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REVIEW

CHROMATOGRAPHY OF COMPLEX PROTEIN MIXTURES

FRED E. REGNIER

Department of Biochemistry, Purdue University, West Lafayette, IN 46907 (U.S.A.)

CONTENTS

1. INTRODUCTION

The first paper on high-performance liquid chromatography (HPLC) of complex protein mixtures appeared in 1976 [1]. During the ensuing decade, two key areas of development advanced HPLC to the point of being a valuable tool for the fractionation of complex protein mixtures. The first of these developments was in the area of column technology. Through the efforts of academic and industrial scientists a wide variety of very fine columns are now commercially available for the separation of proteins by size-exclusion (SEC), ion-exchange (IEC), reversed-phase (RPC), hydrophobic-interaction (HIC), metal chelate, hydroxyapatite, and bioaffinity chromatography. As new columns became avail-

able, the equally important task of optimizing their operation for protein separation was undertaken. This latter endeavor is the subject of this review.

It is logical, in teaching protein chemistry, to emphasize the common features relating to the genetic regulation, biosynthesis, and structure of proteins. As a consequence, we often fail to appreciate the enormous difference between proteins and the implications of these differences in their fractionation. It is the development of strategies to deal with these differences that has driven the recent diversification of protein fractionation into so many areas of biochemistry. This review will examine the features that are unique in the chromatography of some protein classes.*

2. CHROMATOGRAPHY OF SOME PROTEIN CLASSES

Two criteria were used in the selection of classes of proteins for inclusion in this review. First, the body of literature had to be sufficiently large that general conclusions could be made. Second, there had to be unique problems associated with the separation of the particular class of proteins.

2.1. Membrane proteins

Cells and often cellular organelles are enclosed within a lipid bilayer membrane. This non-polar membrane appears to provide two problems for the cell. First, the lipid layer would be a barrier for the transport of polar substances into and out of the cell. Second, it would be difficult to anchor the proteins and carbohydrates necessary for cellular recognition and communication at a lipid bilayer. Nature has solved these problems by developing special groups of proteins such as the peripheral membrane proteins that adsorb at the exterior of the membrane and the integral membrane proteins that penetrate into the membrane. It is probable that peripheral membrane proteins are adsorbed through electrostatic and hydrophobic interactions with both polar fucntional groups of the membrane lipids and portions of integral membrane proteins which protrude out of the membrane. Internal membrane proteins are of three types: transmembrane proteins which completely span the membrane and are exposed to the aqueous environment on both sides; proteins which have both an exposed and membrane embedded portion; and proteins that are completely embedded within the lipid of the membrane.

Peripheral membrane proteins are generally dissociated from the membrane by using salt and EDTA or changes in pH to disrupt the electrostatic forces holding them in place. The characteristics of peripheral membrane proteins are not substantially different than those of other water-soluble proteins [2]. For this reason they do not present unique problems in their chromatographic separation. In contrast, integral membrane proteins are much more challenging. Portions of an integral membrane protein within the bilayer contain many non-polar amino

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*Readers searching for detailed information rather than for generalizations in separation of individual protein categories are deferred to specialized chapters of this volume.

acids which are strongly associated with the hydrocarbon segments of membrane lipids. Releasing integral membrane proteins from the membrane requires a detergent which will both disrupt the membrane and keep the protein in solution once the lipids have been removed.

Although sodium dodecyl sulfate (SDS) and other powerful detergents easily disrupt membranes and solubilize proteins, the objective is often to try to maintain some of the original secondary and tertiary structure of the protein during purification. Accomplishing this usually requires a detergent which is less harsh. Two reviews on the solubilization of functional membrane-bound proteins [3, 4] are suggested for a discussion of this non-chromatographic portion of membrane protein purification. As a note of caution, the reader is reminded that detergents which contain an aromatic residue have substantial absorbance in the UV portion of the spectrum and present detection problems.

Purification of a protein that requires a detergent to keep it in solution presents a challenge to the chromatographer. Detergents are not always compatible with chromatographic processes. Fortunately, the use of detergents and solubilizing agents such as SDS, zwitterionic detergents, non-ionic detergents, organic solvents, urea, and guanidinium hydrochloride has been examined in the SEC of proteins [3–8]. There is also a series of reports on detergent use in the SEC of integral membrane proteins [9–15]. Although SEC is of limited resolving power in the purification of membrane proteins [12], it has sufficient resolution to be used in the selection of detergents for solubilization [9], optimization of detergent concentration [11], and quick estimation of the purity of a sample purified by other techniques [13]. SEC, using sodium cholate, EDTA, dithiothreitol (DTT), and glycerol in the mobile phase to preserve activity, has also been used to study the induction pattern of microsomal cytochrome P-450 [10]. Still another application of SEC has been to monitor the aggregation state of detergent-solubilized sarcoplasmic reticulum adenosine triphosphatase [16]. When SEC is used in the preparative mode it is usually in conjunction with another chromatographic step. The purification of spectrin subunits from erythrocytes by a combination of SEC and RPC is an example [17]. Spectrin is composed of dissimilar 220- and 240-kD subunits which, through dimerization, form the major structural proteins in the cytoskeleton of red blood cells.

IEC has proven to be much more useful in the separation of integral membrane proteins. Although large amounts of organic solvent are occasionally used in the mobile phase to solubilize proteins, as in the case of chloroplast proteins [18], most IEC separations are carried out with either non-ionic detergents [19–23] or the 3-[(3-chloroamidopropyl)dimethylammonio]-1-trifluoroacetic acid (CHAPS) zwitterion [24, 25]. There is strong evidence that the quality of the detergent as a solubilizing agent and its concentration has an impact on resolution in IEC. Using a series of polyethylene glycol alkyl ether (C_mE_n) detergents as a mobile phase additive it was possible to show that both alkyl chain length and the number of glycol residues influenced resolution of *Escherichia coli* K-12 membrane proteins in anion-exchange separations [19] ($C_{12}E_8$ was optimal). With both $C_{12}E_8$ [19] and *n*-octyl-β-D-glucopyranoside (octyl glucoside) [20], it was reported that resolution was better above the critical micelle concentration

of the detergent. This was attributed to diminished protein aggregation on the column. The presence of EDTA in the extracting medium and mobile phase also increased resolution [20]; apparently divalent cations are involved in aggregate formation. All three of these phenomena indicate that complete intermolecular dissociation of proteins is a prerequisite to good chromatographic resolution of membrane proteins.

Occasionally, it is even possible to partially purify peripheral membrane proteins on the basis of differential extraction. A receptor peptidoglycan on the outer portion of the *E. coli* K-12 membrane is solubilized with difficulty by any nonionic detergent but Triton X-100. Following exhaustive extraction by other detergents, IEC of the Triton X-100 extracted material indicates that the peptidoglycan is almost pure [19].

Based on the discussion above it is clear that the single greatest obstacle to the use of IEC in the fractionation of membrane proteins is solute solubility. When effective conditions for solubilization have been selected that completely disrupt intermolecular interactions without disturbing the electrostatic interactions necessary in IEC, it is possible to carry out IEC separations. Unfortunately, it appears that the optimum conditions for both solubilization and chromatography may be protein-specific and a general protocol cannot be provided.

When maintenance of native structure is less important, RPC becomes a viable option for membrane protein fractionation. The problem in RPC is much the same as in IEC and SEC; the more hydrophobic membrane proteins are difficult to solubilize. Although recovery is more of a problem in RPC of membrane proteins than water-soluble proteins [26], it is still widely used. Khorana et al. [27] have made the observation that "the straightforward application of current methods for sequencing water-soluble proteins to large hydrophobic membrane proteins such as bacteriorhodopsin is not feasible". In addition, the extreme hydrophobicity of an RPC column makes elution of very hydrophobic proteins even more difficult. Khorana et al. [27] and Tahagahi et al. [28] have successfully solubilized proteins such as bacteriorhodopsin, cytochrome b_5 and cytochrome b_5 reductase with formic acid. Elution of proteins and peptide fragments from RPC columns was achieved with mobile phases containing 5% formic acid. The fact that formic acid can cleave aspartyl-prolyl (Asp-Pro) bonds, deaminates asparagine and glutamine, aggregates cleavage fragments, slowly destroys tryptophan residues, and is viscous, is a concern. Addition of ethanol to a final concentration of 70% in the extracting solvent diminished these problems.

