PERSPECTIVE: ANALYTICAL BIOTECHNOLOGY

The Role of Quality Control in Biotechnology: An Analytical Perspective

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The role of quality control in biotechnology is examined. The use of living organisms for the manufacture of pharmaceutical products and the complexity of the resulting products are described. Quality control testing is performed not only to identify the final product and determine its purity, potency, stability, and safety, but also to characterize the production organisms and monitor fermentation and cell culture processes and recovery/purification operations. The concerns of industry and the Food and Drug Administration are discussed. A review of the fundamentals of biotechnology including basic terminology and definitions, recombinant DNA and vector production, and cloning and expression processes is presented. Development of quality control testing strategies for biologicals derived from bacteria, yeast, and mammalian cells are discussed. Finally, the analytical methods used in the quality control of biologicals are examined. Efforts to improve or automate these methods are either in progress or provide exciting analytical opportunities for the future.

I. INTRODUCTION

Quality control plays an important role in biotechnology because of the uniqueness of this industry, which is based on the use of living organisms for the manufacture of commercial products (*1*). Although the beginnings of biotechnology can be traced to the fermentation industry, it is at present considered to be represented by the recombinant DNA (rDNA) and monoclonal antibody technologies. During the past 10 years we have seen the development of a number of products that have a profound effect on the quality of human life.

An example of a product derived from rDNA technology is the thrombolytic therapy drug tissue-type plasminogen activator, or rt-PA (*2*), which has been approved by the Food and Drug Administration (FDA) for human use. This enzyme specifically converts plasminogen to plasmin, the active fibrinolytic enzyme that lyses the blood clot causing certain myocardial infarctions, and is expected to save thousands of lives each year. The development of rt-PA required the codevelopment of quality control systems capable of ensuring the lot-to-lot consistency of a glycosylated protein of approximately 64 000 daltons (Da) manufactured from large-scale mammalian cell culture. The emergence of such a control system was a result of the careful blending of traditional quality control systems such as raw material analysis and documented process control with many of the advanced techniques of molecular biology and protein chemistry. This control system was designed to evaluate a broad spectrum of known and/or theoretical product impurities and potential degradation products and was used in the validation of the manufacturing process to remove those impurities of significant concern. Thus, it is apparent that the quality control of recombinant biotechnology products is quite different from that of traditional products.

Prior to the development of many of the analytical methods described in this text, the quality control of most traditional biologicals was based solely on the concept of biological activity, usually in an animal model (e.g., insulin). In addition, electrophoretic or molecular sizing methods (e.g., ultracentrifugation) were used to evaluate the purity of the materials. With the advent of highly purified rDNA products, the requirement for activity measurements is still necessary, and the development of suitable reference materials and standards has become an extremely important issue with international attention.

The complexity of quality control systems is fundamentally a function of the size and structural characteristics of the manufacturing process and the final product. The consistency of manufacture and the ability to ensure that consistency are perhaps the most important parts of ensuring the safety of the products being developed by the biotechnology industry (*1*). This aspect of the quality control of biotechnology products is extremely important because there is little or no history of the long-term safety of such materials in humans. For example, to ensure the quality control of rt-PA requires several hundred in-process and final product assays per lot (Genentech unpublished data)! The analytical methods used range from cell culture virology to high-performance liquid chromatography (HPLC)-peptide mapping to immunological purity analysis (*3*). The future needs for automated instrumentation to accurately measure impurities such as DNA, endotoxin, and residual host organism proteins clearly offer tremendous opportunities for analytical chemists. In addition, the applications of modern high-resolution techniques such as capillary electrophoresis are just now being investigated and are sure to make significant contributions to the quality control of the biotechnology products of the future.

This article is a review of both the fundamentals of biotechnology and some of the current analytical methods used for the quality control of biotechnology products. The rationale and limitations of the use of these methods are also discussed. Emphasis is placed on areas where new analytical methodology is needed to facilitate the development of new products.

II. FUNDAMENTALS OF BIOTECHNOLOGY PRODUCTION

Although the basic concepts of rDNA technology are relatively simple to understand, the actual production or design of rDNA molecules is highly sophisticated and requires detailed knowledge in many areas, including molecular biology,

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microbiology, analytical chemistry, enzymology, and immunology. An attempt has been made in this section to present the highlights of this complex process in simple terms. No attempt has been made to explain all the techniques and methodologies currently used in the biotechnology field. A more in-depth discussion of the principles and techniques of biotechnology can be found in many sources. Several of these are listed here (*4–9*).

A. Terminology. rDNA is hybrid DNA formed by combining pieces of DNA from different organisms. rDNA technology uses bacterial (e.g., *Escherichia coli*), yeast (e.g., *Saccharomyces cerevisiae*), and mammalian (e.g., Chinese hamster ovary) cells as living hosts of genetic material to produce proteins that can be used in therapeutic, diagnostic, industrial, and research applications. *E. coli* and mammalian cells are generally used to manufacture pharmaceuticals while yeasts are used primarily to manufacture industrial enzymes and, recently, recombinant hepatitis B vaccine (surface antigen). Yeasts have been used to lesser degrees in pharmaceuticals because they have been found to be more difficult to work with and they may glycosylate differently than mammalian cells.

The production of rDNA involves inserting a piece of "foreign" (i.e., not normally present in the host cell) DNA into a cloning vehicle called a vector, which is usually a plasmid. Plasmids are closed circular forms of DNA (see Figure 1) that are widely distributed among prokaryotes. The vector is then inserted into a host cell, where the desired end product can be generated by the normal protein production mechanisms of the cell. This, in effect, converts the host organisms into miniature "factories" for the production of a desired protein. Plasmids can replicate independently in the cell and contain and express genetic information (*10*). The foreign DNA is generally a portion of the gene coding for the particular protein desired. The process of protein synthesis is initiated when the genetic code is "copied" to messenger RNA (mRNA) in the presence of the enzyme RNA polymerase in a process called transcription. The mRNA then serves as a template for the assembly of the protein from the specific amino acids that code for each protein. This assembly or translation takes place at the ribosomes in the presence of transfer RNA (tRNA). Therefore, in addition to the foreign DNA coding for the protein, DNA control signals that will function in the host cell for transcription and translation must be added if they are not already present (*10*). Otherwise, the cell will contain all the genetic elements needed to make the protein but will never actually produce any. Care must be taken in designing the controls to ensure that expression of the protein is not at a level so high that it will be toxic to the host cells.

Both prokaryotic and eukaryotic plasmid vectors are in use. The latter type of vector has been derived from yeast and viruses such as the simian virus SV40 (*7*). All useful vectors, whether prokaryotic or eukaryotic, carry an origin of replication for bacteria, enabling replication of itself within the bacteria. They also contain a selectable genetic marker (such as the gene for antibiotic resistance), enabling identification of the recombinant molecule from all other molecules present by growth in an antibiotic-containing medium that eliminates host cells without the genetic marker.

B. Cloning in Bacterial Cells. The actual process of cloning is illustrated in Figure 1. The foreign DNA of interest is prepared. A vector and the foreign DNA are treated with the same restriction enzymes (endonucleases), which cleave the DNA at nucleotide sequences determined by the specific enzymes used. Because the vector and the foreign DNA have been tailored or designed to have complementary base pair ("sticky") ends (i.e., the DNA is homologous), they can be joined together by treatment with DNA ligase. The vector

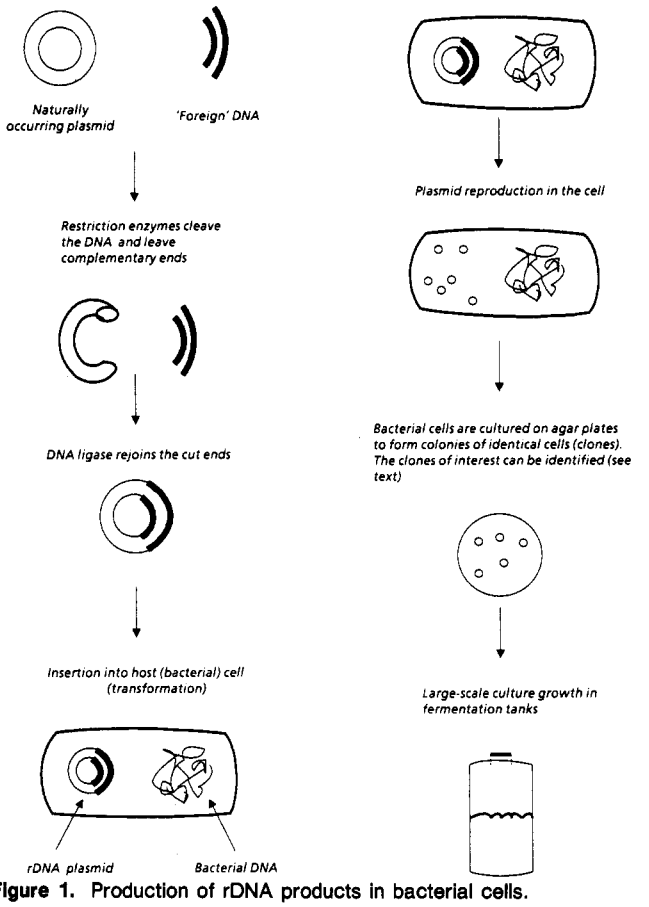

Figure 1. Production of rDNA products in bacterial cells.

