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# PROTEIN/PEPTIDE SEQUENCE ANALYSIS: CURRENT METHODOLOGIES

Ajit S. Bhown

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# Protein/Peptide Sequence Analysis: Current Methodologies

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# PREFACE

The functional properties of a protein molecule have long been known to depend upon its primary structure. It is, therefore, knowledge of the amino acid sequence that allows an investigator to understand: the interaction between an antibody molecule and its antigen; the ability of a hormone to bind to a receptor; the ability of an enzyme to bind and cleave its substrate with the ability for a variety of molecules to form aggregates and, hence, develop a structural framework upon which other biological activities occur. The fascination of protein chemistry, therefore, depends very much upon our ability to gain information regarding the primary structure of protein molecules and how that amino sequence directs the molecular function. Even with the new excitement generated by the very rapid methods allowing DNA sequencing to be transformed into protein structure, we still have an enormous need to determine amino acid sequences on ever-decreasing amounts of available polypeptide samples. Since structure and function are so intimately involved in proteins, even our knowledge of the DNA sequence does not always allow us to make a leap to functional relevance without first knowing and determining at least some parts of the amino acid sequence of a given protein molecule. This book is an attempt to provide in a single source current state-of-the-art methodologies for protein sequence analysis. It is hoped that these various chapters are presented in such a way that both the newcomer and the established protein chemist will find useful information and directions to new techniques.

The contributors to this volume have been carefully selected by the editor from among the world leaders in this field. Each chapter is written in a way that establishes the individual author's particular perspective on the field of his research. Chapter 1 outlines experimental details for the purification of proteins and peptides suitable for sequence analysis by high pressure liquid chromatography. Chapters 2 through 5 describe in detail how the purified protein-peptide can be subjected to sequence analysis. Use of homologous coupling reagents has been described in Chapter 6, while amino acid sequence analysis from the carboxyterminal end by chemical and enzymatic procedures has been discussed in Chapters 7 and 8, respectively. Mass spectrometry as a tool for sequence analysis is discussed in Chapter 9, and Chapter 10 covers the novel methods of amino acid sequence analysis. The last two chapters (11 and 12) deal with the identification procedures of modified (methylated) and derivatized (phenylthiohydantoin) amino acids.

This book offers a rich array of techniques and methods for sequencing proteins and peptides. It should meet the expectations of investigators in protein chemistry who wish to update their knowledge of sequencing techniques, and of those who wish to reacquaint themselves with the best available current technologies.

**J. Claude Bennett**

## THE EDITOR

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Dr. Bhown obtained his Ph.D. degree in 1965 from the University of Rajasthan, Jaipur, India, and his post-doctoral training at the University of Tennessee Medical Center, Knoxville, from 1965 to 1967 and at the University of Alabama at Birmingham from 1974 to 1975. He was on the Teaching Faculty in the College of Medicine in Jodhpur, India from 1967 to 1974. His major research interest has been in the development of techniques for the structural studies of proteins and peptides. Dr. Bhown has presented numerous lectures at international and national scientific meetings and seminars and has published more than 80 scientific papers and chapters.

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In memory of my Parents

*This book is affectionately dedicated to my wife Meera, daughter Sonchita, and son Abhoyjit.*

Chapter 1

# PURIFICATION OF PROTEINS/PEPTIDES FOR STRUCTURAL STUDIES

**K. J. Wilson**

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# I. INTRODUCTION

Since the introduction of the gas phase sequencer,[1] there have been changes and improvements in isolation methodologies used for sample preparation. The manipulations required for any isolation often involve numerous methods which differ in their basic operating principles. These methods frequently introduce contaminates that chemically modify the desired end product and/or further compromise the purity of the sample. In addition, the time required for an isolation, its cost, overall yields, and the availability of laboratory instrumentation are collectively important factors which must be considered prior to the start of an isolation.

Given that there are a number of variables which require consideration, it is often helpful to consider the sequencing results from a random selection of samples which, for the most part, have *not* been "optimally" isolated.