Poliovirus coat proteins were even more difficult to solublize [29, 30]. In this case, it was necessary to use 60% formic acid and 2-propanol as a mobile phase (Fig. 1). Since one of the peptides being examined had three asp-pro pairs which are known to be labile in acid, the possibility of fragmentation was examined. With this particular peptide no cleavage occurred in 24 h when the samples were incubated with a mixture of formic acid–acetonitrile (60:20). Again the addition of organic solvent diminished degradation. In all but a few cases, there was no change in proteins after 6 h of storage in the mobile phase as determined by electrophoresis and chromatography. Antisera prepared against proteins which had been purified by RPC with the 60% formic acid mobile phase reacted with
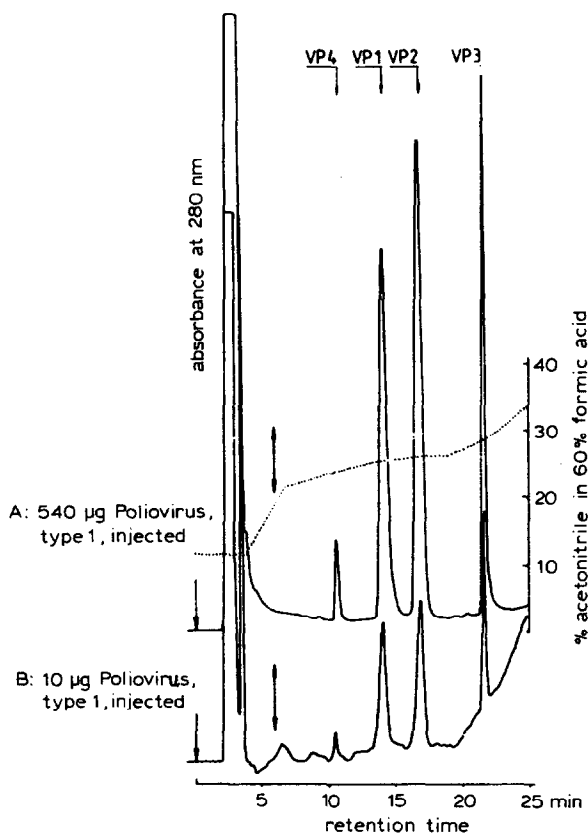
Fig. 1. Elution pattern of poliovirus polypeptides, separated by RPC on a micropreparative (A) and analytical (B) scale [30]. Virus samples (poliovirus, type 1, strain Mahoney) in 3 M cesium chloride were precipitated by 30% formic acid, redissolved in 50 μl of 70% formic acid and injected. Elution conditions: Baker wide-pore C_{18} column (250×4.6 mm), eluted by 60% formic acid with a gradient of acetonitrile, as indicated by dotted line. Flow-rate was 1 ml/min at 23°C. Absorbance range indicated by the arrow is 0.032 for A and 0.002 for B.

intact virus. This indicated that sufficient native structure was recovered when these proteins were returned to a physiological environment that antibodies could be produced which were functional against native viral proteins. Although formic acid is a superior solubilizing agent, it should be appreciated that with silica-based sorbents substantial degradation, i.e. cleavage of the alkyl group from the support, occurred during its use. Inferior performance with other mobile phases may be experienced after use with formic acid because large areas of underivatized silica surface would have been generated.

The most popular mobile phase still contains trifluoroacetic acid (TFA) and either acetonitrile or an alcohol such as ethanol, 2-propanol, or butanol [31–34]. It has also been noted in the TFA system that quite different selectivity can be obtained by using mixtures of acetonitrile and 1-propanol [33]. Selectivity varied almost continuously with the ratio of these two solvents. Separations which could not be achieved at one solvent ratio were easily accomplished at another. Recoveries were typically greater than 90%. Prior to the introduction of formic acid and

TFA, acetate (pH 6.5) with chloroform–methanol was successfully employed in the isolation of mitochondrial sector proteins [35].

Although resolution is generally quite good and recovery high in RPC [33], this is not always the case. Less than 50% of membrane proteins are recovered from an RPC column in some cases [14, 32, 34]. The proteins are either too hydrophobic or insufficiently soluble in the mobile phase for elution from an RPC column.

The introduction of sample heterogeneity by partial oxidation of cysteine during protein extraction has also been noted as a problem in the case of yeast cytochrome c oxidase [33]. This problem may be overcome by pretreating samples with a reducing agent such as DTT.

Sorbent pore diameter could also play a role in RPC of membrane proteins. In the case of red blood cell membrane proteins, the resolution of 1000 Å pore diameter organic resin-based sorbents was superior to 300 Å pore diameter silica-based materials when the columns were eluted with a TFA–acetonitrile mobile phase [26]. Apparently these pore diameter effects are eliminated with mobile phases containing formic acid [28]. It is not clear whether this problem is due to differences in the pore diameter or to differences in the matrix and ligand density.

There is also a report of the use of hydroxyapatite in the purification of membrane proteins [36]. The coronavirus glycoprotein E2, which is responsible for virus attachment to cell receptors and virus-induced cell fusion, is composed of two distinct 90-kD subunits. Although these two subunits have identical electrophoretic mobilities when analyzed by SDS polyacrylamide gel electrophoresis (PAGE), they could be widely separated by hydroxyapatite columns in the presence of SDS. Solutes were eluted in a 0.15–0.5 M sodium phosphate buffer gradient, pH 6.8, containing 0.1% SDS. There was some difference in resolution between hydroxyapatite columns from various suppliers. To avoid column dissolution, it was recommended that the mobile phase contain calcium and phosphate at the solubility product levels of calcium phosphate. However, the formation of a precipitate in the presence of SDS precluded the addition of calcium and phosphate to the mobile phase when SDS was present. This reduced the lifetime of the column. Conventional columns were discarded after a single use whereas HPLC columns had a useful lifetime of twelve to fifteen runs. Erosion of the chromatographic support was accompanied by a gradual increase in headspace and back-pressure.

High-performance bioaffinity chromatography may also be useful in the future. The separation of human plasma membrane proteins of cultured human fibroblasts has been achieved by affinity chromatography on microparticulate silica derivatized with an affinity ligand [37]. Isothiocyanatopropyl silane-derivatized silica was used to immobilize a series of nucleophilic ligands ranging from synthetic triazine dyes to concanavalin A. Both the Procion Orange and mannan columns successfully retained and released plasma membrane proteins as determined by SDS-PAGE.

2.2. Ribosomal proteins

Ribosomes of a typical procaryote, such as *E. coli,* have a relative molecular mass approaching $2.3 \cdot 10^6$, of which 65% of the mass is nucleic acids and 35% is

protein. *E. coli* ribosomes have been fractionated into two major components, a 50S component and a 30S component. The 50S component is known to contain 33 polypeptides ranging in size from 9 to 28 kD, some of which are present in multiple copies. The smaller 30S component contains 21 polypeptides which range in size from 10 to 28 kD with the exception of the 65-kD S1 protein. Some of the ribosomal proteins are very basic and probably adsorb electrostatically to polynucleotides. Although other proteins are associatd with the ribosome during protein synthesis, functional ribosomes may be reconstituted from the 21 30S proteins and 33 50S poteins without addition of other proteins or enzymes.

Ribosomes of eucaryotes are larger and contain more polypeptides than those of procaryotes. A 40S subunit contains 30 polypeptides whereas a 60S subunit contains 40. The relative molcular masses of eucaryotic ribosomal proteins are also generally larger than those of their procaryotic counterparts.

The classical chromatographic methods for purifying ribosomal proteins include SEC and various types of IEC. In addition to these methods, RPC has been found to be useful as will be shown below.

Due to the inherently low resolving power of SEC, it provides the least resolution of ribosomal protein mixtures. At best, no more than seven peaks can be seen in an SEC separation [38, 39]. It is even doubtful that the separation is truly based on size exclusion. Elution profiles are strongly dependent on the column manufacturer and the mobile phase. However, the fact that greater than 90% of the ribosomal proteins were recovered [39] makes the technique acceptable as a first step in fractionation.

Fractionation of ribosomal proteins by RPC is a relatively straightforward process. Alkyl chain length does not seem to be a critical variable but TFA is almost universally selected as the mobile phase pairing agent [38–42]. Although both acetonitrile [38] and 2-propanol [39] have been used as mobile phase displacing agents, it appears that acetonitrile gives lower recovery and allows the formation of protein complexes [39]. For example, one of the 30S ribosomal proteins was found to complex and coelute in seven other protein peaks with acetonitrile. This problem precluded purification of the protein. In contrast, propanol prevented complex formation and the protein could be isolated in pure form.

The RPC system purified 4 of the 21 proteins from the procaryotic 30S subunit to homogeneity, while an additional 15 were purified to a high degree [39]. The 50S proteins were separated into 23 fractions (Fig. 2). Recoveries ranged from 27 to 91% with the average being 70% [41]. The most strongly retained proteins gave the lowest recovery. Proteins non-specifically retained by the column were probably responsible for the "ghosting" phenomenon observed when a blank gradient was run after a ribosomal protein sample. Residual materials were removed from the column by running a series of blank gradients. Analytical columns were capable of fractionating 2–3 mg protein loads when used in the preparative mode [38].

