STUDIES IN CHROMATOGRAPHIC ANALYSIS: APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO PEPTIDE AND PROTEIN CHEMISTRY.

by

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SUMMARY

Separation methods have long played a crucial role in peptide and protein chemistry. Prior to the mid 1970s, protein chemists had at hand a large variety of chromatographic and electrophoretic techniques but, in many cases, these methods were deficient in terms of speed, resolving power, recovery or capacity. Over the past several years, research trends have greatly accelerated with many current studies in the biological and biomedical sciences now requiring considerable experimental flexibility and expertise before a particular biomolecule can be adequately characterised or its biological function accurately determined. Often, the abundance of a particular component in, for example, a tissue extract may be low, the availability of sufficient material limited, and precise and sensitive methods for its analysis, quantitation and isolation poorly documented. Improvements in classical separation techniques were clearly needed. Recent advances in high performance liquid chromatography, affinity chromatography and iso-electric focusing have greatly simplified the task of analysis and isolation of peptides and proteins available only in minute quantities. This thesis comprises 62 accounts of original research devoted to pioneering developments in three areas related to these fields. The papers are set out essentially in chronological order within three chapters.

Chapter 1 deals mainly with the different facets of the reversed phase high performance liquid chromatographic separation of amino acids, peptides and proteins on chemically bonded hydrocarbonaceous silica-based supports. At the time these

studies began, little was known about the suitability of the (then) newly available mesoporous microparticulate silica-based chromatographic media for the separation of amphoteric biomolecules such as peptides or proteins. Early attempts, notably by Shechter, with adsorptively coated controlled-pore glasses for the separation of peptides and proteins under conventional open column procedures had met with only limited success. We now recognise that this was partly due to the nature of the solute-stationary phase interaction under these conditions which generally led to poor peak shape associated with low resolution and recovery. Hydrophobic interaction chromatography, as introduced by Porath, Shaltiel and others, was primarily intended as a low pressure and low resolution technique for the separations of non-polar peptides and proteins on amphiphilic gels prepared by immobilising hydrocarbonaceous ligands such as alkyl- or aryl- groups onto soft hydrophilic gels. With these systems selectivity was achieved through differences in the relative hydrophobicities of the solutes and exploited via selective desorption with eluents of decreasing elutropic strength, for example by lowering the ionic strength or changing the buffer ions to those of greater chaotropicity. Because most agarose- and polystyrene-based amphiphilic supports were found to exhibit low separation efficiencies, and are unsuitable at high mobile phase linear flow velocities, they have attracted little interest for rapid high resolution separation of peptides or polypeptides based solely on relative differences in solute hydrophobicities. Although, useful peptide separations can be achieved by liquid-liquid partition chromatography on crossed-linked dextrans, again these methods are characterised by only modest column efficiencies

and recoveries and are incompatible with gradient elution which thus limits the polarity range of peptides that can be separated by any one phase system. During the mid 1970's, reversed phase high performance liquid chromatography was enjoying spectacular success in the separation of polar low molecular weight organic compounds, including many substances of pharmaceutical or pharmacological importance. In many cases, these separations employed microparticulate alkylsilicas with elution conditions based on the concepts of ion-pair bulk extraction and secondary chemical equilibria as developed by a number of analytical chemist, including Schill, Horvath, Karger and Huber. The early recognition and ample demonstration in my various published works that these principles could be applied under appropriate conditions to amino acid, peptide and protein separation and analysis by reversed phase high performance liquid chromatography has led to many subsequent analytical and preparative developments which have found expression in numerous biological applications (see for example, Chapter I publications 40, 44).

In fact, over the past several years, high performance liquid chromatography has emerged as the preeminent technique of biochemical analysis. The rapid acceptance and growth of the technique can be attributed not only to advances in sophisticated instrumentation, now frequently microprocessor controlled, but also to the advent of stable chemically modified silica-based stationary phases with surface characteristics appropriate to biomolecule separation and to the development of novel elution conditions which permit adequate control over secondary chemical equilibria which polyfunctional biomolecules can undergo in solution. As a consequence of these developments, rapid high

resolution chromatographic separations in protein chemistry have become a reality. Similar advances in nucleic acid chemistry are imminent.

Papers 1-18 describe my initial studies on the use of the fully porous and pellicular alkylsilicas for amino acid and peptide separation using mobile phases containing either hydrophilic or hydrophobic ionic additives. These studies deal mainly with methodological aspects of the use of reversed phase high performance liquid chromatographic procedures and provided some of the earliest practical examples of the use of this new silica-based technology for rapid high resolution separation of amino acids and peptides. Publications 3,13,18,21,25,30,37 record the application of these techniques for the micro- to macro- scale purification of synthetic and native peptides. Papers 4,10,12,26,29,30,33,41,43 describe the development of reversed phase high performance liquid chromatographic methods for the rapid analysis and isolation at high detection sensitivity of peptides derived from the proteolytic digestion of a number of proteins under study in this laboratory, including pituitary protein hormones, haemoglobin variants and enzymes. These peptide mapping and micro-preparative separation techniques have now been very widely applied in many laboratories engaged in the structural study of polypeptides and proteins.

