BIOSEPARATIONS

1. Introduction

The large-scale purification of proteins and other bioproducts is the final production step, prior to product packaging, in the manufacture of therapeutic proteins, specialty enzymes, diagnostic products, and value-added products from agriculture. These separation steps, taken to purify biological molecules or compounds obtained from biological sources, are referred to as bioseparations. Large-scale bioseparations combine art and science. Bioseparations often evolve from laboratory-scale techniques, adapted and scaled up to satisfy the need for larger amounts of extremely pure test quantities of the product. Uncompromising standards for product quality, driven by commercial competition, applications, and regulatory oversight, provide many challenges to the scale-up of protein purification. The rigorous quality control embodied in current good manufacturing practices, and the complexity and lability of the macromolecules being processed provide other practical issues to address (1).

Recovery and purification of new biotechnology products is the fastest growing area of bioseparations. Biotechnology was broadly defined in 1991 by the U.S. Office of Technology Assessment as "any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses." The new biotechnology, introduced in 1970, involves directed manipulation of the cell's genetic machinery through recombinant deoxyribonucleic acid (DNA) techniques and cell fusion. The new biotechnology was first applied on an industrial scale in 1979. Since then it has fundamentally expanded the utility of biological systems, so that biological molecules for which there is no other means of industrial production can be generated. Substantial manufacturing capability is expected to be needed to bring about the full application of this biotechnology (2). The recovery, purification, and packaging of biotechnological products for delivery to the consumer is undergoing unprecedented growth.

Manufacturing approaches for selected bioproducts of the new biotechnology impact product recovery and purification. The most prevalent bioseparations method is chromatography (qv). Thus the practical tools used to initiate scaleup of process liquid chromatographic separations starting from a minimum amount of laboratory data are given.

2. Economic Aspects

The development of biotechnology processes in the biopharmaceutical and bioproduct industries is driven by the precept of being first to market while achieving a defined product purity, and developing a reliable process to meet validation requirements. The economics of bioseparations are important, but are likely to be secondary to the goal of being first to market. The cost of a lost opportunity in a tightly focused market where there is room for only a few manufacturers can be devastating for products which take 5 to 10 years and \$100 to $\$200 \times 10^6$ to develop. After process and product are validated, the cost of change in any

Table 1. **Unit Values and Relative Production Quantities for Selected Approved Biopharmaceuticals, 1990–1991**[a]

Product	Year approved	Selling price, $/g[b]	Quantity for $\$200 \times 10^6$ in sales, kg
human insulin	1982	375	530.0
tissue plasminogen activator	1987	23,000	8.7
human growth hormone	1985	35,000	5.7
erythropoetin (Epogen)	1989	840,000	0.24
GM CSF	1991	384,000	0.52
G-CSF	1991	450,000	0.44

[a] Adapted from Ref. 2 with additional data from Ref. 4.
[b] Values are approximate and are likely to decrease.

portion of the procedure can also be great, if only to satisfy regulatory constraints. Hence, once the manufacturing process is in place, changes are likely to be considered only if significant improvements result.

The three main sources of competitive advantage in the manufacture of high value protein products are first to market, high product quality, and low cost (3). The first company to market a new protein biopharmaceutical, and the first to gain patent protection, enjoys a substantial advantage. The second company to enter the market may find itself enjoying only one-tenth of the sales. In the absence of patent protection, product differentiation becomes very important. Differentiation reflects a product that is purer, more active, or has a greater lot-to-lot consistency.

2.1. Biopharmaceuticals and Protein Products. Purification of proteins is a critical and expensive part of the production process, often accounting for $\geq 50\%$ of total production costs (2). Hence, bioseparation processes have a significant impact on manufacturing costs. For small-volume, very high value biotherapeutics (Table 1), however, these costs may be considered secondary to the first to market principle unless a lower cost competitor surfaces. Annual 1995 sales were $700 million for human insulin (5), $300 million for tissue plasminogen activator, and $220 million for human growth hormone (6). The most successful bioproduct in biotechnology history, recombinant erythropoetin (EPO), had worldwide sales estimated at $1.6 to $2.6 billion in 1995 (5, 7, 8). Epogen is a genetically engineered version of erythropoetin [11096-26-7], which is produced by the kidneys and stimulates blood stem cells to mature into red blood cells. Epogen can reverse the severe anemia often caused by kidney disease. Amgen's sales of this product, together with Neupogen (a recombinant protein that directs blood stem cells to become bacteria-fighting neutrophils), was about $1.8 billion in 1995 (9).

3. Bioproduct Separations

The task of quickly specifying, designing, and scaling-up a bioproduct separation is not simple. These separations are carried out in a liquid phase using macromolecules which are labile, and where conformation and heterogeneous chemical

structure undergoing even subtle change during purification may result in an unacceptable product. A typical purification scheme for biopharmaceutical proteins involves the harvesting of protein-containing material or cells, concentration of protein using ultrafiltration (qv), initial chromatographic steps, viral clearance steps, additional chromatographic steps, again concentration of protein using ultrafiltration, and finally formulation (10).

3.1. Biosynthetic Human Insulin from *E. coli*. Insulin [9004-10-8], a polypeptide hormone, stimulates anabolic reactions for carbohydrates, proteins, and fats thereby producing a lowered blood glucose level.Porcine insulin [12584-58-6] and bovine insulin [11070-73-8] were used to treat diabetes prior to the availability of human insulin [11061-68-0]. All three insulins are similar in amino acid sequence. Eli Lilly's human insulin was approved for testing in humans in 1980 by the U.S. FDA and was placed on the market by 1982 (11, 12).

Human insulin was the first animal protein to be made in bacteria in a sequence identical to the human pancreatic peptide. Expression of separate insulin A and B chains were achieved in *Escherichia coli* K-12 using genes for the insulin A and B chains synthesized and cloned in frame with the β-galactosidase gene of plasmid pBR322 (13, 14). Insulin's small size, 21 amino acids for the A-chain, mol wt = 2300; and 30 for the B-chain, mol wt = 3400, together with the absence of methionine (Met) and tryptophan (Trp) residues, were critical elements both in the decision to undertake cloning of this peptide hormone and in the rapid development of the manufacturing process. The Met and Trp residues, produced as a consequence of engineering and expression in *E. coli*, are hydrolyzed by reagents used during the recovery process. The presence of these amino acids in insulin would have resulted in the hydrolysis and destruction of the product (12).

Recovery and Purification. The production of Eli Lilly's human insulin requires 31 principal processing steps of which 27 are associated with product recovery and purification (13). The production process for human insulin, based on a fermentation which yields proinsulin, provides an instructive case study on the range of unit operations which must be considered in the recovery and purification of a recombinant product from a bacterial fermentation. Whereas the exact sequence has not been published, the principle steps in the purification scheme are outlined in Figure 1**a**.

The fermentation product is a fusion protein where a portion of the Trp protein is connected to proinsulin through a Met residue (Fig. 1**b**). The *E. coli* contains a plasmid having the proinsulin gene connected to the Trp promoter. The Trp operon is turned on when the fermentation media is allowed to become depleted of tryptophan and the production of a fusion protein of proinsulin occurs. An inclusion body, ie, a large body of aggregated protein and nucleic acids occupying about half of the cell volume is formed. Because formation of inclusion bodies causes cell growth to stop, premature formation of inclusion bodies results in lower productivity. Hence, the Trp switch is an important practical tool in maximizing productivity. At this point the fermentation is complete, and protein recovery, dissolution, protein refolding, and purification is carried out (12). Following inclusion body recovery, CNBr, a hydrolytic agent which specifically attacks Met and Trp linkages, cleaves away the fusion protein from the proinsulin (see Fig. 1**b**). No Met or Trp occurs in proinsulin, so the proinsulin

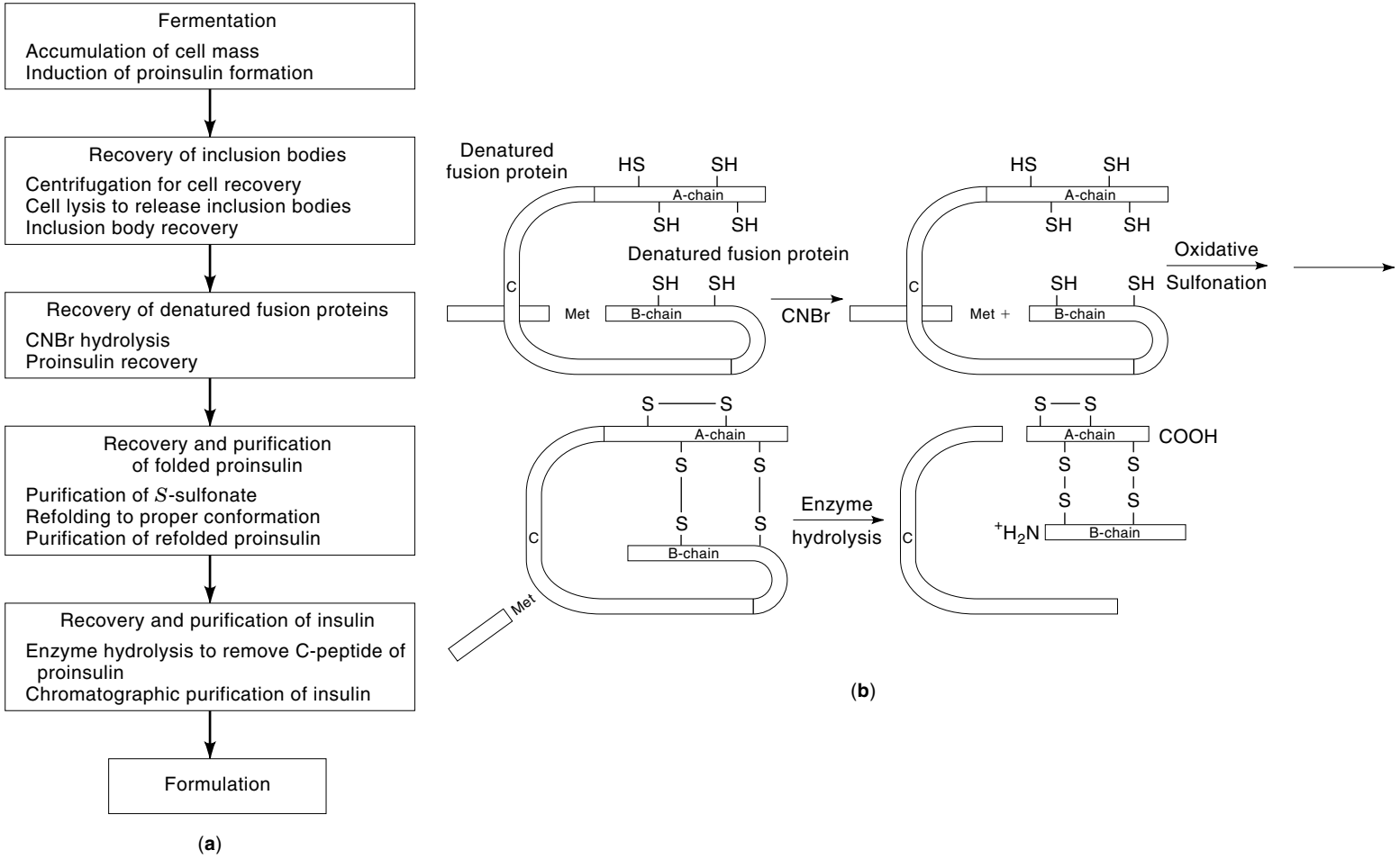

Fig. 1. (**a**) Process flow sheet for human insulin production, recovery, and purification (12); (**b**) corresponding steps in recovery of biosynthetic human insulin.

molecule is left intact. The proinsulin is then subjected to oxidative sulfitolysis, refolding to its proper conformation, purification, and enzyme treatment to remove the peptide connecting the insulin A- and B-chains. The crude insulin consisting of A- and B-chains in their proper conformation is then further purified using a sequence of ion-exchange (qv), reversed-phase, and size-exclusion chromatography (3,12,14).

