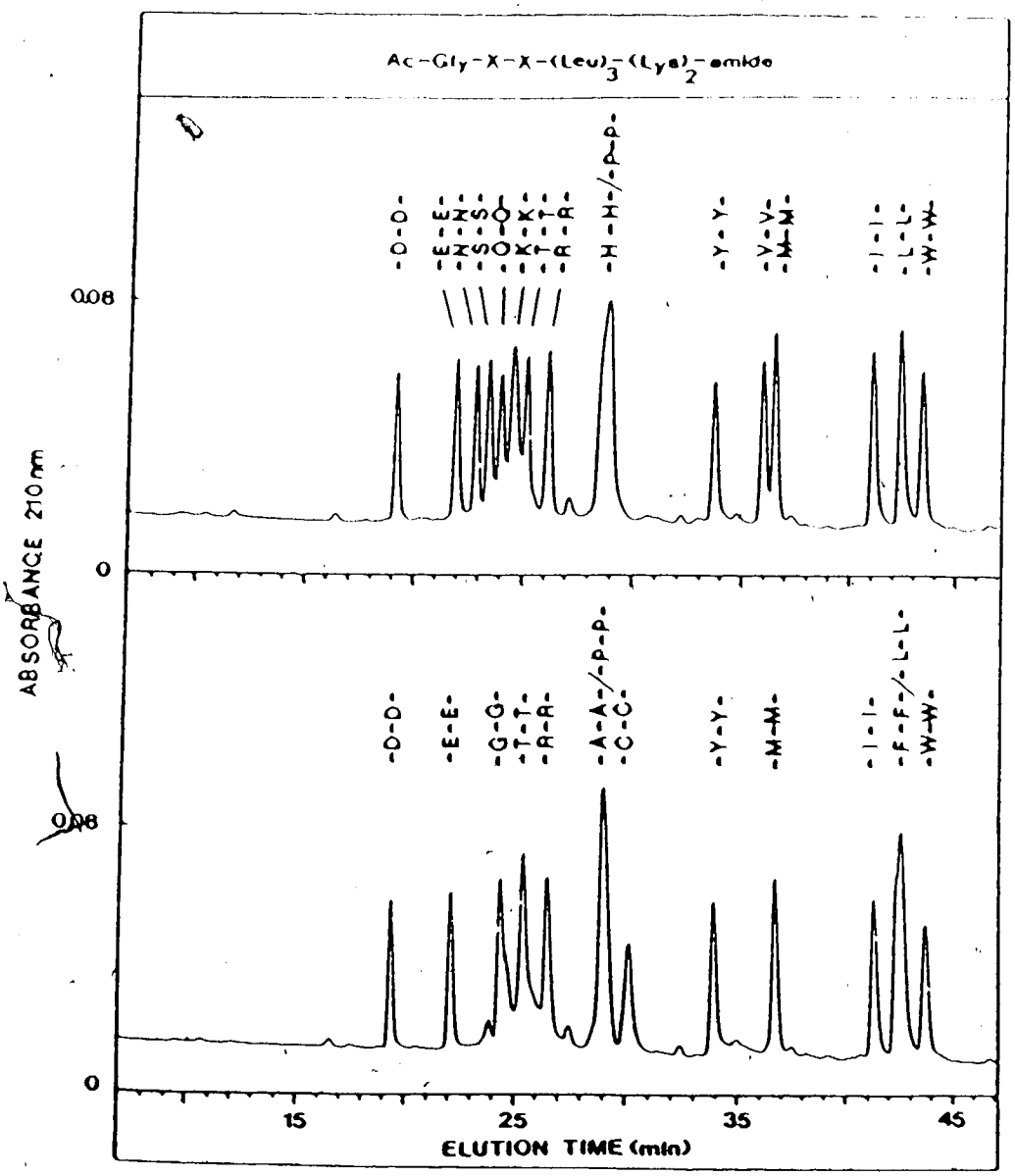


Fig. 31. Chromatograms of the synthetic model peptides, having the sequence Ac-G-X-X-L-L-L-K-K-amide, where position X is substituted by the 20 amino acids found in proteins, at pH 7.0. Conditions: column, SynChropak C-18 (250 x 4.1 mm I.D.); linear AB gradient, at pH 7.0, solvent A consisted of aq. 10 mM $(NH_4)_2HPO_4$-0.1M $NaClO_4$ buffer and solvent B consisted of 0.1 M $NaClO_4$ in 60% aq. acetonitrile, 1.67% B/min (equivalent to 1% acetonitrile/min); flow-rate, 1ml/min, 26°C; absorbance at 210 nm.

residues (Glu, Asp) are completely ionized so that the retention coefficients have a relatively large negative shift.

It is also important to examine the effect of pH on N- and C-terminal groups on a peptide (Bennett, 1983). When the acetyl moiety was changed to a free α-amino group at pH 2.0, a large decrease in retention time for the peptides (compare peptides AC and BC or AD and BD; Fig. 32, top panel) was observed. An additional positive charge on N-terminal groups at low pH is responsible for this decrease. In contrast, the effect of changing the C-terminal amide to an α-carboxyl group showed a much smaller decrease in retention time (compare peptides BC and BD or AC and AD; Fig. 32, top panel). This is due to complete protonation of the C-terminal α-carboxyl group under very acidic conditions (pH 2.0). A chromatogram of a mixture of the four peptides at pH 7.0 is shown in the bottom panel of Fig. 32. Both retention time and elution order are found to be different from those at pH 2.0. The effect of changing the acetyl moiety at the N-terminal to a free α-amino group is shown by comparing peptides AD and BD or AC and BC (Fig. 32, bottom panel). It is common for the pKa of a peptide α-amino group to be near 7 and this partial deprotonation would explain the smaller effect at pH 7.0 compared to that observed at pH 2.0. In contrast, a large decrease in retention time is expected as the C-terminal amide is changed to an α-carboxyl group at pH 7.0 (compare AC and AD or BC and BD; Fig. 32, bottom panel).