thus has been "recombined" and contains the foreign DNA. The foreign DNA will either have replaced the original gene that was present on the plasmid or will have replaced a portion of the gene. In either case, the appropriate controls, as described above, must be present. This modified plasmid can then be inserted into the host cell through a process called transformation. However, not every host cell will receive the transformed plasmid. The host cells are then cultured on agar plates and allowed to multiply. Each cell grows into a colony composed of identical cells, all containing the same plasmids. The genetic marker (see section A above) allows the determination of which clones have the desired gene. Verification that the desired clone has been obtained is conducted by using restriction mapping and DNA sequence analysis.

Once the desired host cell containing the transformed plasmid is produced, some of the cells are frozen by placing them into the vapor phase of a liquid nitrogen freezer so that a "cell bank" of original cells can be maintained for future production of the protein.

C. Expression in Mammalian Cells. Mammalian cells are used when prokaryotic cells such as bacteria either are unable to express the protein of interest or cannot do so in adequate amounts. Typically, mammalian cells are used for the production of high-molecular-weight glycosylated proteins because these cells can perform the posttranslational modifications required. Protein expression in mammalian cells commonly requires additional DNA control signals such as from the simian virus SV40 to ensure proper functioning of the vector in the mammalian cells. These cells must be maintained under highly controlled conditions (*11*).

As described earlier, recombinant plasmids are first grown in bacteria and allowed to replicate, to provide a sufficient supply of recombinant plasmids. These plasmids are then isolated from the bacteria and inserted into the mammalian cells in a process called transfection. In mammalian cells, in contrast to bacterial cells, the plasmid is actually incorporated

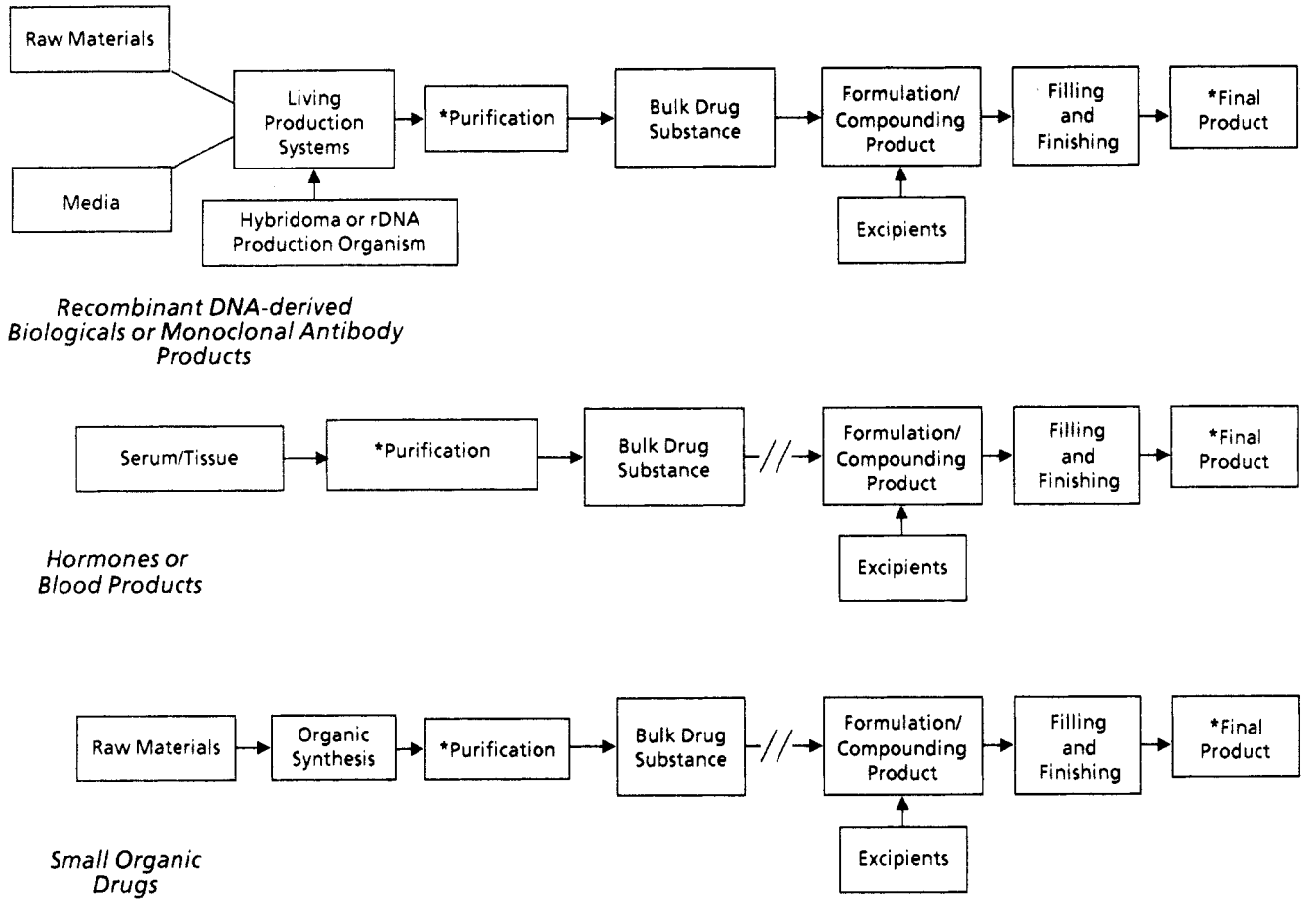

Figure 2. Examples of typical manufacturing processes for the production of pharmaceuticals.

into the chromosome of the host cell. The cells are maintained in culture and screened directly to determine if protein expression is detectable. Antibody staining is often used as an aid in this screening process. Nucleotide sequence analysis cannot be used because the mammalian cell genome is too complicated. Once cells producing the desired protein are identified, some of the cells are frozen by placing them in the vapor phase of a liquid nitrogen freezer to establish a cell bank to maintain the cell line for future use.

III. BIOTECHNOLOGY QUALITY CONTROL AND HOW IT DIFFERS FROM TRADITIONAL QUALITY CONTROL

Biotechnology quality control is defined by both the individual production process and the product itself. The quality control strategies developed for human growth hormone (hGH), for example, which is produced in *E. coli* and has a molecular weight of 22 000, are considerably different from those developed for rt-PA, which is produced in large-scale mammalian cell culture and has a molecular weight of 64 000. The quality control strategies may even vary considerably between two separate processes producing the same final product. This is because of significant concerns that must be addressed systematically about end-product purity, safety, and stability (*12*). Many other aspects of biotechnology quality control, however, are identical with the methods and procedures developed for more traditional pharmaceutical products. Thus, systems for raw material testing and release, manufacturing/process control documentation, environmental monitoring, and aseptic processing are virtually identical with those used for the manufacture of small organic drugs, as presented in Figure 2. Biotechnology quality control differs from traditional control systems in the following three major areas: development and characterization of the production organisms; significant issues relating to the control of fermentation and cell culture processess, which includes the complex issue of media (analytical and performance) testing (see Figure 2); and quality control of the recovery/purification process and final products obtained from biotechnology. Each of these areas is discussed in some detail in the following sections.

A. Development and Characterization of Production Organisms. The development and characterization of the production organisms used for biotechnology products are best addressed by separately examining two major classes of production vehicles (bacteria and yeast, and mammalian cells) and the requirements for their use (*13*, *14*). These requirements are to ensure that the product is as consistent as possible, with no genetically induced variation.