Desamidation of asparagine or glutamine residues can occur readily in either acidic or neutral solutions; disulfide exchange reactions can occur at alkaline pH and cause formation of isomeric monomers or aggregated forms (multimers) of the protein (13). Deamidation products of insulin, referred to as desamido insulin, can form during processing. These insulin variants require high resolution chromatography techniques to remove. Therefore, a multimodal sequence of chromatographic separations for the crude recombinant insulin is required.

Ion-exchange chromatography removes most of the impurities and is followed by reversed-phase chromatography which separates insulin from structurally similar insulin-like components. Then size-exclusion (gel-permeation) chromatography is introduced to remove salts and other small molecules from the insulin. The best pH range for the acetonitrile mobile phase for reversed-phase chromatography is reported to be 3.0–4.0. This is well below the isoelectric pH of 5.4, gives excellent resolution, and minimizes deamidation of the insulin if the residue time in the reversed-phase column is less than several hours (12,14). This sequence (14) follows the principle of orthogonality of separation sequence, ie, each step is based on a different property, in this case charge, solubility, and size, respectively (1). Near the end of the chromatography sequence, the insulin may be concentrated by precipitation to form insulin zinc crystals.

Process Equipment Volumes. Product recovery involves cell lysis, centrifugation, refolding, buffer exchange, chromatography, precipitation, and filtration. Some of these steps are repeated. The volumes of the individual chromatography columns are estimated to range from 50 to 1000 L. These volumes are small compared to other types of chemical recovery processes, but are large in the context of biotechnology manufacturing. For example, the reversed-phase step uses an 80 L column volume for an insulin loading of 1.2 kg per run (3). Assuming the total amount of recombinant insulin produced annually in a typical plant is on the order of 1000 to 2000 kg, this size column would enable processing as much as 30% (>300 kg) of the annual output of insulin.

Yield Losses. The numerous steps incur a built-in yield loss. For example, if only 2% yield loss were to be associated with each step, the overall yield for a purification sequence of 10 steps would be as in equation 1:

$$\eta = 100(1 - L/100)^n = 100(1 - 0.02)^{10} = 81.7\% \quad (1)$$

where η denotes yield, L the percent yield loss, and n the number of steps. If the yield loss at each step were 5%, the overall yield would only be 60%. Maximizing recovery at each step is important.

The purification of human insulin involves five separate alterations in the molecular structure, and hence, changes in physicochemical properties during its recovery and purification. The various forms are fusion protein, denatured

aggregate, denatured monomers, properly folded proinsulin, and finally insulin. Whereas various purification procedures are used repeatedly, thus introducing more steps in the process, the change of removing contaminants is maximized because the contaminants are not as likely to change chemically in the same way as the insulin molecule. The final purification steps rely on multiple properties of the insulin, such as size, hydrophobicity, ionic charge, and crystallizability (13). The final purity level is reported to be >99.99% (15).

3.2. Tissue Plasminogen Activator from Mammalian Cell Culture

Tissue-type plasminogen activator or tissue plasminogen activator [105857-23-6] (t-PA) was originally identified in tissue extracts in the late 1940s (15). Other known plasminogen activators include streptokinase from bacteria, urokinase from urine, and prourokinase from plasma (16). In 1981 the Bowes melanoma cell line was found to secrete t-PA (known as mt-PA) at 100× higher concentrations, making possible the isolation and purification of this enzyme in sufficient quantities that antibodies could be generated and assays developed to lead to cloning of the gene for this enzyme and subsequent expression of the enzyme in both *E. coli* and a Chinese hamster ovary (CHO) cell line (15,17).

Comparison of the melanoma and recombinant forms of the enzymes showed the same activity toward dissolution of blood clots. Comparison to urokinase, another thrombolytic agent, served as the basis for introducing recombinant t-PA into clinical trials in 1984 (17). Two pilot studies demonstrated that mt-PA resulted in thrombolysis without significant fibrinogenolysis. Fibrinogen, the precursor to fibrin, is important to the clotting of blood. Because mt-PA was available in limited quantities, recombinant t-PA (rt-PA) was used to carry out the first significant clinical trial. Doses of 0.375–0.75 mg rt-PA/kg body weight was found to be effective in humans for achieving 70% recannalization. Another pilot study confirmed that a dose of 80 mg over three hours gave the same results (17). The comparison of rt-PA (injected intravenously) to streptokinase IV (injected intracoronary) produced sufficiently favorable results to end the trial early, and make the results public in 1985, resulting in the use of t-PA for heart attacks (15). A trial completed in 1996 showed that t-PA administered within three hours of a stroke caused by a clot in the brain facilitated full recovery of 31% of stroke patients. Hence, another use of rt-PA is likely to develop (18).

Approximately 500,000 Americans suffer strokes each year. Many of the 80% that survive suffer paralysis and impaired vision and speech, often needing rehabilitation and/or long-term care. Hence, whereas treatment using rt-PA is likely to be expensive (costs are $2200/dose for treating heat attacks), the benefits of rt-PA could outweigh costs. In the case of heart attacks, the 10 times less expensive microbially derived streptokinase can be used. There is currently no competing pharmaceutical for treatment of strokes (18, 19). Consequently, the cost of manufacture of rt-PA may not be as dominant an issue as would be the case of other types of bioproducts.

Characteristics of t-PA. Tissue plasminogen activator, a proteolytic, hydrophobic enzyme, has a molecular weight of 66,000, 12 disulfide bonds, four possible glycosylation sites, and a bridge of 6 amino acids connecting the principal protein structures (17,20,21). Only three of the sites (Asn-117–118, -448) are actually glycosylated (16). When administered to heart attack victims it dissolves

clots consisting of platelets in a fibrin protein matrix and acts by clipping plasminogen, an active precursor protein found in the blood, to form plasmin, a potent protease that degrades fibrin (17,21). Whereas plasminogen activator is found in blood and tissues, concentrations are low (17).

The concentration of t-PA in human blood is 2–5 ng/mL, ie, 2–5 ppb. Plasminogen activation is accelerated in the presence of a clot, but the rate is slow. The dissolution of a clot requires a week or more during normal repair of vascular damage (17). Prevention of irreversible tissue damage during a heart attack requires that a clot, formed by rupture of an atherosclerotic plaque, be dissolved in a matter of hours. This rapid thrombolysis (dissolution of the clot) must be achieved without significant fibrinogenolysis elsewhere in the patient.

rt-PA is derived from a biological source, transformed CHO cells, and by definition is a biologic, not a drug. It is generally not possible to define biologics as discrete chemical entities or demonstrate a unique composition. Other biologics include blood fractionation products such as albumin and Factor VIII, and both live and killed viral vaccines.

The process used to make a biologic is closely monitored and regulated by regulatory agencies, because a significant change in the process may result in a product which is different from that previously reviewed and regulated, and hence may require a new license. Process changes made during the investigational new drug (IND) development stage, and before the license is approved, are more easily incorporated into a new product (from a regulatory point of view) than after the license is generated (15).

t-PA Production. Recombinant technology provides the only practical means of rt-PA production. The amount of t-PA required per dose is on the order of 100 mg. Cell lines of transformed CHO cells, selected for high levels of rt-PA expression using methotrexate, are grown in large fermenters (21). The purification steps for rt-PA must therefore separate out cells, virus, and DNA. The literature on the industrial practice of recovery and purification of rt-PA generated by suspension culture of chinese hamster ovary cells is limited (15). Recovering a protein derived from mammalian cells involves a number of steps (15). One possible scheme is shown in Figure 2. The culture medium is separated from the cell by sterile filtration (see MICROBIAL AND VIRAL FILTRATION). This is followed by additional removal by cross-flow filtration, ultrafiltration (qv), and chromatography to remove DNA and remaining viruses. The product protein then undergoes purification by chromatography.

The separation of cells from the culture media or fermentation broth is the first step in a bioproduct recovery sequence. Whereas centrifugation is common for recombinant bacterial cells (see CENTRIFUGAL SEPARATION), the final removal of CHO cells utilizes sterile-filtration techniques. Safety concerns with respect to contamination of the product with CHO cells were addressed by confirming the absence of cells in the product, and their relative noninfectivity with respect to immune competent rodents injected with a large number of CHO cells.

The possibility that DNA from recombinant immortal cell lines such as CHO cells could cause oncogenic (gene altering) events resulting in cancer (22) was a concern during development of the rt-PA purification sequence. Data suggest that DNA, by itself, is inactive *in vivo*; removal of the DNA, however, is still a concern. The goal for rt-PA purification is to reduce the DNA to

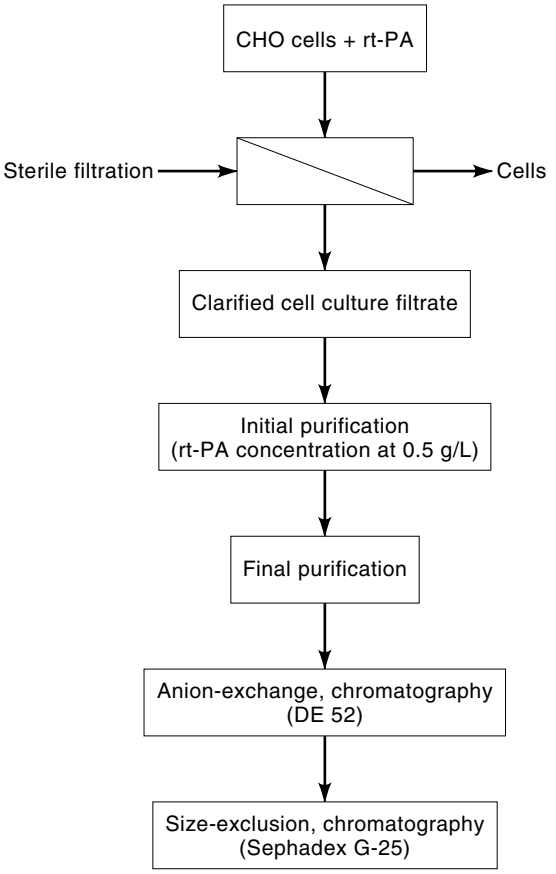

Fig. 2. Outline of possible steps in the recovery and purification sequence for recombinant tissue plasminogen activator derived from recombinant CHO cells.

<10 pg/dose (10^{-11} g/dose). A level of 0.1 pg has been achieved, representing a ~9 log reduction in the DNA, and requiring special assays to detect and quantify these very low levels of DNA in the final product (15).

Sensitive, specific, and if possible, rapid assays for product and potential contaminants are an essential part of separation methods selected for the purification sequence. Ultrafiltration (qv) followed by ion-exchange (qv) chromatography (qv) and then a final round of ultrafiltration concentrate the dilute protein while purifying it (23). Precipitation prior to chromatography could remove unwanted proteins before the sample is injected into the first liquid chromatography column. At the initial purification stage the rt-PA concentration is 0.5 g/L; the DNA is at 0.11 ng/mL. The use of anion-exchange chromatography (DE step) appears to be particularly effective in removing the DNA. Studies using another product, IgM, derived from cell culture showed DNA clearance may be enhanced by predigestion (hydrolysis) of the DNA using nucleases prior to the anionic-exchange chromatography step (24).

Independent Assays for Proving Virus Removal. Retroviruses and viruses can also be present in culture fluids of mammalian cell lines (15,24). Certainly the

absence of virus can be difficult to prove. Model viruses, eg, NIH Rausher leukemia virus and NZB Xenotropic virus, were spiked into fluids being purified, and their removal subsequently validated when subjected to the same purification sequence as used for the product.