TABLE 13. HPLC retention coefficients and a hydrophilicity scale

Amino Acid	Retention Coefficients pH 2.0 (min)	Retention Coefficients pH 7.0	HPLC Hydrophilicity Scale based on pH 7.0 Retention Coefficients
Trp	+ 8.8	+ 9.5	-10.0
Phe	+ 8.1	+ 9.0	- 9.2
Leu	+ 8.1	+ 9.0	- 9.2
Ile	+ 7.4	+ 8.3	- 8.0
Met	+ 5.5	+ 6.0	- 4.2
Val	+ 5.0	+ 5.7	- 3.7
Tyr	+ 4.5	+ 4.6	- 1.9
Cys	+ 2.6	+ 2.6	+ 1.4
Ala	+ 2.0	+ 2.2	+ 2.1
Pro	+ 2.0	+ 2.2	+ 2.1
His	- 2.1	+ 2.2	+ 2.1
Arg	- 0.6	+ 0.9	+ 4.2
Thr	+ 0.6	+ 0.3	+ 5.2
Lys	- 2.1	0.0	+ 5.7
Gly	- 0.2	0.0	+ 5.7
Gln	0.0	- 0.2	+ 6.0
Ser	- 0.2	- 0.5	+ 6.5
Asn	- 0.6	- 0.8	+ 7.0
Glu	+ 1.1	- 1.3	+ 7.8
Asp	+ 0.2	- 2.6	+10.0
α-amino	-6.9, -3.0*	-2.4, 0*	+ 9.1
α-COOH	- 0.8	- 5.2	+14.3

* The charged α-amino group on an N-terminal Arg residue had a smaller effect than it did on N-terminal residue with an uncharged side chain.

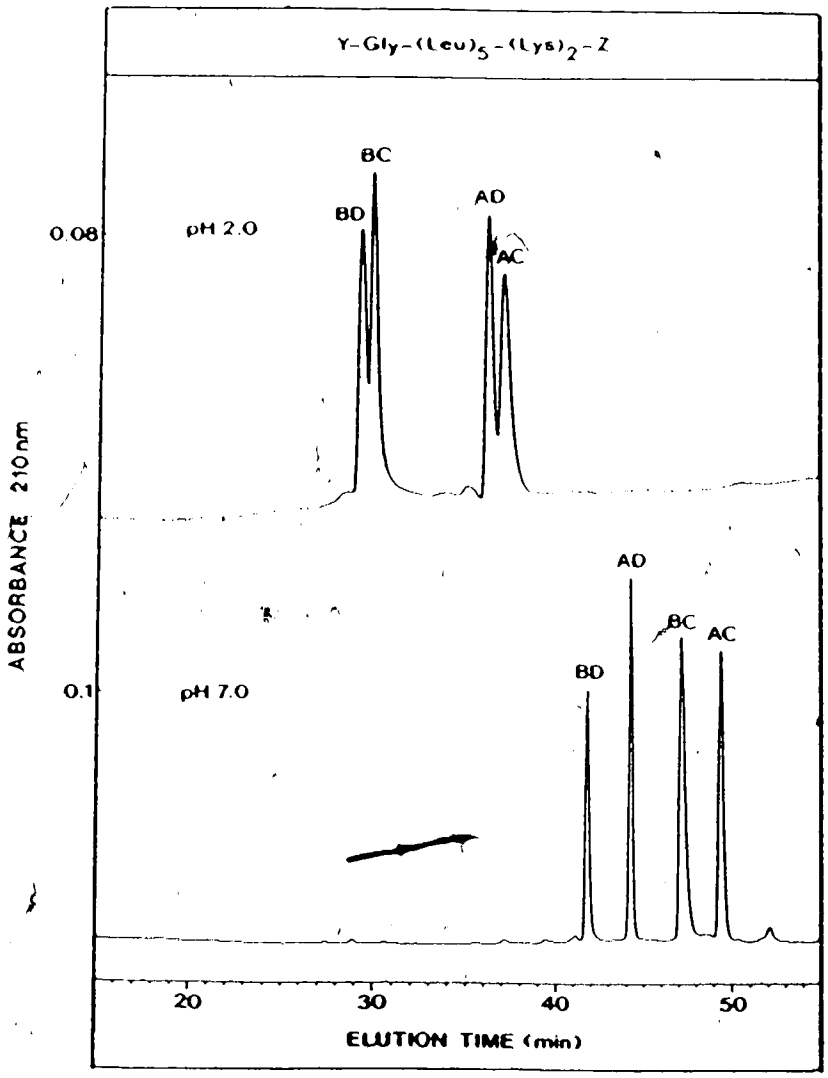


Fig. 32. Chromatograms of four synthetic peptides with the sequence Y-G-L-L-L-L-L-K-K-Z, where Y=N$^{\alpha}$-acetyl (A) or α-amino (B) and Z=$^{\alpha}$carboxyl-amide (C) or α-carboxyl (D). Conditions: SynChropak C-18 (250 x 4.1 mm I.D.); at pH 2.0 (top) and pH 7.0 (bottom); linear AB gradient; at pH 2.0, solvent A consisted of 0.1% aq. TFA and solvent B of 0.1% TFA in acetonitrile, 1% B/min; flow-rate, 1 ml/min; 26 ^{0}C. At pH 7.0, solvent A consisted of aq. 10 mM $(NH_4)_2HPO_4$-0.1 M $NaClO_4$ buffer and solvent B consisted of 0.1 M $NaClO_4$ in 60% aq.acetonitrle, 1.67% B/min (equivalent to 1% acetonitrile/min); flow-rate, 1 ml/min; 26 ^{0}C; absorbance at 210 nm.

In this case, the C-terminal α-carboxyl group would be completely ionized (COO^-) under these conditions (pH 7.0) and would be expected to have a large effect on the hydrophobicity of the peptide. Thus, at pH 7.0 the α-carboxyl group is fully ionized and at lower pH the α-amino group is fully protonated. These charged end groups, in contrast to blocked and uncharged end groups, can drastically affect the retention time of a peptide.

