# Development and Manufacture of Protein Pharmaceuticals

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# Formulation Development of Protein Dosage Forms

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## 1. INTRODUCTION

A formulation scientist assigned the task of developing a stable, elegant, and manufacturable dosage form of a therapeutic protein drug has been given a significant challenge. Most proteins, as natural physiological molecules, are inherently unstable outside the human or animal body. Stability challenges in protein formulation development are typically enormous. The instability of these reactive and complex molecules must be considered not only in the *formulation* process, but also in development of the *packaging system* and the *manufacturing* process. These three areas are intimately and inseparably connected.

Protein dosage forms are also sterile dosage forms. Sterile dosage forms must be essentially free* from microbial contamination (sterile), free from pyrogenic (including endotoxin) contamination, and free from particulate

*The term "essentially free" is preferable over the more absolute term "free" when dealing with the subject of microbiological contamination. Except for products which can be terminally sterilized, which do not include proteins, there is no total and absolute assurance that each unit of product is, in fact, sterile.

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matter contamination (ready-to-use and reconstitutable solutions). Depending on the route of administration, sterile dosage forms must also be isotonic. For example, the intravenous route of administration can tolerate fairly wide ranges of "tonicity" (osmolality or osmolarity) or oncocity* whereas subcutaneous and intramuscular routes may require tighter control of product tonicity. Sterile products administered into spinal fluid or topically applied to the eye must be as close to isotonic as possible because of the potential of irreparable damage of spinal or corneal cells due to extremes in the osmolar concentrations of administered products. In addition, products administered by the injectable or ophthalmic topical routes should be as close to physiological pH (7.4) as possible to minimize pain and tissue irritation or damage.

This chapter was written to provide the basic approaches and techniques used to design and develop dosage forms of proteins. To develop dosage forms means not only to generate a viable formulation, but also to identify a final packaging system, to design and scale up a quality manufacturing method, and to employ valid measurements to assure product quality. In addition, in this era of globalization, formulations must be developed that are acceptable from a regulatory standpoint throughout the world.

Since protein stabilization has already been extensively discussed in many excellent references (Table I), we also intend to cover other issues essential in the complete formulation development of protein products yet not covered elsewhere, such as antimicrobial preservation, packaging components, container–closure integrity, clinical trial manufacturing, and development history reports.

We have reviewed the literature and have selected the articles which provide both intensive analysis and extensive information on solving protein formulation and other product development problems. Advanced injectable (e.g., controlled release, implantable devices, gene delivery) and noninjectable (e.g., pulmonary, oral, buccal) protein formulation research will not be covered in this chapter, but other references are available that deal with these advances (Baker, 1980; Davis *et al.*, 1986; Senior and Radomsky, 2000; Hillery *et al.*, 2001).

## 2. WHY PROTEINS PRESENT UNIQUE CHALLENGES TO THE DEVELOPMENT SCIENTIST

Many texts and articles already discuss the great difficulties scientists experience in protein dosage formulation because of the significant

* Oncotic pressure = osmotic pressure exerted by colloids (e.g., plasma proteins) in a solution.

**Table I**
Major Protein Formulation References

*Stability of Protein Pharmaceuticals, Parts A and B*, T. J. Ahern and M. C. Manning, (eds.), *Pharmaceutical Biotechnology*, vols 2 and 3, Plenum Press, New York 1992
*Formulation concerns of protein drugs*, T. Chen *Drug Dev Ind. Pharm.* **18**:1311–1354 (1992)
*The formulation of proteins and peptides*, M. J. Groves, M. H. Alkan, and A. J. Hickey, in: *Pharmaceutical Biotechnology* (M. E. Klegerman and M. J. Groves, eds.), Interpharm Press, Englewood, CO, 1992
*Protein stability and degradation mechanisms*, B. L. Currie and M. J. Groves, in: *Pharmaceutical Biotechnology* (M. E. Klegerman and M. J. Groves eds.), Interpharm Press, Englewood, CO, 1992
*Stability and Characterization of Protein and Peptide Drugs: Case Histories*, Y. J. Wang and R. Pearlman, Plenum Press, New York, 1993
*Factors affecting short-term and long-term stabilities of proteins*, T. Arakawa, S. J. Prestrelski, W. C. Kenney, and J. F. Carpenter, *Adv. Drug Deliv. Rev.* **10**:1–28, 1993
*Formulation and Delivery of Proteins and Peptides, Design and Development Strategies*, J. L. Cleland and R. Langer, (eds.), ACS Symposium Series 567, American Chemical Society, Washington, DC, 1994
*Formulation, Characterization, and Stability of Protein Pharmaceuticals*, R. Pearlman and Y. J. Wang, Plenum Press, New York, 1996

instabilities of these molecules. Depending on the amino acid types and sequence, proteins are subject to various types of degradation mechanisms, including hydrolysis, oxidation, racemization, and interaction with a variety of solutes and surfaces. These mechanisms are especially critical, because pharmaceutical proteins are very pure and removed from their natural environments where they are most stable (Hanson and Rouan, 1992). Dealing with physical instability (e.g., denaturation, aggregation, and adsorption) often is more a problem with proteins than dealing with their chemical stabilization. Physical instability actually involves solubility problems with large molecules. Although proteins generally contain many polar groups capable of ionization and hydrogen bonding with water, they also can contain many hydrophobic amino acids that under various conditions will preferentially self-associate, leading to aggregation and decreased solubility. Therefore, the development scientist needs to know the structure of the protein and its conformation in solution in order to anticipate potential chemical and physical stability difficulties and then, using principles outlined in this chapter, develop formulation strategies which will overcome these instabilities. Protein formulations may also have significant potential for supporting microbial growth as compared to smaller molecules. The problems associated with protein microbial growth promotion properties are covered in Chapter 3. Table II summarizes some of the primary differences one must recognize in developing protein dosage forms compared to nonprotein dosage forms.

**Table II**
Protein versus Small Molecule Comparison from a Stability Standpoint[a]

| Protein | Small molecule |
|---|---|
| Many potential reactive sites | Few reactive sites |
| Many ionizable sites | Few ionizable sites |
| Buffer effect usually a unique acid/base catalysis | Buffer effect usually general acid/base catalysis |
| Secondary/tertiary/quarternary structure | Lacks "higher order" structure |
| Disperse (colloidal) aqueous systems | Single and continuous phase in solution |
| Temperature effects can be discontinuous (denaturation) | Temperature effects are continuous |
| Readily supports microbial growth | |

[a] Courtesy, in part, of Dr. Lee Kirsch, University of Iowa, Iowa City, IA.

## 3. GENERAL FORMULATION PRINCIPLES FOR PROTEINS

Protein stability, both in the dry state and in solution, is the main reason why formulation science has such a presence in the commercial development of protein dosage forms. Proteins are complex in size and structure and, as macromolecules, contain a large number of functional groups. Generally, their biological activity in solution depends on a specific three-dimensional conformation. Almost every conceivable environmental factor (e.g., temperature, light, water, pH, presence of glass, rubber, or plastic, shear, presence of salts and other solutes, both macromolecules and low molecular weight compounds, detergents, or sanitizing agents, nature of the filling processes, freeze-thawing, freeze-drying) can effect conformational changes and lead to denaturation, aggregation, or adsorption to surfaces. The challenge to formulation scientists is to develop a stable formulation that can be consistently manufactured and is stable in a given packaging system over the shelf life of the product. A reasonable target expiration dating is 18 months to 2 years at ambient temperature or, failing this, at refrigerated conditions. Aqueous, ready-to-use solutions are preferable dosage forms for many reasons (convenience, cost, customer acceptance), but most proteins are not sufficiently stable in solution to allow practical expiration dating. Therefore, most protein dosage forms are solid forms in the commercial package with the solid form being produced by freeze-drying. Stability data should include not only the freeze-dried solid, but also solution stability after reconstitution with an appropriate vehicle.

Most additives in protein formulations are needed for stability purposes. These include buffers to enhance stability against specific acid/base-catalyzed hydrolysis, antioxidants, chelating agents, and inert gases to

enhance stability against oxidative degradation, cryoprotectants/lyoprotectants to enhance stability during freeze-drying of the protein product, surface-active agents to minimize interfacial denaturation, and excipients (e.g., albumin) to minimize protein adsorption to inert surfaces such as glass. The best formulation strategy is to keep the formulation as simple as possible, to have a clear reason for including each additive, and, if possible, to use excipients that have previously been used in Food and Drug Administration (FDA)-approved formulations. Hundreds of articles have appeared in the literature just in the last 20 or so years that report the stabilizing effects of additives on various proteins. We will reference those we feel have the most relevance to the industrial formulation scientist.

It is also important in this age of globalization that the formulation scientist develops a formulation that is acceptable worldwide. This is not an easy assignment, because there are many commonly used additives acceptable in one country, but not another. For example, disodium ethylenediaminetetraacetic acid (DSEDTA) is acceptable for use in injectable products in the United States and Europe, but is not acceptable in Japan. Levels of antimicrobial preservative agent(s) needed to pass the United States Pharmacopeia (USP) preservative efficacy test are much lower than levels required to pass the European Pharmacopoeia (EP) test.

Other additives in protein products (not including controlled drug delivery systems) serve one or more of the following functions:

- Agents for antimicrobial preservation
- Agents for solubility enhancement
- Bulking agents for freeze-dried products
- Agents for achieving isotonicity

Most proteins alone and in final product formulations support the growth of microorganisms. The microbial growth properties of proteins alone and in the final product formulation should be well known and steps should be taken to assure that the antimicrobial properties of the final formulation meet the appropriate acceptance criteria. For multiple-dose products, the addition of an antimicrobial preservative system is required to provide antimicrobial properties to the final product. Although including an antimicrobial preservative in a single-dose product has the advantage of providing additional assurance against introduction of microorganisms during manufacturing, this practice is generally frowned upon by regulatory agencies. Therefore, strict microbial control during manufacturing and the integrity of the packaging system all must be optimized in order to minimize the risk of inadvertent microbial contamination of the final product.

**Table III**
Common Stability and Compatibility Problems with Proteins and Possible Solutions

| Stability problem | Possible solutions |
|---|---|
| Hydrolysis, deamidation (e.g., asparagine deamidation) | pH control, buffers, low ionic strength |
| Oxidation (e.g., methionine oxidation) | Antioxidants, chelating agents, low pH, oxygen-free processing and packaging |
| $\beta$-Elimination | Low pH, chelating agents |
| Transpeptidation | pH control, lower concentration |
| Racemization | pH control, buffers |
| Disulfide exchange | Thiol scavengers (e.g., cysteine) |
| Denaturation during freeze-drying | Cryo-, lyoprotectants |
| Aggregation, precipitation | pH control, surface-active agents, minimize mechanical stress |
| Adsorption to surfaces | Surface-active agents, albumin, presaturation |

The formulation scientist must be aware of the potential for adverse effects of low-level impurities in formulation components and packaging materials on physical and chemical stability of proteins. Impurities such as peroxides from surface-active agents and other polymeric agents, aldehydes from polymer synthesis and degradation, and extractables from rubber closures must be known and controlled to avoid both short-term and long-term adverse effects on product quality.

Table III summarizes common stability and/or compatibility issues with protein dosage forms and suggested approaches for solving these issues. These approaches will be covered in more detail later in this chapter.

## 4. WHY PACKAGING, PROCESSING, AND FORMULATION ARE INTERRELATED

Most publications that deal with protein formulation do not cover aspects of the manufacturing process or packaging. Yet the three are interrelated. A formulation is not stable unless the product can be manufactured consistently at a large scale and packaged in a container/closure system that can maintain sterility and stability for a relatively long period of time. Packaging of proteins is especially challenging because of the inherent interactive nature of proteins with inert surfaces such as glass, rubber, and plastic. For many proteins, adsorption at these surfaces

sometimes results in the surface denaturation and subsequent aggregation of the protein (Cleland *et al.*, 1993). This includes interfacial denaturation at the air–water interface (e.g., the headspace in a vial containing a protein solution). Minimizing foaming caused by agitation during manufacture, as well as during use of the product, may be critical in order to avoid significant loss of protein activity or generation of visible particulate matter.

It is well known now that processing of protein formulations can affect protein stability. Examples include adverse effects of freezing and/or drying that occur during the lyophilization process, mixing/agitation processes, the filtration process, complicated manufacturing procedures requiring longer filling or hold times, and the movement of intermediate product from one location or site to another. In all these examples, the protein formulation must be designed to resist changes in potency, purity, and other physical–chemical characteristics of the protein itself and the finished formulation.

The bottom line message here is simple: A formulation scientist developing a protein (or, for that matter, any) dosage form must consider the formulation, process, and package together, not focus on one aspect exclusive of others. The smart formulation scientist, in fact, not only will consider all aspects of the formula, process, and package, but also will develop close interactions with packaging engineers, polymer scientists, manufacturing experts, and other experts in areas outside of the formulation scientist's direct expertise. ("None of us is as smart as all of us"—Satchel Paige.)

## 5. COMMERCIALLY AVAILABLE PROTEIN DOSAGE FORMS

Table IV summarizes U.S. marketed protein dosage forms approved by the FDA through 2000. The table contains information from the *Physicians' Desk Reference* (2001) on the dosage form, route of administration, and types and quantities of additives. Although preferential interaction experiments (e.g., Arakawa and Timasheff, 1982) can predict which solutes can serve as protein stabilizers, the majority of protein formulation research and development requires a great amount of trial and error to finalize the type and amount of formulation components. Prior "art," in the sense of knowing what has worked before and, particularly for injectable formulations, which additives have a history of safety and regulatory acceptance, greatly assists the protein formulation scientist in developing stable, elegant, and manufacturable dosage forms. Characterization of protein structure, as well as collecting preformulation data as described in Chapter 1, will provide supporting data for stabilizers and other additives that are most

**Table IV**
Current commercial protein forms

| Generic name[a] | Trade name | Manufacturer | Physical form[b] | Route of administration[c] | Excipients[d] |
|---|---|---|---|---|---|
| **Abciximab** (*Antiplatelet prevention of blood clots*) | ReoPro | Centocor Lilly | Solution | IV | Sodium phosphate<br>Sodium chloride<br>Polysorbate 80 (0.001%) |
| **Alglucerase** (*Gaucher disease*) | Cerdase | Genzyme | Solution | IV infusion | Sodium citrate<br>Human serum albumin (1%) |
| **Alteplase** (*Acute MI; pulmonary embolism*) | Activase | Genentech | Freeze-dried (*recons. with D5W or NS*) | IV | L-Arginine<br>Polysorbate 80 (0.011%)<br>Phosphoric acid<br>Incompatible with preservatives |
| **Antihemophilic factor** | Koate-HP<br>Recombinate | Miles<br>Baxter<br>Genetics Institute | Freeze-dried (*recons. with sterile WFI*) | IV | PEG (1500 ppm)<br>Glycine (0.05 M)<br>Polysorbate 80 (25 ppm)<br>Tri-n-butyl phosphate (5 ppm)<br>Calcium chloride (3 mM)<br>Albumin (10 mg/ml) |
| **Anti-inhibitor coagulant complex** (*Vitamin k dependent clotting factors [Heparin]*) | Autoplex T | Nabi | Freeze-dried (*recons. with sterile WFI*) | IV | Polyethylene glycol (2 mg/ml)<br>Sodium citrate |
| **Domase alpha DNAse** (*Cystic fibrosis*) | Pulmozyme | Genentech | Aerosol | Inhalation | Calcium chloride<br>Sodium chloride |

| | | | | | |
|---|---|---|---|---|---|
| **Eptifibatide** (*Acute coronary syndrome inhibits platelet aggregation*) | Integrilin | Cor | Solution | IV | Citric acid<br>Sodium hydroxide to pH 5.25 |
| Erythropoietin (*Anemia*) | Epogen<br>Procrit | Amgen<br>Ortho Biotech | Solution | IV or SC | Sodium chloride<br>Sodium citrate<br>Human serum albumin (0.25%) |
| Filgrastim (Granulocyte CSF) | Neupogen | Amgen | Solution | IV or SC | Sodium acetate<br>Mannitol<br>Polysorbate 80 (0.004%) |
| Hepatitis B vaccine | Engerix-B | GlaxoSK | Solution | IM | 20 mcg adsorbed on 0.5 mg aluminum<br>Thimerosal<br>Sodium chloride<br>Sodium phosphates |
| | Recombinax | Merck | Suspension | IM | 5.40 mcg adsorbed on 0.5 mg aluminum<br>Thimerosal |
| Human chorionic gonadotropin | A.P.L. | Wyeth-Ayerst | Freeze-dried (*recons. with special diluent*) | IM | Benzyl alcohol (2%) (diluent)<br>Phenol (0.2%) (diluent)<br>Lactose |
| Human chorionic gonadotropin | Pregnyl | Organon | Freeze-dried (*recons. with speical diluent*) | IM | Sodium phosphate<br>Sodium chloride (diluent)<br>Benzyl alcohol (0.9%) (diluent) |
| | Profasi | Serono | Freeze-dried (*recons. with bact. WFI*) | IM | Sodium phosphate<br>Mannitol<br>Benzyl alcohol (0.9%) (diluent) |