Viral clearance can be achieved by use of chaotropes such as urea or guanidine, pH extremes, detergents, heat, formaldehyde, proteases or DNA'ses organic solvents such as formaldehyde, or ion-exchange or size-exclusion chromatography. The protein product must be stable at the conditions used to deactivate or remove the virus. Because only the inactivation or removal which can be measured counts as validation, a sequence of orthoganol removal/fractionation steps must be used (1,15,24). For a fluid spiked with 10^6 virus particles/mL, if the sensitivity of detection after each treatment is 10^2 particles/mL, the analytical technique could only show a removal of 10^4 particles/mL. Hence to achieve evidence of 12 logs of clearance ($10^6 - 10^{-6}$), three different mechanisms of analysis would need to be used assuming each gives 4 logs of clearance (20). It is not valid to use the same approach, ie, the same step repeated three times, to achieve the 12 log reduction. Total clearance, based on the product of three separation steps ($10^4 \times 10^4 \times 10^4 = 10^{12}$), requires that the three steps be totally independent or orthogonal.

Another example of virus clearance is for IgM human antibodies derived from human B lymphocyte cell lines where the steps are precipitation, size exclusion using nucleases, and anion-exchange chromatography (24). A second sequence consists of cation-exchange, hydroxylapatite, and immunoaffinity chromatographies. Each three-step sequence utilizes steps based on different properties. The first sequence employs solubility, size, and anion selectivity; the second sequence is based on cation selectivity, adsorption, and selective recognition based on an anti-u chain IgG (24).

Purification of Human Cell-Line t-PA. The sequence of steps making up the initial and final purifications of recombinant t-PA from CHO cells is proprietary. A detailed experimental protocol for t-PA derived from normal, nonrecombinant human cultured cells (ATCC CRL-1459, American Type Culture Collection, Rockville, Maryland), however, is available (16). This provides insights into the types of chromatography steps which might be employed for purification of rt-PA. Human cell t-PA also contains urokinase plasminogen activator (u-PA) which, except for a single glycosylation (at Asn-302), is structurally similar to t-PA and tends to co-purify. The sequence in Figure 3 shows the steps for fractionating the two proteins. The yield is only 20 mg from 1400 L, illustrating the critical role of a recombinant cell line in obtaining both high yields and higher selectivity in producing a specific type of protein.

Because the culture media contained both t-PA and u-PA, this separation required several extra affinity chromatography steps, as well as dialysis/buffer exchange between the different chromatography columns (see Fig. 3). The salts and buffers added during the purification sequence must also be removed from the product at various points, adding significant complexity to the purification sequence. Desalting the buffer exchange constitute significant separation steps in the production of almost all biotechnology protein products.

Adsorption of t-PA to process equipment surfaces consisting of either stainless steel or glass was minimized by adding the detergent polyoxyethylene

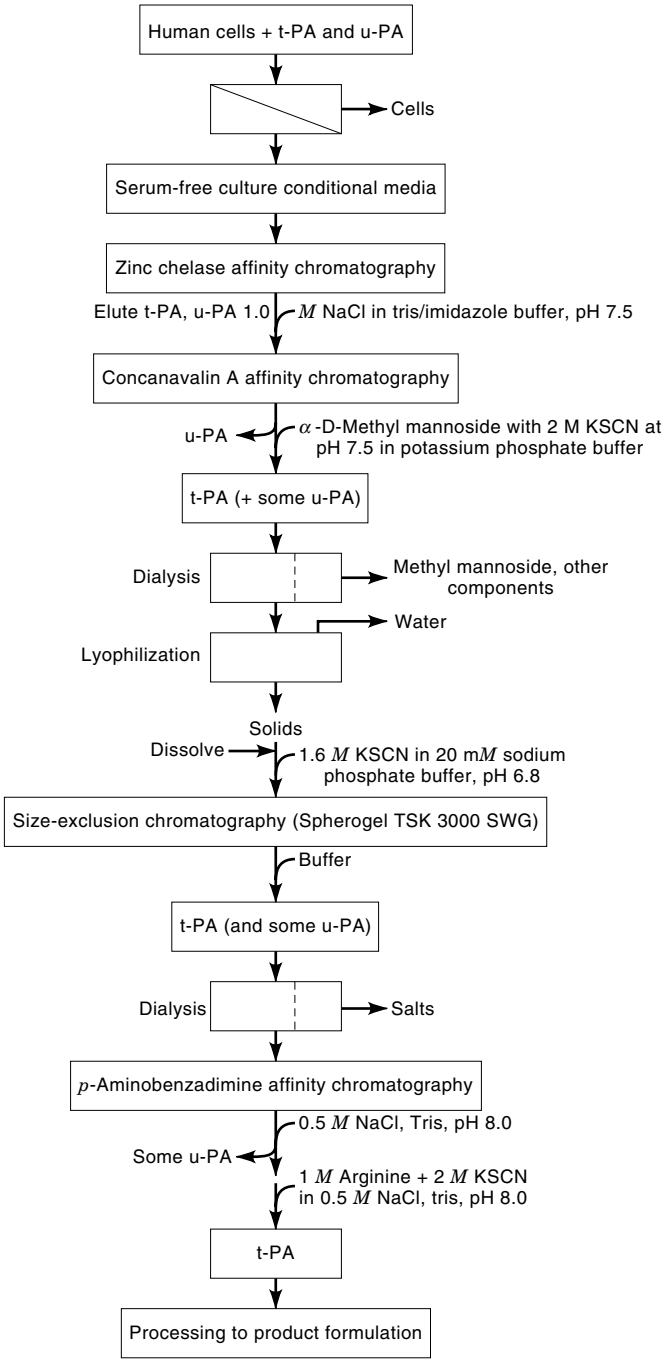

Fig. 3. Overview of purification sequence for the nonrecombinant tissue plasminogen activator (t-PA) which also contains urokinase plasminogen activator (u-PA). Serum-free culture conditional media is from normal human cell line. The temperature for all steps, except for size-exclusion chromatography (22°C), was 4°C. Adapted from Ref. 16.

sorbitan monooleate (Tween 80) to the serum-free culture conditioned media at 0.01% (vol/vol). The equipment was also rinsed, before use, with phosphate buffered saline (PBS) containing 0.01% Tween 80. Hydrophilic, plastic equipment was used whenever possible. All buffers were sterile filtered. Sterile filtration of liquids and gases is usually carried out using 0.2 or 0.45 μm filters.

4. Manufacture of Biologics and Government Regulation

The difference between biologics and drugs is not only a matter of definition, it is also a process design issue. To compensate for the incomplete analytical capability to define biologics, regulatory agencies include parameters of the process used to make biologics in the control and monitoring. Changes in the process may yield a different product from that previously reviewed and approved. A different product requires a new license (15). Thus substantial barriers exist in terms of effort, money, and time to making significant changes in processes used to produce licensed biologies. Process changes are to be expected during the investigational new drugs (IND) phase and before the license is approved, but significant changes are rarely made after licensing. The time which can elapse between conception of an idea for a process change and granting of a new license can be as much as two years and cost several million dollars.

The definition of biologics versus drugs continues to evolve. Assignment is made on a case by case basis (25). Section 351 of the Public Health Service Act defines a biologic product as "any virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product ... applicable to the prevention, treatment, or cure of diseases or injuries in man." Biologics are subject to licensing provisions that require that both the manufacturing facility and the product be approved. All licensed products are subject to specific requirements for lot release by the FDA. In comparison, drugs are approved under section 505 of the FD & C act (21 USC 301–392), where there is not lot release by the FDA except for insulin products. Insulin, growth hormone, and many other hormones have been treated as drugs, whereas erythropoietin (EPO), which also fulfills the criteria of a hormone, was reviewed in the biologic division of the FDA. Insulin is derived from a bacterial fermentation; EPO is obtained from mammalian cell culture. Hormones, for the most part, are expected to be reviewed as drugs.

The design of bioseparation unit operations is influenced by these governmental regulations. The constraints on process development grow as a recovery and purification scheme undergo licensing for commercial manufacture.

5. Protein Chromatography

Proteins and nucleotides are macromolecular biomolecules. Mixtures of biomolecules are fractionated based on differences in charge; molecular weight, shape, and size; solubility in organic solvents; surface hydrophobic character; and types of active sites using ion-exchange, size-exclusion (gel-permeation), and reversed-phase, hydrophobic interaction (surface hydrophobicity), and affinity

chromatographies, respectively. The appropriate separation may be selected from these five basic classes of chromatography. More than 30% of the purification steps for laboratory-scale protein purification procedures use ion-exchange and/or gel filtration, and at least 20% use affinity chromatography (23). This pattern is likely to be consistent with industrial practice. The following represent some chromatography column options for biopharmaceutical proteins (10). Sepharose, Sephadex, and Sephacryl resins are supplied by Pharmacia; Spherodex, Spherosil, and Trisacryl resins are supplied by Sepracor, Inc.; Toyopearl resins are supplied by TosoHaas; Fractogel resins are supplied by E. Merck Separations; Bakerbond resins and silicas are supplied by J. T. Baker. For adsorption, silica may be used.

Ion exchange	*Gel permeation*	*Hydrophobicity*
DEAE Sepharose Fast Flow LC	Agarose, 16%	Toyopearl Bulyl-650 M
DEAE Sepharose Fast Flow HC	Sephadex G25 Medium	Bulyl Spherodex M
DEAE Spherodex M	Sephadex G75	Toyopearl Ether-650 M
DEAE Spherosil M	Trisacryl Plus GF 03 M	Octyl Spherodex M
DEAE Trisacryl Plus M	Trisacryl Plus GF 10 M	Phenyl Spherodex M
Toyopearl DEAE-650 (M)	Trisacryl Plus GF 20 M	Toyopearl Phenyl-650 M
Fractogel EMD DMAE-650 (M)	Sephacryl S-100HR	Bakerbond Hi-Propyl
Fractogel EMD DEAE-650 (M)	Sephacryl S-200HR	
Q Sepharose Fast Flow	Toyopearl HW-50F	
QMA Spherodex M	Toyopearl HW-55F	
QMA Spherosil M		*Affinity*
QMA Trisacryl Plus M		Blue Sepharose CL-6B
Toyopearl QAE-550 C		Red Sepharose CL-6B
SP Sepharose Fast Flow		Blue Spherodex M
SP Sepharose High Performance		Baseline Blue Trisacryl M
SP Sepharose Big Bead		Heparin Sepharose CL-6B
SP Trisacryl Plus M		Heparin Spherodex M
Toyopearl SP-650 M		Toyopearl AF-Chelate-650 M (Copper)
Fractogel EMD SO_3-650 M		Toyopearl AF-Chelate-650 M (Zinc)

Reversed-phase chromatography is widely used as an analytical tool for protein chromatography, but it is not as commonly found on a process scale for protein purification because the solvents which make up the mobile phase, ie, acetonitrile, isopropanol, methanol, and ethanol, reversibly or irreversibly denature proteins. Hydrophobic interaction chromatography appears to be the least common process chromatography tool, possibly owing to the relatively high costs of the salts used to make up the mobile phases.

5.1. Liquid Chromatographs. The basic equipment for liquid chromatography is shown in Figure 4. The column is packed with an adsorbent, ie, the stationary phase. The mixture to be separated is pushed through the column by the eluent or mobile phase (26). Isocratic chromatography, carried out at a constant flow rate, buffer composition, and temperature, is usually associated with size-exclusion separations. Gradient chromatography typically uses a constant flow rate and temperature, but the composition of the element is altered by

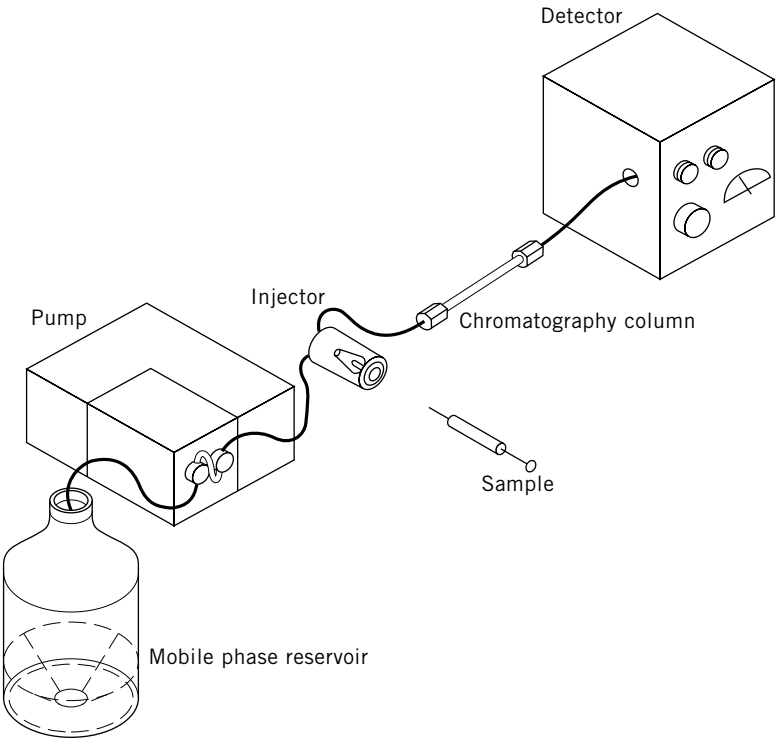

Fig. 4. Schematic of a process liquid chromatography system (16).

mixing two or more buffer reservoirs to achieve a steadily changing salt concentration or changes in pH. The gradients formed are reported in terms of concentration at the inlet of the chromatography column; protein is detected at the column outlet. A chromatogram of the type illustrated in Figure 5 results.