1. Bacteria and Yeast. As discussed earlier, the production host consists of a well-understood and characterized organism such as bacteria (prokaryotes) or yeast (eukaryotes) to which has been introduced a naturally occurring or synthetic segment of DNA that codes for the commercial product desired. A specific lot of the transformed host is used after extensive characterization to produce a master cell bank and manufacturing working cell bank.

Current regulations require that the DNA segment itself must be thoroughly characterized by nucleotide sequence analysis, restriction enzyme mapping, and its own production

Table I. Quality Concerns over Fermentation and Cell Culture Processes

1. genetic stability
2. product yield/product stability (inter- or intracellular)
3. nonhost contamination (phage, mycoplasma, virus, bacteria, yeast, mold)
4. culture conditions (cell viability/growth rate, cell density)
5. fermentation conditions/growth rate
6. environmental considerations
7. media (complex proteins and amino acids)
8. glycosylation of the final product
9. protein folding

history (*15*). A detailed history and description of the host cell and expression vector, including details of the origin and identification of the gene and the construction, genetics, and structure of the expression vector, the method of introduction of the vector into the host cell, and the state of the vector within the host cell, are also required. All flanking control regions of the expression vector must be determined as well as all other relevant expressed sequences. The origin, form, storage, stability, and use of the master cell and working cell bank must be documented. The quality control of such cell banks includes karyology, phenotyping, antibiotic resistance, restriction mapping, sequencing, stability monitoring, and testing for nonhost adventitious organisms (*16*).

2. Mammalian Cells. The general principles described above also apply to cell cultures of higher eukaryotic organisms. Because of the complexity of these organisms, however, it is not always possible to sequence the cloned gene at the production level. Currently, a reasonable solution to the question of genetic stability relies on the use of the technique of peptide mapping of the expressed protein to ensure that the DNA sequence has not mutated. This approach is totally dependent on the resolution and thorough development/validation of the peptide mapping method. Furthermore, the production organism may need to be limited to a finite number of passages or population doublings to ensure genetic stability. The quality control of mammalian cultures also requires a master cell bank and working cell bank as described above. However, testing for specific isoenzymes and immunological markers is usually added to aid in establishing cell identity. Testing continuous cell lines for tumorigenicity in specific animals is also necessary, as is evidence that the cell bank is free from potential oncogenes (*15*, *17*). Data that ensure the absence of adventitious agents such as viruses, bacteria, fungi, and mycoplasmas must be obtained. The issue of endogenous retroviruses is particularly important because these latent viruses may be induced by the production process. The cell lines must be tested for retroviruses and retroviral activity markers such as reverse transcriptase to ensure their absence (*15*).

B. Significant Issues Relating to the Control of Fermentation and Cell Culture Processes. The production process using a living system is the fundamental cornerstone of biotechnology processes, whether that process is fermentation using *E. coli* or yeast or large-scale cell culture using mammalian cells. Therefore, the issues that relate directly to the control of such processes need to be examined in some detail. For example, concerns over the production of proteins in bacteria primarily revolve about systems for ensuring genetic stability, consistent product yield, and evidence of the lack of contamination by nonhost organisms. These same concerns apply to large-scale mammalian cell culture, where, in addition, there are significant issues relating to the use of transformed cell lines such as the putative presence of oncogenic DNA and impurities from media proteins. The most relevant issues are listed in Table I, and examples of these will be discussed in the following sections.

1. Bacterial and Yeast Fermentations. A considerable amount of knowledge has been obtained for the production of recombinant proteins in bacteria and yeast; therefore, the major fermentation issues typically are resolved by the demonstration of consistency in fermentation conditions. Bacterial and yeast fermentations are generally performed over short, well-defined time periods, where growth rate and product expression conditions are very carefully controlled. The presence of any contaminating foreign organism is usually detected on the basis of the effects on growth rate and is cause for termination of the fermentation. The issue of genetic stability of the production plasmid can be addressed by studies that demonstrate by isolation and nucleotide sequence analysis that mutation has not occurred (*18*). This can also be confirmed by the technique of peptide mapping of the expressed protein, which can be performed for each product lot manufactured. Because intra- and intercellular production of the target protein may be desired, it is highly important to optimize the fermentation conditions to avoid or limit the amount of proteolytic processing of the target protein that might occur. This problem is often encountered in *E. coli* fermentations and may lead to recovery difficulties as well as low product yields. Finally, the issue of protein conformation and its effects on potency must be addressed by the fermentation process and analytical optimization efforts.

2. Mammalian Cell Culture. The development of large-scale cell culture techniques for the production of biotechnology products can be readily traced to the vaccine industry. Recent developments such as large-scale cell suspension cultures using recombinant organisms that express the desired product into the media have revolutionized biotechnology. For the first time, it is now possible to produce large glycosylated proteins in quantities sufficient for the marketplace. These developments, however, are complicated by the issues listed in Table I. For example, the genetic stability of cell cultures cannot be addressed as readily as for *E. coli* fermentations by plasmid sequence analysis because the gene that codes for the product is incorporated into the cell genome and is not easily recovered. The only solution, therefore, is to rely on peptide mapping of the expressed protein. This, in turn, places major emphasis on the resolution and sensitivity of the mapping method to detect subtle mutations. Fortunately, such powerful methods have been developed and employed for this purpose in routine quality control analysis. An example of this technique is shown in Figure 3, in which a single amino acid at position 275 in rt-PA has been changed from an arginine to a glutamic acid. Reversed-phase (RP)-HPLC readily distinguishes this change, as shown in the figure. Although the example presented illustrates the present "power" of the HPLC-peptide mapping technique, the development of such a map is a difficult process that must involve chromatographic development and structural identification to be at all useful. It is probable that, in the future, this method may be supplanted by routine HPLC–mass spectrometry procedures.

As described previously for yeast and bacterial fermentations, the absence of nonhost organisms in cell cultures is very important. In addition to the demonstration that bacteria, yeast, and molds are not present in cell cultures, evidence that mycoplasmas and viruses are absent must be presented for each culture (*19*). This necessitates that valid methods be developed to ensure the lack of contamination by mycoplasmas as well as by human or animal viruses. Because these tests require from 14 days to 1 month to perform, significant opportunities exist for the development of improved methods.

The extent of glycosylation is an important consideration in the design of cell culture conditions for the manufacture of glycosylated proteins. The degree of glycosylation may

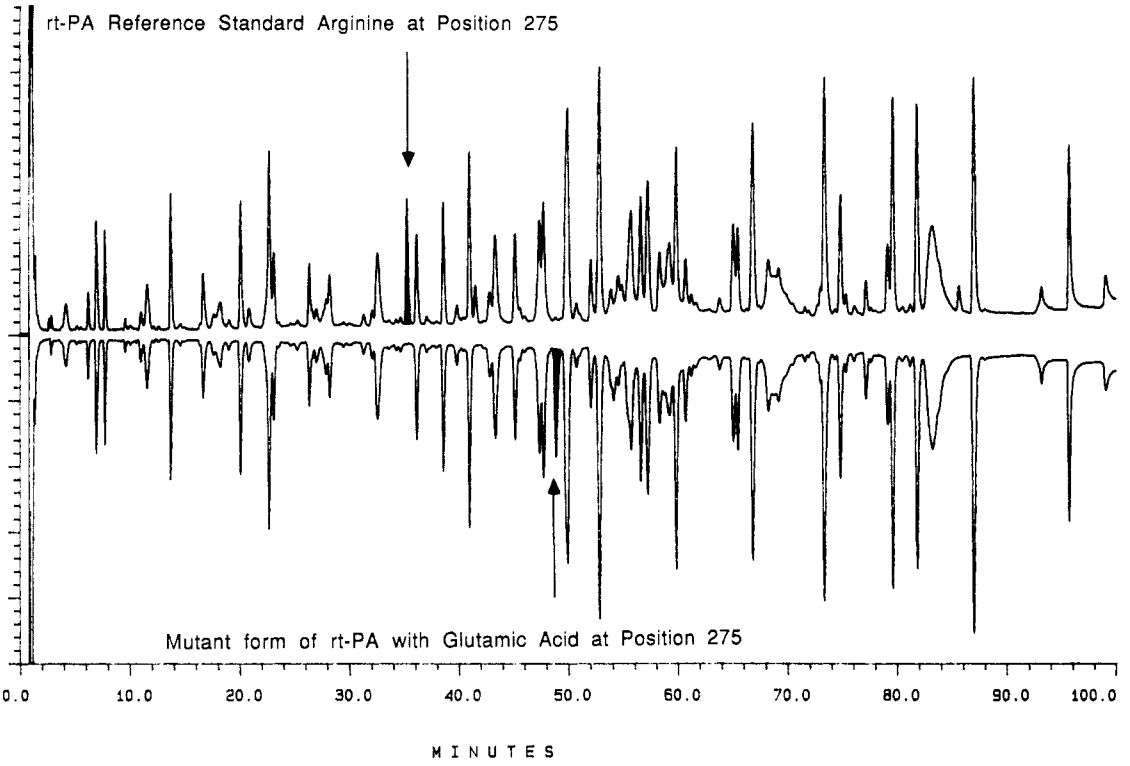

Figure 3. Tryptic map chromatograms of the rt-PA reference standard and a mutant form of rt-PA with a glutamic acid residue in place of the normal arginine residue at position 275. Arrows illustrate the differences in the two chromatograms caused by the substitution.

affect the half-life of the product in vivo as well as its potency. Although difficult to assess, the glycosylation level of a cell culture product can be verified to be consistent if highly reproducible culture conditions exist.