It is amazing that although ribosomal proteins are partially denatured in the process of purification by RPC, active ribosomal subunits may be reconstituted from these proteins [41]. Reconstitution resulted in the production of multiple supramolecular complexes which were fractionated by centrifugation. Only the
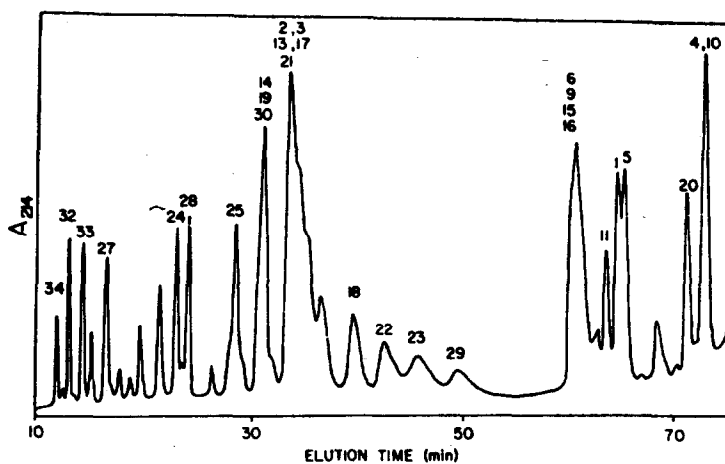
Fig. 2. RPC of TP50 (total protein from 50S ribosomal subunits) [41]. A solution of TP50 (700 pmol) in 60 μl of 0.1% TFA was applied to a SynChrom RP-P column and eluted with the following gradient: 17–34.9% acetonitrile in 20 min; 34.9–36% acetonitrile in 10 min; 36% acetonitrile for 10 min; 36–45% acetonitrile in 10 min; 45–50% acetonitrile in 5 min; 50–75% acetonitrile in 5 min; 75% acetonitrile for 5 min. Absorbance at 214 nm was measured at 0.6 a.u.f.s. Proteins L7 and L12 were present in low amounts in the 50S subunits from which these proteins were extracted and are not shown.

30S and 50S subunits isolated from these mixtures showed activity. Reconstituted 30S subunits had 84% of the activity of the native subunit and an identical composition except for the S-3 protein. The 50S subunit had 52% of the activity of the native subunit and a nearly identical composition to the 33 ribosomal proteins.

Puromycin is known to be an inhibitor of protein synthesis. Photoincorporation studies directed at determining which protein(s) in the ribosome interact with puromycin used RPC to determine that proteins L-23, S-7, and S-14 were labelled [41]. This would imply that puromycin is bound to these proteins in some way during the course of inhibition. It has been suggested that by purifying these labelled proteins and incorporating them into the ribosome a clearer picture of puromycin inhibition could be obtained [41].

Eucaryotic ribosomal proteins have also been examined by RPC. In a study [42] directed at the purification of the S-6 ribosomal protein from rat liver, whole ribosomes were extracted with 6 M guanidinium hydrochloride and the RNA precipitated by acidification. Highly purified proteins were obtained in two chromatographic steps using C_4, C_8, and diphenyl columns.

IEC is equally effective in the purification of ribosomal proteins and in many ways complements RPC and SEC [39]. Urea (5 M) has been found to be an essential mobile phase component [39, 43–45] Due to the basic nature of most ribosomal proteins [38, 39], cation-exchange chromatography is the most useful. In the case of procaryotic ribosomes, proteins from the 30S subunit gave much broader peaks than those from the 50S subunit. The reason for this is not readily apparent.

IEC of the 30S ribosomal proteins on a weak cation-exchange (CM) column

resolved, at least partially, 18 of the 231 proteins [43]. The remaining five proteins eluted in two peaks. One peak contained proteins S-9, S-19, and S-15 while the other contained S-14 and S-18. Through the use of very shallow gradients it was possible to partially resolve even these components. Recovery ranged from 60 to 100% with the average being 90%. The chromatographic behavior of a strong cation-exchange (SP) column with *E. coli* ribosomal proteins was essentially the same as that of the weak ion-exchange (CM) column [44]. Recovery of many proteins approached 100% with the lowest being 80%. Preparative separations on a 150×21.5 mm SP column fractionated 400 mg of protein per 5-h elution cycle. Regeneration between cycles was achieved with a potassium hydroxide wash.

IEC was also useful in the fractionation of 50S proteins; a weak cation-exchange (CM) column resolved 19 of the 32 proteins [39]. The remaining eleven proteins eluted in five peaks. The proteins in each of these five peaks were as follows: L-3 and L-11 coeluted; L-23 and L-24 coeluted; L-1, L-30, and L-13 coeluted; L-21 and L-27 coeluted; and L-15 and L-17 coeluted. The strong cation-exchange (SP) column was superior to the CM column in that it resolved 25 of the 32 proteins from the 50S subunit [44]. A strong cation-exchange (SP) column was also used to resolve the ribosomal proteins in the 60S subunit from *Saccharomyces cerevisiae* [45].

2.3. Cereal proteins

There are four major classes of cereal proteins which are classified according to their solubility. Albumins and globulins which may be extracted by water and dilute salt, respectively, may be treated in separation systems much like the cytoplasmic proteins of bacteria and animals. In contrast, the prolamines are much more hydrophobic, requiring 70% ethanol for extraction from cereals. The gliadins in wheat and zeins in corn belong in the prolamine class. These 30–40 kD storage proteins owe their solubilty properties to a high content of glutamine, proline, and hydrophobic amino acids. Glutelins are the endosperm proteins remaining after the extraction of albumins, globulins and prolamines. Extensive disulfide cross-linking, hydrogen bonding, and hydrophobic association between these high-molecular-mass proteins contributes to their insolubility. Through the use of extremes in pH, detergents, denaturants, reducing agents, and alkylating agents, many of these proteins can be solubilized.

RPC is by far the most successful of the HPLC modes for the separation of cereal proteins [46]. For example, a low-molecular-mass gliadin sample that could be resolved into 46 protein components by two-dimensional electrophoresis produced 36 components (Fig. 3) in an RPC system using a 100-min TFA–acetonitrile gradient at a flow-rate of 1 ml/min [47]. Using a higher flow-rate (3 ml/min) and column temperature of 70°C it has been possible to reduce the separation time to 10–15 min [48]. Although several different organic solvents and pairing agents have been examined, they offered no distinct advantage over TFA–acetonitrile in these profiling studies. Preparative separations using a 250×10 mm RPC column and a 25-mg sample load in conjunction with SEC and cation-exchange chromatography have been used in the purification of gliadins
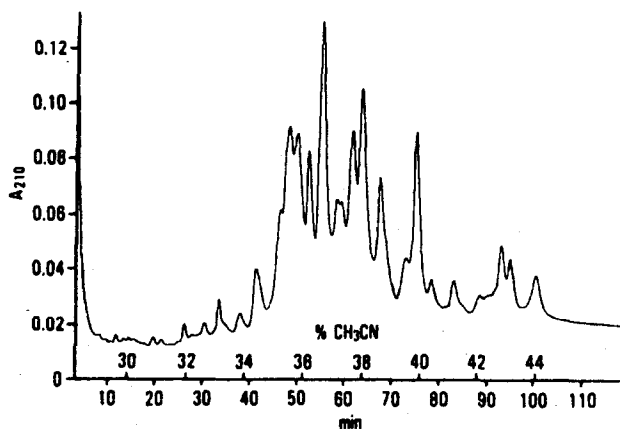
Fig. 3. RPC separation of pyridylethylated low-molecular-mass gliadin proteins from Ponca wheat [47]. A sample of 0.1 mg in 100 μl was applied to a SynChrom RP-P column (250×4.1 mm), and eluted with a linear gradient from 20 to 50% B during 120 min. The column effluent was monitored at A_{210} = 0.1 a.u.f.s. per 10 mV. Solvent A, 15% acetonitrile + 0.1% TFA; solvent B, 80% acetonitrile + 0.1% TFA.

[49, 50]. Intercolumn variation is often a problem in RPC of proteins (see section 2.7). This was not found to be the case with cereal proteins [47], probably due to their lower content of basic amino acids.

At the present time RPC has been applied to the identification of wheat varieties [49], prediction of bread quality [51], genetic studies of wheat [51], and the analysis of corn proteins [52].

High-performance SEC has also seen some use in the separation of cereal proteins. Bietz et al. [53] have used SEC in the separation of both native and reduced wheat gliadins. Reducing conditions confirmed that high-molecular-mass gliadins are composed primarily of low-molecular-mass gliadins joined by disulfide bonds. SDS solubilization of glutenins from single kernels of various wheat varieties provided samples which could be analyzed on SEC columns eluted with a mobile phase containing SDS [53, 54]. These separations revealed marked differences in the relative molecular mass and distribution of proteins among the varieties examined.

High-performance IEC has seen little use in the separation of cereal proteins. Bietz [46] reported a separation of the water-soluble wheat albumins and globulins on a carboxymethyl high-performance cation-exchange column and Batey [55] demonstrated that it was possible to identify wheat varieties using high-performance anion-exchange chromatography.

It appears that HPLC techniques will make it possible to correlate specific endosperm proteins with either functional or nutritional quality of cereal products which will in turn provide geneticists with an analytical tool to be used in breeding programs for cereal grain improvement.

2.4. Hemoglobins, variant and glycosylated

Hemoglobin is a tetrameric protein composed of two sets of identical globin chains and four heme groups. Fetal hemoglobin (HbF), consisting of α- and γ-

chains, is normally present at birth. Early in life HbF is replaced by hemoglobin A_0 (HbA$_0$) which is composed of α- and β-chains and is the dominant hemoglobin in adults. In some segments of the population, there are genetically transmitted variants of these hemoglobins which lead to serious health problems. For example, the substitution of valine for glutamine at the sixth residue in the β-chain leads to the formation of hemoglobin S (HbS) which is responsible for sickle cell anemia. In other cases such as α- and β-thalassemia, synthesis of either the α- or β-globin chain is reduced or absent. Homozygous states of any of the above hemoglobinopathies or combinations of any two abnormalities may result in clinical manifestations especially during pregnancy, surgery, or other noxious situations.