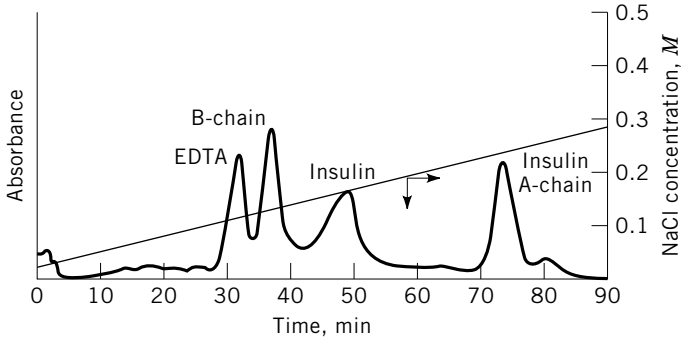

Fig. 5. Anion-exchange separation of insulin, and insulin A- and B-chains, over diethylaminoethyl (DEAE) in a 10.9×200 mm column having a volume of 18.7 mL. Sample volume is 0.5 mL and protein concentration in 16.7 m*M* Tris buffer at pH 7.3 is 1 mg/mL for each component in the presence of EDTA. Eluent (also 16.7 m*M* Tris buffer, pH 7.3) flow rate is 1.27 mL/min, and protein detection is by uv absorbance at 280 nm. The straight line depicts the salt gradient. Courtesy of the American Chemical Society (48).

The concentration profile of the gradient at the outlet of the column can be significantly different from the profile at the inlet when a component (referred to as a modulator) in the eluting buffer adsorbs onto the stationary phase in a non-ideal manner causing the gradient to deform as it passes through the column. What appears to be anomalous peak behavior can result including self-concentration of a peak and the appearance of shoulders or multiple peaks for single sample components known to be homogeneous. This can occur for gradients used in reversed-phase, ion-exchange, or affinity chromatography (27–29).

5.2. Ion-Exchange Chromatography. Ion-exchange chromatography is initiated by eluting an injected sample through a column using a buffer but no NaCl or other displacing salt. The protein, which has charged sites spread over its surface, displaces anions or cations previously equilibrated on the stationary phase, ie, the protein sites exchange with the salt counterions associated with the ion-exchange stationary phase. A protein having a greater number and/or density of charged sites displaces or exchanges more ions and hence binds more strongly than a protein having a lower charge number or charge density.

Proteins deform and change shape in response to the environment. Hence, a protein left on the surface of an ion-exchange resin for a day or longer may slowly start to unfold exposing an increasing number of charged sites to bind with the ion-exchange resin. It is possible that this process can continue until the protein binds so strongly that it is impossible to desorb the protein without dissolving it, in NaOH, for example, and destroying it. To prevent such a situation, ion-exchange chromatography must be completed in a matter of hours or less.

After the column is loaded, proteins of similar size and shape are separated by differential desorption from the ion exchanger by using an increasing salt gradient of the mobile phase. The more weakly bound macromolecules elute first; the most tightly bound elute last, at the highest salt concentration. Figure 5 is an example of an anion-exchange separation (48). Prior to injection of the sample, the column was equilibrated with the 16.7 m*M* Tris buffer; the EDTA stabilized the solubility of the insulin sample injected onto the column. All of the proteins are initially retained on the anion-exchange stationary phase (DEAE 650 M) during loading of the sample onto the column. Subsequent application of the NaCl gradient, formed by the controlled mixing of buffers from two reservoirs of mobile phase, elutes the proteins. One reservoir contains only the 16.7 m*M* Tris buffer; the second contains 0.5 *M* NaCl in the same buffer. Following the elution of the last peak, the column may be flushed using a buffer at a high (2.5 *M* NaCl) salt concentration to verify that all proteins are desorbed. In some cases, a cleaning procedure is performed by passing methanolic NaOH through the column. The column is then re-equilibrated using the salt-free buffer, by pumping approximately 10 column volumes of the buffer through the stationary phase, or until the pH of the effluent and influent are the same to prepare the column for another injection.

Amphoteric Properties Determine Conditions for Ion-Exchange Chromatography. Proteins, amphoteric polymers of acidic, basic, and neutral amino acid residues, carry both negatively and positively charged groups on the surface, the ratio depending on pH (30). The isoelectric point (pI) is the pH at which a protein has an equal number of positive and negative charges. Proteins in solutions at a pH > pI have a net negative charge. Below the pI,

Table 2. **Ion-Exchange Groups Used in Protein Purification**[a]

Name	Abbreviation	Formula
	Weak anion	
aminoethyl	AE	—$C_2H_4NH_3^+$
diethylaminoethyl	DEAE	—$C_2H_4NH(C_2H_5)_2^+$
	Weak cation	
carboxy	C	—COO^-
carboxymethyl	CM	—CH_2COO^-
	Strong anion	
trimethylaminoethyl	TAM	—$CH_2N(CH)_3^+$
triethylaminoethyl	TEAE	—$C_2H_4N(C_2H_5)_3^+$
diethyl-2-hydroxypropyl-aminoethyl	QAE	—$C_2H_4N^+(C_2H_5)_2CH_2CH(OH)CH_3$
	Strong cation	
sulfo	S	—SO_3^-
sulfomethyl	SM	—$CH_2SO_3^-$
sulfopropyl	SP	—$C_3H_6SO_3^-$

[a] Courtesy of IRL Press (30).

proteins have a net positive charge. Many proteins have a pI < 7 and are processed using buffers having a pH of 7 to 8. Thus anion exchangers (positively charged stationary phases) are popular for protein chromatography. Ion-exchange matrices derivatized having negatively charged groups are cation exchangers. These bind positively charged proteins, ie, cations, when the mobile phase pH is <pI.

The selection of the pH of the buffer, as well as the type of ion-exchange (anion or cation) stationary phase is a function of the amphoteric nature of the protein and protein stability as a function of pH. For example, for a protein stable at pH > 6.5, having pI $= 5.5$, an anion exchanger is appropriate when the separation is run in a buffer of pH > 6.5. If this protein were stable at pH $= 5$, and the pI $= 5.5$, a cation exchanger and buffer of pH < 5.0 would be appropriate.

The ion-exchange (qv) groups used to derivatize stationary phases for the purification of proteins are summarized in Table 2. Corresponding buffers are given in Table 3. Strong anion and cation exchangers are almost fully ionized

Table 3. **Buffers for Ion-Exchange Chromatography**[a]

Buffer	p*K*	Buffering range
	Anion exchange	
L-histadine	6.15	5.5–6.0
imidazole	7.00	6.6–7.1
triethanolamine	7.77	7.3–7.7
Tris	8.16	7.5–8.0
diethanolamine	8.80	8.4–8.8
	Cation exchange	
acetic acid	4.76	4.8–5.2
citric acid	4.76	4.2–5.2
Mes	6.15	5.5–6.7
phosphate	7.20	6.7–7.6
hepes	7.55	7.6–8.2

[a] Courtesy of IRL Press (30).

at pH = 3–11 and coincide with the pH range of protein purification. Weak anion and cation exchangers have a narrower pH range over which they are ionized. Anion exchangers are preferred because desorption of the protein is more readily accomplished at lower salt concentrations.

Size Exclusion (Gel-Permeation) Chromatography. Size-exclusion chromatography is often referred to as gel-permeation chromatography because the stationary phases are usually made up of soft spherical particles which resemble gels. Separation occurs by a molecular sieving effect (see MOLECULAR SIEVES; SIZE SEPARATION). The larger molecules, which explore less of the intraparticle void fraction (ie, pores) than smaller molecules, elute first because the former spend less time inside the stationary phase than the latter. Separation can be achieved if the porosity of the stationary phase is properly selected and there is a significant difference in the sizes of the molecules to be separated. This size difference is measured in terms of hydrodynamic ratio. To select the stationary phase having the appropriate pore size requires that the size of the proteins be known.

The apparatus utilized to carry out size-exclusion (gel-permeation) chromatography is analogous to that used for isocratic operating conditions (see Fig. 4). The column is packed with a gel-filtration stationary phase, selected according to the molecular weight of the protein of interest (31). A variety of commercially available gel-filtration matrices facilitates separations ranging from molecular weights of 50 to 10^8 (Fig. 6). However, a single gel having a porosity which is capable of sieving molecules over the entire separation range does not exist.

An example of a size-exclusion chromatogram is given in Figure 7 for both a bench-scale (23.5 mL column) separation and a large-scale (86,000 mL column) run. The stationary phase is Sepharose CL-6B, a cross-linked agarose with a nominal molecular weight range of $\sim 5000 - 2 \times 10^6$ (see Fig. 6) (31).

Buffer Exchange and Desalting. A primary use of size-exclusion chromatography (sec) is for removal of salt or buffer from the protein, ie, desalting and buffer exchange (32). The difference in molecular weights is large; salts generally have a mol wt < 200, whereas mol wts of proteins are between 10,000 and 60,000.

Alternative methods of desalting and buffer exchange include continuous diafiltration, countercurrent dialysis (ccd), a membrane separation technique, and cross-flow filtration, which uses membranes (see MICROBIAL AND VIRAL FILTRATION). Both of those methods rely on filtration at a molecular scale, using membranes having porosities which reject proteins but allow passage of salts. Membrane methods are often preferred for an unspecified protein because these procedures are less costly and enable higher throughput than size-exclusion chromatography. Buffer exchange, used to remove denaturing agents in order to induce refolding of proteins, to remove buffers between purification steps, or to remove buffers and other reagents from the final product, is usually carried out at later steps in a recovery sequence (see Figs. 2 and 3). Equations for calculating separation efficiencies and recovery yields for all three methods for a specific case study using a recombinant protein of 160,000 mol wt are available (32). Size-exclusion chromatography using Sephadex G-25M gel-filtration media in this case had disadvantages compared to the membrane filtration techniques giving 100 × less complete ion removal, 130–1200% greater dilution, 30% higher cost, 66% higher (eluting) buffer requirement, 50% higher space on the plant