(*continued*)

**Table IV**
*(continued)*

| Generic name[a] | Trade name | Manufacturer | Physical form[b] | Route of administration[c] | Excipients[d] |
|---|---|---|---|---|---|
| **Human growth hormone** | Humatrope | Lilly | Freeze-dried (*recons. with special diluent*) | SC | Mannitol<br>Glycine<br>Sodium phosphate<br>Meta cresol (0.3%) (diluent)<br>Glycerin (diluent) |
| | Nutropin | Genentech | Freeze-dried (*recons. with bact. WFI*) | SC | Mannitol<br>Sodium phosphates<br>Benzyl alcohol (0.9%) (diluent) |
| | Nutropin NQ | Genentech | Solution | SC | Sodium chloride<br>Phenol (0.5%)<br>Polysorbate (0.4%)<br>Sodium citrate 10 mM |
| Human insulin | Humulin R | Lilly<br>Squibb-Novo | Solution | SC | Glycerin<br>*m*-Cresol (0.25%) |
| Human insulin | Humulin N | Lilly<br>Squibb-Novo | Suspension | SC | Protamine<br>*m*-Cresol (0.16%)<br>Phenol (0.065%)<br>Sodium phosphate |
| Human insulin | Humulin L | Lilly<br>Squibb-Novo | Suspension | SC | Methylparaben (0.1%)<br>Sodium chloride<br>Sodium acetate<br>Zinc oxide |
| Immune globulin (*Gamma globulin IgG*) | WinRho SDF | Nabi | Freeze-dried (*recons. with accompanying 0.9% sodium chloride*) | IV or IM | Glycine<br>Sodium chloride<br>Polysorbate 80 (0.01%) |

| | | | | | |
|---|---|---|---|---|---|
| Interferon α-2a (*Hairy cell leukemia*) | Roferon-A | Roche | Freeze-dried and RTU Solution | IM or SC | Sodium chloride<br>Phenol (0.3%)<br>Human serum albumin |
| Interferon α-n3 (*Genital warts*) | Alferon N | Interferon | Solution | Intralesional | Phenol (0.33%)<br>Human albumin (0.1%)<br>Sodium chloride<br>Potassium chloride<br>Sodium phosphates |
| Interferon α-2b (*Hairy cell leukemia*) | Intron A | Schering | Freeze-dried (*recons. with bact WFI*) | IM or SC<br>Intralesion | Glycine<br>Sodium phosphates<br>Human serum albumin<br>Benzyl alcohol (0.9%) |
| Interferon α-26 (*Hairy cell leukemia*) | Intron A | Schering | Solution | IM or SC<br>Intralesion | Glycine<br>Sodium phosphates<br>Human serum albumin<br>Parabens |
| Interferon β-1B (*Multiple Sclerosis*) | Betaseron | Berlex<br>Chiron | Freeze-dried | SC | Human serum albumin<br>Mannitol<br>Sodium chloride |
| Interferon γ-1B | Actimmune | Genentech | Solution | SC | Mannitol<br>Sodium succinate<br>Polysorbate 20 (0.01%)<br>Sodium citrate |
| Interleukin-2 (*Metastatic renal cell carcinoma*) | Proleukin | Chiron | Freeze-dried (*recons. with sterile WFI*) | IV Infusion | After reconstitution, dilute in 50 mL D5W<br>Incompatible with preservatives |

(*continued*)

**Table IV**
*(continued)*

| Generic name[a] | Trade name | Manufacturer | Physical form[b] | Route of administration[c] | Excipients[d] |
|---|---|---|---|---|---|
| Interleukin-11 (*Prevention of severe thrombocytopenia; reducing need for platelet transfusions*) | Neumega | Genetics Institute | Freeze-dried (*recons. with sterile WFI*) | SC | Glycine<br>Phosphate buffer to pH 7 |
| Leuprolide Acetate | Lupron | TAP Pharma | Solution | SC | Sodium chloride<br>Benzyl alcohol<br>Acetic acid |
| Leuprolide Acetate | Lupron Depot | TAP Pharma | Freeze-dried | IM | Gelatin<br>DL-Lactic/glycolic acids<br>D-mannitol<br>Reconstituted with diluent containing CMC sodium, D-mannitol, Polysorbate 80 |
| Muromonab-CD3 (*Immuno suppressant*) | Orthoclone OKT3 | Ortho Biotech | Solution | IV | Polysorbate 80 (0.1%)<br>Sodium phosphates<br>Soduim chloride |

| | | | | | |
|---|---|---|---|---|---|
| Rituximab (*Treatment of B-cell non-Hodgkins lymphoma*) | Rituxan | Genentech | Solution pH 6.5 | IV | Sodium chloride<br>Sodium citrate<br>Polysorbate (0.07%) |
| Sargramostim (*Granulocyte macrophage CSF for bone marrow transplant*) | Leukine | Immunex | Freeze-dried (*recons. with sterile WFI*) | IV Infusion | Mannitol<br>Sucrose<br>TRIS<br>Incompatible with preservatives |
| Sermorelin acetate (*Stimulates pituitary gland to release growth hormone*) | Geref Ampuls | Serono | Freeze-dried (*recons. with accompanying 0.9% sodium chloride*) | SC | Mannitol<br>pH adjusted with sodium phosphate buffer |
| Urokinase | Abbokinase | Abbott | Freeze-dried (*recons. with. sterile WFI*) | IV, Intra-coronary Infusion | Mannitol<br>Sodium chloride<br>Human serum albumin |

[a]MI, myocardial infarction; CSF, colony stimulating factor.
[b]recons., reconstituted; D5W, dextrose 5% in water; NS, 0.9% sodium chloride; WFI, water for injection; bact., bacterial; RTU, ready to use.
[c]IV, intravenous; SC, subcutaneous; IM, intramuscular.
[d]CMC, carboxymethylcellulose.

likely to be effective. For example, pH–solubility/stability studies will provide direction on what type of buffers to use, if any. Having knowledge of the structural conformation of a protein in order to predict which amino acids in the protein sequence may be particularly vulnerable to degradation because of exposure to the environment may give the formulation scientist some direction on the stabilizers required. The final selection of excipients, unfortunately, must be a result of much empirical evaluation. However, information such as that given in Table IV summarizes what others have done with their protein products, and thus can give significant guidance to formulation scientists facing the development and stabilization of new peptide and protein dosage forms.

Note that protein dosage forms primarily are divided into three types

1. Ready-to-use solutions
2. Freeze-dried powders that are reconstituted into solutions immediately before administration
3. Ready-to-use suspensions

Proteins are commonly formulated at very low doses (very dilute solutions), although there are examples of relatively high dose protein products, such as formulations of immunoglobulin G (IgG) at 50 mg/ml. In general, dilute solutions are less physically stable than more concentrated solutions (Hanson and Rouan, 1992) and adsorption to surfaces will result in a higher fractional loss of protein. However, in the case of the Neutral Protamine Hagedorn (NPH) formulation of insulin, the rate of formation of higher molecular weight polymers increases as a function of concentration (Brange *et al.*, 1992b). Also, for interleukin 1$\beta$ (IL-1$\beta$), aggregation/precipitation was shown to demonstrate biphasic kinetics (slower rate followed by a more rapid rate) at temperatures lower than 55°C and to be dependent on concentration. When the concentration was increased from 100 to 500 mg/ml, the slower rate was observed to be suppressed and a more rapid degradation was observed (Gu *et al.*, 1991). In general, however, there are surprisingly few literature reports of protein stability as a function of concentration.

## 6. CHEMICAL STABILIZATION

### 6.1. pH, Hydrolysis, and Buffers

The effect of solution pH on stability is probably the most important factor to study in early protein dosage form development. Figure 1

schematically depicts expected stability problems of proteins as a function of pH. pH–stability studies are conducted very early to understand relative protein stability over a pH range, typically from about pH 3 to about pH 10. The relationship of stability and solubility to pH usually follows a pattern of higher solubility resulting in lower chemical stability and lower solubility resulting in lower physical stability. Protein solubility is usually at a minimum at its isoelectric point. Insulin, for example, has an isoelectric point of 5.4, and at this pH it is quite insoluble in water ( < 0.1 mg/ml). Adjusting the solution pH to less than 4 or greater than 7 greatly increases insulin solubility ( > 30 mg/ml, depending on zinc concentration and species source of insulin), but also increases the rate of deamidation at these pH ranges (Brange, 1992). An example of the effect of pH on deamidation and polymerization of insulin is shown in Fig. 2 (Brange and Langkjaer, 1993). In dosage form development, the scientist must first determine what pH range provides acceptable solubility of the protein for proper dosage, then determine whether this pH range also provides acceptable stability. There is usually a trade-off between solubility and stability and it is up to the scientist to identify what pH is optimal for both. When an acceptable trade-off does not exist for a solution formulation, a freeze-dried formulation is usually indicated.

*Hydrolysis* or *deamidation* occurs with proteins containing susceptible Asn and Gln amino acids, the only two amino acids that are primary amides. The side-chain amide linkage in a Gln or an Asn residue has been shown to undergo deamidation to form free carboxylic acid. Deamidation

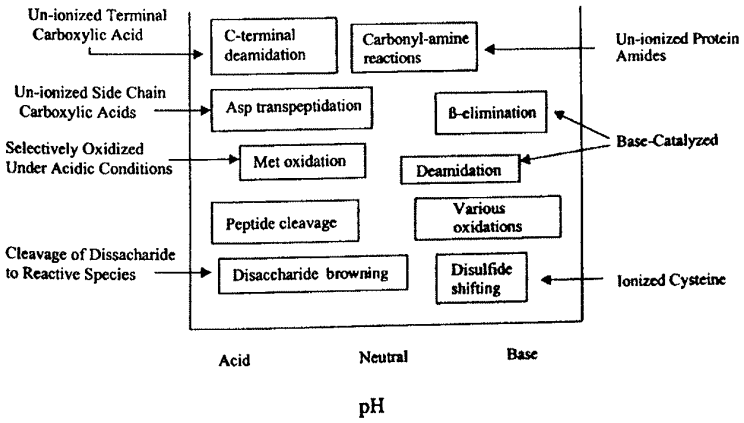


**Figure 1.** Protein reactions as a function of pH. Figure courtesy of Dr. Lee Kirsch.

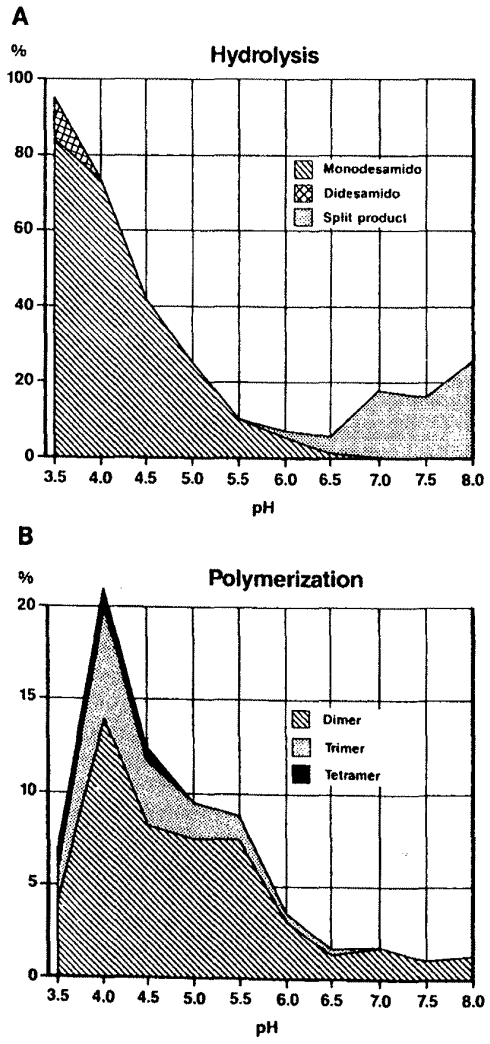


**Figure 2.** Chemical transformation of insulin during storage of rhombohedral insulin crystals (bovine insulin crystals: 0.7% NaCl, 0.2% phenol) as a function of pH during storage at 25°C for 12 months. (A) Formation of the hydrolysis products mono and didesamido insulins and the insulin split product (AB-A9). (B) Formation of covalent dimers and oligomers. Reprinted with permission from Brange and Langkjaer (1993). Copyright 1993 Plenum Press.

can be promoted by a variety of factors, including extremes in pH, temperature, and ionic strength. Many investigators have observed altered forms of proteins which have been attributed to this deamidation process. Robinson and Rudd (1974), Geiger and Clarke (1987), and Clarke *et al.* (1992) reviewed the chemical conditions necessary for hydrolysis of Asn and Gln residues for many proteins. It was shown that neutral or alkaline conditions enhanced the rate of deamidation of proteins mainly at the Asn–Gly sequence. This rate was also found to be higher than the hydrolysis of the amino acid Asn itself. This deamidation proceeds through a five-membered cyclic imide intermediate formed by intramolecular attack of the succeeding peptide nitrogen at the side-chain carbonyl carbon of the Asn residue. The cyclic imide then spontaneously hydrolyzes to give a mixture of peptides in which the backbone is attached via either an Asp ($\alpha$-carboxyl) or an iso-Asp ($\beta$-carboxyl) linkage. Haley *et al.* (1966) showed that Gln also undergoes a similar deamidation reaction via the formation of a six-membered ring. Peptide backbone hydrolysis also has been shown to occur from this cyclic imide (Tyler-Cross and Schirch, 1991).

One approach that has been used to lower the rate of deamidation is to reduce pH, because in general, deamidation is slower at acidic pH than at neutral or alkaline pH. However, caution must be exercised here, because a reduction in pH may lead to cleavage or cyclization at Asp–X residues, where X is usually a residue with a small side chain, such as Gly or Ser. Proteins with Asp–X degradation must be formulated at a higher pH to avoid cyclization (Manning *et al.*, 1991). However, higher pH conditions (i.e., greater than pH 8) may catalyze oxidation, thiol–disulfide exchange, and $\beta$-elimination reactions. In the case of insulin, multiple deamidation sites are observed, where deamidation at the A21 position predominates at acidic pH and deamidation at B3 predominates at neutral pH (Brange, 1992).

*Buffers.* Buffers are used to prevent small changes in solution pH which can affect protein solubility and stability. Buffers are composed of salts of ionic compounds, the most common of which are acetate, citrate, and phosphate. Buffer systems acceptable for use in parenteral solutions are listed in Table V.

The proper selection of buffer type and concentration is done by performing solubility and stability studies as a function of pH and buffer species. In general, it is good practice to keep the buffer concentration as low as practical.