Glycosylated hemoglobins are also of clinical importance. They are minor constituents of the hemoglobin pool in adults which arise from the non-enzymatic glycosylation of HbA. One of these glycosylated hemoglobins, HbA$_{1c}$, has come into wide use as an indicator of glucose regulation in diabetics. HbA$_{1c}$ is normally found in levels of 4–6%, but can be more than double this value in the blood of uncontrolled diabetics. The quantity of HbA$_{1c}$ reflects the average glucose level in a patient's blood over the life span of the erythrocyte. The advantage of HbA$_{1c}$ measurements over blood glucose levels for monitoring diabetics is that they circumvent the physical discomfort of glucose tolerance tests.

In the past, electrophoresis and IEC on carbohydrate columns have been used in hemoglobin determinations. It is shown below that HPLC columns are equally capable of determining hemoglobinopathies with much greater speed and less manipulation. Hemoglobin variants can be determined chromatographically in three ways: direct separation of the intact protein where possible; analysis of the globin chains; and analysis of a globin chain tryptic digest. Since the thrust of this review is on complex proteins, the discussion will be restricted to the separation of intact hemoglobins.

Understandably, a subject of such clinical significance has an extensive literature. The objective here is only to discuss some of the more critical variables in hemoglobin separations that have been examined in the recent literature. Although hemoglobins are easily eluted from SEC columns, this technique is not discussed here because SEC will not discriminate between the various hemogoblin species.

Both cation-exchange and anion-exchange chromatography are effective in the separation of hemoglobins. Using a weak cation-exchange column it has been possible to completely separate hemoglobins Bart's F, A_0, A_2, S, C, D,E,G,SG, Winnipeg, and Sealy in a 30-min gradient elution [56]. The gradient was constructed by varying both pH (from 6.5 to 6.8) and ionic strength. Variant hemoglobins E, D, G, S, and C result from a single amino acid substitution in either the α- or β-chain. They are detectable by IEC because the mutation results in either the elimination of a charge, addition of a charge, or a switch in the sign of a charged group in the hemoglobin. A recent study on strong cation-exchange columns finds them to be equally effective under the same conditions used with the weak cation-exchange column [57]. Both of these IEC systems are vastly superior to conventional electrophoretic and column IEC methods. It should be noted, however, that there are small differences in selectivity between commer-
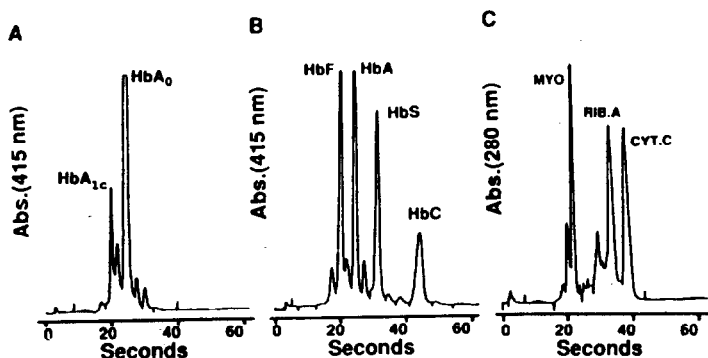
Fig. 4. Very fast chromatography of a HbA$_{1c}$ standard (A), hemoglobin AFSC mixture (B), and a protein standard mixture (C) on an MA7C cartridge column [64]. A and B, buffer A $= 20$ mM bis-Tris (pH 6.0), buffer B $= 20$ mM bis-Tris plus 0.10 M sodium chloride (pH 6.0), gradient from 0 to 100% B in 30 s, at a flow-rate of 5.0 ml/min. C, buffer A $= 20$ mM morpholinoethanesulphonic acid (MES) (pH 6.0), buffer B $= 20$ mM MES plus 0.50 M sodium chloride (pH 6.0), gradient from 0 to 30% B in 30 s, at a flow-rate of 5.0 ml/min.

cial columns. For example, the elution order of hemoglobins E and D is reversed on the weak and strong ion-exchange columns. The reason for this is undetermined. With cation-exchange chromatography alone it is possible to differentiate between normal individuals and those who have hemoglobin Bart's, sickle cell trait, sickle cell disease, hemoglobin E trait, hemoglobin D Los Angeles disease, sickle cell hemoglobin D disease, hemoglobin C trait, hemoglobin C disease, hemoglobin G Philadelphia disease, hemoglobin Winnipeg disease, hemoglobin Sealy disease, and sickle cell hemoglobin G Philadelphia disease. An exact description of how these hemoglobins are related may be found in the International Hemoglobin Variant List [58] (see also pp. 277–304).

Anion-exchange chromatography has also been widely used in the separation of variant hemoglobins [59–61]. A good high-performance anion-exchange column can discriminate between hemoglobins A$_0$, C, S, and F. Separation speed, recovery, ease of sample manipulation, and column lifetime are all comparable to cation-exchange chromatography. However, it is the conclusion of most practitioners that cation-exchange chromatography is more useful because it discriminates between more variant hemoglobins.

Cation-exchange chromatography is by far the most suitable for HbA$_{1c}$. Using gradient elution of either a weak [62] or strong [63] cation-exchange column it has been possible to determine the concentration of HbA$_{1c}$ in a little over 6 min, even in the presence of elevated levels of HbF, HbA$_{1a}$, HbA$_{1b}$ and HbA$_2$. Allowing for column recycling and re-equilibration, it was possible to make four measurements per hour. Full automation of the method gave a between-assay coefficient of variation of 2–3%. Small differences in the selectivity between different manufacturers' columns may require modification of gradient shape to achieve maximum throughput on any particular column. Although the speed of these separations seems remarkable, determination of the HbA$_{1c}$ concentration in a serum sample has recently been achieved in less than 40 s (Fig. 4) using a new non-porous cation-exchange column [64]. The speed of non-porous particle
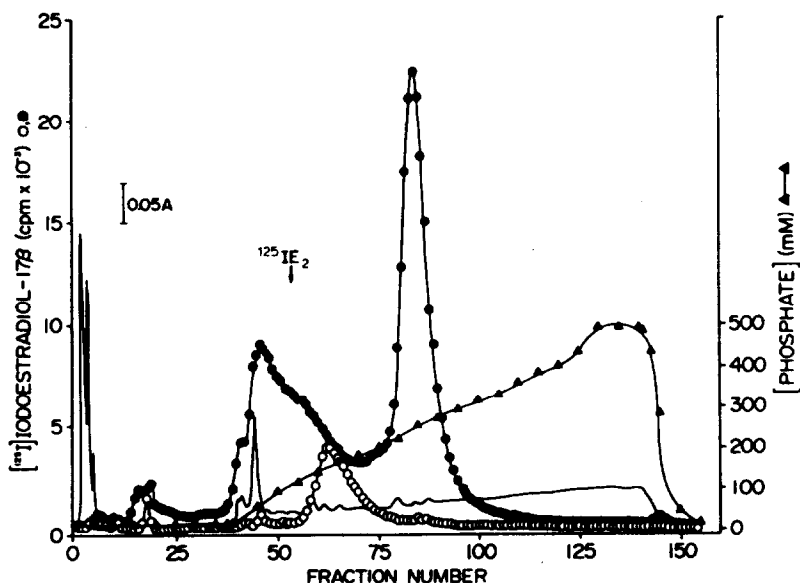
Fig. 5. IEC separation of ionic forms of the estrogen receptors from human breast cancer tissue on AX-300 [64]. Cytosol was prepared from human breast cancer tissue and incubated in the presence (o) or absence (●) of 500-fold excess diethylstilbestrol (DES). Elution was performed at 1 ml/min using a gradient of potassium phosphate (▲) at pH 7.4. The elution of the labeled ligand alone was determined previously under identical conditions and is marked with an arrow. The recovery of total radioactivity from the column was 94% for the aliquot of cytosol incubation in the absence of DES. A total of 1.54 mg of protein was applied in 200 μl. A tracing of species absorbing at 280 nm is given by the continuous line.

chromatographic systems should have a major impact on the utilization of HPLC in quality control and clinical environments.

2.5. Steroid hormone receptors

Steroid hormones are known to penetrate the cell membrane and bind to cytoplasmic receptor proteins. It appears that for a single steroid there may even be multiple receptor proteins which vary with cellular differentiation [65]. Since receptor proteins do not have unique spectral properties, they are generally detected (or assayed) by their ability to bind labelled steroids. Radiolabelling is the most widely used [65, 67–69], but fluorescent labelling has also been reported [66]. The labelling of proteins with a steroid may be done before or after the separation [65–68] as shown in Fig. 5. Post-separation detection is so time-consuming and labor-intensive that it is seldom used. Although much quicker and more widely used, preseparation labelling presents two types of problems. Many cytoplasmic proteins, in addition to specific receptor protein(s), may bind steroids [65–69]. This makes it difficult to differentiate specific steroid receptor protein(s) from those that bind steroid non-specifically. Fortunately, steroid receptor proteins have a higher affinity for labelled steroid than those that adsorb the steroid non-specifically. This difference in affinity is the basis for the second

problem. During passage through the column, weakly bound steroid will begin to dissociate from proteins and either migrate through the column as free steroid or be adsorbed by the sorbent matrix [65]. If dissociation is incomplete, the labelling pattern in the column effluent will be misleading. The degree of dissociation is due in large part to the binding constant of the steroid and the rate of dissociation. One of the ways to overcome this problem is to separate labelled proteins in the presence of a large excess of non-labelled steroid [68, 69]. In those cases where the labelled steroid is weakly associated with protein, the large excess of non-labelled steroid will displace the labelled steroid.