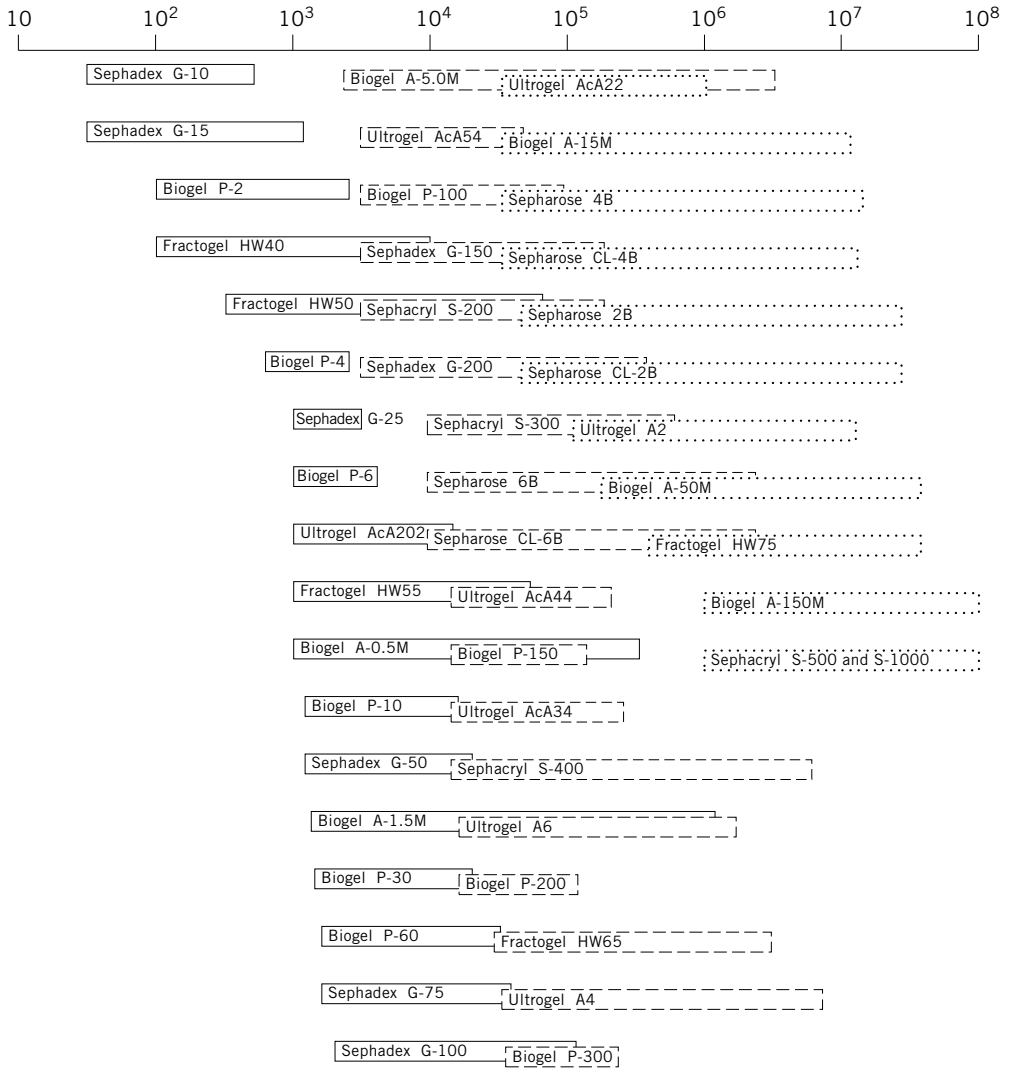

Fig. 6. Fractionation ranges of commercially available gel-filtration matrices: (□) small, (⬚) medium, and (⋯) large (31).

floor, 50% higher operating time, and half the throughput. However, cross- or tangential-flow filtration (tff) required up to 90 passes through a pump whereas sec and ccd were single pass. Other disadvantages of tff include frequent change-out of membranes and relatively large volumes of protein feed being required for laboratory-scale tests, a particular disadvantage for recombinant products. The more recent testing of a novel size-exclusion stationary phase, however, which facilitates rapid preparative (process-scale) separation of salts from protein in less than seven minutes, shows that process size-exclusion chromatography is capable of high throughput while reducing the volume needed to obtain proper refolding of the protein. Salts causing the protein to be denatured were rapidly

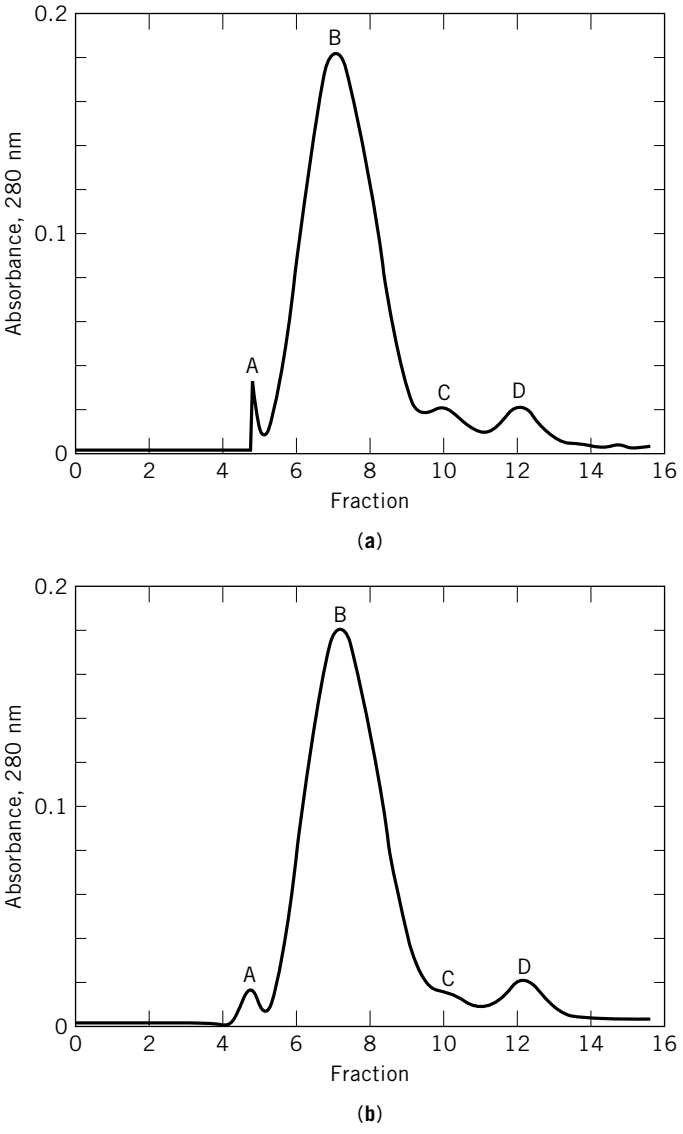

Fig. 7. Chromatograms of size-exclusion separation of IgM (mol wt = 800,000) from albumin (69,000) where A–D correspond to IgM aggregates, IgM, monomer units, and albumin, respectively, using (**a**) FPLC Superose 6 in a 1×30-cm long column, and (**b**) Sepharose CL-6B in a 37-cm column. Courtesy of American Chemical Society (24).

removed and reduced to a level where protein refolding occurred (33). The use of sec is likely to continue to be a widely practiced technology in industry. Rapid size-exclusion columns for the purpose of buffer exchange have been developed which enable desalting to be achieved at linear velocities of 500–600 cm/h (33), significantly increasing throughput and reducing operating time and plant floor space. Further, sec using gel-filtration media on cellulosic-based material has a special niche for the partial and controlled separation of denaturing salts

from recombinant proteins for purposes of refolding. The development of such rapid desalting techniques is important because of the larger volumes of proteins needing to be processed in industry.

Column Size. Size-exclusion chromatography columns are generally the largest column on a process scale. Separation is based strictly on diffusion rates of the molecules inside the gel particles. No proteins or other solutes are adsorbed or otherwise retained owing to adsorption, thus, significant dilution of the sample of volume can occur, particularly for small sample volumes. The volumetric capacity of this type of chromatography is determined by the concentration of the proteins for a given volume of the feed placed on the column.

The volume of the solvent between the point of injection and the peak maximum of the eluting protein is defined as the elution volume, V_e (Fig. 8). The fluid volume between the particles of the stationary phase is the extraparticulate void volume or exclusion volume, V_o. The porosity of the stationary phase, determined by the extent of cross-linking of the polymers which make up the particles of a gel-permeation matrix, determines the extent to which a protein or other solute can explore the intraparticulate void volume. The higher the cross-linking, the smaller the effective pore size and the lower the molecular weight (or size) of the molecule which is excluded from the gel. Hence, the apparent porosities of a gel-permeation column are a function of the molecular probes used to measure it. For a large molecule that is completely excluded, the void volume is equivalent to the extraparticulate void volume, V_o. Molecules that are small enough to penetrate the gel have an elution volume $> V_o$. A small molecule, such as a salt, can potentially explore almost all of the bed volume and has the following elution volume:

$$V_e = V_o + K_d V_s \tag{2}$$

where K_d represents the fraction of the volume of the mobile phase inside the particle, V_s, which can be explored by the molecular probe. For a probe small enough to explore all of the intraparticle void volume, K_d is 1 and the elution volume is $V_o + V_s$. Because the combined volume of the fluid between the particles, V_o, and inside the particles, V_s, cannot exceed the total volumes of the column, V_t, V_e must be less than column volume V_t. All components injected into a size-exclusion column thus elute in one column volume.

The total stationary-phase volume required to process a given feed stream is proportional to the inlet concentration and volume of the feed. For example, for a typical inlet concentration of protein of 10 g/L, in a 100 L volume of feed, a column volume of at least 100 L is needed for size-exclusion chromatography. In comparison, an ion-exchange column having an adsorption capacity of 50 g/L would only require 20 L of column volume for the same feed.

Elution and Sample Volumes. The elution volume, V_e, is measured from the beginning of the injection to the center of the peak maximum for a Gaussian peak, if the sample volume is negligible relative to the elution volume (see Fig. 8**a**). A negligible injection volume is defined as being $\leq 2\%$ of the elution volume. The elution volume for samples larger than this are measured from the halfway point of the volume injected to the center of the eluting peak (see Fig. 8**b**). In samples which are so large that a plateau region is obtained having

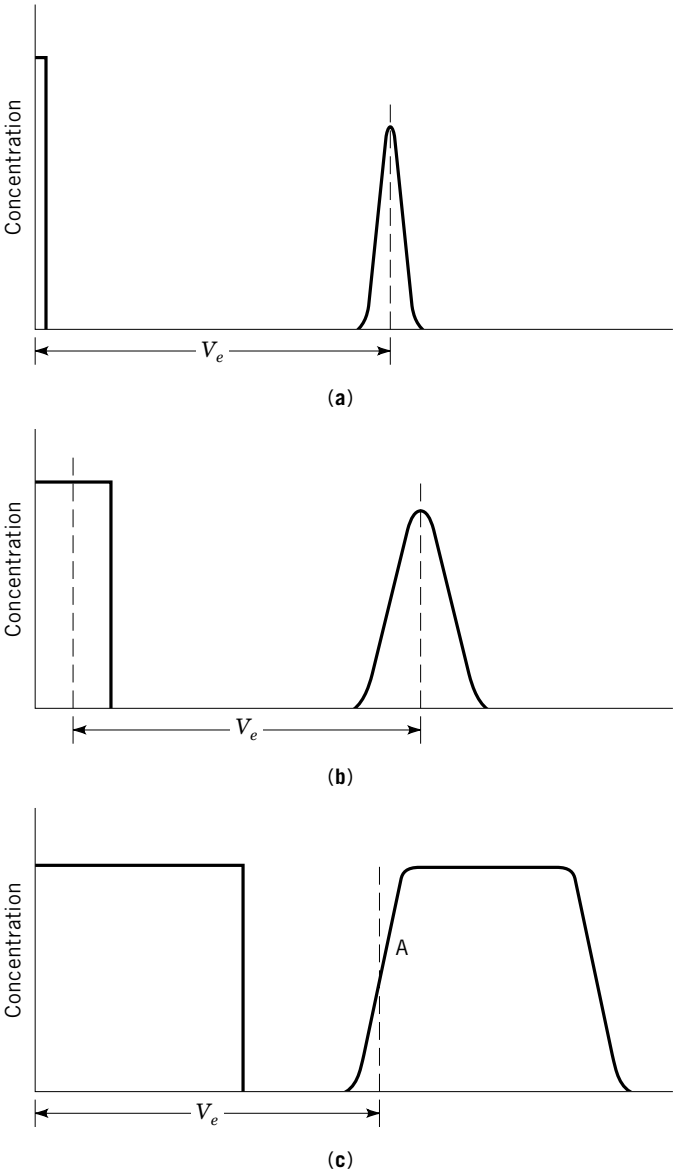

Fig. 8. Representation of measurement of elution volume, V_e, as a function of sample volume: (**a**) <2% of bed volume, (**b**) >2% and (**c**) >2% and giving a plateau region which has the same concentration as the injected sample; A represents the inflection point. See text. Courtesy of Pharmacia (34).

the same concentration as the sample (see Fig. 8**c**), the elution volume is measured from the start of the sample injection to the inflection point of the peak.