Potential problems associated with using buffers include the following.

1. It may be difficult to meet the pH target with a buffer system while preparing solutions during scaleup and full-scale manufacturing. Dilute solutions of strong acids (hydrochloric acid) or bases (sodium hydroxide) usually are required, which may alter the buffer capacity

**Table V**
Buffers Used in Protein Formulations

| Buffer system | $pK_a$ | pH range of use |
|---|---|---|
| Acetate | 4.76 | 2.5–6.5 |
| Citrate | 3.14, 4.8, 5.2 | 2.5–6.0 |
| Glutamate | 9.67($pK_{a3}$) | 8.2–10.2 |
| Glycinate | 2.4,9.8 | 6.5–7.5 |
| Histidine | 1.8, 6.0, 9.2 | 6.2–7.8 |
| Lactate | 3.8 | 3.0–6.0 |
| Maleate | 1.92, 6.23 | 2.5–5.0 |
| Phosphate | 7.2 ($pK_{a2}$) | 6.0–8.2 |
| Succinate | 4.2, 5.64 | 4.8–6.3 |
| Tartrate | 2.93, 4.23 | 3.0–5.0 |
| Tris | 6.2 ($pK_b$ 7.8) | 6.8–7.7 |

of the buffer system and the ionic strength of the solution depending on the buffer type, the concentration of the buffer, and the pH of the formulation (Niebergall, 1990).

2. Increasing buffer capacity to better control pH probably also will significantly increase ionic strength, which, in turn, may cause increase in frequency of pain upon injection due to the increased osmolality.
3. General acid and/or general base buffer catalysis may accelerate the hydrolytic degradation of the protein. An example is given in Fig. 3 (Yoshioka *et al.*, 1993), where the inactivation rate of $\beta$-galactosidase increases with increasing concentration of phosphate buffer up to 0.5 *M*, then decreases, presumably because of higher buffer concentration causing a reduction in water activity. Cleland *et al.* (1993) cite several examples where the rate of protein deamidation was markedly dependent on the buffer anion. One interesting example was reported by Capasso *et al.* (1991) in which the deamidation rate of a small peptide was measured using different buffers. The peptide was most unstable in a phosphate buffer and most stable in Tris buffer.
4. Buffer crystallization during freezing and freeze-drying may change the pH of the freeze-concentrated solution and could affect drug/protein stability (Murase and Franks, 1989; Szkudlarek *et al.*, 1996); (van den Berg and Rose, 1959). The dibasic phosphate salt tends to crystallize during freezing and the degree of pH change depends on a variety of factors, including the original pH, the buffer concentration, and whether the cation of the phosphate buffer is sodium or phosphate. A freezing-induced pH shift may occur with mono-

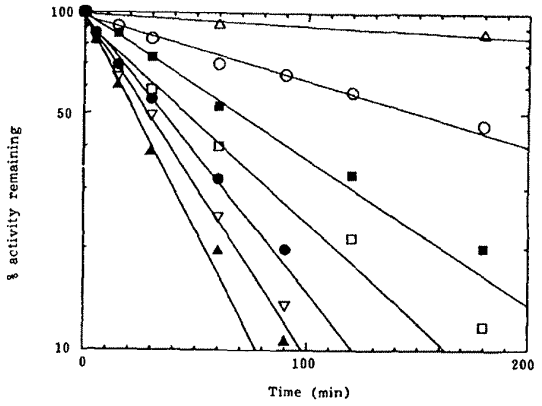


**Figure 3.** Inactivation of $\beta$-galactosidase in pH 7.4 phosphate buffer solution at 50°C as a function of phosphate buffer concentration: (△) 10, (○) 50, (□) 100, (▽) 200, (▲) 500, (●) 700, and (■) 900 m*M*. The concentration of $\beta$-galactosidase was 0.1 mg/ml. Reprinted with permission from Yoshioka *et al.* (1993). Copyright 1993 Plenum Press.

sodium succinate buffer, causing a loss of activity of interferon gamma during freeze-drying (Lam *et al.*, 1996). This was overcome by using glycolate buffer instead of succinate buffer.

### 6.2. Oxidation, Antioxidants, and Other Antioxidation Approaches

Proteins containing methionine, cysteine, cystine, histidine, tryptophan, and tyrosine may be sensitive to oxidative and/or photolytic degradation, depending on the conformation of the protein and resultant exposure of these amino acids to the solvent and environmental conditions such as oxygen, light, high temperature, metal ions, and various free radical initiators. Oxidation of sulfhydryl-containing amino acids (e.g., methionine and cysteine) may lead to disulfide bond formation and loss of biological activity. The free thiol group in a Cys residue of any native biologically active protein not only may oxidize to produce an incorrect disulfide bridge, but also can result in other degradation reactions, such as alkylation, addition to double bonds, and complexation with heavy metals. Atmospheric oxygen has been shown to oxidize Met residues during isolation, purification, and storage (Brot and Weissbach, 1982). The thioether moiety in Met is a weak nucleophile and therefore stays deprotonated even at low

pH. However, it has been shown to be selectively oxidized to Met sulfoxide by agents such as hydrogen peroxide under acidic conditions. Other agents that have been used to catalyze this oxidation reaction are periodate, iodine, chloramine-T, dimethylsulfoxide, and *N*-chlorosuccinimide. In several instances these oxidation processes render the protein moiety biologically inactive as has been shown in corticotropin, α- and β-melanotropins, gastrin, calcitonin, corticotropin releasing factor, and parathyroid hormone (Brot and Weissback, 1992; Deadman *et al.*, 1961; Dixon, 1956; Morley *et al.*, 1965; Riniker *et al.*, 1968; Tashjian *et al.*, 1964; Vale *et al.*, 1981). In addition to these examples, several nonhormonal proteins and peptides have been shown to lose activity due to oxidation of the Met residue. In these instances restoration of biological activity concurrently occurs with the reduction of Met sulfoxide to Met. However, there are also several cases where the oxidation of the Met residue does not seem to affect the biological activity of the pharmaceutical agent.

Human growth hormone, chymotrypsin, lysosyme, insulin-like growth factor I, and glucagon are examples of proteins that will degrade by oxidative mechanisms. Hemoglobin, with its oxygen carrying properties dependent on the reduced state of ferrous iron, is very sensitive to oxidation and, as a commercial product in the deoxy state, must contain antioxidants to maintain stability of the heme groups. Kerwin *et al.* (1999) and Cleland *et al.* (1993) list 61 different proteins that can be oxidized with varying degrees of loss of biological activity following oxidation.

There are several choices of antioxidants that can be used in protein formulations (see Table VI). Those which have been used most frequently in protein formulations are ascorbic acid, salts of sulfurous acid (sodium bisulfite, sodium metabisulfite), and thiols such as thioglycerol and thioglycolic acid. Dithriothreitol, reduced glutathione, acetylcysteine, mercaptoethanol, and thioethanolamine are thiols which usually oxidize too readily to be of practical use in pharmaceutical formulations requiring long-term storage.

Precautions must be applied when considering ascorbic acid as an antioxidant in protein formulations. Li *et al.* (1993) found that ascorbate in the presence of $Fe^{3+}$ and oxygen actually induces the oxidation of methionine in small model peptides. Ascorbate is a powerful electron donor and is readily oxidized to dehydroascorbate. It also generates highly reactive oxygen species, such as hydrogen peroxide and peroxyl radicals. These, in turn, will accelerate the oxidation of methionine. Phosphate buffer compared to other buffer systems [e.g., tris(hydroxymethyl)aminomethane (Tris), *N*(2-hydroxyethyl)piperazine-2-(2-ethanesulfonic acid) (HEPES), and 3-(*N*-morpholino)propanesulfonic acid (MOPS)] accelerated the degradation of methionine in the presence of ascorbic acid. The addition of ethylenediamine-

**Table VI**
Antioxidants and Chelating Agents for Protein Formulations

| Agent | Normal % used in formulation |
|---|---|
| Antioxidants (all water soluble) | |
| Ascorbic acid (including isoascorbic acid, sodium ascorbate) | 0.1–1.0 |
| Sulfurous acid salts (sodium bisulfite, sodium metabisulfite, sodium sulfite) | 0.1–0.5 |
| Thioglycerol | 0.1–0.5 |
| Thioglycollic acid | 0.05–0.2 |
| Cysteine hydrochloride | 0.1–0.5 |
| Chelating Agents (all water-soluble) | |
| Ethylenediaminetetraacetic acid (usually the disodium salt) | 0.05–0.1 |
| Citric acid/sodium citrate | 0.02–1 |

tetraacetic acid (EDTA) did not enhance stability, even though ferric ion and other transition metals were components of the formulation, either purposely added or as trace components of the buffer and peptide. This pro-oxidant effect of ascorbate methionine oxidation was reported to be concentration-dependent and occurred most readily at pH 6–7. It is also known that bisulfite can cause stability problems with certain drugs and proteins. For example, bisulfite will rapidly destroy insulin (Brange, 1987).

For protection against oxidation, use of an effective antioxidant is one of several precautions that must be enacted in formulation development which dramatically affects final product manufacture. Other procedures that contribute to protein stability against oxidative degradation include the following:

- Preparation and storage at low temperatures
- Use of chelating agents to eliminate metal catalysis
- Increase of ionic strength (decreases disulfide bond formation; Shahrokh *et al.*, 1994b)
- Elimination of peroxide and metallic contaminants in formulation additives
- Protection from light
- Awareness of possible interaction of light exposure and phosphate buffer in forming free radicals (Fransson and Hagman, 1996)
- Replacement of oxygen with nitrogen or argon during manufacturing
- Removal of oxygen from the headspace of the final container
- Formulation at the lowest pH possible while still maintaining desired protein solubility and hydrolytic stability, because there is

an inverse correlation between oxidative stability and pH (Akers, 1982)

- Use of a container/closure system that allows no oxygen transmission through the package during distribution and storage
- Assurance that phenolic or other oxidizing cleaning agent residues are minimal in the production environment, including the freeze-dry chamber (Kirsch *et al.*, 1993)

Stabilization of human growth hormone against oxidative degradation will be presented as a case history example at the end of this chapter.

Chelating agents (see Table VI) are used in protein formulations to aid in inhibiting free radical formation and resultant oxidation of proteins caused by trace metal ions, such as copper, iron, calcium, manganese, and zinc. Although organic buffer salts, such as sodium citrate, have some capability of binding trace heavy metal contaminants in protein solutions, the major chelating agent used is DSEDTA. The concentration of DSEDTA usually is very small, typically in the range of 0.01–0.05%. DSEDTA tends to dissolve slowly and is usually among the first of formulation ingredients to be dissolved during compounding before adding other ingredients, including the protein.

### 6.3. Other Protein Chemical Instabilities and Formulation Approaches

At temperatures above ambient temperature, proteins can undergo disulfide cleavage due to $\beta$-elimination from cysteine residues. Such a reaction is known to occur at low temperatures and high pH. Volkin and Klibanov (1987) found that the rate of $\beta$-elimination is greatly accelerated under alkaline conditions. The free sulfhydryl group that is formed from $\beta$-elimination reaction of cysteine can then contribute to other degradation pathways such as disulfide exchange, aggregation, precipitation, and adsorption. In addition to cysteine, other amino acids that undergo degradation via the $\beta$-elimination route under alkaline conditions are Cys, Ser, Thr, and Lys. Sen *et al.* (1977) showed that the rate of $\beta$-elimination in phosvitin directly correlated with hydroxide concentration, and $\beta$-elimination of phosphoserine occurred in the presence of calcium chloride. On the basis of their studies, Lee *et al.* (1977) showed that the antifreeze glycoprotein undergoes $\beta$-elimination in proportion to the hydroxide concentration and temperature. Control of pH, therefore, is essential to prevention of $\beta$-elimination degradation pathways of susceptible proteins.

Sulfhydryl groups and disulfide bonds play a major role in determining the tertiary structure properties of most proteins. Biological activity can be

lost when an interchange of disulfide bonds leads to an altered three-dimensional structure. Various nucleophiles and electrophiles are able to carry out reversible breakage of disulfide linkages. The free thiol group that is generated can then produce incorrect disulfide exchanges. As Srinivasan *et al.* (1990) pointed out, rates of such reactions can be very significant in proteins at elevated temperatures or extremes of pH, and refolding is not usually observed. Liu *et al.* (1991) observed that moisture-induced aggregation of lyophilized proteins such as ovalbumin, bovine serum albumin (BSA), and β-lactoglobulin is caused by incorrect disulfide exchanges in combination with conformational changes. Ryle and Sanger (1955) and Torchinsky (1981, pp. 81–86) were able to successfully prevent disulfide exchanges by using thiol scavengers such as copper ion, *N*-ethylmaleimide, and *p*-mercurybenzoate. Haber and Anfinsen (1962) and Galat *et al.* (1981) observed that proteins with scrambled disulfide bonds can be rearranged to yield native, biologically active species by incubating the protein with mercaptoethanol or cysteine.

With the exception of glycine, all amino acids exhibit chirality at the carbon site to which side chains are attached. Consequently, these amino acids are subject to racemization under basic conditions. These racemizations in proteins can create either a nonmetabolizable enantiomeric form such as D amino acids or introduce peptide bonds at locations inaccessible to proteolytic cleavage. The rate of racemization depends on factors such as inductive and field effects, steric hindrance to solvation, intramolecular solvation, and intramolecular base action. Friedman and Masters (1982) showed that Asp in proteins undergoes racemization at a rate much faster than the free amino acid residue and that the rate of conversion of the L form to D-Asp is more rapid than that of any of the other amino acid side chains. Geiger and Clarke (1987) showed that the racemization of the Asp residue in alrenocorticotropic hormone (ACTH) occurs via a cyclic imide at pH 7.4 and 37°C. Figure 1 graphically summarizes the pH range where the above degradation reactions most likely will occur.

## 7. PHYSICAL STABILIZATION

Physical instability is rarely encountered in formulations of small drug molecules except for poorly water-soluble compounds. Proteins, because of their ability to adopt higher ordered structures, tend to undergo a number of changes structurally, independent of chemical modifications. Physical instability of proteins is often a greater cause for concern and may be more difficult to control than chemical instability. Virtually all protein structures have hydrophobic regions to some extent, and low solubility in water is

regarded as an indication that a material is hydrophobic (Currie and Groves, 1992). Hydrophobic interaction is a major driving force for protein folding, where hydrophobic regions tend to be on the interior of the folded structure. Such exposure will promote aggregation or self-association, possibly leading to physical instability and potential loss of biological activity, because the interaction with the receptor site requires folded structures with the correct conformation.

### 7.1. Denaturation

Denaturation is unique to proteins and occurs when their native tertiary and frequently secondary structure is disrupted. Denaturation can lead to unfolding, and the unfolded polypeptide chain may undergo further reactions. Such inactivation could be association with surfaces and/or interaction with other protein molecules leading to aggregation and precipitation. Denaturation is of two types: (1) reversible denaturation, caused by temperature or exposure to denaturants (urea, guanidine hydrochloride), where, if the denaturing condition is removed, the protein will regain its native state and recover its activity, and (2) irreversible denaturation, where the protein, once unfolded, will not regain its native form and activity. However, there are several instances where a protein which is "irreversibly denatured" is returned to its native state by the use of denaturant followed by dialysis. One example is T4 lysozyme, where activity can be restored by renaturation with guanidine hydrochloride (Wetzel *et al.*, 1988).