Finally, mammalian cell cultures possess complex requirements for growth. Hence, they are affected by multiple growth factors derived from various components in the culture media. Alteration of this delicate balance of media components can cause major changes in large-scale mammalian cell culture production, resulting in poor cell growth and product yields. Quality control of each lot of basal medium and its components with rigid release criteria is therefore of utmost importance. Although this has been addressed by a combination of analytical methods such as amino acid analysis, infrared (IR) spectroscopy, HPLC, and atomic absorption spectroscopy, the development of "media maps" offers the analytical chemist an exciting challenge. In lieu of such media mapping methodology, the only recourse is the small-scale performance testing of media.

In the testing of basal medium and its components, the test systems consist of a scaled-down mimic of the production processes. In each of the test systems, the appropriate mammalian cell system is cultured simultaneously in two sets of the appropriate basal medium with components. One of the sets is the reference material, and the other set is the test in which the component under investigation is varied from the reference material. For example, a test serum lot would be substituted for a reference material serum lot in a completed medium with all other components remaining unchanged.

Under testing conditions, both the reference material and the test lots are similarly prepared at the same time. Cell growth performance is monitored in parallel over an appropriate period followed by quantitative product yield determinations.

C. Quality Control of the Recovery/Purification Process and Final Products Obtained from Biotechnology. The recovery of protein products obtained from either fermentation or cell culture is based on efficient protein separation techniques such as molecular sizing, ion exchange, chromatofocusing, affinity chromatography, and the use of highly specific monoclonal antibodies. The recovery process begins with the isolation of the desired protein, often in a highly impure state, from the fermentation or cell culture medium. The obvious advantage of cell culture and yeast-derived products at this stage is that many of these proteins are expressed directly into the medium, thus requiring only the separation of the cells to achieve a significant purification. In the case of *E. coli*-derived products, lysis of the bacteria is required to recover the desired protein. In both cases, it is important to achieve a rapid purification of the desired protein because both lysed *E. coli* and cell culture organisms release proteases that may "clip" the desired product even when they are carefully monitored. Such trace proteases are a major concern in the purification of recombinant products because they may be extremely difficult to remove and may complicate the recovery process and/or significantly affect the stability of the final product.

The recovery process is usually designed to purify the final product to at least the 97–99 plus percentage level. The purity requirement for any given product depends on any number of factors, although products intended for chronic use are normally required to be of much higher purity than those destined for single-use purposes (*20*). Biotechnology products have certain impurities that the recovery processes are designed to eliminate or minimize. These impurities are trace amounts of DNA, residual host proteins, pyrogens, and residual cellular proteins from the media. A list of the most common impurities of concern and suitable assay methods of detecting them is presented in Table II.

Typically, recovery processes are developed specifically to achieve the desired purification of the final product from key impurities such as DNA, host proteins, and pyrogens. The recovery process equipment is also designed to be readily sanitized and often requires extensive process validations to demonstrate that the final product made by using these processes is consistent from lot to lot. Nevertheless, extensive in-process testing is required to guide the recovery operations.

Table II. Common Impurities of rDNA-Derived Biologicals

impurities	detection method
endotoxin	LAL,[a] rabbit pyrogen
host cell proteins	SDS-PAGE,[b] immunoassays
other protein impurities (media)	SDS-PAGE, HPLC,[c] immunoassays
DNA	DNA hybridization, UV spectrophotometry
protein mutants	HPLC-tryptic mapping
formyl methionine	HPLC-tryptic mapping
oxidized methionines	amino acid analysis, HPLC-tryptic mapping, Edman degradation analysis
proteolytic clips	IEF,[d] SDS-PAGE (reduced), HPLC, Edman degradation analysis
deamidation	IEF (standard comparison), HPLC
monoclonal antibodies	SDS-PAGE, immunoassays
amino acid substitutions	amino acid analysis, HPLC-tryptic mapping
viruses (endogenous)	CPE,[e] HAd,[f] electron microscopy, reverse transcriptase activity

[a] Limulus Amebocyte Lysate. [b] Sodium dodecyl sulfate polyacrylamide gel electrophoresis. [c] High-Performance Liquid Chromatography. [d] Isoelectric focusing. [e] Cytopathic effect. [f] Hemadsorption.

Tests such as protein content, endotoxin by Limulus Amebocyte Lysate (LAL), bioburden, and potency or activity are performed at key steps in the recovery process. Because these tests are performed at multiple points, it is a great advantage to develop automated analytical methods from which the data generated can be transmitted to the recovery plant via computer. The use of robotics for endotoxin analysis and a microcentrifugal analyzer for potency determination of rt-PA (*21*) are examples of such automated methods.

The result of the recovery process is a bulk product that may be lyophilized or maintained in a solution containing the excipients required by the final product formulation. The quality control that is generally performed on this bulk product is usually concerned with the demonstration of purity. Testing performed at this stage, therefore, typically consists of assays for residual host proteins and endotoxins and sensitive methods to ensure that molecular changes have not occurred to the product during recovery. Thus, assays such as peptide mapping, amino acid analysis, Edman sequence analysis, electrophoresis, HPLC purity profiles, host cell protein enzyme-linked immunosorbent assays (ELISAs), and LALs are performed at this stage to confirm the consistency of manufacture. Total protein content and potency measurements are also performed at this point to ensure that the final processing of the bulk into vials or ampules will yield a product that will meet specifications.

The final product is usually packaged in vials or ampules after sterile filtration, filling, and, often, lyophilization. The quality control tests that are performed at this point, therefore, are concerned primarily with those factors that may be affected by the final processing operations. Sterility, pyrogenicity, particulate testing, content uniformity, identity, excipient chemical content, potency, and protein content are typical examples of such final product testing.

IV. ANALYTICAL METHODS OF MAJOR IMPORTANCE IN BIOTECHNOLOGY

The quality control testing of biologicals prior to the development of rDNA-derived products focused primarily on safety issues and potency determinations. This was due, in part, to the impurities in biologicals manufactured from tissue and blood sources, the extremely limited amount of the purified active agent, and the relatively limited number of analytical techniques available before the 1970s. However, with the advent of biotechnology, quality control in the pharmaceutical industry has taken on new meaning and broader scope. rDNA technology has stimulated the protein chemist in academia and industry to provide a wide variety of analytical methods. These include HPLC, peptide mapping, ELISAs, polyacrylamide gel electrophoresis using sensitive staining or detection techniques, and DNA hybridization. Immunoassays for the detection of host cell protein impurities at the parts per million (ppm) level as well as sensitive assays in the picogram to nanogram level for residual DNA (DNA hybridization) have been developed.

The analytical methods currently in use for the analysis of biologicals are discussed in the following sections with respect to assays for the determination of identity and structure, purity, potency, stability, and safety. Although these methods are, for the most part, general procedures, their applicability to a specific biological depends not only on the biological itself but also on the formulation and excipients used to solubilize and stabilize the product.

A. Biochemical Identity/Structural Methods. The application of multiple structural methods is the only way to ensure that a biotechnology product is thoroughly identified and characterized. A combination of classical and modern physicochemical characterization methods such as amino acid analysis, Edman degradation (partial sequence) analysis, peptide mapping, HPLC, carbohydrate analysis, circular dichroism (CD), and optical rotatory dispersion (ORD) are employed by quality control staff for this purpose (*22*). International regulatory agencies require that analytical methods be used for drugs and biologicals to demonstrate their molecular identity. This identification must be performed on a lot by lot basis. In addition, inherent in some identity methods is the ability to ensure the absence of significant levels of impurities. Methods such as peptide mapping and quantitative ELISAs have been developed for this purpose.