Table 1 summarizes such results from samples submitted over a 12-month period to our Custom Sequencing Facility. The percentage of each group that was successfully sequenced (defined by a major-interpretable sequence) differed considerably: 51% of the proteins and 74% of the peptides.

Such results suggest that the analytical methods used for determining protein homogeneity and/or quantity are, at best, wanting. The high peptide heterogeneity (16%) supports a similar conclusion. The lower "contamination" (12%) noted for proteins speaks well of the isolation methods used for this sample set.

Insufficient amount and/or the absence of an available α-amino group could not be differentiated and the quantity estimates supplied with the samples were assumed to be correct. However, the obviously low initial yields from what could be considered a "typical" set of proteins suggest that either quantity estimates were inflated or significant amino-terminal blockage had occurred during isolation, or both. Since peptide amounts were, for the most part, unknown, the appropriate calculations could not be done.

As indicated in Table 1, the final steps for purifying proteins were based predominately (57%) on conventional chromatographic methods. This is not unexpected, since the majority of samples came from laboratories experienced in protein preparation. Although the isolation methods used were not necessarily among the more "modern" ones, including polyacrylamide gels and the various modes of HPLC, they appear to have been adequate.

Conversely, the almost exclusive use of reverse-phase chromatography (RPC) for peptide isolation (despite considerable contamination problems, 26% being unsequenceable for one reason or another) suggests that this method has found wide acceptance.

A survey of the literature has indicated that gas phase sequencer users are also employing conventional methodologies quite effectively (Table 2). It was interesting to see that affinity chromatography was being used almost as frequently as the ion exchange methods, and that size exclusion was being used for both isolation and desalting. The preference for an electrophoretic method is similar to the ratios noted in Table 1. The frequent use of various forms of HPLC for characterization (fragment isolation) was also supported in this survey.

The aim of this review is to indicate those methodologies found to be convenient sample preparation schemes by us and other gas phase sequencer users. Some of the techniques reviewed are designed so that sample volumes and sizes are compatible with the cartridge design of gas phase instruments. Since there are only a few laboratories that are employing all of the techniques, every attempt is made to give sufficient detail so that the appropriate literature can be located. Note that the topics covered and a person's familiarity with each are considered to be of *absolute* importance when sequencing at high sensitivities of 25 pmol or less.

**Table 1**
**CUSTOM SEQUENCING SUMMARY**

| | Proteins | Peptides |
|---|---|---|
| Total number samples | 75(100) | 69(100) |
| Number successfully sequenced | 38(51) | 51(74) |
| Insufficient amount/blocked | 28(37) | 7(10) |
| Heterogeneous | 9(12) | 11(16) |
| % Initial yield | 30 ± 20[a] | ND |
| Last step in purification | | |
| Conventional | 43(57) | 2(3) |
| SDS-PAGE | 24(32) | — |
| HPLC | 8(11) | 67(97) |

*Note:* Values in parentheses represent percentages of total. ND, not determined, insufficient data.

[a] Calculated only on those samples where sufficient data were available.

**Table 2**
**ISOLATION/CHARACTERIZATION METHODS EMPLOYED IN PREPARING PROTEINS/PEPTIDES FOR HIGH-SENSITIVITY STRUCTURAL INVESTIGATIONS**

| Isolation | | Characterization | |
|---|---|---|---|
| Chromatography, liquid | | High-performance liquid chromatography | |
| Affinity | 28 | Ion-exchange | 19 |
| Chromatofocusing | 1 | Reverse-phase | 86[a] |
| Hydrophobic | 1 | Size-exclusion | 14 |
| Ion-exchange | 37 | | |
| Reverse-phase | [a] | | |
| Size-exclusion | 52 | | |
| Electrophoresis, PAGE | | | |
| Isoelectric focusing | 4 | | |
| Preparative SDS-PAGE | 27 | | |

*Note:* A survey of 109 papers which have employed the Applied Biosystems 470A Gas Phase Sequencer from 1982 through mid-1984 (6 in 1982, 23 in 1983, and 80 in 1984).

[a] Values under Characterization and Isolation are combined.