Estrogen receptor proteins have been examined with SEC [66, 67], anion-exchange chromatography [68], and high-performance chromatofocusing (HPCF) [65–68]. HPCF is a form of IEC in which a pH gradient is generated along the length of the column using mobile phase buffers and the buffering capacity of a weak anion-exchange sorbent [65].

At least three isoforms of estrogen receptor protein have been identified with IEC and HPCF [65–68]. Anion-exchange chromatography on 300, 500, and 1000 Å pore diameter sorbents indicated that each sorbent had slightly different properties, particularly in regard to the retention of free steroid [68]. The reason for this was not determined. In a direct comparison with soft-gel chromatofocusing media, 300 and 500 Å pore diameter HPCF sorbents were judged to be superior in resolution [68]. It is interesting that none of the chromatofocusing columns behaved in exactly the same way. These differences were attributed to the buffering properties of the sorbents. Although greater than 90% of the receptor activity was recovered when HPCF was carried out over the pH range of 8.3–4.5 in 60 min, there was some variability between samples. Sodium molybdate was shown to produce an irreversible size and charge stabilization of the receptor [65].

Previous isoelectric focusing studies have indicated that the estrogen binding proteins have a pI of 7–8. It is interesting that none of the estrogen receptors eluted from the HPCF column in this pH range. This suggests surface charge heterogeneity in the estrogen receptor proteins.

The human uterine progesterone receptor has also been studied with both anion-exchange chromatography and HPCF [69] using three different tritiated progesterones and an azoanalogue of progesterone. The function of the analogue was to covalently label the steroid-specific binding site of the receptor. Labelled cytosol components were separated on an anion-exchange column in both the presence and absence of a high molar excess of the respective unlabelled competitor steroids. After elution with a sodium chloride gradient, labelling was determined in each fraction and the elution profiles were superimposed. One specifically labelled and two non-specifically labelled peaks were located. The same specifically labelled peak was detected by chromatofocusing. When this covalently labelled peak was subjected to SDS-PAGE, 45-kD and 27-kD proteins were detected.

2.6. Immunoglobulins

Immunoglobulins (Ig) are produced by plasma cells derived form B-lymphocytes. On the basis of relative molecular mass and chemical properties, five major

classes of immunoglobulins have been recognized IgG (150 kD), IgM (950 kD), IgA (300 kD), IgD (160 kD), and IgE (190 kD). Within each major immuno-globulin class there can also be subclasses which are of similar relative molecular mass. IgM and IgA are sufficiently different in relative molecular mass that they may be differentiated from each other and the other immunoglobulins by SEC [70, 71]. In contrast, IgG, IgD, and IgE are sufficiently close in relative molecular mass that no real differentiation is to be expected with SEC.

The basic structural unit of all antibodies is composed of two light chains and two heavy chains joined by disulfide bridges. In the cases of IgG, IgE, and IGD, the antibody is composed of a single structural unit, i.e. two heavy and two light chains. The light-chain pair may be of either the κ- or λ-type. Although these two types of light chains are approximately equal in relative molecular mass, they differ chemically. The heavy-chain pair is identical within any antibody class but varies in relative molecular mass between classes. In the cases of IgM and IgA, the antibody is a multimer of the basic structural unit.

The basic structural unit of an immunoglobulin has a "Y" shape and is biva-lent. IgG is frequently cleaved with papain to produce two F_{ab} and an F_c fragment. The F_{ab} and F_c fragments are sufficiently different in molecular properties that they may be separated by either SEC or IEC. The F_{ab} fragment is essentially one of the arms of the Y which contains an unmodified light chain, retains full immu-nological activity, and is monovalent. The F_c fragment, in contrast, has no immu-nological activity but is that portion of the antibody which binds to the *Staphylococcus aureus* protein A. The use of protein A in antibody immobilization will be discussed below. Reduction of the basic immunoglobulin structural unit with mercaptoethanol in the presence of 6 M urea produces a mixture of heavy and light chains which vary two-fold or more in relative molecular mass and are separable by SEC [71].

Of particular importance in chromatography is the fact that immunoglobulins are glycoproteins. Structural studies indicate that fucose, mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid are attached in the F_c region through serine, threonine, and asparagine residues. Apparently carbohy-drate groups are attached to the heavy chains in a stepwise manner at different subcellular sites during transport of the protein through plasma cells. This pro-cess is not always complete and accounts for the chromatographic heterogeneity in antibodies.

Six general types of high-performance columns have found application in the separation and study of immunoglobulins: size-exclusion, anion-exchange, cation-exchange, hydrophobic-interaction, hydroxyapatite, and protein A bioaffinity columns. Classical dye columns are also used in preliminary fractionations prior to high-performance separations. Although SEC is useful in the separation of (1) IgM and IgA from IgG, IgD, and IgE [70, 71], (2) IgG from F_{ab} and F_{ab} fragments [71], (3) heavy and light chains [71], (4) antibodies from antigen–antibody complexes [72], and (5) serum proteins such as albumin and transferrin from immunoglobulins [73], it is of no value in the fractionation of antibody sub-classes and idiotypes.

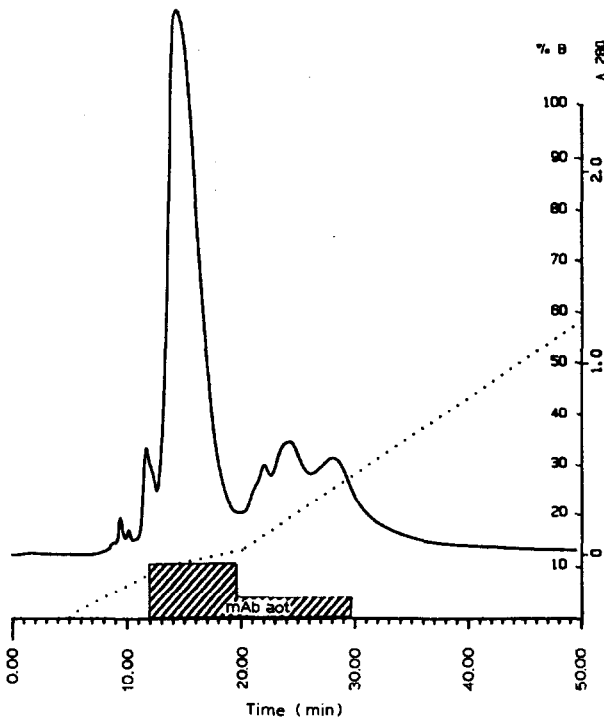Historically, anion-exchange chromatography has been a major tool in anti-

Fig. 6. IEC of high sample load (25 mg protein) of ammonium sulfate-precipitated ascites fluid [75]. The hatched areas indicate antibody activity. The separation was achieved on a gradient-eluted Mono Q HR 5/5 anion-exchange column (50×5 mm). Mobile phase A was 0.02 mol/l Tris–HCl (pH 8.0). Mobile phase B was 0.02 mol/l Tris–HCl (pH 8.0) containing 1.5 mol/l sodium chloride. The gradient was generated over 50 min at a flow-rate of 1 ml/min.

body purification. The new high-performance IEC columns are sufficiently similar to the old gel-type DEAE columns that they may be substituted in older purification procedures, such as in the fractionation of IgG from serum proteins on DEAE cellulose [74]. When the fractionation of ascites fluid on a high-performance anion-exchange column is preceded by either an ammonium sulfate precipitation [70] or dye-column step [75], monoclonal antibody (mAb) collected from the anion-exchange column is pure. Up to 25 mg of prefractionated sample per run was processed (Fig. 6) on an analytical column [75].

Without the ammonium sulfate precipitation step, both IEC and HIC columns are often required to obtain high purity. For example, direct fractionation of mouse mAb IgG_1 sample on an anion-exchange column produced an antibody with 7% transferrin contamination [76]. Anion-exchange columns may also be used in the fractionation of IgG subclasses. Although mouse IgG_1 and IgG_{2a} coelute, IgG_{2b} and IgG_3 may be resolved from the other antibodies. IgM and IgG from sheep and cattle [70] and F_{ab} and F_c fragments from IgG [76] have also been fractionated by anion-exchange chromatography. One of the difficult problems in the purification of some IgG samples on an anion-exchange column is the elimination of albumin and transferrin. This problem has been circumvented with a strong

cation-exchange column [71]. Contaminating proteins from the anion-exchange column often have a lower pI than IgG and elute much earlier on the strong cation-exchange column. There is also a report of the use of HPCF columns for the fractionation of IgG and IgM [77].In addition, the technique was used in the fractionation of submilligram quantities of normal and abnormal IgG of differing pI.