Sample dilution in gel permeation can be 10-fold or more for small volumes. Hence, proper representation of the inlet concentration (eg, see Fig. 8**a**), would require that the injection pulse be much higher because the area under the eluting peak and under the injected sample should be the same. Similarly, dilution of

Table 4. **Properties of Standard Proteins**[a]

Protein	Mol wt	pI	Radius, nm	Asymmetry, f/fo[b]
bovine serum albumin	66,000	5.74	3.50	1.29
ovalbumin	45,000	5.08	2.78	1.16
α-lactalbumin	14,200	4.57	2.30	1.18
myoglobin	16,900	7.10	2.40	1.18

[a] Adapted from Ref. 53.
[b] Frictional coefficients of f, solvated protein, and fo, nonsolvated sphere.

the injected sample would occur in the cases represented by Figures 8**b** and 8**c**, although the difference in heights between injected pulse and the maximum of the eluting peak would be smaller, because diffusion of the solute away from the center of mass occurs at the leading (left side) and tailing (right side) edges of the peak. If the peak is broad enough, ie, the sample volume is large enough, the solute at the center of the peak is not diluted owing to diffusion and the peak maximum is therefore equal to that of the injected sample.

Distribution Coefficients. Gel-permeation stationary-phase chromatography normally exhibits symmetrical (Gaussian) peaks because the partitioning of the solute between mobile and stationary phases is linear. Criteria more sophisticated than those represented in Figure 8 are seldom used (34).

The elution volume, V_e, and therefore the partition coefficient, K_D, is a function of the size of solute molecule, ie, hydrodynamic radius, and the porosity characteristics of the size-exclusion media. A protein of higher molecular weight is not necessarily larger than one of lower molecular weight. The hydrodynamic radii can be similar, as shown in Table 4 for ovalbumin and α-lactalbumin. The molecular weights of these proteins differ by 317%; their radii differ by only 121% (53).

Some types of size-exclusion phases, based on silica or macroporous polymeric materials, having rigid pores and defined pore size distributions. The dominant types of gels used in industry are dextran cross-linked epichlorophydran (Sephadex), agarose prepared from agar (Sepharose), or allyl dextran cross-linked with *N,N′*-methylenebisacrylamide (Sephacryl). These materials imbibe significant quantities of water and have bed volumes ranging from 2 to 3 mL/g dry weight of stationary phase for Sephadex G-10 (nominal molecular weight cutoff of 10,000) to 20–25 mL/g dry weight of stationary-phase Sephadex G-200 (nominal molecular weight cutoff of 200,000). Their structures resemble a cross-linked spider web, where the extent of cross-linking or association between hydrated polymer chains, rather than specific pore sizes, determine the apparent pore size distribution. The hydrophilic character of the polymers which make up these gels require cross-linking to prevent dissolution. The hydrophilic character is compatible with the majority of industrially relevant proteins, most of which can be denatured by hydrophobic surfaces but preserved in active confirmation at hydrophilic conditions. This property can be offset by poor flow properties, however, particularly for lightly cross-linked gels, because these gels are soft and have a tendency to compress when flow rates exceed a threshold which decreases with decreasing extents of cross-linking. Hence, Sephadex G-10 has the highest cross-linking and flow stability, and the lowest specific bed volume,

Table 5. **Comparison of Gel-Permeation Stationary Phase**[a]

Sephadex G-X[b]	Specific volume water mL/g dry gel	Permeability, K_o	Operating pressure[c], kPa[d]	Flow rate[c] water, mL/(cm^2 · h)
10	2–3	19	[f]	[f]
15	2.5–3.5	18	[f]	[f]
25	4–6	9–290[e]	[f]	[f]
50	9–11	13.5–400[e]	[f]	[f]
75	12–15		160	77
100	15–20		96	50
150	20–30		36	23
200	30–40		16	12

[a] Adapted from Ref. 34.
[b] Corresponds to the nominal cutoff value for wt $\times 10^3$, eg, G-10 has a mol wt cutoff value of $\sim 10,000$.
[c] Value is maximum unless otherwise noted.
[d] To convert kPa to cm H_2O, multiply by 10.2.
[e] Depends on particle size (dp); as dp increases, K_o increases.
[f] May be calculated using Darcy's law: $U = K_o\ (\Delta P/L)$, where U is linear flow as mL/(cm^2·h), L is bed length in cm, ΔP is pressure drop over gel bed in kPad, and the maximum pressure is 30.4 kPad (310 cm H_2O).

but also has the lowest effective pore size or porosity, limiting its sieving capabilities. Sephadex G-200 has the lowest cross-linking and flow stability and the highest specific bed volume and effective pore size. Sephadex 200 is useful for separating high molecular weight proteins, but at relatively low flow rates (Table 5).

The distribution coefficient, K_d, represents the fractional volume of a specific stationary phase explored by a given solute, represented by equation 3:

$$K_d = \frac{V_e - V_o}{V_s} \tag{3}$$

where V_o is the void volume, V_s is the volume of the solvent (usually acqueous buffer) inside the gel which is available to very small molecules, and V_e is the elution volume of a small volume of injected molecular probe. The measurement of V_s is difficult, requiring use of an ion or small molecule which freely diffuses into all of the fluid volume inside the gel particles and then is readily detected at the outlet of the column. D_2O and radioactive ^{23}Na have been used. The latter is detected by a refractive index detector. An indirect measurement of V_s is more convenient and adequate. The column void volume (Fig. 9) may be measured using a soluble, high molecular weight target molecule which, because it does not explore any of the internal fluid volume of the stationary phase, it only distributed in the mobile phase. Blue dextran, a water-soluble, sulfonated, blue-colored dextran having mol wt $> 669,000$, manufactured by Pharmacia, and DNA (Type III from salmon tests) have been employed (26). The total column volume, V_t, can be calculated from the dimensions of the bed, although the direct measurement of column volume using water displacement before packing is more accurate. (It should be noted that total column volume is also represented by V_c in the recent literature). The difference, $V_t - V_o$, is then taken as an approximation of V_s. On this basis, K_{av}, the fraction of stationary phase volume

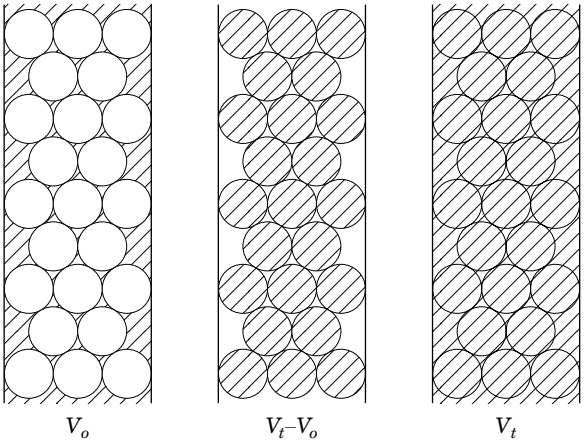

Fig. 9. Diagrammatic representation of V_t and V_o. $V_t - V_o$ includes the volume of the solid material forming the matrix of each bead. Courtesy of Pharmacia (34).

available for a given solute species, is defined as in equation 4:

$$K_{\text{av}} = \frac{V_e - V_o}{V_t - V_o} \tag{4}$$

The constant K_{av}, is not a true partition coefficient because of difference, $V_t - V_o$, includes the solids and the fluid associated with the gel or stationary phase. By definition, V_s represents only the fluid inside the stationary-phase particles and does not include the volume occupied by the solids which make up the gel. Thus K_{av} is a property of the gel, and like K_d it defines solute behavior independently of the bed dimensions. The ratio of K_{av} to K_d should be a constant for a given gel packed in a specific column (34).

Selectivity curves result from measured values of K_{av} plotted vs log (mol wt) enabling molecular weight determination of globular proteins having similar asymmetry factors. A sphere has an asymmetry factor of 1; an ellipsoid has a factor >1 (51). Such curves are linear (Fig. 10), the intercept increasing with increasing porosity (decreasing cross-linking). Extrapolation of these curves through the x-axis yields the molecular weights of probes which should be completely excluded from the gels because these target molecules are larger than the largest pores. Theoretically, the K_{av} for a given molecular probe should have a value between 0 and 1. A completely excluded molecule has $K_{\text{av}} = 0$; molecules able to completely explore the fluid inside the stationary-phase particle have $K_{\text{av}} = 1$. If K_{av} is less than zero, then channeling owing to a poorly packed bed is a probably cause. If the K_{av} is greater than 1, an interaction (adsorption) of the molecular probe with the stationary phase is a likely explanation.

Gel-permeation media are extremely versatile and may be used for separation of particles such as viruses (Fig. 11) as well as proteins (34). Separations of proteins and other particles having sizes equivalent to a molecular weight of 40×10^6 are possible using the agar-based Sepharose-type gel. This particular

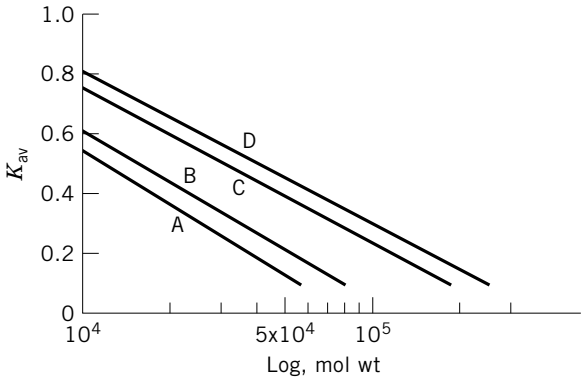

Fig. 10. Selectivity curves A–D for Sephadex G-75, G-100, G-150, and G-200, respectively, for globular proteins. Courtesy of Pharmacia (34).

gel has a limited temperature range for operation, however. It melts upon heating to 40°C (34).

The gels having a larger extent of cross-linking, particularly Sephadex G-10, G-15, and G-25, may retain through weak adsorption some types of aromatic molecules, and consequently impart reversed-phase properties to the gel. This may be the result of the weakly hydrophobic character of the cross-linking agents used in the synthesis of these gels. A recently introduced product, based on crosslinked agarose combined with crosslinked dextran (Superdex), exhibits low nonspecific interactions as well as enhanced resolution (34). An excellent overview of gel permeation chromatography is given in Reference 51.

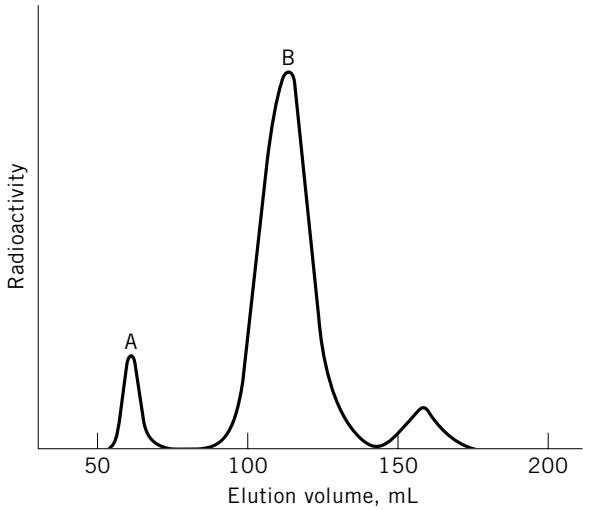

Fig. 11. Separation of ^{32}P-labeled A, adenovirus Type 5, and B, poliovirus Type 1 on Sepharose 2B where the column is 2.1×56 cm; eluent, 0.002 *M* sodium phosphate, pH 7.2, containing 0.15 *M* NaCl; and flow rate, 2 mL/(cm^2 · h). Courtesy of Pharmacia (34).