## II. CHROMATOGRAPHIC METHODS

### A. Reverse-Phase Chromatography (RPC)

There are numerous reasons why RPC is suited for sample preparation. Among these are the short separation times that range between 30 and 120 min and the high sensitivities that can be achieved using either UV or fluorescence detectors for preparing microgram or smaller amounts of material. In addition, sample work-up is usually simple and the general methodology is suitable for most peptides and many proteins.

The disadvantages of RPC are few, but extremely important. For example, column-to-

**Table 3**
**CHROMATOGRAPHIC VARIABLES IMPORTANT IN REVERSE-PHASE SEPARATIONS OF PROTEINS AND PEPTIDES**

| | Proteins | Peptides |
|---|---|---|
| Support | | |
| Phase | $C_3$—$C_{18}$, CN, phenyl | $C_4$—$C_{18}$ |
| Pore size (Å) | 300—500 | 100—300 |
| Particle size (μm) | 3—20 | Same |
| Column | | |
| Length (cm) | 3—25 | Same |
| Diameter (mm) | 1—4.6 | Same |
| Conditions | | |
| Temp (°C) | 5—35 | Ambient |
| pH | 2—8 | Same |
| Buffers | Varied depending on protein activity/ stability | Normally including a volatile component (TFA, etc.) and low pH |
| Organic modifier | Acetonitrile, 1- or 2-propanol, mixtures with butanol or others | Acetonitrile or propanol |
| Flow rate (mℓ/ min) | 0.1—2.0 | Same |

column reproducibility, from the same vendor or from different vendors, is frequently poor. Of equal importance is the initial equipment cost for mid- to high-sensitivity applications, and the fact that many proteins/enzymes are not compatible with the chromatographic conditions often used.

There are also problems associated with sample preparation which directly affect the overall yield. These include possible introduction of contaminates by the chromatographic step and the probability of chemically modifying the sample of interest. Sample losses on surfaces (glass, polypropylene, etc.) by either adsorption from a liquid or following a drying/ lyophilization step often reduce overall recoveries. As will be discussed later (Table 8), there are general guidelines that allow one to minimize such interactions or losses for many samples.

*1. Chromatographic Variables*

RPC is based on the hydrophobic differences between the sample being chromatographed and the bonded phase on the support material. There are a number of variables which effect these associations. The most important are listed in Table 3.

The utility of an RPC separation, under a defined set of experimental conditions, is dependent on the factors:

1. Column retention and selectivity
2. Material or mass recovery
3. How the chromatographic conditions affect recovery of biological or enzymatic activity

Since there are a number of variables which must be considered both prior to and following chromatography (see Table 3), it is frequently necessary to look at a series of well-defined experiments which optimize at least one of these three factors. One can often achieve high

mass and activity recoveries, but not increase the overall purification (specific activity, biological or enzymatic); alternatively, retention and selectivity might be optimized at the expense of sample recovery.

Sample recoveries are usually not a serious problem given that "correct" chromatographic parameters can be found. For example, many of the earlier RPC isolations were carried out on samples that were either minimally affected by the chromatographic conditions or denatured during chromatography, with renaturation occurring during sample recovery by lyophilization, dialysis, or neutralization.

Comparisons of columns from various vendors have shown that differences do exist and the methods used by such vendors for column characterizations do not yield useful information as far as protein chromatography is concerned.[2] In fact, the product descriptions (particle size, pore size, carbon coverages, etc.) are often obscure or even incorrect, and it is virtually impossible to determine a support's particular efficacy. This information is disconcerting to the protein chemist that simply wants something that "will work" and not be forced into carrying out testing on each newly purchased column to determine its utility. It is even more of a concern when he realizes that even the same column from a given vendor can generate quite different results. There are other reports on nonspecific factors thought to contribute to the mass loss of sample: adsorption by stainless steel tubing,[3] frits,[4] or by the packing material itself.[5] These studies have been carried out on only a limited number of samples and it is not clear how generally applicable the results are to peptide/protein RP-HPLC.