To test the effect of different organic solvents on the retention coefficients at pH 7.0, the model peptide mixture was chromatographed in pH 7.0 buffer containing isopropanol, acetonitrile and methanol (Fig. 33). As seen in Fig. 33, the relative elution order of the peptides using all three solvents is almost identical. The discrepancies observed for a few residues are probably due to differences in column selectivity and column aging. However, much superior resolution and selectivity are observed with acetonitrile as the eluting organic solvent compared to the alcohols. The retention coefficients of amino acid residues derived from the acetonitrile solvent system were used to derive the hydrophilicity scale.

A hydrophilicity scale was determined as follows: the amino acid residue with the maximum retention coefficient was assigned a hydrophilic value of -10 and the amino acid residue with the minimum retention coefficient a hydrophilic value of +10 (see Table 13). The remaining amino acid residues were scaled proportionally. In a similar manner,

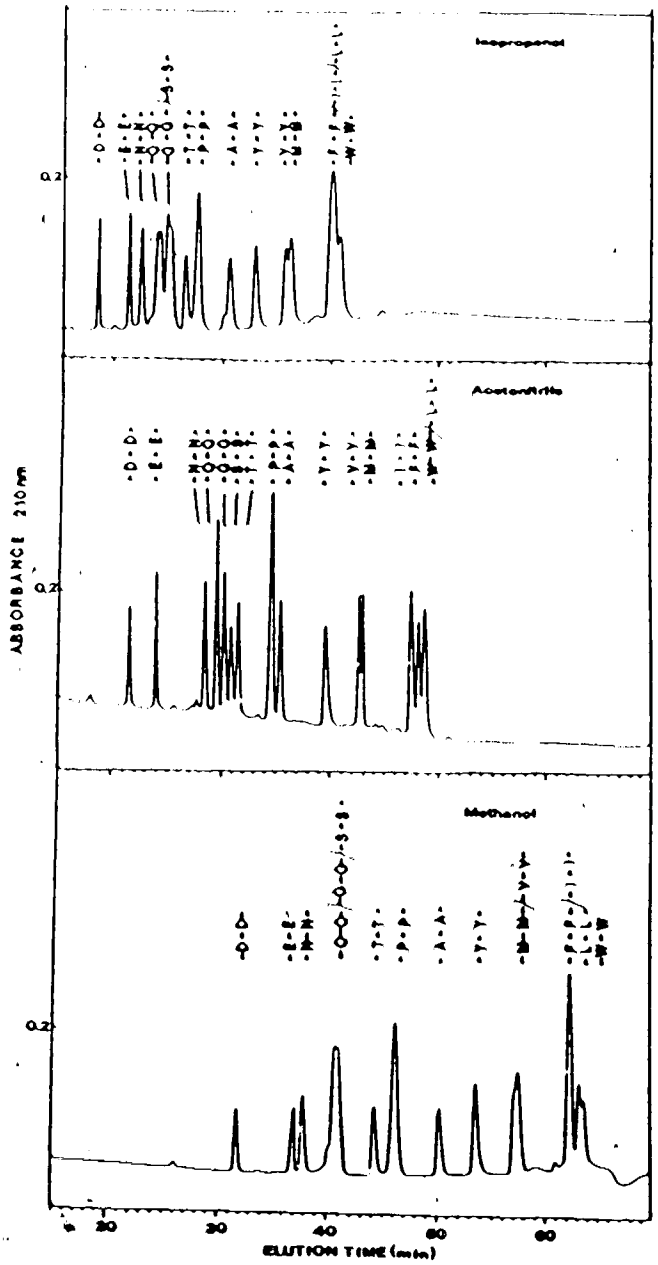


Fig. 33. Chromatograms illustrating the effects of three different organic solvents on the separation of the 16 synthetic model peptides at pH 7.0. Conditions: column, SynChropak C-8 (250 x 4.1 mm I.D.); at pH 7.0. Organic solvents: top, isopropanol; middle, acetonitrile; bottom, methanol, linear gradient (1.67 B/min), where A= aq. 10 mM $(NH_4)_2HPO_4$/ 0.1M $NaClO_4$ buffer and solvent B= 0.1M $NaClO_4$ in one of the above three organic solvents; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm.

other hydrophilicity parameters were scaled for a direct comparison. The results are summarized in Table 14. The large discrepancies among the parameters may result from different experimental methods in which the parameters were determined.

B. Determination of a Protein Surface Profile

In the folded protein, hydrophobic residues tend to be buried in the interior with polar residues at or near the surface. Some amino acid residues have more of a chance to become accessible residues than others. Since antigenic sites are likely to be on the surface of a protein, hydrophilicity parameters have been used to predict antigenic sites.

The surface profile for a protein was determined by summing the parameters for each residue of a seven residue segment and assigning this sum to the fourth residue. This procedure was repeated by shifting the segment by one residue from the N- to the C-terminus. A seven residue segment was found to be an appropriate unit to generate a reasonable surface profile. The summed values were then plotted against the residue number to provide a protein surface profile which described the relative hydrophilic as well as hydrophobic regions on a protein. To objectively interpret the profiles, the following arbitrary set of rules have been defined:

1. The average surface hydrophilicity is defined as the

Table 14. Comparison of the HPLC parameters with the parameters derived from partition and free energy transfer data of the amino acids.