Reversed-Phase Chromatography. Stationary phases for reversed-phase chromatography consist principally of silica particles, silica supports having a hydrocarbon bonded phase, or polymeric materials based on either vinyl or styrene–divinylbenzene copolymers (35–41). Mobile phases commonly used in reversed-phase chromatography are aqueous methanol, 2-propanol, and acetonitrile. These solvents are often mixed with acidic buffers containing small amounts of acids such as trifluoroacetic acid or hexafluorobutyric acid. These acids reduce the pH of the mobile phase to 3 and give sharper peaks by suppressing ionization of silanol groups of silica-based reversed-phase supports, and minimizing ionic effects (42).

The most prevalent type of stationary phase is made of silica or another type of inorganic support derivatized and bonded with an octadecyl (C_{18}) or octyl (C_8) coating. Nonderivatized silicas are sometimes utilized for process chromatography. Much like ion-exchange chromatography, the organic component, sometimes referred to as a modifier, is mixed with water or buffer to form an increasing gradient of the modifier. This gradient serves to elute the components, which are initially adsorbed onto the stationary phase in order of increasing hydrophobic character. Methanol is used as the modifier for eluting weakly adsorbed, hydrophilic peptides; 2-propanol is used to elute strongly adsorbed, hydrophobic peptides. Acetonitrile is widely used for separations of proteins and many other types of molecules because this solvent exhibits favorable mass-transfer properties, lower viscosity (and back-pressure) than the other solvents, and good eluting strength. Methanol has a higher aqueous heat of mixing than the other solvents, and this can lead to solvent degassing and bubble formation in the column. Bubbles interfere both with the operation of the column, causing peak dispersion, and detection of the peaks at the column outlet. Bubbles give anomalous peaks in spectrophotometric detectors and refractive index detection (42).

Product Monitoring and Peptide Mapping. Reversed-phase chromatography is widely used for analysis of proteins. Historically, the principal use of reversed-phase chromatography has been in the analysis and process separations of peptides, amino acids, and organic compounds which are characterized by lower molecular weight and solubility in acetonitrile or alcohol gradients. These mobile phases denature proteins and some polypeptides. Hence, reversed-phase chromatography is infrequently used for process-scale purification of proteins. Rather the excellent protein resolving power of this type of chromatography is employed on an analytical scale using columns packed with 2–10 mL of stationary phases having 1–5 μm particle sizes and for sample volumes which typically range from 1 to 10 μL.

One purpose in monitoring a protein product is to detect the presence of a change in which as little as one amino acid has been chemically or biologically altered or replaced during the manufacturing process. Variant amino acid(s) in a protein may not affect protein retention during reversed-phase chromatography if the three-dimensional structure of the polypeptide shields the variant residue from the surface of the reversed-phase support (20). Reversed-phase chromatography discriminates between different molecules on the basis of hydrophobicity. Because large proteins may contain only small patches of hydrophobic residues, these patches may not correlate to the molecular modifications which a

reversed-phase analytical method seeks to detect. The reversed-phase method must therefore be completely validated, and preferably combined with controlled chemical and/or proteolytic hydrolysis followed by chromatography or electrophoresis (see ELECTROSEPARATIONS) of the cleared protein to give a map of the resulting peptide fragments (20,43).

A peptide map is generated by cleaving a previously purified protein using chemicals or enzymes. Hydrolytic agents having known specificity are used to perform limited proteolysis followed by resolution and identification of all the peptide fragments formed. Identification of changes, and reconstruction of the protein's primary structure, is then possible. Reagents and enzymes which cleave specific bonds are discussed in the literature (44).

An example of a peptide map generated by trypsin hydrolysis of recombinant tissue plasminogen activator (rt-PA) is shown in Figure 12. The chromatogram shows the resolving power or reversed-phase high performance liquid chromatography in separating peptides obtained from t-PA in which the disulfide bonds had been reduced and alkylated prior to enzyme hydrolysis. The small peptides formed have little or no three-dimensional structure. Hence, measurable shifts in elution profiles occur when there are variant amino acids because a single amino acid change in a peptide has a larger effect on its solubility and retention than the same change has in a protein. The replacement of arginine at position 275 in a normal t-PA molecule with glutamic acid results in a significant peak shift (see Fig. 12) (43), showing how tryptic mapping can be a suitable method for monitoring lot-to-lot consistency of this particular recombinant product (20).

Reversed-phase high performance liquid chromatography has come into use for estimating the purity of proteins and peptides as well. However, before employed, a high performance liquid chromatographic (hplc) profile of a given protein must be completely validated (43).

Insulin Purification. An example of the purification of recombinant product by reversed-phase chromatography is recombinant insulin, a polypeptide hormone. Insulin consists of 51 amino acid residues in two chains and is relatively small. Reversed-phase chromatography is used after most of the other impurities have been removed by a prior ion-exchange step (see Fig. 1) (12). The method utilizes a process-grade C_8 reversed-phase support (Zorbex) having a particle size of 10 μm (14). Partially purified insulin crystals, dissolved in a water-rich mobile phase, are applied to the column and then eluted in a linear gradient generated by mixing 0.25 *M* aqueous acetic acid to 60% acetonitrile. The acidic mobile phase gives excellent resolution of insulin from structurally similar insulin-like components. The ideal pH is from 3.0 to 4.0, below insulin's isoelectric point, pI = 5.4. Under mildly acidic conditions insulin may deamidate to monodesamido insulin, but if the reversed-phase separation is done within a matter of hours, the deamidation can be minimized.

This reversed-phase chromatography method was successfully used in a production-scale system to purify recombinant insulin. The insulin purified by reversed-phase chromatography has a biological potency equal to that obtained from a conventional system employing ion-exchange and size-exclusion chromatographies (14). The reversed-phase separation was, however, followed by a size-exclusion step to remove the acetonitrile eluent from the final product (12,14).

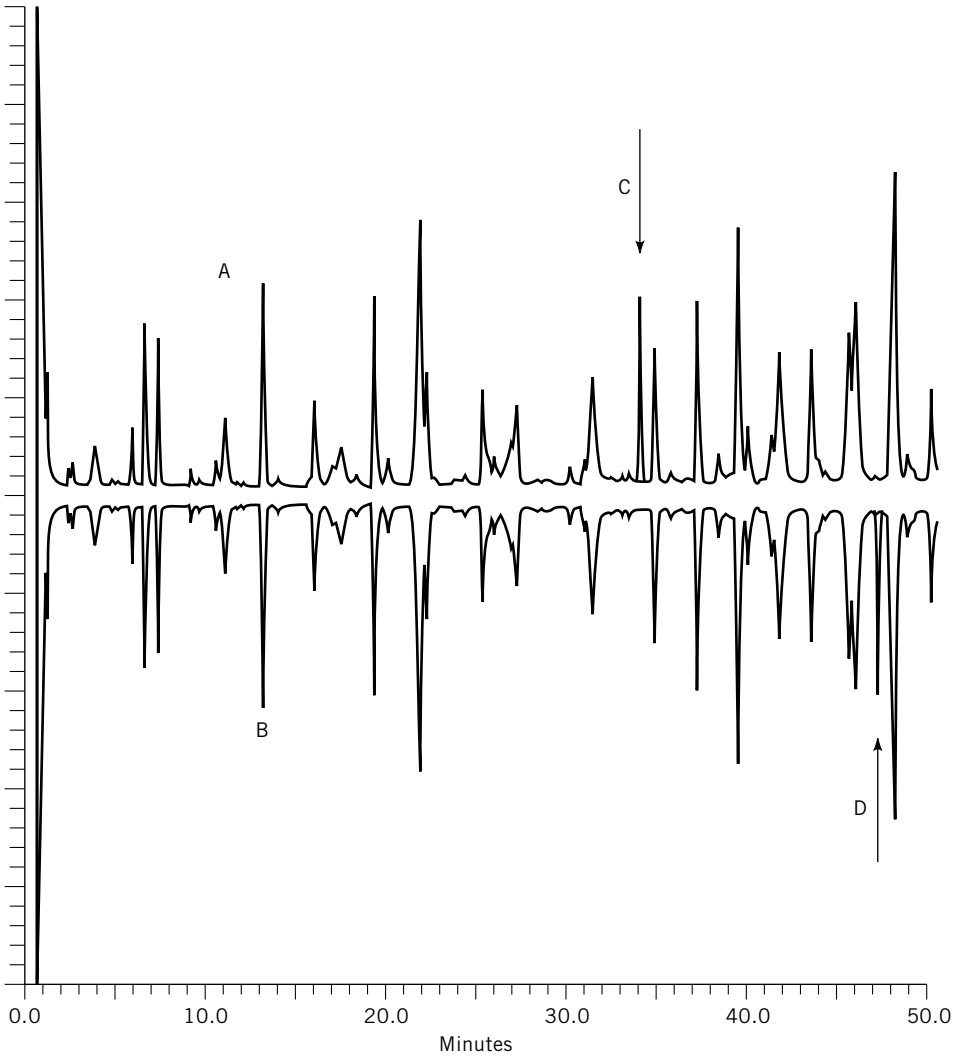

Fig. 12. Tryptic map of rt-PA (mol wt = 66,000) showing peptides formed from hydrolysis of reduced, alkylated rt-PA. Separation by reversed-phase octadecyl (C_{18}) column using aqueous acetonitrile with an added acidic agent to the mobile phase. Arrows show the difference between A, normal, and B, mutant rt-PA where the glutamic acid residue, D, has replaced the normal arginine residue, C, at position 275. Courtesy of Marcel Dekker (43).

Whereas recombinant proteins produced as inclusion bodies in bacterial fermentations may be amenable to reversed-phase chromatography (42), the use of reversed-phase process chromatography does not appear to be widespread for higher molecular weight proteins.

Reversed-Phase Process Chromatography. Polypeptides, peptides, antibiotics, alkaloids, and other low molecular weight compounds are amenable to

process chromatography by reversed-phase methods. There are numerous examples of bioproducts which have been purified using reversed-phase chromatography. The manufacture of salmon calcitonin, a 32-residue peptide used for treatment of post-menopausal osteoporosis, hypercalcemia, and Paget's disease of the bone, includes reversed-phase chromatography. This peptide, commercially prepared on a kilogram scale by a solid-phase synthesis, is then purified by a multimodal purification train. Reversed-phase chromatography is the dominant technique used by Rhône-Poulenc Rorer (45).

Another example is the purification of a β-lactam antibiotic, where process-scale reversed-phase separations began to be used around 1983 when suitable, high pressure process-scale equipment became available. A reversed-phase microparticulate (55–105 μm particle size) C_{18} silica column, with a mobile phase of aqueous methanol having 0.1 *M* ammonium phosphate at pH 5.3, was able to fractionate out impurities not readily removed by liquid–liquid extraction (37). Optimization of the separation resulted in recovery of product at 93% purity and 95% yield. This type of separation differs markedly from protein purification in feed concentration (≈50–200 g/L for cefonicid vs 1 to 10 g/L for protein), molecular weight of impurities (<5000 compared to 10,000–100,000 for proteins), and throughputs (≈1–2 mg/(g stationary phase · min) compared to 0.01–0.1 mg/(g · min) for proteins).

Reversed-phase separation was also found to purify diastereomer precursors used in the chemical synthesis of the insect sex phermone of *Limantria dispar*, a pest which attacks oak trees. The liquid chromatography columns tested had dimensions of up to 15 cm id by 130 cm long, and were able to purify up to 708 g of starting material in 4.1 L sample using a column having 23 L of stationary phase. The throughput is estimated to have been on the order of 0.2–0.4 mg/(g · min), where separation was obtained using a gradient of hexane and diethyl ether.

Small Particle Silica Columns. Process-scale reversed-phase supports can have particle sizes as small as 5–25 μm. Unlike polymeric reversed-phase sorbents, these small-particle silica-based reversed-phase supports require high pressure equipment to be properly packed and operated. The introduction of axial compression columns has helped promote the use of high performance silica supports on a process scale. Resolution approaching that of an analytical-scale separation can be achieved using these columns that can also be quickly packed. These columns consist of a plunger fitted into a stainless steel column. The particles are placed into the column in a slurry. The plunger then squeezes or compacts the bed in an axial direction to give a stable, tightly packed bed. This type of column must be operated at pressures of up to 10 MPa (100 bar), but also gives excellent resolution in run times of an hour or less (36).