These early studies raised several fundamental questions as far as the manner by which solute selectivities and peak efficiencies were influenced by stationary phase and mobile phase characteristics. Papers 19,20,32,35,36,38,39,40,44-47 go some way towards evaluating these issues, other manuscripts delineating the influence of the stationary phase ligand density, alkyl chain

length, and particle porosity have been submitted or are in preparation. Collectively, these studies were directed towards more detailed investigations on the physico-chemical basis of amino acid, peptide and protein retention to hydrocarbonaceous silica-based stationary phases. My initial goal here was to illuminate the mobile phase effects of pH (20,35), ionic strength and pairing ion interactions (20,24,27,36,45) and the organic solvent modifier (27,32,38,46) on retention and selectivity. In subsequent studies, stationary phase effects have been similarly systematically evaluated (main list, papers 100,103-105). The recognition and evaluation of these conditional effects was essential if the full potential of these high resolution separation methods is to be realised. This field of research has become at the present time one of the most actively pursued in biochemical analysis and will undoubtedly continue this vigorous course until both the thermodynamic distribution phenomena and the associated kinetic processes are fully understood. The demonstrations (paper 20,27) that peptide retention behaviour under regular reversed phase conditions can be evaluated in terms of solvophobic theory was followed by more detailed studies on the dependency of the logarithmic capacity factors on the mole fraction of the organic modifier(ψ) and surface tension of isocratic mobile phases (papers 32,38,46). These studies provided the first detailed description of a general bimodal relationship between the logarithmic capacity factor and ψ, a phenomenon presumably due to the interplay of solvophobic and silanophilic retention processes. This aspect was further explored in publication 46 in which procedures for the optimisation of gradient elution separations of peptides on alkylsilicas with

water-rich eluents were established. One important consequence of these studies on mobile phase effects was the demonstration that the retention of a peptide or related solute to an alkylsilica support reflects the effective hydrophobic contact area, a finding in accord with the theoretical solvophobic model. In attempts to utilise this finding as the basis of a predictive approach, topological analysis of the amino acid side chain and end groups of small peptides was undertaken (paper 39). This study provided further evidence in favour of the earlier hypothesis (papers 10,19,27) that hydrophobic group contributions of the amino acid residues in small peptides without conformational constraints have an essentially additive effect on peptide retention to alkylsilicas for $\psi < 0.5$.

The advantages of high performance liquid chromatography in peptide and protein chemistry are further illustrated in the studies on phosphorylated rat caseins (33), growth hormone variants (41), POMC-related peptides (42), haemoglobin variants (43) and summarised in publications 40,44 and 47. Papers 5,14,17,35 describe methods for the reversed phase high performance liquid chromatographic analysis of the thyroid hormones. These studies provided the first demonstration of the capability of these methods to resolve all the known natural iodothyronines and related iodo-amino acids. Finally, publications 22,31 give accounts of researches on the high performance gel permeation chromatography of proteins on two different types of microparticulate hydrophilic support media.

Biospecific affinity chromatography is potentially the most selective form of chromatographic separation currently available for proteins. The technique takes advantage of the molecular

specificity of a biomolecule for a suitable ligand immobilised onto an inert matrix. The key to its successful use is in the chemistry involved with the activation of the matrix, the ability to couple ligands in a suitably active form and the conditions required for the desorption of specifically bound components. Historically, the reaction of polysaccharide gels with cyanogen bromide at high pH has been the most widely used method of matrix activation. However, ligand immobilisation procedures based on cyanogen bromide activated polysaccharide gels often suffer from limited stability of the positively change N-substituted isourea linkage between the ligand and matrix and relatively low activation levels. These characteristics have prompted much interest in alternative methods of activation of polysaccharide gels, including the oxirane approach of Sundberg and Porath, and the use of sec-triazines, pyrimidines and benzoquinones. Chapter II considers specifically a new method for the activation of hydroxylic polymers by 1,1' -carbonyldiimidazole and related heterocyclic carbonylating reagents and the application of this new class of activated matrix in biospecific affinity chromatography. Papers 48,51,52,53 describe the characteristics of the CDI- activated agarose, celluloses and other types of support media, and the stability and other properties of immobilised ligands. Papers 49,51 describe application of these new affinity media to protein purification. Notable features of this new approach are the high activation levels which can be achieved, the excellent ligand stability over a wide range of pH and eluent conditions, and the absence of additional charge groups due to the activation procedure. These activated matrices and derived affinity chromatographic procedures have achieved wide

acceptance and usage following their commercialisation by Pierce Chemical Company, Rockford, Illinois. Studies on affinity purified antithyroglobulin autoantibodies are described in paper 50.

Chapter III encompasses papers related to the application of multicomponent buffer mixtures for the generation of natural pH gradients under electrofocusing or chromatofocusing conditions. The physicochemical basis for the formation of natural pH gradients under these conditions has been discussed in terms of dissociation theory for polyprotic substances. Papers 55-61 describe the characteristics of a series of natural pH gradients generated in layers of granulated gels using mixtures of amphoteric and nonamphoteric buffer reagents. These studies, which were specifically designed with preparative applications in mind, significantly extend earlier observations notably by Chrambach and co-workers on the formation of pH gradients under analytical isoelectric focusing conditions using mixtures of simple amphoteric buffers in place of proprietory ampholytes such as the Ampholines. Also included in this section are experimental studies on the purification of pregnancy associated α_2-macroglobulin (paper 57), thyroid protein antigens (paper 58), interleukin I (paper 59) and the potent hemagluttinin, maclurin (paper 60). The chapter concludes with an investigation on the use of multicomponent buffer systems in the chromatofocusing of proteins (paper 62).