1. Amino Acid Analysis. Amino acid analysis is a useful tool for obtaining compositional information on peptides and small proteins, but is much less valuable for proteins in excess of 15000–20000 Da. However, for larger proteins, a correlation of the results from product and reference material analyses would furnish evidence for chemical identity. Amino acid analysis may also be useful in fermentation development for evaluating side reactions that are a result of media-based phenomena (e.g., norleucine substitution for methionine).

Amino acid analysis usually involves the complete hydrolysis of a protein to its constituent amino acids using 6 M HCl for 24 h in evacuated vessels. Traditionally, the amino acid hydrolysate is separated by ion-exchange chromatography followed by reaction with ninhydrin and UV detection at 440 and 570 nm. More recently, RP-HPLC has been used to separate the amino acid hydrolysate following precolumn derivatization with *o*-phthaldialdehyde (*23*) or postcolumn fluorescent derivatization (*24*). The amino acid composition of the sample is then determined by comparing the results to a reference standard, which contains the 18 regularly occurring amino acids at known concentrations. Protein hydrolysis with acid causes the destruction of tryptophan, and this amino acid is therefore not determined unless methanesulfonic acid or other hydrolysis conditions are used.

2. Partial Sequence Analysis (N- and C-Termini). Most quality control sequencing of proteins is performed by using Edman degradation analysis to elucidate the first eight to 10 amino acids of the *N*-terminus (*25*). This method is used to confirm the complementary DNA (cDNA)-predicted amino acid sequence and to evaluate the potential extent of proteolytic clips. Edman degradation is based on the coupling

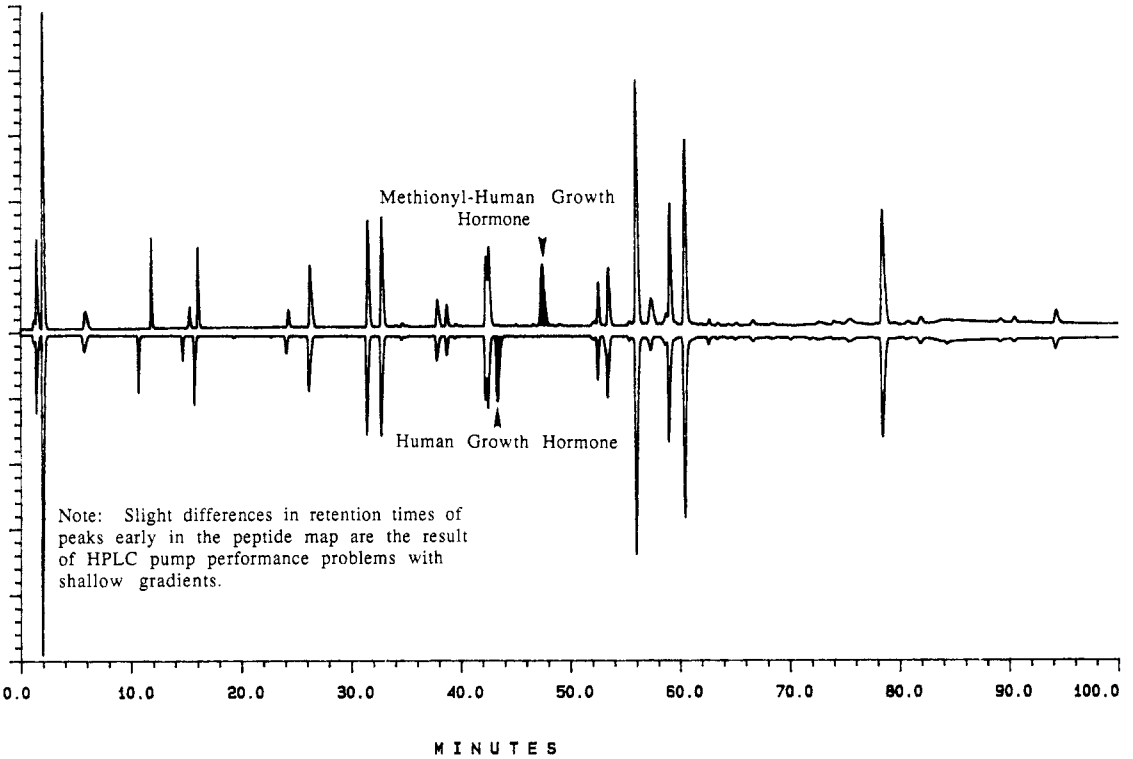

Figure 4. Tryptic map chromatograms of methionyl-human growth hormone and human growth hormone showing the ability of the tryptic map to differentiate the two materials. The *N*-terminal peptide differences are highlighted by arrows.

reaction of the *N*-terminal amino acid of a protein with phenyl isothiocyanate. The derivatized amino acid is cleaved from the protein in a subsequent reaction, exposing the next amino acid for further cycles of coupling and cleavage. Thin-layer chromatography (TLC) or, more recently, HPLC is used to separate and identify the amino acids by comparison to known derivatized amino acid standards.

The carboxy or *C*-terminal amino acids can be determined by the sequential degradation of the protein based on the reaction with ammonium thiocyanate followed by HPLC separation of the resulting thiohydantoin derivatives (*26*, *27*). Other novel approaches have been developed to partially sequence the *C*-terminus.

Because of several limitations in each method, the partial sequencing of the *N*- and *C*-termini is mostly useful to demonstrate homogeneity and, thus, the chemical identity of the protein product. Based on the limitations described above, the development of HPLC methods for the analysis of intact proteins and their peptidyl fragments is a major goal of the analytical chemist and offers an area for considerable future development.

3. Peptide Mapping. Peptide mapping can be used to determine the entire primary structure of a protein (*28*) and can provide additional information such as the position of glycosylated peptides. Consequently, this technique is particularly important in the quality control of biotechnology protein products. Peptide mapping is used routinely to compare the protein structure of a product to that of a reference material or to those of previous lots to confirm correctness of the primary structure and to ensure no lot-to-lot variation in protein structure. This technique is invaluable in demonstrating the genetic stability of proteins obtained from mammalian cell culture.

Peptide mapping is accomplished either directly or, when required, by initially reducing and carboxymethylating any disulfide bonds present in the protein. This is followed by cleaving the protein into much smaller peptides, usually 25 residues or less, by either an enzymatic or, occasionally, a chemical method that cleaves the protein at specific sites. The resultant peptides can then be easily separated by RP-HPLC, ion-exchange chromatography, two-dimensional TLC, or gel electrophoresis. A map or "fingerprint" is obtained, which allows for differentiation between proteins of similar, but not identical, primary structure.

Several different proteolytic enzymes may be employed for the purpose of peptide mapping. Most common is trypsin, which reproducibly and quantitatively cleaves on the *C*-terminal side of only arginine and lysine residues. Trypsin will also, less frequently, cleave the protein at other sites such as a region of several adjacent basic amino acids, but this is usually not a significant problem. Therefore, the peptides liberated by trypsin should be identical from lot to lot. Other proteolytic enzymes for producing peptide fragments are V8 protease, chymotrypsin, subtilisin, and clostripain (*29*).

Chemical cleavage of proteins can be accomplished at specific residues such as tryptophan, methionine, and cysteine. In general, this type of cleavage method has seen limited use because of the relatively large peptide fragments produced, which tend to be hydrophobic and difficult to chromatograph with HPLC.

The peptides generated by any cleavage method must be completely (or nearly completely) resolved by some chromatographic technique. Gradient elution RP-HPLC is usually the method of choice in most laboratories. A C_8 or C_{18} bonded-phase silica column of 125–300-Å pore diameter can be used with good resolution of most peptides. A sophisticated HPLC system with a highly accurate and reproducible pumping system is required; equipment maintenance and column temperature control are also essential to prevent variable peptide retention times. The elution solvents most widely employed are acetonitrile and propanol in acidic conditions obtained with trifluoroacetic or phosphoric acids.