Protein A bioaffinity chromatography is the second major method used in the purification of IgG antibodies. With the recent commercial availability of high-performance protein A columns [78], a rapid and efficient tool has been added to the arsenal of high-performance columns available to the immunologist. When a protein A column is eluted with a pH gradient ranging from 8 to 2, the IgG subclasses from mice elute in the order $IgG_3 < IgG_2 < IgG_1$. It has also been reported that protein A columns can separate the IgG_{2a} and IgG_{2b} antibodies of mice [79]. Protein A columns have also been used to prepare immunosorbent matrices by cross-linking antibodies after adsorption [80]. The principal limitation of protein A columns is that they do not bind all antibodies.

In a direct comparison of IEC, HIC, and hydroxyapatite columns in the fractionation of mouse monoclonal antibodies directed against coagulation factor VIII, Pavlu et al. [75] reported that the resolution of both the IEC and HIC columns was superior to the hydroxyapatite. However, this is only one case. Hydroxyapatite columns are widely used in the purification of antibodies where they generally eliminate greater than 95% of the non-immunoglobulin proteins and give 75% or greater recovery [81–83].

As noted above, HIC has also been used with success in the fractionation of immunoglobulins [73, 75]. In view of the fact that ammonium sulfate fractionation has been used widely in antibody purification, it is possible that HIC could accomplish this step with greater resolution. The principal limitation of HIC today is that it has not been applied widely enough to make general conclusions concerning its broad utility.

2.7. Histones

The chromosomes of eucaryotic cells contain a family of small, basic proteins which are tightly associated with DNA and function to package it into nucleosome structural units. There are five major classes of histones which are designated H1, H2A, H3B, H3, and H4 and differ in both relative molecular mass and amino acid composition. For example, H1 is a 21-kD protein containing 29% lysine wheras H2A and H3B have 14.5 and 13.7 kD, respectively, and are approximately equal in arginine and lysine content. These three histones also vary considerably among eucaryotic species, i.e. there is little sequence homology. In contrast, the 15.3-kD H3 histone and 11.3-kD H4 histone are arginine-rich and almost identical among species. A striking feature of the H4 histone is that the basic residues seem to be clustered at one end of the molecule where there is a net positive charge of 16. The fact that arginine and lysine constitute approximately one fourth of the amino acid residues in histones presents both an opportunity and challenge in their separation.
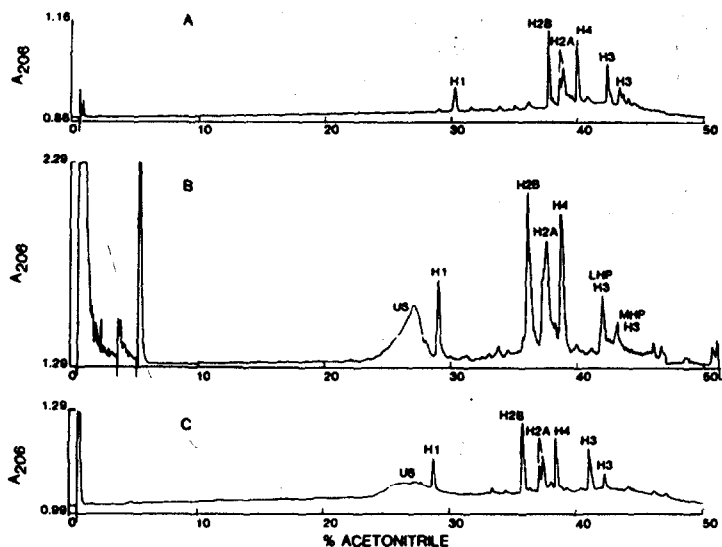
Fig. 7. HPLC of CHO nucleoproteins on a μBondapak CN column equilibrated with water containing 0.2% TFA [89]. (A) Injection of 250 μg of whole histone dissolved in 100 μl water containing 0.2% TFA. (B) Injection of 8.5·10^7 nuclei, dissolved in 1 ml of 6 M guanidine hydrochloride (Gu·HCl) containing 0.2% TFA. (C) Injection of 3·10^7 nuclei, dissolved in 0.1 ml of 6 M Gu·HCl containing 0.2% TFA and then diluted with 1 ml of water containing 0.2% TFA to precipitate DNA. The DNA was removed by centrifugation and the supernatant fluid (1.1 ml) was injected. Following injection of the above samples, all three columns were eluted with a 0–50% linear gradient of acetonitrile containing 0.2% TFA in 300 min at 1 ml/min. LHP and MHP refer to less-hydrophobic and more-hydrophobic variants, respectively.

Fractionation of all the major classes of histones has been achieved by RPC using a C$_{18}$ or CN column [84–90]. There are, however, some special requirements. The first is the use of end-capped columns. Non-end-capped columns gave very poor recovery in the case of C$_{18}$, C$_8$, and CN columns. Since this is not always the case with proteins, it is probably due to the very basic nature of histones. The second requirement was the use of TFA in the mobile phase. Using 0.3% TFA in an aqueous-to-acetonitrile gradient on a Waters Radial-Pak μBondapak C$_{18}$ column it was even possible to split the H2A and H3 histones into less hydrophobic (LHP) and more hydrophobic (MHP) variants [85]. When these two requirements are met, histones may be fractionated and recovered in greater than 95% yield. Although the CN column will not separate (MPH)H2A and H4 components, Gurley et al. [86] favor this column for analytical work because histones elute at lower organic solvent concentration and better sensitivity is obtained.

Histones are known to undergo post-translational modifications such as acetylation. Using labelled histones it was found that RPC was ineffective in resolving histones varying in degree of modification [87].

A variety of C$_{18}$, C$_8$, and CN silica-based columns and the polystyrene–divinylbenzene-based PRP-1 resin column were examined in the fractionation of histone mixtures [88]. As in the case of cytosolic proteins, not all columns of the same type of bonded phase were equally effective. Resolution of the

(LHP)H2A, (MPH)H2A, and H4 triplet seems to be the most sensitive (Fig. 7). In fact, there were even large interlot differences between columns. The exact reason for these variations between columns has not been determined. Although of comparable selectivity, the PRP-1 column gave poorer resolution of histones than the silica-based columns.

Resolution of the (MHP)H2A and H4 doublet is also sensitive to mobile phase composition [89]. At 0.1% TFA there was little resolution of the doublet on any of the columns tested. However, at 0.3% TFA it was possible to resolve this pair on the Radial-Pak μBondapak C_{18} column. Although increasing TFA concentration increased histone retention on all columns, the retention difference between (MPH)H2A and H4 increased to the point that they were resolved at 0.3% TFA.

Using a CN column and perchlorate-extracted proteins from the chromatin of Chinese hamster cells, it has been possible to isolate two H1 histone variants (H1 and H1^0) and the high-mobility group (HMG) non-histone proteins. Additional preparative studies on Drosophila H2a histones produced a Drosophila-specific D2 histone [90]. Through the use of column switching, these studies have shown that it is now possible to fractionate the total nuclear proteins of whole nuclei in one step. This will substantially diminish quantitation problems with small samples and aid in studies correlating histone concentrations with cellular changes.

2.8. Lipoproteins

Technically there are two types of lipoproteins: (1) those in which the lipid is covalently bonded to the protein and (2) those in which the lipid and protein form a non-covalently associating complex. There is only a single reference to aminoacylated peptides and proteins in which the acyl group(s) are long-chain fatty acids [91]. In contrast, there is abundant literature on the separation of lipoproteins. Excellent review articles on the separation of plasma lipoproteins [92] and apolipoproteins (apo) [93] have appeared recently. The function of the plasma lipoproteins is to transport phospholipids, neutral lipids, and cholesterol esters. Since the density of triglyceride (TG) is lower than that of protein, the density of lipoproteins decreases as they are loaded with TG. This is the basis for classifying plasma lipoproteins. High-density lipoproteins (HDLs) contain approximately 3% TG and are of the highest density. The low-density lipoproteins (LDLs) are of intermediate density with 10% TG while the very-low-density lipoproteins (VLDLs) contain 60% TG. In addition to the lipoproteins, plasma contains chylomicrons. Chylomicrons are particles of approximately 96% TG with a thin layer of protein coating the outer surface. The fact that the concentration ratio of lipoproteins in plasma may be related to atherosclerosis is the reason that so much work has been done on the separation of lipoproteins and determination of their lipid composition.