Amino Acid Residues	Parameters*						
	HPLC[a]	Hydrophobicity[b]	Global[c]	Hydrophobicity[d]	π[e]	Hydropathy[f]	Hydration Potential[g]
Trp	-10.0	-10.0	-10.0	-6.6	-10.0	+1.0	+1.4
Phe	-9.2	-7.2	-5.0	-5.0	-7.1	-6.1	-5.1
Leu	-9.2	-5.0	-3.7	-10.0	-6.6	-6.4	-9.6
Ile	-8.0	-5.0	-3.9	-8.5	-7.1	-10.0	-9.6
Met	-4.2	-3.4	-2.2	-2.4	-3.7	-4.2	-4.2
Val	-3.7	-4.1	-3.1	-3.1	-3.7	-9.3	-9.4
Tyr	-1.9	-6.6	-5.0	-8.3	-2.1	+1.9	+1.7
Cys	+1.4	-2.5	-1.7	+5.0	-2.1	-5.5	-4.5
Ala	+2.1	-0.9	-0.1	+7.2	+1.9	-4.0	-9.3
Pro	+2.1	+0.6	+1.1	+2.3	-0.6	+3.5	+1.0
His	+2.1	-0.9	-0.2	+7.9	+3.0	-7.1	+8.9
Arg	+4.2	+10.0	+10.0	+7.9	+10.0	+10.0	+10.0
Thr	+5.2	-0.6	-0.1	+4.8	+2.2	+1.5	+0.9
Lys	+6.0	+10.0	+9.2	+6.1	+5.8	+8.6	+7.9
Gly	+6.0	+0.6	+1.1	+8.8	+3.8	-0.5	-10.0
Gln	+5.7	+1.25	+1.6	+10.0	+5.1	+7.8	+7.7
Ser	+6.5	+1.5	+2.1	+5.8	+4.0	+1.8	+1.2
Asn	+7.0	+1.25	+1.6	+9.4	+7.5	+7.8	+8.1
Glu	+7.8	+10.0	+9.2	+6.5	+7.7	+7.8	+8.9
Asp	+10.0	+10.0	+8.7	+7.2	+8.5	+7.8	+10.0

*These parameters are derived experimentally from free amino acids or their derivatives and from peptide data.

[a] This thesis. [b] Hopp and Woods (1981). [c] Fraga (1982). [d] Bull and Breese (1974). [e] Fauchere and Pliska (1983). [f] Kyte and Doolittle (1982). [g] Wolfenden et al. (1979).

the mean of the profile values for a protein using a particular parameter set.

2. Any residues with a profile value greater than 25% above the average surface hydrophilicity value are defined as surface sites. The 25% value is calculated as 25% of the difference between the maximum value in the plot and the average surface hydrophilicity value.

3. In determining the profile of a protein with a free N-terminal amino group or a C-terminal carboxyl group, the experimental value of 9.7 (amino group) and 14.3 (carboxyl group) is added directly to the value for the N- or C-terminal amino acid residue. The coefficients listed in Table 13 were calculated for the Nα-acetyl and Cα-amide groups. For all parameters other than the HPLC parameters determined in this study, no account is made for free N- or C-terminal residue.

The surface profiles for lysozyme are shown in Fig. 34A, 34B and 34C which were determined by applying the HPLC, Janin accessibility and Karplus and Schulz mobility parameters respectively. Table 15 shows the hydrophilicity scales derived from the accessibility and mobility parameters. The accessibility parameters, determined from X-ray crystallographic data, describe the fractions of

buried and accessible residues in 22 proteins. The mobility parameters were derived from the X-ray crystallographic data of the average temperature values of all Cα-atoms of each amino acid in 31 proteins. A couple of points can be drawn from the results: Firstly, the intensity of all the maximum profile values varies with every parameter set. To compare these profiles with antigenic sites on the proteins, a criterion was made for the profiles derived from different sets of parameters. In this study, residues 25%, 50% and 75% above the average surface hydrophilicity (see rules) were chosen as possible antigenic sites. Secondly, no single parameter set is able to predict all antigenic sites. For example, strong antigenic sites for residues 98-102 and 115-120 were assessed by both HPLC parameters and those of Karplus and Schulz (Fig. 34A and 34C) compared to very weak antigenic sites for the same regions predicted by the Janin parameters (Fig. 34B). Another example is shown by comparing the profiles for residues 15-22. A strong antigenic site was predicted by the Janin parameters but a very weak antigenic site predicted in the same region using the HPLC parameters and the Karplus and Schulz parameters did not show a value above the 25% cut-off. Similar surface profiles were also determined for the other three proteins, myoglobin, cytochrome *c* and influenza haemagglutinin.

To improve the accuracy of prediction of antigenic sites, three parameters were combined to generate a composite surface profile. They were the HPLC hydrophilicity