The peptide map can provide information on the *N*- and *C*-terminal peptides, the positions of disulfide bonds (by examining the sequence of the peptidyl fragments before and after reduction), and the positions of carbohydrate-containing peptides. Tryptic mapping can distinguish an rDNA-derived methionyl-hGH from pituitary-derived hGH as shown in

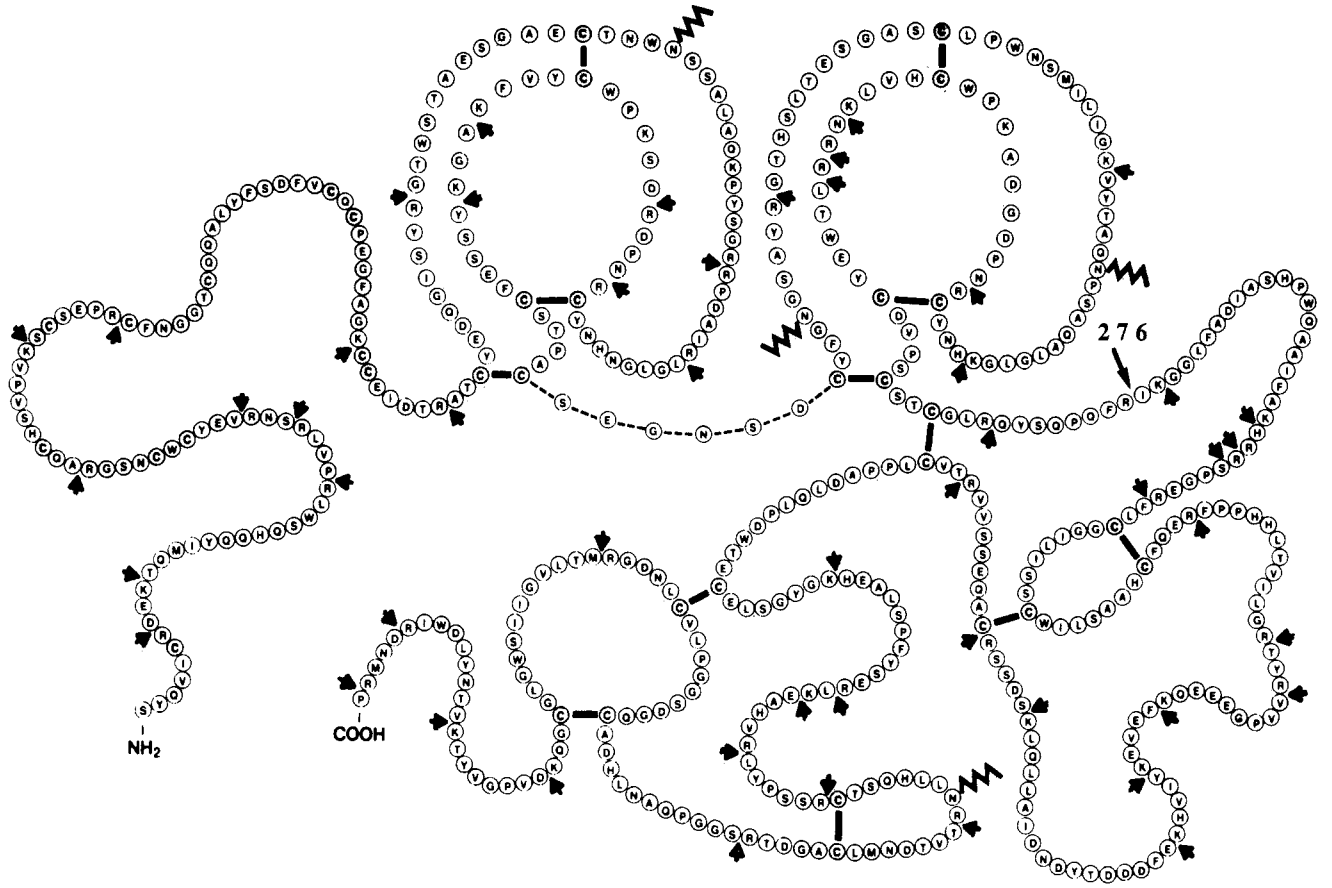

Figure 5. Structure of rt-PA. Arrows indicate the tryptic sites on the molecule giving rise to some 57 tryptic peptides. The solid lines indicate the position of disulfide bonds.

Figure 4. The extra methionyl group at the *N*-terminal is the result of the AUG start codon required by the molecular biology of the *E. coli* system (*30*). The effect of the extra methionyl residue on the retention of the *N*-terminal peptide is dramatic; the peptide with the extra methionyl residue is more retained on the RP-HPLC column (*31*). A concern in the rDNA manufacture of proteins is the potential for a single point mutation or mistranslation of the cDNA sequence, which would cause single or multiple amino acid substitutions in a protein. Here again, tryptic mapping can indicate such substitutions in the primary structure of a protein as shown for rt-PA in Figures 3 and 5. By purposeful creation of a mutant rt-PA with a glutamic acid instead of an arginine residue at position 275, a new peak is observed in the tryptic map of the mutant rt-PA at approximately 48 min that is not present in normal rt-PA. In addition, the peak at 35 min in normal rt-PA is missing in the mutant form.

4. Carbohydrate Analysis. Glycoproteins are an important class of commonly occurring proteins containing covalently linked carbohydrate units (i.e., oligosaccharides). These sugars are attached to the protein in any of the following three ways: (a) through an *O*-glycosidic bond, (b) through the *N*-glycosidic bond of asparagine, and (c) rarely, through an *S*-glycosidic bond. The determination of the number and positions of carbohydrate attachments is usually accomplished by (a) digestion of the protein with trypsin, (b) isolation of the carbohydrate-containing peptides on a lectin-affinity column, and (c) sequential degradation of the bound peptides using amino- or carboxypeptidases such that only the linked amino acid is left and can be identified during sequencing. Neutral sugar and sialic acid content can be quantitated in the intact protein by relatively simple colorimetric assays. Complete characterization of the carbohydrate units, which would require elucidation of the sequence of monosaccharides, intersugar linkages, and the anomeric configuration of the glycosidic bonds, is usually not required for the routine analysis of glycoprotein biologicals.

5. Optical Spectrophotometry. Information about secondary or tertiary conformational modifications in proteins can be obtained by using various optical spectrophotometry methods such as ORD and CD. Generally, these two methods are not very sensitive to subtle conformational differences when used in the region of peptide bond absorption (i.e., 200–250 nm). However, comparison of the ORD and CD spectra of an rDNA-derived biological with those of a reference material would indicate structural equivalence. CD spectra more sensitive to minor conformational differences can be obtained in the region of aromatic amino acid absorption (i.e., 250–320 nm). The CD spectra of rDNA-derived methionyl-hGH and pituitary-hGH compared in both absorption regions indicated identical solution conformations for these two forms of hGH (*32*).

B. Purity. The absolute purity of a biological or any drug substance is extremely difficult to evaluate and is only of academic significance in most cases. Purity determinations are inherently defined by the specificity and sensitivity of the methods used to detect the suspected impurities. Historically, the purity of biologicals has been significantly lower than that of other pharmaceutical drug products. However, rDNA technology and the codevelopment of sophisticated preparatory and analytical methods have produced biologicals with purity levels approaching 99% or greater as estimated by multiple analytical methods. Specific impurities of concern, such as DNA or pyrogens, may be required to be controlled at the nanogram or picogram per dose level.

It is critical to use analytically valid methods for the determination of purity because the purity of a biological is defined by specifying the assay method used. The lack of an extensive clinical data base of rDNA-derived biologicals has resulted in a conservative approach to establishing purity

levels for these products. Therefore, it is especially important to use the most sensitive and reliable purity methods available.

1. Electrophoretic Assays. This group of assays is among the most powerful for estimating purity because of simplicity, speed, and high resolution and because only a small probability exists that any of the analyte will be lost during separation and analysis. As in many modern biochemical methods, only microgram quantities of sample are required, there is high sample throughput, and usually more than one piece of information is obtained. The use of one electrophoresis method is often insufficient for an adequate purity determination. Therefore, two or more methods, which separate proteins by combinations of molecular size and charge, are often employed to evaluate the level of purity more thoroughly.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by their molecular weight (*33*, *34*), is used extensively for protein characterization (i.e., purity, molecular weight, and composition). SDS, which is an anionic detergent, disrupts the noncovalent intermolecular bonds that hold proteins and parts of proteins together. The protein separation that occurs in the presence of SDS is based mainly on molecular weight because a net negatively charged complex results from the association of SDS with proteins in a fixed ratio of 1.4 g of SDS/g of protein (*35*). The method is combined with various staining techniques such as Coomassie Brilliant Blue and silver stains, depending on the quantitative or qualitative nature and sensitivity of protein detection required. Coomassie Brilliant Blue is a valuable stain for obtaining quantitation of the relative intensity of bands and the state of aggregation of a protein. However, the method is not sensitive enough for some applications because it has a sensitivity limit at approximately the 100-ng level of a single protein impurity.