*2. Protein Separation by RPC*

More definitive factors that affect both recovery and selectivity are the pore size of the support[5-10] (100 to 300 Å being optimal) and the choice of the organic modifier used for sample elution.[5,7,10] The most popular modifiers have been either acetonitrile or propanol, with several recent reports suggesting that mixed organic phases have distinct advantages. For example, combinations of propanol-butanol, acetonitrile-propanol, or even propanol-2-methylbutanol have proven effective.[11,12]

The actual pH during chromatography is of equal importance and has been illustrated in a number of publications.[13,14] The effects of parameters, including the pH, aqueous buffer, or organic component(s), on activity recovery can often be determined by simple incubation experiments. It is imperative that one not only perform these experiments prior to chromatography, but that the recovery yields from each be compared. Only through such evaluations can the factors, specific to a given support material, be identified and, perhaps, understood.

Other factors long considered unimportant, such as column temperature and sample-bonded phase contact time, have recently been investigated. Using soybean trypsin inhibitor,[15] papain,[16] lysozyme,[17,18] and ribonucleae A[19] as model proteins, it was demonstrated that both irreversible and reversible denaturation can occur during chromatography. Again, the denaturation was a function of the chromatographic conditions.

Native and denaturated species that create two or more peaks can often be separated, and the kinetics of unfolding can be determined.[14-18] The ability to detect both the native and denatured forms is a function of the refolding kinetics. The slower the refolding the greater the separation. Conversely, the faster the refolding the higher the probability of eluting a sharp peak. Distorted peaks are observed when the half-life of refolding was comparable to the elution time of chromatography.[18]

Another investigation[20] studied the optical changes which occurred in a number of proteins as a function of propanol concentration and pH. By comparing UV, circular dichroism, and/or fluorescence emission spectral changes, it was possible to account for greater than 80% renaturation. These results were consistent with the induction of an altered, though highly ordered, conformation.

Since RPC can detect very slight hydrophobic alterations in a protein, the presence of multiple peaks concerns the protein chemist interested in determining a primary structure. One of the most obvious detectable modifications that occurs is the substitution of an amino acid at one or more positions in a protein.[21,22] Others are chemical modifications such as glycosylation,[23] or oxidation of amino acid side chains.[24] The presence of disulfides can also be detected and has been employed to determine the disulfide bridging in proteins, including interferon,[25] T4 lysozyme,[26] and human growth hormone.[27] Similarly, various mixed disulfide forms of the high mobility group proteins[28] and recombinant α-interferons[29] have been isolated and characterized.

As previously discussed, denaturation caused by the chromatographic conditions can lead to peak multiplicity. For example, $Ca^{2+}$-binding proteins such as calmodulins, parvalbumins, etc. can chromatograph as broad, ill-defined peaks.[30] These proteins elute as sharp, well-defined peaks when either $CaCl_2$ or its specific chelator, EGTA (ethyleneglycoltetracetic acid), is added to the buffers. The elution positions for this particular class of proteins can be modulated through changes in the pH of the chromatography buffer. A decrease in the buffer pH from 7 to 2.5 causes increased retention. Since it is known that bound $Ca^{2+}$ is lost at low pH, it stands to reason that the molecular structure is altered and the "open" configuration (lacking $Ca^{2+}$) interacts more strongly with the support.

Therefore, when a particular cofactor is required by the protein being isolated, it should be either removed from the sample or added to the buffers prior to chromatography. An example of the latter case was the need to add $CaCl_2$ to the buffers when chromatographing trypsin and α-chymotrypsin.[31] For both enzymes the recovery yields were significantly increased following the addition.

*3. Peptide Separation by RPC*

The application of RPC for the separation of peptides is now the method of choice in most labs for either mapping or isolation for sequence analysis. The chromatographic conditions are listed in Table 2.

The ligands most frequently used are either $C_8$ or $C_{18}$ on 100- or 300-Å pore-size supports. Various authors have noted slight improvements of one particle size over another for specific applications, i.e., the use of a 3-μm particle for hydrophilic peptides. In general, 5- and 10-μm supports work equally well. Figure 1 illustrates how the reduction in column length affects peptide separation. With the exception of the most hydrophilic components, there are basically no differences in selectivities as a function of column length.