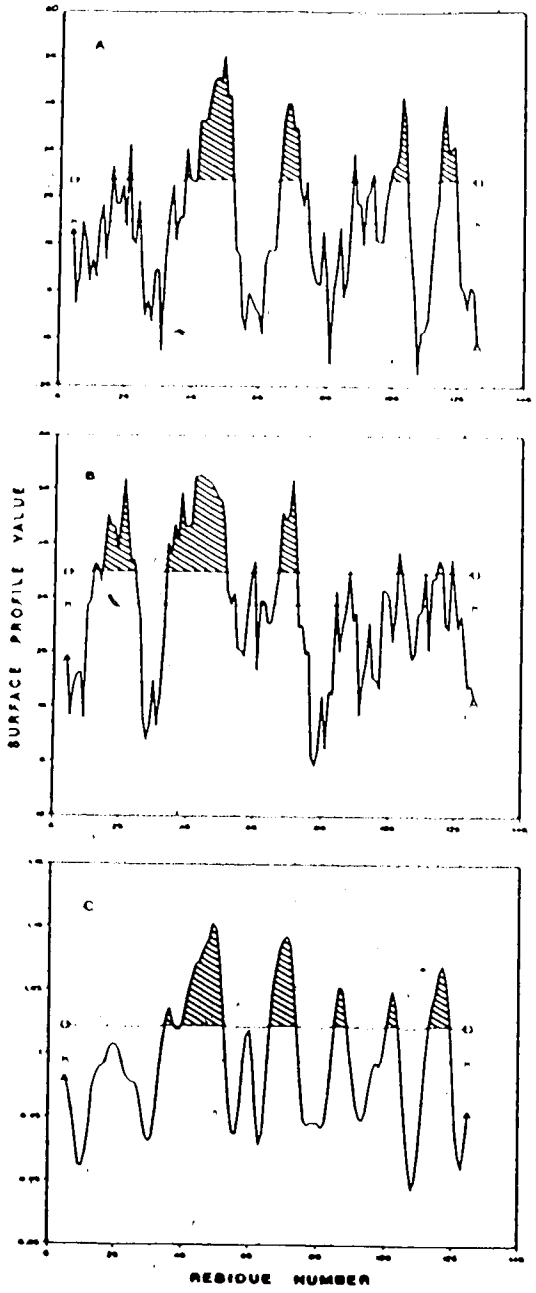


Fig. 34. Surface profile plots for lysozyme. Panel A: A surface profile calculated with the HPLC parameters (Table 13). Panel B: A surface profile calculated with the accessibility parameters (Table 15). Panel C: A surface profile calculated with the B-value parameters (Table 15). The dotted line represents the mean protein hydrophilicity value and the solid line represents the 25% cutoff value described in this thesis. The hatched areas defines those regions of the primary sequence used to obtain the composite surface profile plot (see Fig. 35), showing the predicted surface sites of lysozyme.

Table 15. Hydrophilicity scales for amino acid residues derived from the accessibility and mobility parameters

Amino Acids	Parameters			
	Accessibility[a]	B-values[b] set 1	set2	set 3
Trp	+3.2	-10.0	-5.1	-10.0
Phe	+0.5	-9.6	-5.6	-1.2
Leu	-0.3	-6.5	-1.3	-0.7
Ile	-3.4	-3.7	-7.3	-4.6
Met	+1.9	-8.2	-10.0	-9.9
Val	-2.5	-5.3	-4.2	-1.3
Tyr	+8.0	-7.0	-4.0	-7.3
Cys	-10.0	-7.1	-8.6	-0.4
Ala	+2.7	-0.5	-2.6	-3.0
Pro	+7.5	+0.6	+9.6	+0.1
His	+6.7	-5.3	-2.1	-2.8
Arg	+9.8	-0.7	+4.6	-2.3
Thr	+7.1	+2.1	+6.6	+0.3
Lys	+10.0	+3.8	+9.4	+10.0
Gly	+2.3	+7.8	+5.8	-0.5
Gln	+8.9	+9.6	+4.6	-3.5
Ser	+6.7	+10.0	+6.4	-0.5
Asn	+8.4	+5.7	+2.7	0.0
Glu	+8.9	+3.8	+5.3	+0.7
Asp	+8.4	-1.1	+10.0	+0.1

** These parameters are derived from protien data.

[a] Janin (1979). [b] Karplus and Schulz (1985).

(this study), Janin accessibility and β-value parameters (Karplus and Schulz). For example, a composite surface plot for lysozyme was made by rescaling the surface sites (hatched areas in Fig. 34A, 34B and 34C) for each profile from 0 to 100 where the maximum surface site value in each plot was set equal to 100 and the 25% surface site value was set to 0 (Fig. 35). Each scaled plot was superimposed on the other. Tables 16 and 17 list the predicted surface sites for lysozyme and cytochrome *c* derived from the composite surface profiles. When comparing the surface sites with the antigenic sites derived from immunological studies in Tables 16 and 17, predicted surface sites generally correlate to the antigenic sites. Obviously, some exceptions are observed. For example, the N- and C-terminal antigenic sites were missed for lysozyme and two surface sites (15-30, 68-78) other than the known antigenic sites were predicted for cytochrome *c* by using the composite surface profile. Moreover, the predicted surface sites are comparable to the surface accessible residues determined by X-ray crystallography shown in Tables 16 and 17. In general, there is a good correlation of the predicted surface sites between these two methods.

In summary, the composite profiles which combined the HPLC, accessibility and mobility parameters provided a better predictive method for some proteins. The predicted surface sites for lysozyme and cytochrome *c* were found to correlate well with the known antigenic and X-ray determined

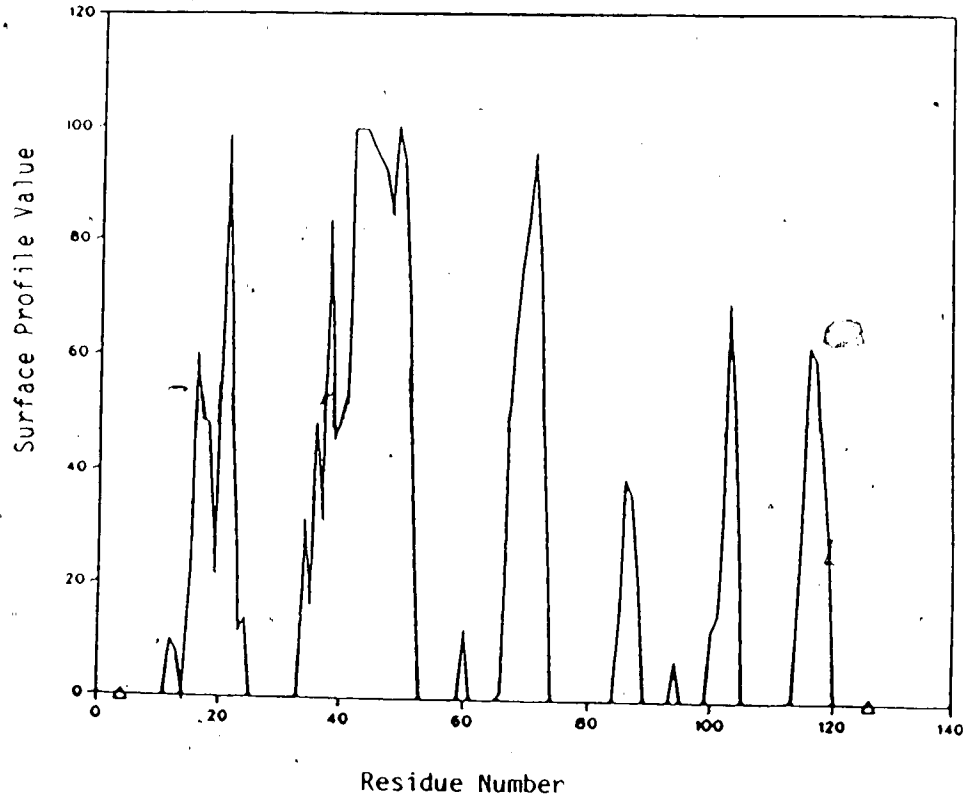


Fig. 35. A predicted composite surface profile for lysozyme. The profile was obtained by combining the hatched areas in Fig. 34A, 34B and 34C. The surface sites for each profile in Fig. 34A, 34B and 34C were scaled from 0 to 100 where the maximum surface site value in each plot was set equal to 100 and the 25% surface site value was equal to 0 in this composite profile.