For stabilizing proteins against denaturation in solution, Arakawa and Timasheff (1982, 1984) and Arakawa *et al.* (1993) showed that reversible denaturation can be decreased by the use of additives such as salts that bind to nonspecific binding sites on the proteins. Dahlquist *et al.* (1976) demonstrated increased thermal stability of thermolysin by the binding of ions to specific sites on the protein. Roe *et al.* (1988) reported the ability of $Zn(NO_3)_2$ to significantly increase the thermal stability of superoxide dismutase. Gekko and Timasheff (1981) concluded that the preferential hydration of proteins observed at all conditions in the presence of a glycerol–water mixed solvent system is a prerequisite for stabilizing the native structure of several globular proteins. Pace and Grimsley (1988) found the stability of ribonuclease $T_1$ to increase in the presence of 0.1 *M* NaCl, $MgCl_2$, and $Na_2HPO_4$. Through genetic engineering they were then able to introduce appropriate amino acid substitution in ribonuclease $T_1$. Due to these newly created specific cation/anion-binding sites on the protein, the stability profile of ribonuclease $T_1$ was enhanced significantly. Pantoliano *et al.* (1988) were

able to introduce negatively charged side chains such as Asp in the vicinity of the weakly $Ca^{2+}$ binding site of subtilisin. Such modifications caused an increase in binding affinity for $Ca^{2+}$, thereby increasing the thermal stability of subtilisin.

### 7.2. Aggregation

Protein aggregation is caused mainly by hydrophobic interactions resulting from denaturation. When the interior hydrophobic region of a partially or fully unfolded protein is exposed to water, this creates a thermodynamically unfavorable situation due to the fact that a hydrophobic interior is now exposed to a hydrophilic aqueous environment. Consequently, the decrease in entropy from structuring water molecules around the hydrophobic region generates a driving force for the denatured protein to aggregate, mainly through the exposed hydrophobic regions. Thus, solubility of the protein is also compromised (Franks, 1994). In some cases self-association of protein subunits, either native or misfolded, may occur under certain conditions and this may lead to precipitation and loss in activity (Brange and Langkjaer, 1993; Brange *et al.*, 1992a,b; Mitraki and King, 1989; Shahrokh *et al.*, 1994a; Silvestri *et al.*, 1993). The protein antithrombin aggregates during denaturation with guanidine hydrochloride in a manner which proceeds through an intermediate, partially unfolded state. Subsequent aggregation occurs slowly; therefore, it is possible to return antithrombin to its native state by dialyzing the partially unfolded form, which aggregates slowly. However, once aggregation occurs, the native state cannot be reformed by this approach (Fish *et al.*, 1985). Irreversible aggregation due to denaturation can be prevented by the use of surfactants, polyols, or sugars.

### 7.3. Adsorption

Proteins exhibit a certain degree of surface activity; that is, they adsorb to surfaces due to their innate nature of being amphiphilic polyelectrolytes. Consequently, biological activity may be either reduced or totally lost if such adsorption occurs during manufacturing, storage, or use of the final product. Protein adsorption, besides causing loss of activity, may also be a precursor to aggregation, denaturation, and precipitation phenomena. The process of adsorption depends on protein–protein interactions, time, temperature, pH and ionic strength of the medium, and the nature of the surface. Norde (1995) reviewed the general principles underlying protein

adsorption from aqueous solution onto a solid surface. Interactions that determine the overall adsorption process between a protein and a surface include redistribution of charged groups in the interfacial layer, changes in the hydration of the sorbent and the protein surface, and structural rearrangements in the protein molecule. Surface denaturation, which commonly takes place at the liquid/solid and the liquid/air interface, has been shown by Lenk *et al.* (1989) to involve conformational changes such as loss of $\alpha$ helices to $\beta$ sheets and certain random structures. These structural changes, which are determined by the nature of the interfaces, are similar to those observed with the aggregation phenomenon caused by heat, high pressure, and chemical denaturants. Strategies often used to overcome protein denaturation due to adsorption are include the following:

- Increase protein concentration
- Modify (e.g., siliconize) the surface of the glass containers, providing a resistant barrier to protein–surface interaction
- Decrease the rate of mixing when it is known that shear will affect protein adsorption
- Add excipients such as surfactants that have higher surface activity
- Add macromolecules such as albumin and gelatin [although one must realize the increased concern for using these natural materials because of their potential for endotoxin and/or bovine spongiform encephalopathy (BSE)* contamination].

The literature is replete with problems encountered while delivering insulin, because of its ability to adsorb onto the surfaces of delivery pumps, glass containers, and the inside of the intravenous bags (Brennan *et al.*, 1985; Iwamoto *et al.*, 1982; James *et al.*, 1981; Lougheed *et al.*, 1983; Massey and Sheliga, 1988; Sato, *et al.*, 1984; Twardowski *et al.*, 1983a–c). Insulin adsorption usually is finite once binding sites are covered and such adsorption is usually not clinically significant.

Adsorption to filters and tubing can often be overcome by first saturating the surfaces with excess protein solution, then discarding the filtrate or wash. This approach will waste valuable protein and so may not be a good choice. In some cases, adsorption can be minimized by using certain additives. For example, Oshima (1989) showed that surface denaturation of chymotrypsin can be prevented by the addition of 0.1 *M* NaCl as well as by coating the surface of the container with either lecithin or BSA. Adsorption of urokinase to glass can be prevented by the addition of

*Also known as "mad cow disease," a chronic generative disease affecting the central nervous system of cattle. First diagnosed in Great Britain in 1986, this transmissible disease could be a contaminant in cow-sourced pharmaceutical excipients such as gelatin, bovine serum albumin, polysorbate 80, and others.

0.25% gelatin to the container surface (Patel, 1990). Calcitonin adsorption to glass syringes can be prevented by benzalkonium chloride or benzethenium chloride, presumably due to coating of glass silanol groups by these positively charged antimicrobial preservatives (Kakimoto *et al.*, 1985).

### 7.4. Precipitation

Precipitation of protein occurs subsequent to denaturation and is a consequence of aggregates combining to form large particles. The mechanism of aggregation leading to precipitation is beyond the scope of this chapter; the reader is referred to Glatz (1992). Brennan *et al.* (1985) established the tendency of insulin to precipitate when loaded into a long-term infusion device. Massey and Sheliga (1988) determined that the presence of large headspace in vials, high concentration of zinc, high pH, and the presence or absence of additives were causative factors for accelerating insulin precipitation in NPH formulations (called "frosting") and concluded that denaturation occurred at the air–water interface, which in turn led to precipitation.

## 8. FORMULATION APPROACHES FOR SOLVING PHYSICAL STABILITY PROBLEMS

### 8.1. Surfactants

Surfactants, as their name implies, are surface-active agents, which can exert their effect at solid–liquid, liquid–liquid, and liquid–air interfaces because of their chemical composition, which includes both hydrophilic and hydrophobic groups. These materials reduce the concentration of proteins in dilute solutions at the surface of the solution where they can be adsorbed and/or denatured. Surfactants can bind to hydrophobic interfaces in protein formulations and packaging. Glass, rubber, or plastic adsorption of proteins is well documented (Chawla *et al.*, 1985; Suelter and DeLuca, 1983). Proteins on the surface of water will aggregate, particularly when shaken, because of unfolding and subsequent aggregation of the protein monolayer. Surfactants can denature proteins, but also can stabilize proteins against surface denaturation.

Generally, ionic surfactants tend to denature proteins. Nonionic surfactants usually do not denature proteins even at relatively high concentrations (1% w/v) (Cleland *et al.*, 1993). Most parenterally acceptable

nonionic surfactants come from either the polysorbate (sorbitol–polyethylene oxide polymers) or polyether (polyethylene oxide–polyproplyene oxide block co-polymers) groups. Polysorbates 20 and 80 are the only known surfactant stabilizers in marketed protein formulations (see Table IV). However, other surfactants used in protein formulations for clinical studies and/or found in the patent literature include polysorbate 20, Pluronic F68, and other polyoxyethylene ethers (e.g., the "Brij" class) (Wang and Hanson, 1988).

Surfactants are well known to prevent the denaturation and aggregation of insulin (Chawla *et al.*, 1985; Lougheed *et al.*, 1983; Massey and Sheliga, 1988; Sato *et al.*, 1984). However, the choice of surfactant and the final concentration optimal for stabilization are quite dependent on a variety of factors, including other formulation ingredients, for example, sugars, protein concentration, headspace in the container, the type of container, and test methodology.

Recombinant human growth hormone (rhGH) will aggregate readily under mechanical and thermal stress. Aggregation from mechanical stress can be substantially reduced in the presence of surfactants (Katakam *et al.*, 1995). Tween 20 (polysorbate 20) in a molar ratio $>4$ with rhGH will effectively inhibit aggregation due to weak binding to hydrophobic patches on the protein's surface (Bam *et al.*, 1998). Mechanical stress may cause proteins to be more exposed to air–water interfaces, where denaturation is more likely to occur than in the bulk phase of water. Surfactants will preferentially compete with proteins for accumulation at the air–water interface and keep the protein from undergoing interfacial denaturation resulting from agitation. Pluronic F-68 and Brij 35 will stabilize hGH at their critical micelle concentrations (0.1% and 0.013%, respectively), whereas stabilization with polysorbate 80 requires a concentration of 0.1%, higher than the critical micelle concentration (cmc) value for polysorbate 80 of 0.0013%. The reasons for these differences in stabilizing concentrations are not clear, but may simply reflect differences in interactions between different surfactants and proteins. It is interesting to note that these surfactants do not stabilize hGH against aggregation due to high-temperature stress.

Further substantiation of the important role of surfactants, particularly polysorbate 80, in protecting proteins against surface-induced denaturation during freezing was reported by Chang *et al.* (1996). These investigators found a strong correlation between freeze denaturation (quick freezing of the protein) and surface denaturation (shaking the protein in solution). Proteins that tend to denature under these conditions are protected by the addition of polysorbate 80 (0.1%). Other surfactants—Brij 35, Lubrol-px, Triton X-100, and even the ionic surfactant sodium dodecyl sulfate—also protected the protein from denaturation, although these surfactants have not yet been approved for use in injectable formulations. The authors

pointed out that surfactants may protect proteins from denaturation during the freezing step only, and that other stabilizers, for example, sucrose, may be needed to further protect the protein during freeze-drying.

Peroxides are known contaminants of nonionic surfactants. Knepp *et al.* (1996) reported the peroxide levels of polysorbate 80 obtained from different manufacturers using a colorimetric titration method. Levels ranged from less than 1 to more than 27 mEq/kg. They found that peroxide levels increased upon storage at ambient temperatures, probably due to headspace oxygen and/or the container/closure interface allowing ingress of air. Peroxides in polysorbate can result in oxidative degradation of proteins. Obviously, formulators need to screen sources of polysorbate 80 or other polymeric additives used in protein formulations for peroxide contamination and establish peroxide specifications for using the additive. Also, as a precaution, incorporation of an antioxidant can help to overcome the potential for nonionic surfactants to serve as oxidative catalysts for oxygen-sensitive proteins.

Studies on protein–surfactant interactions (Bam *et al.*, 1995; Katakam *et al.*, 1995) have used electron paramagnetic resonance (EPR) spectroscopy to determine the binding stoichiometry of the surfactant to the protein and, thus, the potentially optimal amount of surfactant to use to stabilize the protein against surface denaturation and other physical instability reactions. Protein/surfactant interactions are also discussed in Chapter 3.

### 8.2. Albumin

Serum albumin is a widely used stabilizer in protein formulations for minimizing protein adsorption to glass and other surfaces (Wang and Hanson, 1988). Albumin competes with other proteins for binding sites on surfaces, but why this is so is not clear. It also is used as a protectant in several lyophilized formulations and has stabilizing effects on other proteins (Wang and Hanson, 1988), yet the mechanism by which it is an effective stabilizer is not understood. Examples of commercial protein formulations containing albumin are found in Table IV.

Because albumin is a natural protein, concerns have been raised about potential contamination of albumin with BSE (Fischer, 1995) if, in fact, bovine serum albumin were to be used rather than human serum albumin. There are also concerns with respect to vial contamination of natural proteins. Epidemiological data collected in the United Kingdom suggest a link between BSE and the feeding to ruminants (e.g. cattle, sheep, goats) of animal feed containing protein derived from transmissible spongiform encephalopathy (TSE)-contaminated tissues. Additionally, the data suggest

that exposure to BSE may account for outbreaks of Creutzfeldt–Jakob disease (v-CJD), a central nervous system disease, in humans in the United Kingdom. The formulator should review materials such as enzymes and excipients used in the manufacture of the drug substance and drug product to ensure that there are no concerns with potential TSE contamination. Examples of animal source materials include, in addition to albumin, also gelatin, glycerol, and polysorbate 80. However, it should be noted that synthetic sources of polysorbate 80 are available. Ideally, the use of synthetic versions of these materials would eliminate concerns over potential disease transmission. If animal source materials are used in the manufacture of the product, then assurance must be provided to show what steps are being taken to prevent transmission of BSE. This could include sourcing of material from BSE-free countries and/or using processing such as high temperatures and pressure to achieve inactivation of TSE agents. Several regulatory agencies, such as those of the European Union, the FDA, and the Therapeutic Drugs Administration (Australia), critically reviewed setting guidelines in this area (Federal Register, 1997).

### 8.3. Sugars and Polyhydric Alcohols

The formulation scientist can select from a wide variety of excipients that can be effective in stabilizing proteins against denaturation due to extremes in temperature. Mannitol (polyol), propylene glycol and polyethylene glycol (polyhydric alcohols), and sucrose, lactose, trehalose, and maltose (dissacharides) are examples. However, at the time of the writing of this chapter, no trehalose-containing protein products had been approved by the FDA. The most effective stabilizers are those that remain amorphous after freeze-drying. Mannitol tends to crystallize during freeze-drying, for example, and thus might not be the best choice as a stabilizer. On the other hand, sugars such as sucrose are usually very effective stabilizers, because sugars tend not to crystallize during freeze-drying. Wang (1992) reviewed the application of these excipients in stabilizing proteins that are subjected to high temperatures ($\sim 60°C$) for short periods of time to pasteurize the protein, particularly those derived from blood. Effects of these solutes in stabilizing proteins during freeze-drying are covered in the following section.

Sucrose has been implicated as the cause of renal failure after the administration of intravenous immunoglobin preparations (Winward and Brophy, 1995). One immunoglobin preparation that did not contain sucrose did not cause acute renal failure, whereas all other preparations with sucrose resulted in this problem. It was pointed out by Winward and Brophy (1995) that acute renal failure was a problem years ago in patients who had

received infusions of sucrose when sucrose was used as an osmotic diuretic. It was recommended that sucrose-containing intravenous immunoglobin preparations be avoided in patients with preexisting renal impairment or if the administration of high daily doses is anticipated.

### 8.4. Cryoprotectants

Proteins are frozen for long-term storage and freeze-dried for increased stability, and can be exposed to freezing conditions inadvertently during distribution of the final product. Some proteins can be deactivated or denatured by freezing. Freezing can produce a "hostile environment" for a protein because of solute concentration, increase in ionic strength, shift in pH, and a loss of hydrophobic interactions that stabilize the native protein (Pikal, 2002). At low temperatures, *freeze* (Franks, 1988) or *cold* (Privalov, 1990) denaturation of proteins can be a problem. Protein instability under very cold conditions may be overcome by the use of certain additives, called cryoprotectants, that protect the protein during freezing.