The eluting "strength" of the organic solvent needs to be sufficiently high to cause even the very hydrophobic fragments to elute. For this reason the most commonly used buffers contain either acetonitrile or propanol and, less frequently, methanol.[7,10] It is not, however, possible to substitute one organic for the other and expect the same resolution from a given column.[7] The most commonly utilized solvent system contains one of the perfluoralkanoic acids, usually 0.05 to 0.5% TFA or even HFBA (heptafluorobuteric acid). The volatile nature of these buffers makes them especially convenient to use.[32-34]

Ambient temperature and flow rates in the range of 1 mℓ/min are most frequently employed. One of the least recognized variables available for significantly altering the elution characteristics is the wide pH range over which separations can be performed. Often a simple pH adjustment from 2 to 6 or 7 will allow one to separate peptides or detect contamination without having to use other modes of HPLC or changing to another buffer system.[35]

*4. Increased Sensitivities with RPC*

Figure 2 illustrates the separation of peptides generated by exposing a 150,000-dalton protein to trypsin for 2 hr prior to RPC. Since less than 100 pmol of sample was available, a 1-mm microbore column was used for preliminary mapping and determining gradient

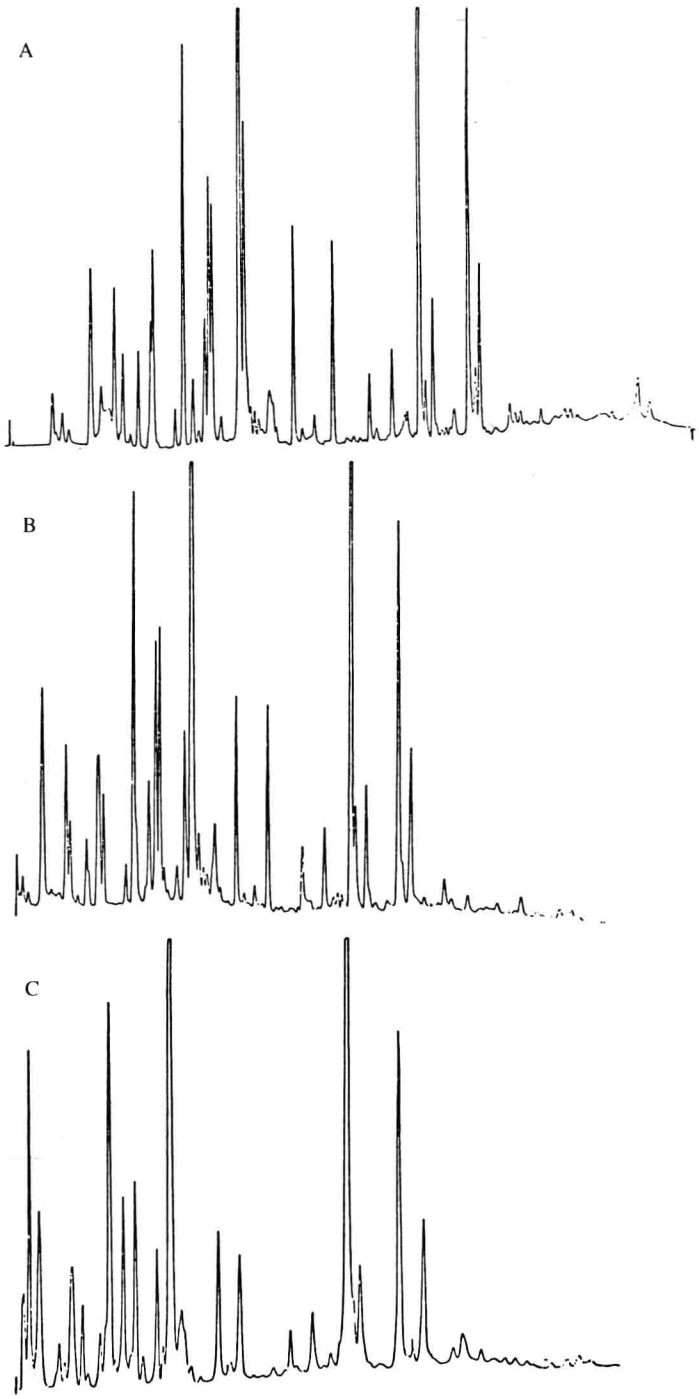


FIGURE 1. Peptide separations as function of column length. Apomyoglobin was tryptically digested and then 500 pmol amounts chromatographed on a series of Aquapore RP-300 cartiridge columns. All columns had internal diameters of 2.1 mm, but lengths varied from 220 mm (A) to 100 mm (B) to 30 mm (C). The solvent systems used were A buffer, 0.1% TFA; B buffer, 60% acetonitrile in 0.1% TFA. A gradient from 0 to 100% B was developed at 200 $\mu\ell$/min over 45 min.

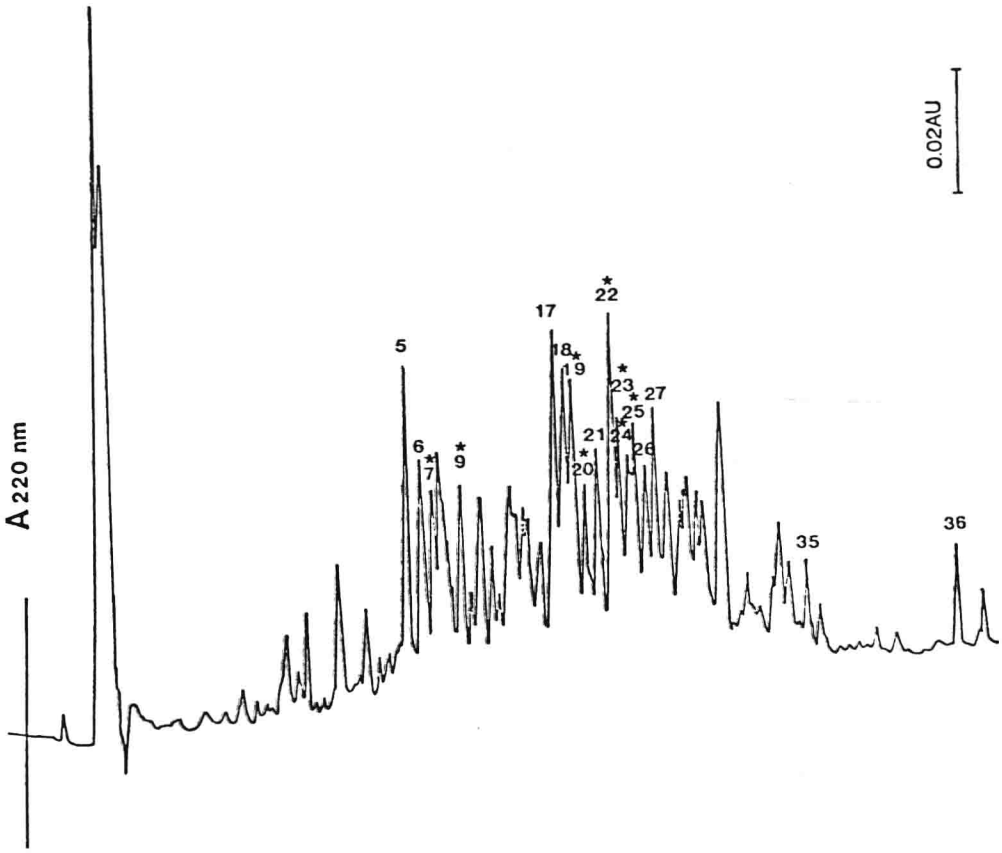


FIGURE 2. Preparative isolation of peptides. The tryptic fragments from a 150,000 dalton protein (approximately 100 pmol) were chromatographed on a 2.1 × 220-mm Aquapore RP-3000 column at 200 μℓ/min. Buffers were A, 0.1% TFA and B, 60% acetonitrile in 0.1% TFA.

profiles. Subsequently, a narrow-bore column was used for the indicated separation. Each peak was collected and those numbered were sequenced in a gas phase instrument. With the exception of the peaks denoted by an asterisk, all were found to contain multiple peptides. Given the size of the protein one would expect more fragments than were detected. Such results suggest that either the digestion was incomplete or the majority of the peaks contain multiple peptides, or both.