Table 16 Comparisons of predicted surface residues of lysozyme with immunological and X-ray crystallographic data.

PREDICTED SURFACE RESIDUES	EXPERIMENTAL ANTIGENIC SITES DETERMINED BY IMMUNOLOGICAL STUDIES[a]	SURFACE ACCESSIBLE RESIDUES DETERMINED BY X-RAY DATA[b]
----	1, 5, 7	1-2
12-13, 15-24	13-14, 19-24	13-14, 18-22
34-52	33, 34, 45, 48	33-34, 43-48
60	62	61-62
66-73	64-80	67-68
85-88	87-89	85-87
94, 100-104	93-97, 102, 103	101, 103
113-119	113, 114, 116, 117-121	112-114, 116-119
----	125	125-126, 128-129

a Atassi (1984); Benjamin et. al., (1984); Amit et. al., (1985)

b Brookhaven Data Bank, # 253 (RS16 Hen Egg-White Lysozyme)

TABLE 17. Comparisons of predicted surface residues of cytochrome C with immunological and X-ray crystallographic data.
(FROM HORSE AND ALBACORE)

PREDICTED SURFACE RESIDUES		EXPERIMENTAL ANTIGENIC SITES DETERMINED BY IMMUNOLOGICAL STUDIES[a]	SURFACE ACCESSIBLE RESIDUES DETERMINED BY X-RAY DATA[b]
ALBACORE	HORSE[c]	HORSE	ALBACORE
4-8	4-7	1-4	2-5, 7-8
10, 15-16, 19-28	15-16, 19-30	----	11-13, 21-23, 25-28
38-55	38-44, 50-56	44, 46-50	44-45, 53-55
63-64	61-64	60-62	60-62
68-78	68-73, 76-78	----	72-73, 76-77
86-93	86-92	89-92, 96	86-88
100	100-101	103, 104	99-100

a Atassi (1984); Benjamin et. al., (1984)

b Brookhaven Data Bank, # 149 (Oxidized Albacore Cytochrome C)

c. primary sequence from Dickerson et. al. (1971)

accessible sites.

REFERENCES

Ben-Naim, A. (1980) *"Hydrophobic Interactions"* (Plemun Press New York).

Bennett, H.P.J. (1983) *J. Chromatogr.*, 266, 501-510.

Bennett, H.P.J., Browne, C.A., Brubaker, P.L., and Solomon, S. (1979) *Chromatogr. Sci. Series*, 18, 197-210.

Bennett, H.P.J., Browne, C.A., and Solomon, S. (1980) *J. Liquid Chromatogr.*, 3(9), 1353-1365.

Bennett, H.P.J., Hudson, A.M., McMartin, C., and Purdon, G.E. (1977) *Biochem. J.*, 168, 9-13.

Berendsen, G.E., and de Galan, L. (1980) *J. Chromatogr.*, 196, 21-37.

Berry, V.V. (1982) *J. Chromatogr.*, 236, 279-296.

Bij, K.E., Horvath, C., Melander, W.R., and Nahum, A., (1984) *J. Chromatogr.*, 203, 65-84.

Bishop, C.A., Hancock, W.S., Brennan, S.O., Carrell, R.W., and Hearn, M.T.W. (1981) *J. Liquid Chromatogr.*, 4(4), 599-612.

Browne, C.A., Bennett, H.P.J. and Solomon, S. (1982) *Anal. Biochem.*, 124, 201.

Bull, H.B., and Breese, K. (1974) *Arch. Biochem. Biophys.*, 161, 665-670.

Cantor, C.R., and Schimmel, P.R. (1980) in *"Biophysical Chemistry"* (W.H. Freeman and Company, San Francisco) Part I.

Cooke, N.H.C., and Olsen, K., (1980) *J. Chromatogr. Sci.*, 18, 512-524.

Cooke, N.H.C., Archer, B.G., O'Hare, M.J., Nice, E.C., and Capp, M. (1983) *J. Chromatogr.*, 255, 115-123.

Colin, H. and Guiochon, G. (1977) *J. Chromatogr.*, 141, 289-312.

Colin, H. and Guiochon, G. (1978) *J. Chromatogr.*, 158, 183.

Deibler, G.E., Boyd, L.F., Martenson, R.E., and Kies, M.W. (1985) *J. Chromatogr.*, 326, 433-442.

Edsall, J.T. and McKenzie, H.A. (1983) *Adv. Biophys.*, 16, 53-183.

Fauchere, J.L., and Pliska, V. (1983) *Eur. J. Med. Chem.*, 18, 369-375.

Fausnaugh, J.L., Kennedy, L.A., and Regnier, F.E. (1984) *J. Chromatogr.*, 317, 141-155.

Fraga, S. (1982) *Can. J. Chem.* 60, 2606-2610.

Geng, X., and Regnier, F.E. (1984a) in *"Peptide structure and function: proc. 8th. Amer. Pep. Symp."* (Hruby, V.J., and Rich, D.H., eds.). 727-734.

Geng, X., and Regnier, F.E. (1984b) *J. Chromatogr.*, 296, 15-30.

Gisin, B.F. (1973) *Helv. Chem. Acta*, 56, 1476-1482.