Arakawa *et al.* (1991, 1993; Arakawa and Timasheff, 1982, 1984) showed that additives that stabilize proteins in solution are preferentially excluded from the surface of native proteins. Based on the use of equilbrium dialysis techniques to calculate interaction parameters between a protein and an additive in water (described by Arakawa *et al.* (1993), and references contained therein), a wide variety of solutes (Table VII) can serve as protein cryoprotectants. These include sugars (sucrose, lactose, and glucose), amino acids (glycine, alanine, and proline), amines (betaine and trimethylamine *N*-oxide), polyols (mannitol and sorbitol), and certain salts (ammonium, sodium, and magnesium sulfate). The presence of these additives in a protein solution creates a thermodynamically unfavorable environment, because the chemical potentials of both the protein and the additive are increased (Arakawa *et al.*, 1993). An entropically unfavorable state occurs when solute molecules are preferentially excluded from contact with the protein. There is a good correlation between preferential exclusion and the effectiveness of an additive to serve as a cryoprotectant. Thus, the native structure of the protein is stabilized, because denaturation of the monomer or dissociation of polymeric oligoproteins would lead to greater area of contact between the protein and the solvent and therefore make a thermodynamically unfavorable situation worse. If a solute binds to the protein, the chemical potential is decreased and the free energy of denaturation decreases, leading to increased stability of the denatured protein and decreased stability of the native protein. Solutes known to bind

**Table VII**
Common Cryoprotectants and Lyoprotectants for Proteins in Finished Dosage Forms

| General Classification | Specific Examples |
|---|---|
| Sugars | Sucrose, trehalose, lactose, maltose |
| Amino acids | Glycine, arginine |
| Polyols | Glycerol, sorbitol, mannitol |
| Polymers | Polyethylene glycol, dextran, polyvinylpyrrolidone |

to proteins in this manner include magnesium chloride, guanidine hydrochloride, and urea. Exceptions are known to exist; for example, polyethylene glycol (PEG) is strongly excluded from the protein surface, but may cause protein destabilization, not by denaturation, but by causing protein molecules to concentrate in smaller volumes and increasing potential for aggregation (Arakawa and Timasheff, 1987; J. C. Lee and Lee, 1987). PEG in dilute solutions of low molecular weights (1000–8000) can provide stable protein solutions by forming a water layer around the protein and separating molecules into soluble monomers (Currie and Groves, 1992). PEG generally is an excellent cryoprotectant (Carpenter and Crowe, 1988).

### 8.5. Lyoprotectants

Some proteins are not stable during the drying part of the freeze-drying process. During drying, the protein's hydration shell is lost, which can result in irreversible loss of biological activity of some proteins (Liu *et al.*, 1991; Pikal *et al.*, 1991a, b). Many effective cryoprotectants offer no protection during dehydration of proteins. This is because the preferential exclusion of solutes, so important when water is present (either in frozen or liquid solution), is not applicable when water is removed from the system. Two different mechanisms for lyoprotection have been proposed, the water-replacement theory (Arakawa *et al.*, 1993; Carpenter and Crowe 1989) and the vitrification theory (Franks, 1990). The water-replacement theory states that a good stabilizer essentially serves as a water substitute by hydrogen-bonding to the dried protein. Many stabilizers are sugars, which hydrogen-bond to water like proteins hydrogen-bond to water. As water is removed during freeze-drying, the sugar replaces water by forming hydrogen bonds to the surfaces of the protein, and keeps the protein in its native, folded state. The vitrification theory, on the other hand, proposes that lyoprotection by solutes is related to glass formation in both the freeze concentrate and the dried state where the

protein and solute, both amorphous glasses, are immobilized together and cannot easily degrade. However, this does not explain why dextran, an amorphous glass-former, does not stabilize and in fact destabilizes human growth hormone (Pikal, 1991) and catalase (Tanaka *et al.*, 1991). More research is needed for reaching a final conclusion on the true mechanism of stabilization of proteins during drying and storage in the dry state.

Common lyoprotectants include mannitol, sugars such as lactose, maltose, trehalose, and sucrose, and amino acids such as glycine, histidine, and arginine. A recent study of lyoprotection of a monoclonal antibody showed histidine buffer at pH 6 to be an effective stabilizer against aggregation, with high concentrations of trehalose and sucrose providing additional protection (Andya *et al.*, 1996). Sucrose was found to be a superior lyoprotectant to trehalose in minimizing conformational changes during the freeze drying of hydrophobic proteins (Kreilgard *et al.*, 1996).

Effectiveness of these stabilizers in the solid state depends at least in part on the following factors:

- The glass transition temperature of the freeze-dried solid (Her and Nail, 1994; Izutsu *et al.*, 1993a; Jang *et al.*, 1995; Pikal, 2002).
- Moisture content (Ahlnek and Zografi, 1990; Bell, *et al.*, 1995; Hageman, 1988; Hsu *et al.*, 1988; Yoshioka *et al.*, 1993).
- The ability of the excipient to remain in the amorphous state (Arakawa *et al.*, 1991, 1993; Costantino *et al.*, 1998; Izutsu *et al.*, 1993b, 1994a).

In fact, these three factors are all interrelated, in that moisture acts as a plasticizer, lowering the glass transition temperature, which increases molecular mobility and promotes crystallization of amorphous solids.

Figure 4 (Izutsu *et al.*, 1993a) provides a good example of the importance of the crystalline versus the amorphous state of a formulation and its ability to stabilize a protein. The open symbols represent amorphous cakes and the closed symbols represent crystalline cakes. As long as the cake remained amorphous, particularly at higher concentrations of mannitol and phosphate buffer, the activity of $\beta$-galactosidase was preserved. However, once the mannitol crystallized, activity decreased. Costantino *et al.* (1998) also demonstrated this effect on the stabililization of recombinant human growth hormone, where bulk additives that did not crystallize (lactose, trehalose, cellobiose, methyl $\alpha$-D-mannopyranoside) during and after freeze-drying were more effective stabilizers against protein aggregation than excipients that did crystallize (mannitol, sorbitol)

To summarize, in pharmaceutical dosage forms, most proteins are too unstable in solution to be marketed as ready-to-use solutions. Therefore, most commercially available protein products as well as proteins in

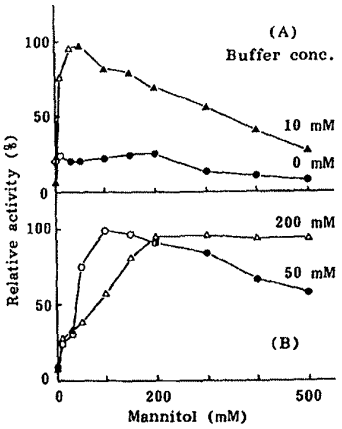


**Figure 4.** Effect of mannitol on the residual activity of freeze-dried β-galactosidase. Aqueous solutions of the enzyme (2 μg/ml) containing no (◇, ○, and ● in A), 10 m*M* (△ and ▲ in A), 50 m*M* (○ and ● in B), and 200 m*M* (△ and ▲ in B) sodium phosphate buffer solution were freeze-dried. The open and filled symbols represent atmorphous and crystallized cakes, respectively, analyzed by X-ray diffraction crystallization was observed in some samples). The open diamond denotes a collapsed cake which could not be analyzed by X-ray diffraction. The values are means of three separate experimetns. Reprinted with permissed from Izutzu *et al.* (1993b). Copyright 1993 Plenum Press.

development are freeze-dried for long-term stability. Stability problems potentially still exist for proteins in solution (both ready-to-use and after reconstitution) and in the solid state. Chemical instability problems (most frequently hydrolysis and oxidation) can be dealt with from a formulation approach by precise control of pH (buffers), use of antioxidants and chelating agents, storage at low temperatures, and control, of moisture content in the solid state (through processing and packaging). Physical instability problems (aggregation, precipitation, adsorption), occurring both in solution and solid states, can be minimized or prevented by employing additives such as sugars, amino acids, polyols, and surfactants.

## 9. ADDITIVES FOR ANTIMICROBIAL PRESERVATION

Antimicrobial preservative (AP) agents are required for parenteral products that are intended for multiple-dose use. Many protein products, because of their expense and course of therapy, are packaged as multiple-

dose products, with the AP either formulated with the protein or, more commonly, formulated in a special diluent vial to be used to reconstitute a freeze-dried solid. The most common APs used in protein dosage forms are phenol, *m*-cresol, benzyl alcohol, and methyl and propylparaben. Examples of use of these preservatives are listed in Table VIII.

Use of antimicrobial agents requires passing an antimicrobial efficacy test (AET). Unfortunately, the USP and the British and/or European Pharmacopoeia (BP/EP) tests for AET have different acceptance criteria. Table IX summarizes the differences between the two tests. The USP requires a bacteriostatic preservative system, where as the BP/EP requires a bacteriocidal system. For example, the USP requires a three-log reduction in the bacterial challenge by the 14th day after inoculation, while criterion A of the BP/EP test requires the same three-log reduction within 24 hr. This difference in compendial requirements for antimicrobial preservative efficacy has caused many problems in the formulation of protein dosage forms. Passing the BP/EP antimicrobial efficacy test requires the use of relatively high amounts of phenol or cresol or other AP, which may have an impact on the stability of the formulation and could result in sorption of the preservative into the rubber closure. The formulator must keep in mind that increasing the concentration of APs may have a negative impact on protein physical stability (precipitation, aggregation, etc.). Increasing AP levels will increase the hydrophobicity of the formulation and could affect the aqueous solubility of the protein.

It is well known that APs not only protect insulin formulations against inadvertent microbial contamination, but also have a significant effect on stability of Lente formulations and prolong the duration of action of NPH

**Table VIII**
Antimicrobial Preservative Agents for Protein Products

| Type | Concentration (%) | Antimicrobial activity[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Gram + | Gram − | Fungi | Yeast | PH | Comment |
| Benzyl alcohol | 0.1–3.0 | + + + | + + + | − | − | 3–6 | Not effective pH > 7 |
| Cresol | 0.1–0.3 | + + | + + | + + | + + | 4–10 | Most effective |
| Methylparaben | 0.08–01 | + + | + + | + + | + + | 3–9 | Slowly soluble |
| Propylparaben | 0.001–0.02 | + + + | + + + | + + + | + + + | 3–9 | Very slowly soluble |
| Phenol | 0.2–0.5 | + + | + + | + + | + + | 4–10 | Most effective |
| Thimerosal | 0.1–04 | + + | + + | + + | + + | 4–8 | Japan will not allow |

[a] + + +, most effective; + +, moderately effective; −, poor.

**Table IX**
Comparison of USP 25 and EP 4th Edition Requirements for Antimicrobial Efficacy Testing

| Time after Inoculation with microorganisms | Log reduction in microorganism count | | |
|---|---|---|---|
| | USP 25 | EP Criterion A | EP Criterion B |
| 6 hr | Not required | 3 (bacteria) | Not required |
| 24 hr | Not required | No recovery (bacteria) | 1 (becteria) |
| 2 days | Not required | No recovery (bacteria) | Not required |
| 7 days | 1 (bacteria), no increase (fungi) | No recovery (bacteria), 2 (fungi) | 3 (bacteria) |
| 14 days | 3 (bacteria), no increase (fungi) | No recovery (bacteria), no increase (fungi) | 1 (fungi) |
| 21 days | No increase | No increase | Not required |
| 28 days | No increase | No recovery (bacteria), no increae (fungi) | No increase (bacteria), no increae (fungi) |

formulations (Brange, 1987). Phenolic preservatives also have a profound effect on the conformation of insulin in solution (Wollmer *et al.*, 1987) and the self-assembly of the LysPro insulin hexamer (Birnbaum *et al.*, 1997). Preservation of Lente suspension with phenol is not possible without adversely affecting the crystal shape and physical stability of these formulations. NPH formulations, on the other hand, require phenol to obtain the desired crystal and subsequent prolonged duration of action. Methylparaben (methyl 4-hydroxy benzoate) is the preservative of choice for the Lente formulations, because it does not affect the crystal morphology while still providing sufficient antimicrobial protection. However, methylparaben is known not to be as effective an antimicrobial preservative as other preservative systems (Akers *et al.*, 1984; Allwood, 1982) and is absorbed to some extent into the rubber closure of Lente preparations (Brange, 1987).

Antimicrobial preservatives are known to interact with proteins and can cause stability problems such as aggregation. For example, phenolic compounds will cause aggregation of human growth hormone (Kirsch *et al.*, 1993, Maa and Hsu, 1996). Benzyl alcohol, above certain concentrations and depending on other formulation factors, will interact with recombinant human interferon-$\gamma$, causing aggregation of the protein (Lam *et al.*, 1997). These examples point out the need for the formulation scientist to understand the importance of potential effects of preservative type, and concentration and other formulation additives on the interaction with proteins in solution.

**Table X**
D Values against Staphylococcus aureus for Different Antimicrobial Preservative Systems in Insulin Solutions[a]

| Antimicrobial preservative system | *D* (hr) |
|---|---|
| Phenol 0.2% + *m*-cresol 0.3% | 0.5 |
| *m*-Cresol 0.3% | 0.6 |
| Phenol 0.5% | 0.8 |
| Benzyl alcohol 2.0% | 0.8 |
| Phenol 0.2% + *m*-cresol 0.2% | 1.3 |
| Methylparaben 0.2% + benzyl alcohol 1.0% | 1.4 |
| Chlorobutanol 0.5% | 1.8 |
| Phenol 0.2% + *m*-cresol 0.1% | 2.2 |
| *m*-Cresol 0.2% | 2.2 |
| Methylparaben 0.2% + propylparaben 0.02% | 3.0 |
| Benzyl alcohol 1.0% | 4.2 |
| Methylparaben 0.1% | 9.5 |
| Methylparaben 0.1% + propylparaben 0.01% | 12.3 |
| Phenol 0.2% | 16.2 |

[a]Reprinted with permission from Akers *et al.* (1984). Copyright 1984 American Pharmaceutical Association.

In determining the appropriate AP agent or agents, the model described by Akers *et al.* (1984) might be an appropriate model to follow. The authors used insulin as the protein to be preserved and combined insulin with different types of AP agents either alone or in combination. These formulations were challenged with the five USP antimicrobial efficacy test organisms and *D* values* determined. The *D* value determination allows a single quantitative estimate of the AP effectiveness of a certain agent or combination of agents in a specific formulation against a specific microorganism. An example of the *D*-value data obtained for insulin formulations with different AP systems against *Staphylococcus aureus* is given in Table X with the AP systems listed in order of effectiveness (e.g., 0.2% phenol + 0.3% *m*-cresol was the most effective AP system).

There are instances where a manufacturer, because of concerns regarding aseptic processing and sterility assurance of the product throughout its shelf life, will add an AP agent in the protein formulation even though it is intended only for a single-dose injection. This is very controversial. Regulatory agencies worldwide object to this practice if there

*$D$ value = time required for a one log reduction in the microbial population due to the effect of the antimicrobial preservative system. The smaller the $D$ value, the greater is the effect of the preservative on the microorganism in question.

is opinion that the use of APs in single-dose injections is practiced in order to "cover up" for inadequate aseptic manufacturing practices and controls.

Many countries require that AETs be performed for routine stability protocols and for special stability studies. In addition there may be requests from agencies to do AETs on containers that have been used (i.e., penetrated; partial volume withdrawn) to demonstrate that the product can still kill microorganisms. In mid-1995, the Australian Drug Evaluation Committee (ADEC) passed resolutions that, in light of safety concerns with regard to contamination and cross-contamination, the use of injectable products in multidose packages is discouraged. In order to support the use of a multidose product and the shelf life once a package has been reconstituted or opened for use, preservative efficacy data are required for approval.

## 10. OTHER ADDITIVES

### 10.1. Osmolality (Tonicity) Agents

Salts or nonelectrolytes (e.g., glycerin) are added to protein formulations in order to achieve an isotonic solution. Nonelectrolytes often are preferred over salts as tonicity adjusters, because of the potential problems salts can cause with respect to protein stability (Pikal, 1990). Generally, solutions containing proteins administered intravenously do not have to be precisely isotonic, because of immediate effects from dilution by the blood. This cannot necessarily be the case for proteins administered by intramolecular and subcutaneous routes, because dilution of the injected product occurs less readily. Intrathecal and epidural injections into the cerebrospinal fluid require very precise specifications for the product to be isotonic and at physiological pH. This is because extremes in osmolality and/or pH can damage or destroy cells, and cerebrospinal fluid cells cannot be reproduced or replaced (Cradock *et al.*, 1977).