Silver stains significantly improve the detection limit of single protein bands to the 0.2–2-ng range, depending on the protein (*36*). This extrapolates to a 40–400-ppm detection limit with a sample load of 5 μg of protein. Silver staining is a very sensitive technique that has seen widespread use in the biotechnology arena. The main disadvantages of this method are that it is generally nonquantifiable and variations have been observed in binding of silver from protein to protein. The method is used qualitatively to determine if low levels of new impurities are present in a given lot of a biological by directly comparing the silver-stained electrophoretic gel of a sample to the reference material under reduced and nonreduced conditions. Some of the quantitative difficulties described can be circumvented by using an internal (intragel) standard such as bovine serum albumin, which is run on the gel at two known absolute amounts. The amounts of an impurity in a biological can then be estimated by comparing the staining intensities of the sample and internal standard.

A sample may contain a significant amount of a family of impurities that individually remain below the detection limit of the assay or that comigrate and, therefore, lie directly "beneath" another protein band. A multiantigen ELISA must be used in conjunction with electrophoretic and chromatographic methods for a complete estimation of protein purity.

In isoelectric focusing (IEF), proteins migrate in an electrical field on the basis of their charge. Each protein continues to migrate until it reaches a pH value in the gradient of the IEF support where its net charge is zero and the migration stops. Altered forms of the protein and other proteins will migrate to different locations on the gel because the migration of a protein is dependent on its amino acid composition. Proteins on IEF gels may be visualized by using the same stains as used in SDS-PAGE. However, electrophoretic patterns on IEF gels are more complicated to interpret than those of SDS-PAGE gels. Consequently, IEF gels are used primarily to ensure the homogeneity of a protein as demonstrated by a single band with the correct theoretical isoelectric point (pI) and to evaluate the stability of a biological product. IEF can provide information on glycoproteins, which appear as many bands because of changes in the apparent charge on the molecule as a result of the sialic acid residues of the carbohydrate. The technique can also aid in estimating the degree of protein deamidation (i.e., glutamine or asparagine residue deamidation) with time because a more acidic pI is obtained as a consequence of a new carboxylic acid group.

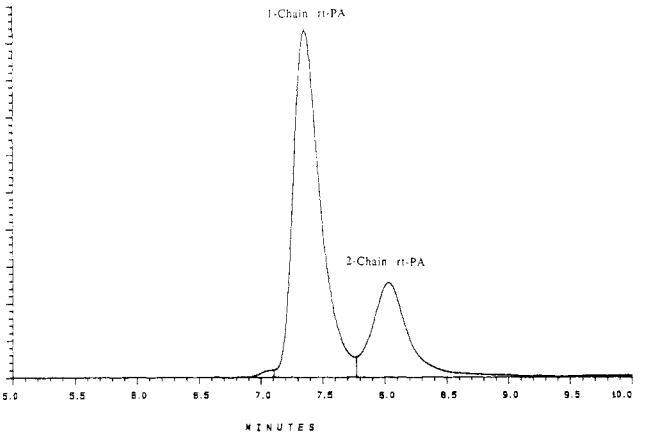

Figure 6. Chromatogram of rt-PA after reduction showing the one- and two-chain forms obtained by using a Dupont GF-250 column in a mobile phase containing 0.1% SDS.

Occasionally, other techniques are combined with electrophoresis to aid in obtaining additional information such as protein identification. Immunoelectrophoresis and immunoblotting are two combinations that have been used in this capacity. Immunoblotting has been used to demonstrate that a faint band observed on a silver-stained SDS gel of hGH is hGH-related and not an *E. coli* impurity (*32*).

2. HPLC. Estimations of protein purity have been obtained with size-exclusion chromatography (SEC), ion-exchange chromatography, and RP-HPLC. As a result, HPLC is an important tool in the quality control of biologicals.

SEC separates proteins by their effective hydrodynamic volume and can be performed on proteins in the native state or in the presence of detergents. With the development of stable, rigid silica-based chromatographic supports (e.g., TSK-SW and GF-250 columns), the analysis of the native or denatured molecular weight distributions of proteins by HPLC-SEC has become common practice. This methodology has the advantages of speed of analysis, high resolution, and rapid quantitation, which is typically in the 0.1–0.01% range. To counter any association of the protein with the chromatographic support, the ionic strength of the mobile phase must be high enough to allow separation on the basis of size only.

Low-molecular-weight fragments can also be accurately and reproducibly quantitated with HPLC-SEC. The amount of clipping in rt-PA can be determined following reduction and chromatography on a Dupont GF-250 column using a mobile phase containing 0.1% SDS (see Figure 6). The two peaks shown in this figure represent the one-chain form (1-527) and the cleaved two-chain forms (1-276, 277-527).

RP-HPLC is another valuable chromatographic technique for protein purity and stability estimates by the quality control chemist. Protein separation is based on the relative differences in the hydrophobicity of various proteins and their interaction with a hydrocarbon backbone chemically bonded to a silica support (*37*, *38*). Although the method is theoretically capable of providing separation based on small changes in the hydrophobicity of the protein, separation may not actually occur. Unless the hydrophobic areas include the region of the protein in which molecular modifications are present, RP-HPLC will

not be able to detect them. Denaturation of the protein as a result of its interaction with the chromatographic sorbent can produce artifact peaks and complicate the chromatography. Therefore, a RP-HPLC method that is to be used for purity or stability analysis must be validated prior to use.

3. Immunoassays. These assays include the radioimmunoassay (RIA) and ELISA for detection and quantitation of low levels (ppm) of protein impurities originating from the host cell that may be present in an rDNA-derived biological. These protein impurities represent a large number of potential impurities rather than a single impurity. The RIA or ELISA method must, therefore, detect as many of these impurities as possible. The development of such a broad-spectrum method with its inherent circular assumptions occurred on the basis of the simple rationale that nothing better has been proposed with the required sensitivity, selectivity, and ability to detect the theoretical range of impurities possible. The development of such a multiantigen ELISA will be described in some detail. However, this area offers significant opportunities for new technology to be developed. A generic multiantigen ELISA is not possible, and specific assays must be developed for a specific product and/or purification process. This type of assay must also be reevaluated for its utility each time the host cell system or purification process is modified.

The development of a valid multiantigen ELISA is a theoretically simple process that, in reality, is both lengthy and somewhat difficult to accomplish. The first major requirement is a pool of host cell protein impurities that is representative of the manufacturing process (prior to final purification) and is devoid of the product or target protein. This preparation of host cell impurities then serves as the immunogen for production of polyclonal antibodies (in goats or rabbits) and later aids in the affinity purification of the crude polyclonal antibody fraction. It must be demonstrated at some point in the development of the ELISA that the affinity-purified antibodies bind to most or all of the observed host cell protein impurities. Finally, horseradish peroxidase (HRP) or alkaline phosphatase is coupled to a portion of the purified polyclonal antibodies to make an enzyme conjugate that serves as the second layer of a double-antibody sandwich ELISA.

Basically, in the double-antibody sandwich ELISA technique, a layer of purified antibodies to the host cell protein impurities is coated onto microtitration plates. Then, the product protein is added, and after incubation, the plates are washed to remove the product protein but none of the bound impurities. The enzyme conjugate is added and incubated. An appropriate substrate is added for color development, which is analyzed with a spectrophotometric plate reader. The concentration of the host cell impurity proteins is determined from a standard curve.

4. DNA Hybridization (Dot Blot) Assay. The technique of DNA hybridization, which is commonly referred to as dot blot analysis, is used to estimate the DNA content of rDNA-derived biologicals (*39*). The method typically can determine DNA at the nanogram to femtogram level and is the most sensitive routine DNA assay currently available. Dot blot analysis is valuable as a purification process assay to demonstrate that a low level of DNA has been attained at an early step in the manufacturing process. Process validation studies should have established the DNA to be even further reduced at the final product stage.

The dot blot assay relies on the hybridization of cellular DNA from the biological sample with specific ^{32}P-labeled DNA probes obtained from the DNA of the host cell (*40*). The hybridization is performed on a nitrocellulose filter that is subsequently placed between two X-ray films and exposed to produce an autoradiograph. The DNA of the sample is estimated by a visual comparison of the dot intensity of the sample to that of diluted DNA standards.