Glajch, J.L. (1986) *LC magazine (Liquid Chromatogr. and HPLC)*, 4(2), 84-86.

Glazer, A.N., Delange, R.J., and Sigman, D.S. (1975) in *"Chemical Modification of Proteins"* (Work, T.S., ed., Amer. Elsevier Publishing Company, INC. New York, Ny).

Grego, B. and Hearn, M.T.W. (1984) *J. Chromatogr.*, 336, 25-40.

Green, N., Alexander, H., Olson, A., Alexander, S., Schinnick, T.M., Stutcliffe, J.G., and Lerner, R.A. (1982) *Cell* 28, 477-487.

Hancock, W.S., Bishop, C.A., Meyer, L.J., Harding, D.R.K., and Hearn, M.T.W. (1978a) *J. Chromatogr.*, 161, 291-298.

Hancock, W.S., Bishop, C.A., Prestidge, R.L., Harding, D.R.K., and Hearn, M.T.W. (1978b) *Science*, 200, 1168-1170.

Hancock, W.S., and Sparrow, J.T. (1981) *J. Chromatogr.*, 206, 71-82.

Hancock, W.S., and Harding, D.R.K. (1984) in *"Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins"* (Hancock, W.S., ed., CRC press, Inc.) Vol. II, pp3-21.

Hatefi, Y., and Hanstein, W.G. (1969) *PNAS USA*, 62, 1129-1136.

Hearn, M.T.W., and Hancock, W.S. (1978) *Chromatogr. Sci.*

Series, Vol. 12, 243-271.

Hearn, M.T.W., Bishop, C.A., Hancock, W.S., and Harding, D.R.K. (1979a) *J. Liquid Chromatogr.*, 2(1), 1-21.

Hearn, M.T.W., Grego, B., and Hancock, W.S. (1979b) *J. Chromatogr.*, 185, 429-444.

Hearn, M.T.W. (1980) *J. Liquid Chromatogr.*, 3(9), 1255-1276.

Hearn, M.T.W., and Grego, B. (1981) *J. Chromatogr.*, 203, 349-363.

Hearn, M.T.W., and Grego, B. (1984) *J. Chromatogr.*, 296, 61-82.

Hennion, M.C., Picard, C and Caude, M (1978) *J. Chromatogr.*, 166, 21.

Hermodson, M., and Mahoney, W.C. (1983) *Meth. Enzymol.*, 91, 352-359.

Heukeshoven, J., and Dernick, R. (1982) *J. Chromatogr.*, 252, 241-254.

Hodges, R.S. and Merrifield, R.B. (1975) *Anal. Biochem.*, 65,

241.

Hodges, R.S., Saund, A.K., Chong, P.C., St.-Pierre, S.A. and Reid, R.E. (1981) *J. Biol. Chem.*, 256, 1214-1224.

Honegger, A., Hughes, G.J., and Wilson, K.J. (1981) *Biochem. J.*, 199, 53-59.

Hopp, T.P., and Woods, K.R.(1981) *PNAS USA*, 78, 3824-3828.

Horvath, C. (1983) *LC mag. (Liquid Chromatogr. and HPLC)*, 1(9), 552-558.

Horvath, C., Melander, W., and Molnar, I. (1976) *J. Chromatogr.*, 125, 129-156.

Hughes, G.J., Winterhalter, K.H., and Wilson, K.J. (1979) *FEBS LETTERS*, 108(1), 81-86.

Ingraham, R.H., Lau, S.Y.M., Taneja, A.K., and Hodges, R.S. (1985) *J. Chromatogr.*, 327, 77-92.

James, S. and Bennett, H.P.J. (1985) *J. Chromatogr.*, 326, 329-338.

Janin, J. (1979) *Nature*, 277, 491-492.

Janssen, P.S.L., van Nispen, J.W., Hamelinck, R.L.A.E., Melgers, P.A.T.A., and Goverde, B.C. (1984) *J. Chromatogr. Sci.*, 22, 234-238.

Johnson, E.L., and Stevenson, R. (1978) in *"Basic Liquid Chromatography"* (Varian Associates, Inc. Palo Alto, California.

Johnson, C.S., Moyes, D., Schroeder, W.A., Shelton, J.B., Shelton, J.R.and Beutler, E. (1980) *Biochimica et Biophysica Acta*, 623, 360-367.

Jones, B.N., Lewis, R.V., Paabo, S., Kojima, K., Kimura, S., and Stein, S. (1980) *J. Liquid Chromatogr.*, 3(9), 1373-1383.

Karch, K., Sebestian, I. and Halasz, I. (1976) *J. Chromatogr.*, 122, 3-16.

Karplus, P.A. and Scnulz, G.E. (1985) *Naturwissenschaften*, 72, S.212.

Kyte, J., and Doolittle, R.F.(1982) *J. Mol. Biol.* 157, 105-132.

Lau, S.Y.M., Taneja, A.K., and Hodges, R.S. (1984) *J. Chromatogr.*, 317, 129-140.

Lewis, R.V., Fallon, A., Stein, S., Gibson, K.D., and Udenfriend, S. (1980) *Anal. Biochem.*, 104, 153-159.

Li, J.B., and Cotter, R.L. (1986) *J. Analysis and purification*, pp.30-36.

Lochmuller, C.H., and Wilder, D.R. (1979) *J. Chromatogr. Sci.*, 17, 574-579.

Lundanes, E., and Greibrokk, T. (1978) *J. Chromatogr.*, 149, 241-254.

Lundanes, E., and Greibrokk, T. (1984) in *"Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins"* (Hancock, W.S., ed., CRC press, Inc.) Vol. II, pp.49-52.

Lundblad, R.L., and Noyes, C.M. (1984)in *"Chemical Reagents for Protein Modification"* Vol. I, pp.31-33.

Mahoney, W.C., and Hermodson, M.A.'(1980) *J. Biol. Chem.*, 255, 11199-11203.