### 10.2. Bulking Agents for Freeze-Drying

These materials not only serve to give mass and pharmaceutical elegance to freeze-dried solids, but also may stabilize the protein during and after freeze-drying. Mannitol, the most commonly used bulking agent for freeze-dried formulations, tends to crystallize during freeze-drying (Pikal *et al.* 1991b) and will dominate X-ray diffraction patterns obtained from protein formulations containing mannitol (Oliyai *et al.*, 1994),

provided that mannitol is at a sufficiently high concentration relative to other solutes. Sugars such as lactose and sucrose have been used as bulking agents in freeze-dried protein dosage forms. An example is the use of lactose in glucagon freeze-dried formulations.

### 10.3. Suspending Agents

Formulations for injectable suspensions generally require a suspending agent to enable the insoluble drug to be easily resuspended and allow the withdrawal of a uniform dose. Although there are not many protein suspensions commercially available, suspension dosage forms of insulin are the most widely used formulations of insulin. NPH insulin employs protamine as a complexing agent, which forms a suspension of insulin by stoichiometrically binding to insulin molecules (Brange, 1987). Lente insulin suspensions employ high levels of zinc ion to suspend insulin in solution. There are many other suspending or complexing agents known to form stable suspensions of insulin (Brange, 1987).

Bovine somatotropin (BST) is another example of a protein that is available as a suspension for once-a-month injection in cattle. However, unlike insulin, this suspension uses a vegetable oil (e.g., sesame oil) as the liquid phase.

### 10.4. Solubilizing Agents

Drug solubility may be improved by the use of solubilizing agents such as glycerin, propylene glycol, polyethylene glycol (300 or 400), and ethanol. Cyclodextrin derivatives have received considerable attention in the literature for their effective solubilization of a wide variety of molecules (Brewster *et al.*, 1989, 1991; Hora *et al.*, 1991, 1992; Rajewski and Stella, 1996; Stratton, 1991; Thompson, 1997). Cyclodextrins are crystalline, cyclic oligosaccharides derived from starch and have six (alpha), seven (beta) or eight (gamma) glucose units. Cyclodextrins are "bucket-like" toroid molecules having a rigid structure with a central cavity (Nash, 1994). The internal surface of the cavity is hydrophobic and the outer surface of the torus is hydrophilic. Thus, hydrophobic molecules can form an inclusion complex within the internal cavity and be rendered soluble in an aqueous solution. The cyclodextrin derivative having the most promise as an excipient in parenteral formulations is 2-hydroxypropyl-$\beta$-cyclodextrin. This derivative has been shown to increase solubility and stability of several

proteins (Brewster *et al.*, 1989, 1991; Hora *et al.*, 1991, 1992; Simkins, 1991; Stratton, 1991). Relatively high cyclodextrin concentrations (10–20%) are required to stabilize these proteins. A relatively new and very promising cyclodextrin is an anionic $\beta$-derivative with a sodium sulfonate salt separated from the hydrophobic cavity by a butyl ether spacer group, Captisol™ (CyDex, Overland Park, KS). Captisol has been shown to increase drug stability (Jarvinen *et al.*, 1994) and solubility (Badawy *et al.*, 1995; Gorecka *et al.*, 1996; Okimoto *et al.*, 1995) of parenteral drugs including insoluble peptides (Johnson *et al.*, 1994). Thompson (1997) has written an excellent review of the safety evaluation and regulatory status of Captisol and other cyclodextrins. Safety of these excipients by the parenteral route of administration is still in question.

There is always a desire for newer excipients that can aid in solubilization, stabilization, and preservation of parenteral formulations. However, new excipients must undergo significant safety and regulatory hurdles. Expensive toxicological studies must be performed to convince drug manufacturers and regulatory authorities of long-term safety of the new excipient. Investment in these studies frequently presents a barrier to further study of new excipients.

## 11. PACKAGING

The packaging system is vital to successful marketing and use of a parenteral dosage form. All parenteral packaging components must be characterized according to appropriate compendial (USP, EP, Japanese pharmacopeia[JP]) requirements for glass, rubber, and/or plastic. These requirements include testing for reactivity with water and other solvents and safety evaluation of whatever is extracted into these solvents. Effect of sterilization methods (dry heat for glass; steam heat under pressure for rubber closures; gas or gamma radiation for plastic) on the integrity and potential leachables from packaging components must be determined. The International Conference on Harmonization (ICH) (Federal Register, 1994) stability guidelines require definitive stability studies to be performed in the final container/closure system, which has to have been thoroughly tested with the product formulation and found not to have any major physical–chemical stability issues.

All manufacturers of packaging materials are required to have a drug master file (DMF) on their components used in a registered product filed with the FDA (U.S. Food and Drug Administration, 1994). A DMF for packaging components is a submission to the FDA that contains information about the materials used for packaging for one or more human drug products. It allows

detailed information about the packaging component to be referenced while maintaining confidentiality of the component or formulation. The pharmaceutical industry is not allowed to access these files. Sometimes this is unfortunate, because the packaging manufacturer may change a component of the container or closure formulation that is not divulged to the drug product manufacturer. Therefore, it is very important to determine lot-to-lot variability of packaging materials and to use good practices in purchased material quality control to assure that the package system originally selected always meets the desired specifications.

Because of their tendency to adsorb to surfaces, proteins can be difficult to stabilize in the appropriate package. As previously discussed, proteins are known to adsorb to glass, rubber and plastic surfaces, with insulin being the most studied protein for its adsorptive properties (Kraegen *et al.*, 1975; Mitrano and Newton, 1982; Petty *et al.*, 1974; Seres, 1990; Twardowski *et al.*, 1983a–c). Protein formulations may also react adversely with extractables from rubber and plastic formulations, although we could not find any published information on this possibility.

### 11.1. Glass

The USP categorizes glass into three types (see Table XI), differentiated by the amount of alkali leachables titrated from the glass when subjected to a high-temperature stress condition (USP, 1995, pp. 1781–1782). Type I borosilicate glass is the most resistant, least reactive (fewest potential leachables), highest quality, and most expensive glass available for injectable products and is normally the type of glass used for ready-to-use injectable solutions. For protein formulations, particularly those marketed as solution products, Type I glass should be used because of these properties. Type III soda-lime glass is of a lower quality as far as alkaline leachables are concerned, but normally is quite acceptable for solid state products such as freeze-dried preparations. Type II soda lime glass compositionally is the same as Type III glass, except that Type II glass receives a surface coating of sulfur dioxide which forms a sodium sulfate film, which can be easily washed off. This treatment renders Type II glass more resistant to alkali leachables, although it is not as pure as Type I glass. Type II glass is used for large-volume parenteral solutions.

Glass is prepared either by a process known as blow molding or by a process where the final glass container is formed from glass tubing. Blow molding processes usually are used to form glass for large vials (usually greater than 50 ml) and bottles, whereas tubing glass processes are used to make ampoules, smaller vials (<50 ml), syringes, and cartridges.

**Table XI**
Types of Glass Used in Protein Dosage Forms[a]

| Type | General description | Type of USP test | Limits | |
|---|---|---|---|---|
| | | | Size (ml) | Amount of 0.02 *N* acid (ml) |
| I | Highly resistant, borosilicate glass<br>Very little alkali leachables<br>Used for most protein products<br>Most expensive type of glass | Powdered glass | All | 1.0 |
| II | Treated (sulfur dioxide) soda-lime glass | Water attack | 100 or less | 0.7 |
| | Sodium sulfate forms on glass because of reaction between sulfuric acid and sodium ions<br>Used for large-volume bottles and acidic-to-neutral small-volume solutions | | >100 | 0.2 |
| III | Soda-lime glass<br>About 10 times less resistant to alkali leachables than type I glass<br>Used for dry powder small-volume products<br>Cannot be terminally sterilized with final product | Powdered glass | All | 8.5 |

[a]See *United States Pharmacopeia*, 24th ed. (2000), pp. 1930–1931.

Glass particulates are known to be a major source of particulate matter in parenteral solutions. Regardless of the quality of glass, the reputation of the manufacturer, the method of manufacture, or the method of cleaning, glass particulates are unavoidable. Glass particulates are generated most commonly during sterilization and depyrogenation of the glass containers (Whyte, 1983). Barium sulfate is a known Type I glass particulate contaminant in parenteral solutions formed from the leaching of the barium oxide component of glass and sulfate ions, the sulfate coming from either a sulfate salt or a bisulfite antioxidant excipient (Boddapatti *et al.*, 1980). Procedures for the isolation and identification of glass extractables are available (Parenteral Drug Association, 1986).

Proteins are packaged not only in glass vials, but also in glass cartridges and, potentially, in glass syringes. Normally, glass vials are not siliconized, but glass cartridges and syringes must be siliconized in order for the rubber-tip plunger rod to be moved easily through the lumen of the

glass barrel. Studies must be done to assure that there is little or no interaction between the silicone on the glass and the protein or other formulation ingredients. For cartridge manufacture, the silicone is added before heat sterilization of the glass container, with the high temperature (usually above 250°C). Some believe that this will "chemically bond" the silicone to the glass, although personal communications with silicone oil manufacturers indicate that such bonding is physical, not chemical. In any case, there is always the possibility of excessive silicone not being completely bonded on the glass and thus the potential for "free" silicone to interact with the product.

### 11.2. Rubber

Rubber is used as the closure of glass vials and bottles, at the tip of the plunger in syringes, and the septum in glass cartridges, and can be a component of intravenous administration sets. Types of elastomeric material acceptable for parenteral products are listed in Table XII along with some general properties and other comments. Natural and butyl rubber are the two most commonly used elastomers for rubber closures of parenteral vials, bottles, and other containers. Elastomers are classified as saturated or unsaturated, with the degree of unsaturation affecting elastomer physical and chemical properties. Rubber closures and other rubber devices are composed of a wide variety of ingredients, examples of which are given in Table XIII.

Rubber closures are most commonly formed in steel molds under extremely high pressures (tons per square inch) and vulcanized (cured) at temperatures greater than 200°C. Mold surfaces where the molten rubber is placed are sprayed with an aerosolized release agent. This process is not perfect; gaps in the spray pattern will leave open areas where the rubber will stick tightly to the mold. When the rubber sheet is removed from the mold, tiny tears can occur in the rubber, which, in turn, can be the source of leachables and particulates. It is not known how prevalent this problem may be. However, the reality of this type of manufacturing problem causes some manufacturers to coat rubber closures with Teflon, silicone, nonsiliconized coatings such as HelvoPure (Helvoet Company), or Purcoat (West Company), or other polymeric material in order to minimize particulate and other potential incompatibilities with the product and improve the machinability of the rubber closure process. However, rubber closure coatings increase the cost of the closure, may not form as tight a container/closure seal (unpublished observations; they can especially be a problem for

**Table XII**
Types and Selected Physical and Chemical Properties of Rubber Elastomers[a,b]

| | Butyl/ halobutyl | Natural | Neoprene | NBR | Silicone | Fluoro elastomers | Urethane | EFDM | Polybutadiene |
|---|---|---|---|---|---|---|---|---|---|
| Chemical name | Isobutylene–isoprene copolymer | *Cis*-1,4,poly isoprene | Polychloro-prene | Butadiene–acrylonitrile copolymer | Poly-dimethyl alloxane | Fluro-rubber | Polyester isocyanate | Etylene propylene diene monomer | *Cis*-polybutadiene |
| Moisture vapor resistance | Excellent | Good | Fair | Fair | Poor | Good | Poor | Fair | Fair |
| Gas transmission resistance | Excellent | Good | Fair | Good | Poor | Good | Poor | Fair | Fair |
| Coring | Fair | Excellent | Good | Fair | Poor | ND | Excellent | Fair | Fair |
| Compression recovery | Poor | Excellent | Good | Good | Poor | Good | Excellent | Good | Good |
| Shelf life | Good | Fair | Good | Fair | Excellent | Excellent | Excellent | Excellent | Fair |
| Automatic handling ease | Poor | Good | Fair | Good | Fair | ND | Fair | Fair | Good |
| Heat resistance | Excellent | Good | Good | Good | Excellent | Excellent | Poor | Very Good | Good |
| Resistance to | | | | | | | | | |
| Water | Excellent | Good | Fair | Good | Excellent | Good | Poor | Good | Good |
| Animal oil | Excellent | Poor | Good | Excellent | Good | Excellent | Excellent | Fair | Fair to Poor |
| Vegetable oil | Excellent | Poor | Good | Excellent | Excellent | Excellent | Excellent | Fair | Fair to Poor |
| Mineral oil | Poor | Poor | Good | Excellent | Fair | Excellent | Excellent | Poor | Poor |
| Aliphatic solvents | Poor | Poor | Good | Poor | Poor | Excellent | Excellent | Good | Poor |
| Aromatic solvents | Good | Good | Poor | Good | Poor | Excellent | Poor | Fair | Poor |
| Chlorinated solvents | Poor | Poor | Poor | Poor | Poor | Excellent | Good | Poor | Poor |
| Dilute Acid | Good | Good | Good | Good | Fair | Fair | Poor | Good | Fair |
| Alkali, dilute | Good | Good | Good | Good | Good | Good | Poor | Good | Fair |
| Abrasion resistance | Fair | Good | Fair | Good | Fair | Good | Excellent | Good | Fair |
| Resillence | Poor | Excellent | Good | Good | Good | Fair | Good | Good | Good |
| Ozone resistance | Excellent | Poor | Good | Fair | Excellent | Excellent | Good | Good | Fair |
| Radiation resistance | Fair to Poor | Good | Good | Good | Fair to Good | Fair to Good | Fair | Fair | Poor |

[a]Reproduced with permission from Smith and Nash (1992). Copyright 1992 Marcel Dekker, Inc.
[b]Ratings expressed in this guide are average values for typical rubber formulations using these base elastomers. Ratings can vary significantly with specific rubber formulations. NBR, Acrylonitrile–butadiene rubber; EPDM, ethylene–propylene–diene terpolymer.

**Table XIII**
Additives in Rubber and Plastic formulations

| Rubber | Plastic |
|---|---|
| Curing (vulcanizing) agent: forms crosslinks to shape rubber, e.g., sulfur, zinc oxide, phenolic resins, peroxide | Lubricant: improves processibility, e.g., stearic acid, paraffin waxes, polyethylene waxes |
| Accelerator: increases curing rate, e.g., 2-mercaptobenzothiazole, amines, thiols, ureas | Stabilizer: retards degradation, e.g., epoxy, organotions, mixed metals |
| Activator: increases efficiency of accelerator, e.g., zinc oxide, stearic acid | Plasticizer: enhances flexibility, resiliency, melt flow, e.g., phthalates |
| Antioxidant: resists aging, e.g., phenol, diethiocarbamates, paraffin waxes | Antioxidant: prevents oxidative degradation, e.g., butylated hydroxy toluene thioesters |
| Plasticizer: aids shaping process | Antistatic agents: minimizes surface static charge, e.g., quaternary ammonium compounds |
| Filler: modifies hardness, e.g., carbon black, chromium oxide, iron oxide | Slip agents: minimizes coefficient of friction |
| Lubricant: paraffin, mineral oil, fatty oil | Dye, pigment |
| Pigment | |

vacuum-sealed or gas-flushed vials), and may themselves be sources of particulates (DeLuca and Knapp, 1993).

Qualifying a rubber closure formulation for use as the primary closure for a protein product is an aspect of product development that should not be taken lightly. Physical–chemical properties of the elastomer itself, such as water vapor transmission, hardness, and oxygen transmission, must be known. Usually butyl rubber derivatives have properties more suitable for labile proteins, for example, low water vapor transmission coefficient. Rubber leachate toxicities must be determined according to compendial tests. Besides generation of particulates and coring, other mechanical properties that need to be known include seal integrity, hardness, penetrability or insertion force (force required to penetrate the rubber with a needle), and resealability (ability of the rubber to reseal after needle puncture, vitally important for multidose vial products). Examples of protocols for such tests can be found in a bulletin published by the Parenteral Drug Association (1982).