Assays to detect the presence of adventitious agents such as viruses (especially endogenous viruses), mycoplasmas, bacteria, and fungi are also performed in the quality control testing of rDNA-derived biologicals.

C. Potency. Certainly not of least significance in this discussion of assays for rDNA-derived biologicals are the potency assays, which measure the activity of the product and, therefore, ensure that it is efficacious. Essentially, there are three major types of potency assays: animal model assays, cell-line-derived assays, and in vitro biochemical assays. Each of these assays currently has application in the quality control testing of biological products. Regardless of the type of potency assay employed, it is desirable and, in some cases, necessary, to use a biomimetic assay (i.e., an assay that mimics the proposed biological effect of the product).

1. Animal Model Assays. Biomimetic assays in animal models have been developed for routine use in quality control (*22*). Although these assays have a relatively long history of use, they have several major disadvantages such as the large number of animals, appropriate animal facilities and handlers required, the high cost of analysis, a long analysis time (i.e., several days to weeks), and poor reproducibility of results. They are, however, in use mainly because a cell-line-derived or in vitro assay has not been developed and demonstrated to be of equal or greater significance. It is hoped that the biotechnology industry and the FDA will actively pursue the replacement of these animal model bioassays with more reproducible in vitro biochemical or cell-line-derived assays. The potency of methionyl-hGH is determined with a rat weight gain bioassay. Hypophysectomized female rats are monitored for weight gain over an 11-day period after daily injections with two doses of methionyl-hGH (*41*). The relative potency of the test sample is obtained by statistical comparison of the activity of the sample to that of a reference material.

2. Cell-Line-Derived Bioassays. This group of assays is comparatively easier to perform, gives results faster (1–3 days), and is considerably less expensive and less wasteful of resources than the animal model assays. Cell-line-derived bioassays provide information on the effect of the biological product in a living system, but they are also imprecise as a consequence of the variances of living cells. However, they can be automated and therefore can be repeated sufficiently to provide relatively reproducible and accurate results. A human lung carcinoma cell (A_{549}) assay that is performed on tissue culture plates is used to determine the antiviral (encephalomyocarditis virus) activity of human γ-interferon.

3. In Vitro Assays. This group of assays does not rely on a living model, but is usually based on the chemical action of a biological product. These methods are comparatively simple, fast, precise, and accurate. The activity of rt-PA, for example, can be determined with an in vitro clot lysis assay that can be automated and can provide the required results within hours (*21*). A synthetic fibrin clot is formed in the presence of plasminogen as a result of the action of the enzyme thrombin on fibrinogen. When rt-PA is added, the plasminogen is converted to the active enzyme plasmin, which then lyses the synthetic clot. The assay endpoint is followed spectrophotometrically or visually by noting the release of entrapped air bubbles. The assay has an accuracy of 99% with a relative standard deviation (RSD) of 5%. Another advantage of this type of assay, because of its precision and accuracy, is that it can be used to provide reliable estimates of the stability of the product.

V. ANALYTICAL OPPORTUNITIES FOR THE FUTURE

As described throughout this review, there is a seemingly endless number of opportunities for the development of new

or improved analytical methods for the biotechnology industry. Because of the complexity of the manufacturing processes and the fact that current "state of the art" analytical methods are often highly labor-intensive, extensive opportunities exist for improvements in automation, particularly in the area of on-stream monitoring. Many of the time-honored protein purity estimation techniques such as electrophoresis, amino acid analysis, and sequencing have already been automated to some extent, although much work is needed in the area of highly complicated sample preparation. Efforts in the application of robotics to this problem have already been described (*21*). Particular emphasis should be placed on the development of on-stream analysis methods for endotoxins and protein concentration in the routine monitoring of recovery processes. Routine methods are also needed for determining the consistency of glycosylation of mammalian cell culture products. The following sections describe some of the obvious areas for which there exist opportunities for future analytical development.

A. HPLC. The development of new HPLC methods for the analysis of proteins continues to offer the greatest near-term opportunities for advancement. In almost every possible area, from identity tests to purity analysis and stability monitoring, HPLC methods using size-exclusion, reversed-phase, hydrophobic interaction, and ion-exchange continue to be developed in order to replace biochemical methods such as SDS-PAGE and IEF. In addition, the coupling of HPLC methods with procedures such as mass spectrometry and immunochemistry offers the opportunity for a tremendous increase in the information obtainable from the analysis of proteins. It is clear that the use of HPLC for the purity analysis of proteins is a fertile area for development, particularly with respect to quality control operations.

B. Biomimetic Potency Assays. A very fruitful area of endeavor is the development of biomimetic assays for the determination of the biological activity of biotechnology products. As described above, potency or activity assays generally suffer from poor accuracy and precision. The opportunity for biologists, microbiologists, and chemists to work together to develop biomimetic assays that are truly meaningful as well as efficient, accurate, and precise provides a new area of collaboration for these divergent disciplines. Rugged, reliable bioassays are vitally important to the development and approval by regulatory agencies of new biotechnology products because literally thousands of such assays must be performed to develop, validate, and control new rDNA products. These assays are typically used for stability monitoring and require accuracies of 95–100% with RSD values of not more than 5%.

C. Electrophoresis. Electrophoretic methods such as SDS-PAGE and IEF are valuable techniques but are also very labor-intensive and slow. Although more rapid, automated systems for both SDS-PAGE and IEF have been developed, they often appear to lack the reproducibility and resolution of the large slab gels. One answer to this problem is the recently introduced technique of capillary electrophoresis (*42*), which offers many of the advantages of HPLC (speed and resolution) but is still not developed fully at this time. This technique appears to be particularly interesting as a potential quality control replacement for traditional gels.

D. Robotics. Robotics offers the advantage of performing highly repetitive, complicated sample preparations, which are common in the biotechnology industry. Unfortunately, current robotic systems are often slower than their human counterparts, and their application must be carefully considered in the quality control environment. Some applications such as isotopic labeling are ideally suited for robots. In addition, high-volume repetitive assays such as Karl Fischer moisture determinations for lyophilized products are suitable for robotic analysis.

E. Carbohydrate Mapping. The manufacture of high-molecular-weight glycosylated proteins is currently in its infancy with respect to rapid and reliable methods to ensure the consistency of glycosylation. Techniques such as hydrolytic assays of sialic acids and neutral sugars are too simplistic to ensure the level of consistency required for a pharmaceutical product. Methods of carbohydrate mapping similar to peptide mapping may solve this problem; however, they need to be developed and evaluated. No current techniques exist to address this issue.

F. DNA. Current regulatory safety concerns over trace amounts of oncogenic DNA in mammalian cell culture products require that cellular DNA be limited to 10–100 pg/dose (*43*). With the possible exception of vaccines, there are tremendous problems associated with the measurement of DNA at these levels in the presence of large amounts of protein. In addition, the commonly used technique of DNA hybridization is tedious, labor-intensive, and time-consuming. Thus, alternate methods that improve sample preparation and exhibit improved sensitivity for specific DNA need to be developed.

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ARTICLES

Different Methods of Graphite Electrode Treatment and Their Effect on the Electrochemical Behavior of a Small Adsorbing Biological Molecule, 2,6-Diamino-8-purinol

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Effect of the method of electrode treatment and the electrode material on the behavior of the adsorbing small biological molecule 2,6-diamino-8-purinol was evaluated. Electrochemical oxidation, polishing, and laser activation were the methods of treatment that were compared and glassy carbon and rough pyrolytic graphite were the electrode materials. The results indicate that effects such as chemistry of the surface rather than microscopic surface area may determine the behavior of this adsorbing molecule.

The chemistry and structure of graphite surfaces play a key role in heterogeneous electron transfer (*1*). Active surfaces, i.e., surfaces at which kinetics of electron transfer are effectively catalyzed, can be produced by different methods. Improved electrochemical behavior is observed for compounds and at surfaces that are very different. This indicates that different mechanisms may be responsible for the effective catalysis (*2*). It has been suggested that a decrease in hydrophobicity of activated surfaces (*2*) may be the major factor in the observed catalytic behavior. It has also been proposed that activation follows desorption of impurities from the electrode surface (*3*, *4*).

As a result of activation of glassy carbon electrodes by electrochemical treatment (*5*, *6*), polishing with alumina (*3*) and laser activation (*4*), background capacitance can increase. Increase in background capacitance has been related to an increase in adsorption (*7*). For example, when alumina-polished and heat-treated glassy carbon electrodes were com-

0003-2700/88/0360-2557$01.50/0