Majors, R.E. and Hopper, M.J. (1974) *J. Chromatogr. Sci.*, 12, 767-779.

Manalan, A.S., Newton, D.L. and Klee, C.B. (1985) *J. Chromatogr.*, 326, 387-397.

Mant, C.T. and Hodges, R.S. (1986) *LC mag. (Liquid Chromatogr. and HPLC)*, 4(3), 250-254.

McNair, H.M. (1984) *J. Chromatogr. Sci.*, 22, 521-535.

Meek, J.L. (1980) *PNAS USA*, 77, 1632-1636.

Meek, J.L., and Rossetti, Z.L. (1981) *J. Chromatogr.*, 211, 15-28.

Meek, J.L. (1983) *J. Chromatogr.*, 266, 401-408.

Melander, W.R., Kalghatgi, K., and Horvath, C. (1980) *J. Chromatogr.*, 201, 201-209.

Molnar, I., and Horvath, C. (1977) *J. Chromatogr.*, 142, 623-640.

Monch, W., and Dehnen, W. (1977) *J. Chromatogr.*, 140, 260-262.

Monch, W., and Dehnen, W. (1978) *J. Chromatogr.*, 147, 415-418.

Moore, S. (1963) *J. Biol. Chem.*, 238, 235.

Nice, E.C., Capp, M.W., Cooke, N., and O'Hare, M.J. (1981) *J. Chromatogr.*, 218, 569-580.

Nozaki, Y., and Tanford, C. (1971) *J. Biol. Chem.*, 246, 2211-2217.

O'Hare, M.J., and Nice, E.C. (1979) *J. Chromatogr.*, 171, 227-232.

Parker, J.M.R. and Hodges, R.S. (1985) *J. Prot. Chem.*, 3, 465.

Pearson, J.D., Lin, N.T., and Regnier, F.E. (1983) *Anal. Biochem.*, 124, 217-230.

Pietrzyk, D.J., Smith, R.L., and Cahill, W.R. (1983) *J. Liquid Chromatogr.*, 6(9), 1645-1671.

Poll, D.J., Knighton, D.G., Harding, D.R.K., and Hancock, W.S. (1982) *J. Chromatogr.*, 236, 244-248.

Quarry, M.A., Grob, R.L., and Snyder, L.R. (1984) *J. Chromatogr.*, 285, 19-51.

Regnier, F.E. (1983) *Science*, 222, 245-252.

Regnier, F.E., and Gooding, K.M. (1980) *Anal. Biochem.*, 103, 1-25.

Rekker, R.F. (1977) *"The Hydrophobic Fragmental Constant"* (Elsevier Scientific Publishing Company, Amstredam).

Rivier, J., and Burgas, R. (1977) *Chromatogr. Sci. Series*, Vol. 10, pp.147-161.

Rubinstein, M. (1979) *Anal. Biochem.*, 98, 1-7.

Rubinstein, M., Stein, S., and Udenfriend, S. (1977) *PNAS USA*, 74, 4969-4972.

Rzeszotarski, W.J. and Mauger, A.B. (1973) *J. Chromatogr.*, 86, 246-249.

Sasagawa, T., Ericsson, L.H., Teller, D.C., Titani, K., and Walsh, K.A. (1984a) *J. Chromatogr.*, 307, 29-38.

Sasagawa, T., Okuyama, T., and Teller, D.C. (1982) *J. Chromatogr.*, 240, 329-340.

Sasagawa, T., and Teller, D.C. (1984b) in *"Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins"* (Hancock, W.S., ed., CRC press, Inc.)

Vol. II, pp.53-65.

Schaaper, T., and Teller, D.C. (1980) *J. Chromatogr.*, 195, 181-186.

Snyder, L.R. (1979) *J. Chromatogr.*, 179, 167-172.

Stewart, J.M. and Young, J.D. (1984) *"Solid Phase Peptide Synthesis"* (Pierce Chemical Company)

Sturtevant, J.M. (1977) *PNAS USA*, 74, 2236-2240.

Su, Suew-Jane, Grego, B., Niven, B., and Hearn, M.T.W. (1981) *J. Liquid Chromatogr.*, 4(10), 1745-1764.

Tanford, C. (1978) *Science*, 200, 1012-1018.

Tanford, C. (1973) in *"The Hydrophobic Effect: Formation of Micelles And Biological Membranes"* (John Wiley & Sons, Inc.)

Tam, J.P., Heath, W.F., and Merrifield, R.B. (1983) *J. Amer. Chem. Society.*, 105, 6442.

Terabe, S., Konaka, R. and Inouye, K. (1979) *J. Chromatogr.*, 172, 163-177.

Tinoco, I., Sauer, Jr. K., and Wang, J.C. (1978) in *"Physical Chemistry"* (Prentice-Hall, Inc.) p.85-90.

Van Der Zee, R., and Welling, G.W. (1982) *J. Chromatogr.*, 244, 134-136.

Vigh, G., Varga-Puchong, I., Hlavay, J., and Papp-Hites, E. (1982) *J. Chromatogr.*, 236, 51-59.

Wehr, C.T., Correia, L. and Abbott, S.R. (1982) *J. Chromatogr. Sci.*, 20, 114-118.

Wilson, K.J., Honegger, A., Stotzel, R.P., and Hughes, G.J. (1981) *Biochem. J.*, 199, 34-41.

Wilson, K.J., Wieringen, E.V., Klauser, S., and Berchtold, M.W. (1982) *J. Chromatogr.*, 237, 407-416.

Wilson, K.J. (1985) *"An Introduction to Protein/ Peptide Isolation Strageies For Primary Structure Determinations"* (Applied Biosystems).

Wolfenden, R.V., Cullis, P.M., and Southgate, C.C.F. (1979) *Science (Washington, D.C.)* 206, 575-577.

Woods, A.H., and O'Bar, P.R. (1970) *Science*, 167, 179-181.

( ) Yamashiro, D. and Li, C.H. (1973) *J. Org. Chem.*, 38, 2594.