Approximately 100 residues were identified and most peptides were sequenced to the carboxy termini, starting with initial yields ranging from 5 to 50 pmol. This information, subsequently used for cloning, could only be derived by sequencing smaller peptides and not the intact protein. In the latter case only a few residues are generally identified due to the quick build-up of PTH background caused by nonspecific acid cleavage of the polypeptide backbone. The reasoning behind not attempting some type of either limited chemical or enzymatic cleavage was that aggregation frequently causes problems with subsequent separations.

In the above example and as Figure 2 illustrates, increased sensitivities were achieved by reducing the column internal diameter. Several possibilities are available to the protein chemist when higher detection sensitivities are required:

1. Decrease to lower UV wavelengths
2. Increase the detector sensitivity

3. Implement one of the stream-splitting, post-column reaction systems such as fluorescamine[36,37]
4. Reduce the internal diameter of the column

The first two possibilities are often used, but limited by either the absorbance and/or refractive index of the buffers commonly employed. Stream-splitting is less frequently implemented due to the expense and problems associated with maintaining the additional equipment. However, most of the HPLC units presently available can carry out gradient elutions on 2-mm ID columns without much difficulty. They are usually not optimized, however, to perform at full-scale expansions of greater than 0.05 AUFS at flow rates of 100 to 200 μℓ/min with proper mixing. When the column diameter is further reduced to 1.0 mm (microbore), problems increase. Pumping accurately and mixing at flows of 25 to 50 μℓ/min are very difficult. Also, the dead volumes must be sufficiently small so that the desired gradient reaches the separating column.

The series of chromatograms in Figure 3 illustrates the usefulness of reducing column ID and flow rate proportionately, while maintaining the sensitivity of the detector constant at 0.1 AUFS and reducing the amounts injected. The quantities chromatographed were 1000 and 100 pmol on the 4.6-mm ID support; 200 pmol on a 2.1-mm ID support, and 100 pmol on a 1-mm ID support.

It is clear that the sensitivity has been drastically increased (compare Figure 3B with 3D). Although not as obvious, the elution volumes of the individual peaks have decreased by a factor of approximately 20-fold, from approximately 500 μℓ with the 4.6-mm ID column to 30 μℓ with the 1.0-mm ID column. This results in simplified sample collection, handling, and concentration.

This significant reduction in elution volume provides an advantage to the gas phase sequencer user in that the glass fiber support disk can be used directly, either before or after polybrene precycling, as a fraction collector. This is accomplished by collecting the two to four drops of liquid containing the eluted peak onto the disk supported in an appropriate container, such as a 1.5-mℓ Eppendorf tube. The results presented in Table 4 indicate how sequencing yields can vary as a function of the collection method.

Similar increases in sensitivity are possible during protein HPLC on reduced ID columns (Figure 4A). Under these chromatographic conditions sample loss does not appear to be a problem (Figure 4B), and as Nice et al.[40] have shown, these smaller columns can be conveniently used for sample trace enrichment. Also, when appropriately excited, the native fluorescence of tryptophan in samples can provide a detection method with a higher sensitivity than direct absorbance measurements at low UV wavelengths. This latter point made by the above authors[40] is also one that we have substantiated.

One application for such a microbore system is illustrated in Figure 5 for the chromatography of a protein which was available in only very limited amounts. The sample, recombinant interleukin 2, was chromatographed at the 6-pmol level (approximately 120 ng). Two major components are clearly discernible as are a number of smaller "background" peaks which, due to their absence in the blank, must represent contaminates of the protein sample.

## B. Ion Exchange Chromatography (IEC) and Chromatofocusing

### *1. IEC Chromatographic Variables*

The recent availability of suitable anion and cation supports has provided both IEC and chromatofocusing as alternatives to RPC. The conditions required to elute proteins from an IEC matrix are usually compatible with maintaining native biological or enzymatic activities. Variables to be considered when performing such elutions are pH, the nature of the anion and cation of the particular salt being employed as the eluant, and the gradient rate.[41-46]