Formulation scientists must conduct comprehensive preformulation screening studies in order to select the most compatible rubber closure formulation for the protein product being developed. Screening studies should include accelerated stability studies with various closures, including one or more coated closures, at high temperatures, exposure to light, and positioning of vials in inverted positions. By using stability-indicating assays

both for the protein and, if present, antimicrobial preservative agent, primary and alternative rubber formulations can be selected from these preformulation studies for long-term stability studies.

Some rubber formulations are known both to contain components that *leach* into a pharmaceutical product (e.g., zinc, 2-mercaptobenzothiazole) and to adsorb components from the product formulation (*sorption*). *Permeation* of water vapor and other gases through rubber materials can sometimes readily occur. Rubber may also be a primary source of particulate matter and may be "cored" (i.e., a piece or pieces of rubber material gorged out by the needle) easily if the method of penetrating the rubber with a needle is not done carefully. These potential problems when using rubber closures are discussed in more detail below.

### 11.2.1. LEACHING

There are several types of substances which can leach from rubber closures into pharmaceutical solutions. Metal ions, such as zinc from cure activators (Boyett and Avis, 1976) and aluminum from fillers (Milano *et al.*, 1982), have been found to be common extractables. Phosphate buffer salts used in parenteral formulations can complex zinc and aluminum extractables and form insoluble precipitates. Other potential extractables from rubber formulations include other metals (aluminum, calcium, magnesium, cadmium, lead, mercury), sulfur from the curing agents, accelerators such as mercaptobenzothiazole (Danielson *et al.*, 1984), carbon black, lubricants (stearic acid), antioxidants, and various plasticizers. Latex leachables, although predominately originating from natural rubber latex gloves, catheters, and condoms, may also leach from natural rubber elastomeric closures (MacCracken, 1996; Towse *et al.*, 1995; Vassallo *et al.*, 1995), although the level of extractables and the associated health risk remains to be determined. Standard operating procedures are available for formulation development scientists to extract, isolate, and identify extractable materials from rubber closures (Parenteral Drug Association, 1980). To minimize leaching, butyl and halobutyl rubber formulations are the best choice, although natural rubber imparts desirable mechanical properties such as resealability after multiple needle punctures. It is important to keep in mind that any type of elastomeric formulation used in pharmaceutical packaging systems has the potential for producing leachable materials.

Stability studies of the final product in the final container/closure system must include data from samples stored in the inverted position in order to allow maximum contact of the product with the rubber closure to

be sure that either product adsorption or rubber leachables will not occur to a significant extent. This also simulates what may happen during shipping and long-term storage of a commercial product when the container may fall over and the solution come into contact with the rubber surface for a long time.

### 11.2.2. SORPTION

Formulation components, particularly the active drug substance and the antimicrobial preservative, can be adsorbed (surface adsorption), even absorbed (migrate through the rubber) by rubber materials. Sorption depends on a number of factors, including physical and chemical characteristics of the drug (molecular weight, concentration, partition coefficient), drug product (composition, pH, surface tension, volatility), and rubber material (hydrophobicity, surface roughness, surface charge), temperature, extent of product–rubber surface contact on storage, and composition of formulation ingredients. The authors' personal experiences indicate that antimicrobial preservatives are the most difficult components of the product formulation, including the protein itself, to stabilize against rubber sorption. Phenol, *m*-cresol, and benzyl alcohol are especially troublesome. Prevention of some loss of antimicrobial preservatives over product shelf life is almost impossible. Therefore, the formulator must determine how much preservative can be lost over the shelf life of the product yet still pass the appropriate compendial preservative efficacy test(s).

### 11.2.3. PERMEATION

Selection of rubber closures depends on the water vapor and oxygen transmission coefficients of these materials. Freeze-dried protein solids must be protected from exposure to water vapor in order to maintain protein stability. Rubber formulations known to have low water vapor transmission coefficients (butyl and halobutyl) are the best choices for freeze-dried vials. Specific data on water vapor transmission of a particular rubber formulation can be obtained from the manufacturer. Rubber formulations that minimize water vapor transmission can still be sources of water adsorbed from the closure washing process or the closure sterilization process. Obviously, it is imperative to dry rubber closures as thoroughly as possible in order to avoid moisture problems originating from the washing and sterilization process. Coating rubber closures with a hydrophobic surface like Teflon may aid in preventing

absorbed water from migrating from the rubber closure into the product, although Teflon itself also is porous. Another, less costly, alternative is to use rubber formulations (see Table XII) that do not readily absorb water that with time might be desorbed into the product.

### 11.2.4. PARTICULATE MATTER

Rubber closures can be a source of particulate matter in one or more of several ways. If closures are not cleaned properly, foreign particulates will be carried into the final packaged product and can be loosened from the closure surface into the solid or liquid product. Specially engineered rubber closure washing machines must be used, which are designed to wash and rinse closures with minimal abrasion and directing drainage of solutions away from clean closures (Huber, Capsolut, DCI Smeja Pharma-Technik). Particulates can be generated from rubber closures in the form of leachable materials that may interact with components of the protein solution to form insoluble precipitates. Finally, particulates can occur from rubber closures when needles are inserted inappropriately causing rubber pieces to be gouged out and to fall into the product solution. Practitioners are trained to insert needles with the "bevel edge up" in order to minimize the potential for coring.

### 11.2.5. LATEX TOXICITY

Natural rubber latex has been under regulatory scrutiny recently because of increased concern regarding latex hypersensitivity reactions in humans (Lang, 1996; Slater, 1994; Wilkinson and Beck, 1996). The *Pharmacopeial Forum* (1996) proposed a ban on natural rubber in elastomeric closures effective November 15, 1999 (it was later rescinded). The source of the allergic reaction is certain proteins in the rubber tree, *Hevea brasiliensis*. Items such as rubber gloves, catheters, and other thin-walled items, which are the primary sources for latex rubber exposure to humans, are prepared from a liquid form of natural rubber ("latex") by a dipping process. Elastomeric closures are made from a "hard rubber" by a molding process. It is believed that the latex protein(s) causing the allergic reactions are more readily extractable from items made using the dipping process. Since elastomeric closures are not prepared in this manner, there is no evidence that latex reactions are originating from natural rubber closure formulations. However, scientifically sound studies on the health risk associated with natural rubber latex extractables from pharmaceutical closures are needed.

### 11.3. Plastic

Plastic or polymeric materials are used in many different aspects of manufacture and use of protein dosage forms. Process tubing, syringes, the container system (e.g., plastic bottles or bags), filters, and other devices are all composed of polymeric materials. Plastic surfaces have the potential to adsorb protein or leach out impurities when the protein solution and the polymeric material are in contact with the surface. Plastic impurities may be unpolymerized monomers of the plastic material, many of which have known human toxicities (e.g., vinyl chloride, acrylonitrile, styrene, and benzene) (Liebe, 1995). Like rubber closures, plastics contain many additives, such as lubricants, stabilizers, plasticizers, antioxidants, antistatic agents, slip agents, and dyes, any or all of which potentially can form leachable material in the protein product it holds.

With the advances in form-fill-seal technology, where sheets of plastic are sterilized and formed into the final container, and the container is filled with product and sealed, all within the same apparatus, future protein products may be packaged in plastic containers more routinely than in glass containers.

Insulin binding to plastic is well known. Hirsch *et al.* (1977, 1981) were the first to report on the clinical significance of insulin adsorption to the internal surface of poly(vinyl chloride) (PVC) intravenous tubing. Insulin also binds to polyolefin infusion containers, although the binding is not as great as to PVC surfaces (Hirsch *et al.*, 1981). Insulin and other proteins will bind to some extent to almost any type of plastic surface; therefore, formulation scientists must take precautions to ensure that such binding can be prevented or minimized. For process tubing protein adsorption can be minimized by the following procedures:

- Prerinsing the tubing with the formulation to saturate potential sites
- Using tubing that has minimal binding properties
- Using tubing only once, to avoid the development of areas that can serve as "seeds" for protein binding and particulates if tubing is used again and not cleaned properly

Usually it is not worth the cost of cleaning validation to reuse tubing. For plastic containers containing proteins, protein adsorption can be minimized by adding albumin or nonionic surfactants to compete for the binding sites or using a plastic container material that has lower surface hydrophobicity.

Table XIV summarizes the types of plastic packaging materials currently available along with comments about their advantages and disadvantages.

**Table XIV**
Plastic Materials Used with Proteins in Processing and Packaging

| Material | Advantage | Disadvantage | Application |
|---|---|---|---|
| Polyvinylchloride | Inexpensive | Plasticizer migration | In-process tubing, finished product bags |
| Polyethylene | Inert, no additives | Fragile | Disposable syringes, IV infusion bags |
| Polypropylene | Rigid | Too hard | Disposable syringes, IV infusion bottles |
| Ethyl vinyl acetate | Inexpensive | Poor sterilization props | Infusion bags, plastic tubing |
| Polyurethane | No migration, strong | Darkens easily | Short-term implants |
| Silicone | Resilent | Poor bonding | Pump tubing, in-process tubing |

Table XII, already referenced for rubber closures, also provides information about typical formulation ingredients of plastic materials.

Formulation scientists must understand how their products will be used commercially. If the product is not going to be injected directly from its primary package, but instead be (a) diluted or admixed with intravenous diluents in glass or plastic containers, or (b) infused through plastic intravenous administration sets, filtered with in-line intravenous filters, and/or placed into plastic syringes, then data must be generated for understanding the compatibility of the protein formulation when in contact with these different products and surfaces. Preparation of dilute protein solutions for infusion may require an agent such as human serum albumin to minimize protein binding to the surface of the large container.

Sterilization of plastic materials most commonly is accomplished by either ethylene oxide gas or gamma irradiation. Tests must be done on sterilized polymeric material to determine potential leachables. For example, gamma irradiation of ethylene vinyl acetate will produce acetic acid leachate, which could affect the final pH of the administered product. Gamma irradiation of plastic material also forms free radicals, which may cause discoloration of certain plastics and affect cosmetic appearance as well as ability to see evidence of particulate matter.

### 11.4. Silicone

Silicone generally is required to lubricate the rubber closure to provide surface lubricity such that closures or stoppers will not "clump" during and

after autoclaving and will "flow" in high-speed stoppering equipment. Silicone coating may also facilitate the proper fit of closures into the necks of vials, thus increasing the integrity of the container/closure interface. Insufficient surface lubricity can cause "clumping" or "twinning" of stoppers resulting in a shutdown of the filling line. Not only can this be costly, it can also jeopardize assurance of sterility, because of human intervention on the filling line to remove poorly siliconized or nonsiliconized stoppers. Glass vials of suspension products sometimes may be siliconized to facilitate product drainage and help to assure uniformity of the withdrawn dose. Silicone also is used in cartridge delivery systems where a rubber plunger must be pushed easily inside the glass barrel to accurately deliver the right volume of solution and dose of protein. Quantities of silicone applied to rubber closures are considered extremely low with respect to potential chemical and biological incompatibilities of this relatively inert material (Riffkin, 1968).

Silicone represents a hydrophobic surface that can cause problems with some proteins. Glass surfaces coated with silicone, originally in the expectation of decreasing protein adsorption to glass, in fact were found to increase the adsorption of a variety of proteins, including insulin, globulin, and lysozyme (Mizutani, 1981). Only albumin did not bind to the siliconized glass surface as much as it did with nonsiliconized glass, an observation leading to the use of albumin in minimizing adsorption of other proteins to glass.

### 11.5. Delivery Devices

Convenience of use drives technology development for protein pharmaceutical products in two important ways: development of more convenient product presentations (e.g., insulin cartridges and pens) and development of sustained-release formulations (e.g., microspheres). Cartridges and delivery pens are becoming a primary administration system for injection of insulin (Fig. 5).

Delivery devices greatly aid in the convenience, ease, and accuracy of administration of drugs by injection. However, there are many issues that must be resolved concerning the compatibility of the drug product and the device, the reproducible use of the device (unless it is a disposable system), and manufacturing quality concerns whereby the device needs meet to strict performance criteria lot after lot. For example, with insulin device delivery systems, stability of insulin in the device must be demonstrated, because the device normally is stored and used under

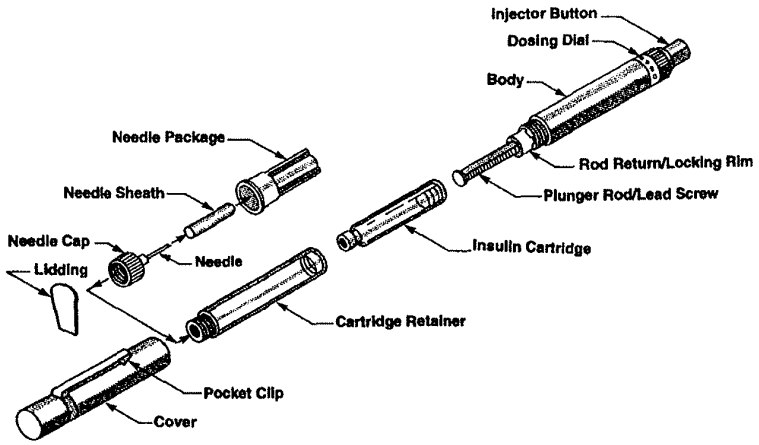


**Figure 5.** A cartridge-pen delivery system for insulin and other protein formulations.

ambient temperature conditions, whereas insulin in vials is stored refrigerated. Repeated use of the same device with a large number of insulin cartridges must assure that dose accuracy and delivery does not change over time. Compatibility of the needle system of the device with the cartridge rubber septum must be demonstrated. Finally, if suspensions are administered by these devices, procedures must be in place and data available to validate that resuspension and dose homogeneity are achieved.

### 11.6. Container/Closure Integrity

Increased concerns in recent years have focused on the integrity of the container/closure interface of sterile products in order to maintain sterility of the product over its shelf life. A number of methods are available for detecting and quantitating leakage in sterile product packaging systems (Chrai *et al.*, 1994; Guazzo, 1994; Morton *et al.*, 1989). A brief description and advantages and limitations of each of these methods are listed in Table XV. The microbial challenge test continues to be the most commonly used leak test for container/closure integrity. Newer methods, such as the application of mass spectrometry for leak detection of sterile packaging

systems, are generating significant interest from quality assurance experts in industry and from regulatory agencies. Kirsch (1997a,b,c) reported excellent correlation between leakage rates (0.00000001–0.05 $cm^3$/sec of helium as measured by mass spectroscopy) and the microbial challenge test results. The critical leak rate, where samples started failing the microbial challenge test, was approximately 0.000003 $cm^3$/sec, corresponding to a pinhole leak in the range of 0.1–0.3 μm.

## 12. INTERFACE BETWEEN FORMULATION DEVELOPMENT AND MANUFACTURING

All unit operations—compounding, mixing, filtration, sterilization, filling, stoppering, freeze-drying, and in-process storage—can affect protein quality and stability. In fact, the purpose of using some of the stabilizers discussed previously is to protect the protein not only during shelf life, but also during its manufacture. Cryo- and lyoprotectants are examples of stabilizers used to protect proteins from degradation during freeze-drying procedures. Surface-active agents and albumin help to minimize binding of proteins to surfaces during manufacture. It has already been emphasized in this chapter that one cannot separate formulation from processing to obtain a quality-finished product having all the current good manufacturing practice (cGMP) values (safety, identity, strength, purity, and quality).