The HPLC analysis of serum lipoproteins is based primarily on SEC and selective detection of lipids [92]. At the present time all of the work reported has been done on the TSK columns of the polymer-based (PW) and silica-based (SW) series. The molecular mass range of chylomicrons and lipoproteins in a human serum sample is such that tandem SEC columns, often of different pore sizes,
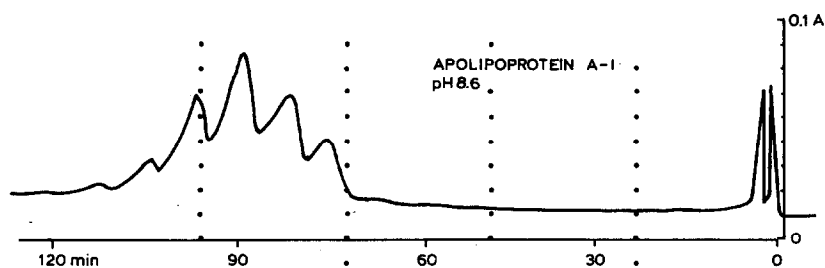
Fig. 8. Separation of apoA-I by reversed-phase HPLC in alkaline conditions [105]. Aquapore RP 300 column.

must be used to gain sufficient resolution of the lipoprotein classes for quantitation [94–97]. For example, three peaks are obtained on the G5000PW + G3000SW + G3000SW three-column set. Chylomicrons and VLDLs elute first as a mixure followed by LDLs in the second peak and HDL_2 and HDL_3 in a partially resolved third peak. The G5000PW column alone is capable of partial resolution of chylomicrons, VLDLs and, LDLs but incapable of resolving HDL_2 from HDL_3. In contrast, coelution of the first three components is experienced on the G3000SW columns with partial resolution of the HDL species. The three-column set noted above is a compromise to achieve the best, albeit incomplete, resolution of the mixture.

Lipoprotein detection was based on A_{280} while simultaneous selective detection of either total cholesterol, triacylglycerides, or phospholipids was achieved in a set of post-column enzymatic reactions. For example, total cholesterol was determined by mixing the column effluent with cholesterol esterase, cholesterol oxidase, a substrate dye for peroxidase, and peroxidase. This mixture was then passed through a $20\ 000 \times 0.4$ mm PTFE reactor and the colored product determined at A_{555} [98]. Triacylglycerides were determined in much the same way except that the glycerides were hydrolyzed with lipoprotein lipase and the glycerol oxidized with glycerol oxidase [99]. Hydrogen peroxide was determined by peroxidase, as above. Choline-containing phospholipids were determined by mixing the effluent with phospholipase D and choline oxidase. Hydrogen peroxide was determined with the peroxidase system noted in both cases above [100, 101]. Excellent correlation was seen between these flow reactors and the more conventional techniques for determining lipid components [92].

The lipid-free forms of serum lipoproteins are termed apolipoproteins. The apolipoproteins apoA-I and apoA-II are obtained by the delipidation of HDLs. In contrast, apoC-I, apoC-II, and apoC-III are produced by delipidation of either HDLs or VLDLs. Both the 28-kD apoA-I and 17-kD apoA-II have been purified by either SEC in 6 M urea [102], anion-exchange chromatography in 6 M urea [103], or RPC using 1% triethylammonium phosphate at pH 3.2 [104]. With an extended gradient, the anion-exchange column shows that there are two isoforms of apoA-I. In contrast, the apoA-I isoforms cannot be resolved on an RPC column (Fig. 8) [105]. The apoC apolipoproteins range from 6.6 kD (apoC-I) to 8.5 kD (apoA-II) and 8.8 kD (apoA-III). Being smaller and more nearly the same

molecular weight, SEC is ineffective in their resolution. They may, however, be resolved both by anion-exchange chromatography in 6 M urea [103] and RPC [104]. With the proper column and gradient conditions, apoC-III was fractionated into three isoforms.

There are two other major classes of apolipoproteins, apoB and apoE. ApoB is the product of either VLDL or LDL delipidation whereas apoE is derived from VLDLs. Both apoB and apoE have been chromatographed on SEC columns in the presence of 4 M guanidinium hydrochloride [106]. It should be noted that the use of high concentrations of urea can lead to the carbamylation of lysine residues in a protein and the production of artificial isoforms [107]. If possible, this may be prevented by working at acidic conditions.

2.9. Isoenzymes

It is common in nature to find different proteins which catalyze the same reaction. These different forms are often referred to as isoenzymes. Three different types of isoenzymes will be discussed here: multimeric enzymes that vary in subunit composition; enzymes that are of the same primary structure but vary in degree of post-translational modification; and those that are of different primary structure.

Lactate dehydrogenase (LDH) is a typical example of a multimeric isoenzyme. Aggregation of the dissimilar 35-kD [M] and [H] subunits into a 140-kD tetramer produces five isoenzyme species (M_4, M_3H, M_2H_2, MH_3, and H_4) which have been separated by high-performance anion-exchange chromatography [108]. With the addition of computer-aided data acquisition and analysis and a post-column reaction detector that allowed LDH activity to be monitored in the column effluent, it has been possible to build an automated high-performance anion-exchange chromatographic system for the detection of myocardial infarction [109–111]. Greater than 95% of the activity of all five isoenzymes was recovered on clinical serum samples with a 1% relative standard deviation. Resolution apparently depends more on mobile phase pH than on the type of ion-exchange column. There was relatively little difference in resolution between weak and strong anion-exchange columns whereas increasing mobile phase pH from 7 to 8 increased resolution [112].

Creatine phosphokinase (CPK) is a second isoenzyme system that has been used in monitoring myocardial infarction by high-performance anion-exchange chromatography [108–110]. The dissimilar [M] and [B] subunits aggregate into a dimer to produce three CPK isoenzymes: MM,MB and BB.Again a post-column reaction detector is used to monitor the activity of eluted enzymes and correlates elevations in the MB isoenzyme with myocardial infarction. A separation of CPK isoenzymes is shown in Fig. 9.

Hexokinase (HK) and alkaline phosphatase (AP) isoenzymes from murine tissue extracts have also been separated by HPLC [113]. Weak anion-exchange chromatography resolved the pancreatic and duodenal forms of AP in the presence of 4 M urea. In the absence of urea, these isoenzymes eluted as an aggregate. It has recently been shown [114] that the heterogeneity in duodenal AP is appar-
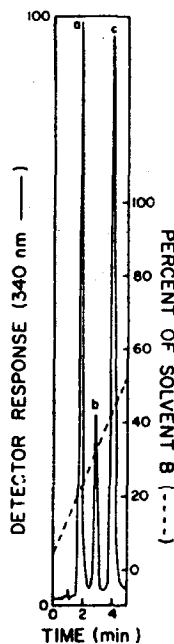
Fig. 9. Separation of creatine phosphokinase (CPK) isoenzymes using a post-column enzyme detector [108]. Column, 250×4 mm I.D. stainless steel; packing, DEAE-Glycophase/CPG (250 Å pore diameter, 10 μm particle size); temperature, 25°C. Solvents: (A) 0.05 M Tris, 0.05 M sodium chloride, 10^{-3} M mercaptoethanol, pH 7.5; (B) 0.05 M Tris, 0.3 M sodium chloride, 10^{-3} M mercaptoethanol, pH 7.5; flow-rate, 4 mm/s (3 ml/min). Peaks: a = CPK_3; b = CPK_2; c = CPK_1.

ently due to differences in glycosylation. Using a concanavalin A (Con A) column, which discriminated on the basis of mannose content, two rat duodenal enzymes have been identified. It is probable that these isoenzymes are formed as a result of post-translational modification of the AP primary structure. Canine plasma isoenzymes of AP have also been reported which can be separated by anion-exchange chromatography [115]. These enzymes differ in that one is corticosterone-inducible and stable to 65°C while the other is more temperature-sensitive and non-inducible.

Malate dehydrogenase (MDH) occurs in almost all eucaryotic cells in mitochondrial (m-MDH) and cytosolic (c-MDH) forms. A rapid 100-fold purification of the beef heart MDH isoenzymes was achieved in a single pass through a strong cation-exchange column [116]. HIC did equally well in a descending 30 to 0% ammonium sulfate gradient where c-MDH eluted first followed by m-MDH. Additional successful applications of high-performance anion-exchange chromatography in isoenzyme purification are the fractionation of β-N-acetylhexosaminidase from human liver into the A and B forms [117], γ-butyrobetaine hydroxylase from human kidney into three forms [118], cyclic-AMP-dependent protein kinase isoenzymes from rabbit gastric mucosa parietal cells into two forms [119], and glutathione S-transferase (GST) from human heart, lung, and erythrocytes into a major and two minor isoforms [120]. Although homogeneous by

biospecific affinity chromatography and isoelectric focusing, the isoenzymes of GST apparently had sufficient charge asymmetry that they could be further fractionated by IEC.

2.10. Glycoproteins

Glycoproteins are produced by post-translational modifications. As a consequence, they have a protein core to which various mono- and oligosaccharides ranging up to 50 units are attached in a stepwise manner at different positions in the primary structure. Although glycoproteins may exist as a single pure species, it is common to find the same polypeptide with varying degrees of glycosylation. Covalent coupling of a carbohydrate to the protein is either through an O-glycosidic linkage to serine or threonine or an N-glycosidic linkage to asparagine. As much as 50% of the mass of a glycoprotein may be carbohydrate.

Because of their contribution to chromatographic retention in IEC, it is also important to note that glucosamine, galactosamine, and the sialic acids are constituents of glycoproteins. When glycosylation with these monosaccharides is incomplete, it means that a variety of charged species with the same polypeptide backbone will be found.

There are many papers on the isolation of peptide cleavage fragments of glycoprotein by both SEC and RPC. In view of the fact that this review is dealing with intact glycoprotein structure, papers on structure elucidation and the separation of cleavage fragments will not be examined.