Hydrophobic Interaction Chromatography. Hydrophobic interactions of solutes with a stationary phase result in their adsorption on neutral or mildly hydrophobic stationary phases. The solutes are adsorbed at a high salt concentration, and then desorbed in order of increasing surface hydrophobicity, in a decreasing kosmotrope gradient. This characteristic follows the order of the lyotropic series for the anions: phosphates > sulfates > acetates > chlorides > nitrates > thiocyanates. Anions which precipitate proteins less effectively than chloride (nitrates and thiocyanates) are chaotropes or water structure breakers,

and have a randomizing effect on water's structure; the anions preceding chlorides, ie, phosphates, sulfates, and acetates, are polar kosmotropes or water structure makers. These promote precipitation of proteins. Kosmotropes also promote adsorption of proteins and other solutes onto a hydrophobic stationary phase (46). These kosmotropes have other beneficial characteristics which include increasing the thermal stability of enzymes, decreasing enzyme inactivation, protecting against proteolysis, increasing the association of protein subunits, and increasing the refolding rate of denatured proteins. Hence, utilization of hydrophobic interaction chromotography is attractive for purification of proteins where recovery of a purified protein in an active and stable conformation is desired (46,47).

Salt Effects. The definition of a capacity factor k' in hydrophobic interaction chromatography is analogous to the distribution coefficient, K_{av}, in gel permeation chromatography:

$$k' = \frac{V_e - V_o}{V_o} \tag{5}$$

However, because protein retention owing to adsorption can occur, the value of k' can be greater than one, ie, elution of the most retained peak need not occur after one column volume of mobile phase has passed through the column. The retention behavior of lysozyme on a polymeric hydrophobic interaction support follows the preferential interaction parameter of the lyotropic series of anions (Fig. 13). The preferential interaction parameter is a measure of the net salt inclusion or exclusion of the hydration layer. The higher the value, the larger the disrupting effect of the salt. This analysis led to derivation and experimental validation of the capacity factor for lysozyme with respect the lyotropic number of the anion for a hydrophilic vinyl polymer support having an average particle diameter of 30 μm, and average pore size of 100 nm. This capacity factor has the following

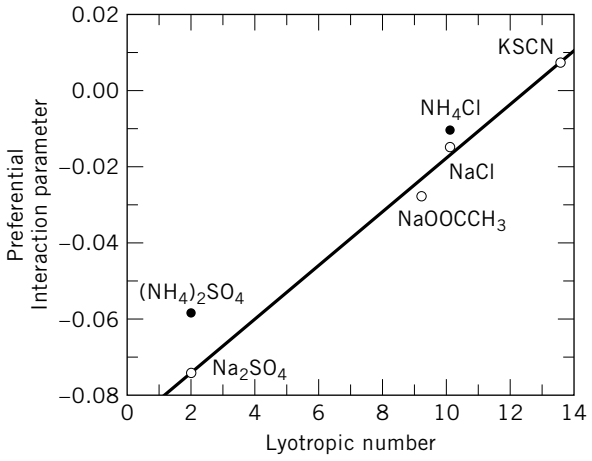

Fig. 13. Preferential interaction parameter vs lyotropic number for lysozyme on (○) bovine serum albumin and (●) Toyopearl. Courtesy of American Chemical Society (47).

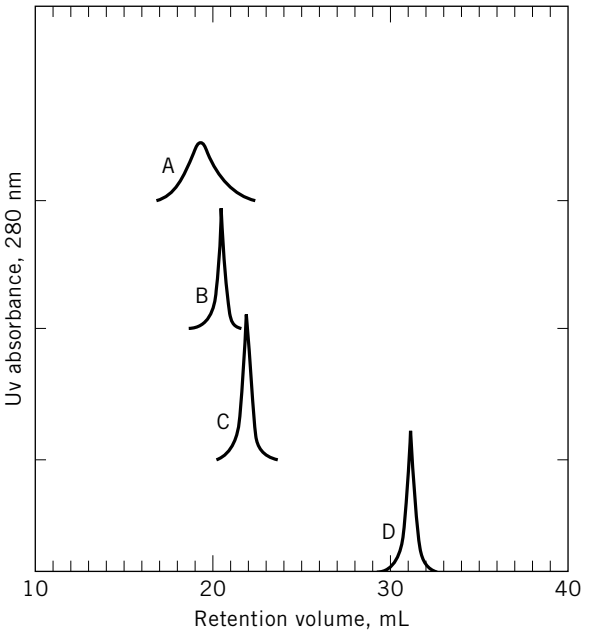

Fig. 14. Chromatographic retention of 20 μL of a 3 mg/mL solution of lysozyme on Toyopearl HW-65S using a 50 cm × 8 min ID column in 1.3 *M* ammonium salt, 20 m*M* Tris mobile phase at 1 mL/min for A, NH_4I; B, NH_4Cl; C, NH_4OOCCH_3; and D, $(NH_4)_2$-SO_4. Courtesy of American Chemical Society (47).

form:

$$K' = a[C]^d\,[N_x - b] + h \tag{6}$$

where *a, b, d*, and *h* are protein specific parameters, N_x is the lyotropic number, and *C* is salt concentration in *M*. Hydrophobic interaction parameters can then be estimated for experimental peak retention data as changes in retention time upon a change in salt or salt concentration. An example of how salt type and concentration affect retention of lysozyme is illustrated in Figure 14. A similar functional relation was found for myoglobin with respect to a hydrophilic vinyl polymer derivatized using butyl groups (47).

Various types of proteins have been purified using hydrophobic interaction chromatography including alkaline phophatase, estrogen receptors, isolectins, strepavidin, calmodulin, epoxide hydrolase, proteoglycans, hemoglobins, and snake venom toxins (46). In the case of cobra venom toxins, the order of elution of the six cardiotoxins supports the hypothesis that the mechanism of action is related to hydrophobic interactions with the phospholipids in the membrane.

The recovery of recombinant chymosin from a yeast fermentation broth showed that large-scale hydrophobic interaction chromatography could produce an acceptable product in one step. Chymosin, which used to be obtained from the lining of the stomachs of calves, is used in cheesemaking, and its cost is an issue. Because the capacity of the hydrophobic interaction stationary-phase is limited, an alternative method has been developed in which the enzyme is extracted into

a two-phase polyethylene glycol (PEG) salt system. The partition for the chymosin into PEG coefficient is 100, and hence enables efficient recovery of this in one step. Together with a subsequent ion-exchange step, this method gives a suitably purified chymosin. The use of hydrophobic interaction chromatography may have helped to indicate that two-phase extraction is a viable approach (10).

5.3. Affinity Chromatography. The concept of affinity chromatography, credited to the discovery of biospecific adsorption in 1910, was reintroduced as a means to purify enzymes in 1968 (49). Substrates and substrate inhibitors diffuse into the active sites of enzymes irreversibly or reversibly binding there. Conversely, if the substrate or substrate inhibitor is immobilized through a covalent bond to a solid particle of stationary phase having large pores, the enzyme should be able to diffuse into the stationary phase and bind with the substrate or inhibitor. Because the substrate is small (mol wt < 500) and the enzyme large ($>15,000$), the diffusion of the enzyme to its binding partner at a solid surface can be sterically hindered. The placement of the substrate at the end of an alkyl or glycol chain tethered to the stationary phase's surface reduces hindrance and forms the basis of affinity chromatography. This concept has also been applied to ion-exchange chromatography under the names of tentacle or fimbriated stationary phases.

The realization that enzymes could be selectively retained in a chromatography column packed with particles of immobilized substrates or substrate analogues led to experiments with other pairs of binding partners. Numerous applications of affinity chromatography developed, given the specific and reversible yet strong affinity of biological macromolecules for numerous specific ligands or effectors. These interactions have been exploited for purposes of highly selective, but often expensive protein purifications, recovery of messenger ribonucleic acid (mRNA) in some recombinant DNA applications, and study of mechanisms of protein binding with effector molecules (49).

Minimization of Nonspecific Binding. The purpose of affinity chromatography is the highly selective adsorption and subsequent recovery of the target biomolecule. Loss of specificity occurs when macromolecules, other than the targeted materials, adsorb onto the stationary phase owing to hydrophobic or ionic interactions. For example, a spacer arm, which allows the binding ligand on the column to be located away from the matrix surface, can improve accessibility and reduce steric hindrance to the immobilized ligand, often decreasing selectivity. Hexamethylenediamine, a common spacer arm used initially in affinity chromatography, has been the source of strong, nonspecific binding. This hydrophobic character has been decreased by interposing an ether or secondary amine, such as 3,3-diaminodipropylamine, in the middle of the spacer arm.

The ideal matrices for anchoring binding ligands are nonionic, hydrophilic, chemically stable, and physically robust. The most popular matrices are polysaccharide based, principly owing to their hydrophilic character and history of use as size-exclusion or gel-permeation gels (see Fig. 6), although glass beads, polyacrylamide gels, cross-linked dextrans (Sephadex), and agarose synthesized into a bead form have all been used (49). In particular, agarose such as Biogel A (Bio-Rad), Ultragel A (IBF), and Sepharose (Pharmacia) are popular (49, 50). Cross-linked agaroses (Sepharose CL and Sepharose FF by Pharmacia) are physically more stable than Sepharose and are suitable for attaching affinity ligands. Both

forms of the agaroses have an open porosity which allows proteins to readily diffuse inside. Affinity chromatography results are usually reported in terms of specific activity of the final product (activity per mg of material) and the amount of biomolecule recovered (% yield).

Activation of the Stationary-Phase Surface. Activation of polysaccharide, silica, or polyacrylamide stationary phases involve the formation of a reactive intermediate, covalently attached to the surface, to which a difunctional alkyl-, aryl-, or glycol spacer is subsequently joined. The other end of the spacer is subsequently reacted with the ligand. Cyanogen bromide, CNBr, has been widely used to activate agarose and dextran gels (49). The attachment of ligands, and sometimes activation of supports, is generally carried out in the laboratories of the chromatography process developers because fully prepared affinity stationary phases are not as widely available as stationary phases for the other types of chromatography.

Multistep Processes. An excellent synopsis and industrial viewpoint of affinity chromatography and its fit with other bioseparations unit operations is available (49, 50). Ligands range from the low molecular weight components, eg, arginine and benzamidine, which both bind trypsin-like proteases, triazine dyes, and metal chelates; to high molecular weight ligands, eg, protein A, immunoglobins, and monoclonal antibodies. The blood factor VIII, purified by monoclonal affinity techniques, was approved by the U.S. FDA in 1988. Limitations of affinity chromatography as an industrial separation technique can be due to leaching of bound ligands from the column into the product at ppm levels, nonspecific interactions resulting in contamination of the target molecule, and failure of the affinity ligand to differentiate all variant forms of a protein or polypeptide (52). For example, polyclonal antibodies do not distinguish desamido-insulin, which contains a deamidated asparagine, from insulin. Because many antibody preparations cannot differentiate between minor structural changes in proteins, affinity chromatography must be followed by other separation steps, and does not provide a one-step purification.

Receptor Affinity Chromatography. Receptor affinity chromatography is a selective form of immunoaffinity chromatography which is based on antigen-antibody interactions (52). Protein or polypeptide ligands used in preparing receptor affinity supports are themselves products of fermentation of recombinant microorganisms and are subjected to a separate sequence of purification steps, prior to being reacted with a functionalized stationary phase to form the affinity support. The resulting affinity chromatography columns are expensive when viewed on the basis of cost of support/unit volume of stationary phase. The cost/benefit ratio would still be attractive because process-scale columns can be small (volumes on the order of 1–10 L). Moreover, as with other types of affinity chromatography, purification of dilute but highly active protein is possible.

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