Since so little work has been done on the retention of glycoproteins in the various modes of chromatography, a discussion of column selection must be based on some speculation. As a beginning, it can be said that column selection depends to a large extent on the objective of the research. When the objective is to study the protein portion of the glycoprotein, a technique should be chosen that shows little discrimination among all of the possible carbohydrate moieties coupled to the polypeptide. In contrast, when the objective is to study either glycosylation or the structure of the fully assembled glycoprotein or even perhaps the fate of a glycoprotein in the biological system, a technique should be chosen that gives maximum discrimination between all of the components of the glycoprotein.

When the objective of the separation is to discriminate among differences in glycosylation, RPC should be chosen. Unfortunately there are little data to support this conclusion except common logic and some work on glycopeptides. The retention behavior of glycosylated and non-glycosylated peptides of the same amino acid sequence showed very similar behavior in RPC [121]. However, glycosylated peptides eluted slightly earlier. When the amount of glycosylation was the same between two peptides, the one with multiple sites of glycosylation eluted first. This is to be expected. Retention of polypeptides in RPC is based on the hydrophobic contact area between the solute and alkyl ligands on the sorbent [122]. Hydrophilic portions of a molecule are directed outward toward the solution and do not participate directly in the adsorption process. Their contribution is simply to decrease the partition coefficient by making the compound more soluble.
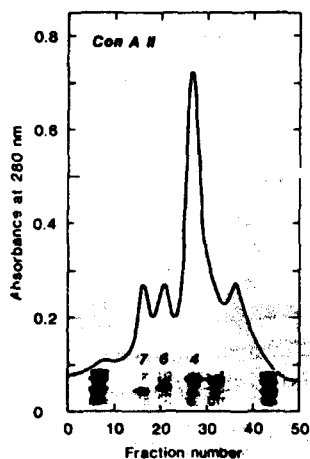
Fig. 10. Chromatofocusing of Con A II [123]. The mono P column was equilibrated with 25 mM methylpiperazine and 10 mM sodium chloride, pH 5.4. A gradient was formed by Polybuffer (1:10), 10 mM sodium chloride, pH 4.0. Flow-rate, 1 ml/min.

Differentiating among variations in the carboyhydrate portion of a glycoprotein is much more difficult. When the differences are the result of either steric shielding of charged groups or the addition or deletion of a charged species, i.e. an amino sugar or sialic acid, ion-exchange columns will be useful. For example, multiple glycosylated species of hemoglobin (HbA_{1a}, HbA_{1b}, HbA_{1c}, and HbA_{1d}) and some antibodies can all be separated by IEC [63]. However, as the degree of complexity in the glycoprotein increases through the introduction of charge in multiple-oligosaccharide chains, heterogeneity of charge within any one oligosaccharide, and steric shielding of charge in the polypeptide backbone by the carbohydrate, the ability of the ion-exchange system to discriminate among these myriad species will be overwhelmed.

It was noted above in the discussion of the fractionation of glycosylated immunoglobulins that hydroxyapatite columns provide selectivity that could not be obtained with ion-exchange columns [81]. If any part of this discrimination was due to the carbohydrate portion of the molecule, then hydroxyapatite would be useful in the fractionation of glycoproteins when the objective is to discriminate between different carbohydrate moieties on the polypeptide.

Biospecific affinity chromatography of glycoproteins on lectin columns is another technique for discriminating between carbohydrate groups in a glycoprotein. For example, α_1-antitrypsin (α_1-protease inhibitor) has two major and three minor fractions with isolectric points between 4.4 and 4.7. The molecule consists of a single polypeptide chain with 394 amino acid residues and three carbohydrate chains, giving a relative molecular mass of 51 000. Con A-Sepharose separates (Fig. 10) these proteins into three fractions based on the mannose content of the oligosaccharides [123]. Future use of lectin columns for the fractionation of glycoproteins will probably increase.

3. FUTURE OPPORTUNITIES AND PROSPECTS

The discussion above would indicate that HPLC has attained a secure position in the arsenal of techniques available to the protein chemist for the purification and characterization of proteins. As in the past, further developments in the field will depend very much on the needs of both the life science research community and the biotechnology industry. At the present time there are several trends in protein chemistry and biotechnology which will have an impact on developments in HPLC. Protein characterization on an ever diminishing scale will continue to drive the development of microvolume columns and the equipment needed to drive them in the gradient elution mode. The fact that microvolume columns contain much less sorbent allows higher recovery of small samples and provides less sample dilution. Diminished sample dilution is important because it enhances both detection sensitivity and solute recovery. Continuing effort must also be made in eliminating non-specific adsorption on column frits and other surfaces in the instrument system itself.

Proteins synthesized in genetically engineered organisms are often contaminated with structural variants that result from expression errors during synthesis, incomplete post-translational modification, or chemical modification during isolation. These variants may be very similar in structure to the native species and be present in small quantities. The possibility that variant forms of a therapeutic protein could be harmful to a person during long-term consumption requires the development of systems for monitoring and eliminating non-native proteins from therapeutic materials. At the present time our understanding of the ability of liquid chromatography (LC) systems to discriminate between similar proteins is incomplete. This is based largely on a lack of understanding of the mechanism by which proteins are adsorbed at surfaces. Much more data must be collected on the ability of a series of different types of LC columns to discriminate among mutant proteins before it will be possible to assess the utility of LC in this difficult task and to design higher-resolution separation systems.

The scale-up in protein production initiated by biotechnology has had an impact on preparative separations. There is a need for larger systems that are economical and of high resolving power and throughput. It would seem that the most logical way to design preparative systems is to take all of the concepts and materials used so successfully in analytical separations and simply make a much larger analytical system. This approach will be very satisfactory if the columns are not overloaded and economics is not a serious question. However, when overloading and economics are inserted into the equation much of what we know in analytical separations begins to fail. Questions of loading capacity, economics, and the kinetics of loading large quantities of solute were never considered when designing the current generation of high-performance sorbents. There is a strong possibility that a generation of true preparative packing materials will emerge in the next five years. It is likely that these materials will be mechanically stable to more than 100 bar, have 1.5 to 2 times the loading capacity of analytical materials, be available at 20% of the cost of current HPLC sorbents, have a dynamic loading capacity that is equal to 70% of that obtainable in the static mode, have good

chemical stability from pH 2 to 11, and be easily cleaned after they have been fouled with protein. Much more study will be devoted to understanding the chromatographic process in the overloaded state. An appreciation of displacement effects and even the use of displacement chromatography on the process scale is a distinct possibility, particularly with smaller polypeptides. There is sufficient difference between preparative and analytical chromatography of proteins so that, with time,they will evolve as separate, but related fields.

The large-scale commercialization of protein production requires that much more rapid and specific methods be developed for monitoring product purity at all stages of the purification process, monitoring the composition and purity of raw materials used in the manufacturing process, and circumventing lengthy biological assays. It is in these very-high-speed and specific systems that bioaffinity materials such as immunosorbents and receptor protein sorbents will be extremely useful. It is also likely that through the use of non-porous sorbents it will be possible to obtain the complete chromatographic analysis of a mixture in less than 30 s. Since most of these separations require the generation of a gradient, this capability must be incorporated into new LC instruments.

4. SUMMARY

This review has shown that a variety of chromatographic techniques are available for fractionating proteins. Fortunately, high-quality columns of every type described in this review are commercially available. Most water-soluble proteins may be eluted from size-exclusion, hydrophobic-interaction, ion-exchange, metal chelate, and bioaffinity columns with ease. When this is the case, high recovery and retention of biological activity are the norm. The exception is reversed-phase chromatography where the organic solvents and acids used in polypeptide elution denature many proteins.

When problems do occur, they are generally the result of unique structural features of the protein. Very hydrophobic proteins have presented the biggest problem in that they are difficult to solubilize, particularly with retention of biological activity. It has been found that zwitterionic and non-ionic detergents are the most suitable solubilizing agents, but urea has also been used in cases where hydrophobic interacts are not as strong. Unfortunately, there is still an element of trial-and-error in selecting the most suitable solublizing agent.

Heterogeneous glycosylation of proteins also presents a problem. Both neutral and charged monosaccharides can be incorporated into proteins through multiple steps at several sites. Thus, there is the potential in a sample for a large number of glycoprotein species which have the same polypeptide backbone and differing amounts of oligosaccharide. A problem arises when size-exclusion, ion-exchange, hydrophobic-interaction, reversed-phase and bioaffinity systems begin to discriminate between these very similar glycoprotein species. Chromatographic peaks can become very broad, due to incomplete fractionation, and the polypeptide chain of interest can be associated with multiple peaks. The separation of glycoproteins requires much more study before logical procedures can be suggested for column selection and operation.

Aggregated species are another class of proteins which present occasional problems. Multimeric proteins are adsorbed to sorbents by a series of forces, among which are hydrogen bonding, hydrophobic interactions, and electrostatic forces. These forces are also responsible for the maintenance of quaternary structure in proteins. When the same forces dominate both retention of protein structure and adsorption at the sorbent surface, the quaternary structure of the protein can be disrupted during elution.

Very basic proteins also present a problem in some cases. Columns with residual negative charges, such as a silica-based reversed-phase column, adsorb anionic species so strongly that they are difficult to elute. This is the case with ribosomal and nucleoproteins. The solution is to use exhaustively end-capped columns which diminish electrostatic interactions.

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