### 12.1. Front-End Analysis of Manufacturing Methods

Early inclusion of personnel representing manufacturing technical services and quality control/quality assurance (QC/QA) are desirable for development of a robust manufacturing process, methods for in-process and final product quality control, and effective technology transfer to manufacturing. For proteins, development issues which may be encountered during processing (chemical, physical, and microbiological) should be addressed in the design and evaluation of the biopharmaceutical manufacturing process. For example, few proteins can withstand the rigors of terminal sterilization; thus, effective aseptic filtration sterilization procedures must be developed. Factors such as viscosity can be critical in filtration, especially if the product must be maintained at subambient temperatures. In-process excursions of temperature, pH, and agitation also need to be considered. Furthermore, exposure to trace reactive substances

**Table XV**
Container/Closure Integrity Methods[a]

| Test | Basic principle | Advantage | Disadvantage |
|---|---|---|---|
| Visual inspection | Look for leaks | Simple<br>Inexpensive | Insensitive<br>Operator-dependent<br>Qualitative |
| Bubble test | Submerge package in liquid, pressurize and/or temperature cycling to accelerate leakage, improvement in sensitivity | Simple<br>Inexpensive<br>Location of leaks can be observed | Relatively insensitive<br>Operator-dependent<br>Wets package seal<br>Qualitative |
| Pressure vacuum decay | Change in pressure or vacuum is measured inside the package or outside in a sealed package chamber | Relevant to package performance<br>Quantitative<br>Clean, nondestructive | Sensitive to temperature and, atmospheric pressure |
| Dye tests | Movement of dye across a seal is visually or instrumentally detected | Widely accepted in the industry<br>Easy<br>Inexpensive<br>No special equipment required for visual detection | Qualitative<br>Destructive<br>Slow |
| Chemical tracer tests | Solution containing a tracer chemical is applied to one side of package seal; pressure or vacuum is applied as a driving force; chemical leakage detected by appropriate instrument | More sensitive and quantitative than dye test<br>Correlates to liquid leakage<br>Operator independent | Destructive |
| Microbial challenge | Containers are media-filled and the seal is either challenged directly with microorganisms or is allowed to sit in ambient storage environment; presence of microbial growth is visually confirmed | FDA-condoned<br>No special equipment required | Insensitive<br>Media fills are expensive due to production down time and labor costs |

**Table XV**
*(continued)*

| Test | Basic principle | Advantage | Disadvantage |
|---|---|---|---|
| | | | Ambient storage challenge requires large storage area |
| Weight change | Container is filled with liquid or dessicant, sealed, stored at various stress conditions, and reweighed over time | Easy<br>Directly relates to closure performance<br>Quantitative<br>Inexpensive | Time-consuming<br>Leak location not detected |
| Helium mass spectrometry | Helium is place either inside or outside of the container; vacuum is applied to seal interface and migrating helium is detected by mass spectrometry | Inert gas<br>Extremely sensitive test<br>Rapid test time<br>Quantitative | May confuse helium diffusion with leakage |
| Gas detection tests | Test tracer gas is placed on one side of container seal; inert carrier gas is passed along opposite seal side; tracer gas is detected either by a coulombic detector (oxygen) or by a photoelectric sensor (water or carbon dioxide); instruments designed to pierce containers and test package headspace for oxygen or carbon dioxide are another type of gas detection method | Directly relates to package performance<br>Does not pick up false leaks as helium detection can | Slow<br>Often fixture-dependent |
| Vacuum-ionization test | High-voltage, high-frequency field is applied to vials sealed under vacuum; the field causes residual gas to glow; glow intensity is function of vacuum level | On-line nondestructive test<br>Rapid | Some protein decomposition has been reported |
| Electrical conductivity/ capacitance tests | High frequency, high voltage is applied to seal; increase in conductivity correlated to presence of liquid along the seal | Both permit 100% inspection<br>Not operator-dependent<br>Clean<br>Nondestructive | On-line capacitance test not yet available |

[a]See Guazzo (1994).

such as sanitizing agents (formaldehyde, hydrogen peroxide, phenolic compounds) has been shown to cause protein degradation (Kirsch *et al.*, 1993). Quality of excipient raw materials can have a significant impact on protein product stability. A recent example was provided by Dubost *et al.* (1996), who found that reducing sugar impurities in mannitol were responsible for the oxidative degradation of a cyclic heptapeptide. Physical or chemical stability can be affected at several points during the process (freezing step or primary drying of freeze-drying process), as demonstrated for proteins such as hGH (Pikal *et al.*, 1991a,b).

"Seamless" manufacturing of proteins is a relatively new technology that should be considered for proteins that cannot be crystallized and where the amorphous form is exceedingly difficult to handle. In seamless manufacturing, the protein "bulk solution" is not stored, but is processed continuously from the bulk production facility directly to the finished dosage form manufacturing area. In situations where continuous processing cannot be done, proteins may be isolated in bulk in frozen granules by a process called "cryogenic granulation" (Ryan *et al.*, 1995; Schmidt and Akers, 1997). Cryogenic granulation or cryogranulation is a process whereby a solution containing a protein is poured onto a stream of liquid nitrogen, where the droplets rapidly freeze and are carried through a series of descending helical trays and deposited as small, discrete frozen pellets.

Selection of equipment will be dictated by the formulation, the critical product attributes, the primary packaging, the production scale, and the site of manufacture. For example, for those drug substances which are heat-labile, it may be necessary to control the temperature during manufacture. Certain proteins and/or excipients may also require special equipment considerations such as glass versus 316 stainless steel. If oxidation is a problem, equipment will be needed to allow for manufacture in an oxygen-free environment and, possibly, protection from light. Mixing of proteins during the compounding stages must be accomplished without generating foam; therefore, special mixing apparatus must be designed to mix thoroughly without excessive foaming.

During this front-end analysis, a preliminary control strategy should be developed. The strategy encompasses the critical product quality attributes and the means by which these attributes can be assured during manufacturing, shipping, distribution, and product use. The control system which is ultimately in place will typically have the following key elements:

1. Release and expiry specifications (or retest intervals) for drug substance, excipients, drug product, and packaging components.

Specifications need to reflect USP, EP, and JP monographs. Preliminary specifications should be based on previous experience (laboratory and clinical scale) and should be set to control the product, but be sufficiently flexible to allow for uncertainty with a process under development.

2. Methods required to evaluate and characterize the protein active pharmaceutical ingredient and the formulation for potency, purity, impurities, and safety. The rationale behind selection of each method should be based on scientific principles. The methods used depend on the complexity of the protein.
3. In-process controls, according to which, samples are routinely collected during the manufacture of the clinical materials. For example, samples may be collected as a function of mixing time, before and after sterile filtration, and at different holding times in the process. Each process step should be evaluated with respect to criteria for forward processing (CFP), proven acceptance ranges (PAR), and the methods for analysis.
4. Instructions for handling product during shipping and distribution. Studies will need to be conducted to evaluate sensitivity to vibration, special handling, aberrant temperature conditions such as freezing or elevated temperatures, and high relative humidities for solid-state products.

## 12.2. Critical Control Parameters for Protein Product Manufacture

In the development of a protein as a commercial product, process screening studies are essential for assessing parameters which may impact the chemical, physical, and microbiological stability of the product as well as overall manufacturability. Each unit operation in the manufacturing process should be evaluated in order to identify critical process parameters (CPP). Optimization studies should then be conducted to establish acceptance criteria (both target levels and ranges). A thorough understanding of the process and how it will perform over a broad range of conditions, including interaction effects among the variables, is critical in order to develop a robust process. The process, furthermore, has to be validated such that the product can be manufactured on a routine and consistent basis without the need for reprocessing or the occurrence of recurring manufacturing deviations. Data to support worst-case limits should be generated in the lab during the development phase. The

development scientist must keep in mind that the scale of the process may influence results. For example, it is commonly assumed that development of the freeze-drying cycle changes greatly on going from lab scale to pilot scale to production scale. Ideally, experiments can be performed at pilot scale using equipment representative of the production process. In addition, the sequence of steps in the process, acceptable environmental conditions (temperature, humidity, air, etc.) for the unit operation, and acceptable excipient ranges should be evaluated.

For solutions, the following process conditions may impact product quality:

1. *Agitation* (rate, duration): High rates may result in undesired foaming, denaturation, aggregation, or oxidation. Kim *et al.* (1994) reported on the effect of high shear force on the $\alpha$ helix-to-$\beta$ sheet conversion of insulinotropin resulting in a reduced solubility of the protein. Thus, agitation or mixing rates will affect the conformation and solubility of this protein, thus requiring special control of these rates.
2. *Compounding sequence:* Order of addition of excipients and drug substance must be determined. Normally, excipients are added and dissolved before drug substance is added (Harwood *et al.*, 1993). This allows sufficient time for excipients to dissolve while minimizing time in solution for unstable drug substances.
3. *pH adjustments:* Prolonged exposure to acid or basic pHs can cause protein degradation.
4. *Filtration:* A membrane is selected which offers low protein binding as confirmed by protein assays on the solutions before and after filtration to detect any losses (Hawker and Hawker, 1975). This is discussed in detail in Chapter 5.
5. *Filtration and filling:* The temperature may need to be controlled to prevent chemical degradation during filling. Rates of filtration and filling should be considered to prevent shearing of the protein, although this possibility is remote. Effects of filtration and filling rates were studied for human growth hormone (Hsu *et al.*, 1988; Pikal *et al.*, 1991b). Shear forces encountered during processing were found not to cause aggregation of human growth hormone. However, aggregation of proteins during filtration and filling can be caused by interactions of the protein with polymeric hydrophobic surfaces, such as those composing sterilizing filters and process tubing for filling equipment, suspected to be the cause of human growth hormone aggregation during processing (Hsu *et al.*, 1988).

Insulin aggregation and fibrillation can be caused by interaction of the protein in solution with plastics (Thurow and Geisen, 1984) (see previous discussion on insulin binding to plastic surfaces).

6. *Freeze-drying:* Denaturation of a protein solution may occur because of pH shifts or ionic strength changes during freezing (Orii and Morita, 1977). The freezing rate, concentration, endpoint pH, and time and temperature of holding prior to lyophilization may affect the chemical and physical stability of the product (see Chapter 6).
7. *Environmental conditions:* Sparging of nitrogen into solution to remove dissolved gases or use of a nitrogen overlay to replace the air headspace during filling to retard the oxidation of oxygen-labile formulation may be required (Brown and Leeson, 1969).
8. *Materials compatibility:* During the manufacture, the drug is exposed to various materials such as stainless steel, filters, tubing, and pump diaphragms. It is important to ensure that the formulation components are compatible with these materials.
9. *Time and temperature:* Throughout manufacture, critical holding times and temperatures need to be established, not only for protein stability protection purposes, but also to assure microbiological control, particularly to prevent endotoxin contamination.

See Chapter 7 on quality assurance and quality control for additional details on this topic.

## 12.3. Clinical Trial Supplies

As the transfer of a process from laboratory scale into the clinical phase occurs, the development scientist needs to be involved during the early production of clinical trial supplies as well as during any subsequent formulation and process changes. The development scientist can obtain valuable insight into the conditions of manufacturing as well as provide key input to the manufacturing groups about the rationale for formulation and process decisions. These lots also provide an opportunity to collect information under larger scale manufacturing conditions than in laboratory-scale experiments. In-process testing should be routinely conducted during the manufacture of clinical supplies. The data are used to evaluate the process and lead to process improvements, which ultimately support the transfer into the manufacturing phase at full scale.

## 13. QUALITY CONSIDERATIONS DURING FORMULATION DEVELOPMENT

### 13.1. Early Product Assessment

All known physical and chemical properties of a protein should be viewed in light of how these properties will affect final quality of the finished dosage form. The development scientist should build quality into the process so that it can be validated and data exist to support "worst-case" limits (e.g., extremes in pH, excipient levels, time/temperature limits). Process design should be evaluated to assure control of quality parameters.

### 13.2. Documentation

An essential element of validation is establishing thorough documentation that a process will consistently meet its predetermined specifications and quality attributes. Ongoing documentation is essential during formulation development. Development history reports are required during preapproval inspections and are generally a high-level overview describing the history of the drug product from preliminary studies to the commercial formulation and the process submitted in the regulatory document. There may be one report or several to describe the various steps of development (e.g., fermentation, granule isolation, drug substance, drug product, methods development). It is as important to document what did not work as what worked and why decisions were made. Where necessary, bioequivalence of lots used in clinical trials should be demonstrated. A method history report should contain information on regulatory commitments during the development process and give the history of each method. Rationale for specifications, reference standards, and cleaning methods should be provided. For product development reports, lot rejections, deviations, and resolution of the issues need to be covered. Typical contents of a parenteral drug product development history report include the following:

- Preformulation data
- Selection of excipients supported by preformulation studies
- Antimicrobial characteristics of the product
- Rationale and basis for packaging component choices
- Description of container/closure integrity studies

- Overview of the manufacturing process with a description of each unit operation, acceptance criteria for all critical steps of the process, and a history of results for each step
- Scale-up studies
- Stability overview, including product use studies

Development history reports include, but are not limited to, method histories, formulation, primary packaging, process development, and control strategy.

Technical reports prepared on an ongoing basis aid in compiling the development history reports and are key in the information transfer to the manufacturing sites. Typical information contained in these periodic reports are the rationale for decisions, description of what worked and what did not work, and information for solving manufacturing problems.

### 13.3. Stability Studies

Stability testing of protein and peptide dosage forms should follow the ICH guidelines (U.S. FDA, 1996; ICH, 1995). Table XVI summarizes the requirements of this guideline. Three batches or more of the final product in the final container/closure system that represents the finished product manufacturing scale must be put on stability testing. The batches should use different lots of bulk material. A minimum of 6 months of data at desired storage conditions must be available at the time of submission (less than 6 months of data for products that will have less than 6 months dating). Product expiration dating is based on real-time data, not extrapolated from accelerated stability studies. If different volumes and/or strengths of the same formulation are to be tested, a matrix system or bracketing may be permitted. Details of matrixing and bracketing are found in the guideline.

Stability testing must use methods that are stability-indicating and validated. Methods should monitor changes in potency, purity, and other product characteristics* as a function of time and storage conditions as defined in the stability protocol. Data on final product in containers maintained in an inverted or horizontal position and all different container/closure combinations must be obtained. Multiple dose containers must have data to support stability during simulated use, for example, repeated

*Visual appearance, visible particulates in solutions. pH, moisture level of powders, sterility testing or container/closure integrity, degradation, if any, of additives.

**Table XVI**
Summary of Guideline for Stability Testing of Biotechnological and Biological Products[a]

| Selection of batches | Guidance for stability studies for regulatory submissions |
|---|---|
| A. Drug substance | At least three batches representative of production scale |
| | A minimum of 6 months of stability data at time of submission (unless storage period will be less than 6 months) |
| | Pilot-plant-scale batch data acceptable at submission as long as there is commitment to place first three manufacturing-scale batches into long-term stability program after approval |
| | Storage containers should represent actual holding containers to be used during manufacture; containers of reduced size acceptable, provided they are constructed of same material and use same type of container/closure system |
| B. Intermediates | Identify intermediates and generate in-house data and process limits to assure final product stability |
| C. Drug product | At least three batches of final container product representative of final product at production scale |
| | Different batches of bulk material should be studied in final product |
| | A minimum of 6 months of data at time of submission (unless storage period will be less than 6 months) |
| | Product expiration dating based on real-time/real-temperature data |
| | Continuing stability updates should occur during review process |
| | Quality of final product must be representative of quality of material used in clinical studies |
| | Pilot-plant-scale batch data acceptable at submission as long as there is commitment to place first three manufacturing scale batches into long-term stability program after approval |
| D. Sample selection | Matrix system and/or bracketing is acceptable when product consists of different fill volumes, units, or mass |

| Stability-indicating profile | Guidance |
|---|---|
| A. Protocol | Include detailed protocol for assessment of stability of both bulk drug substance and drug product to support proposed storage conditions and expiration dating periods; include statistical methods, specifications, test intervals |
| B. Potency | Potency must be compared to an appropriate reference standard |
| | Perform at appropriate intervals as defined in the protocol and report in units of biological activity calibrated against some recognized standard |
| C. Purity/molecular characteristics | Purity should be assessed by more than one method |
| | Limits of acceptable degradation should be documented and justified, taking into account levels observed in material used in preclinical and clinical studies |
| | Usual methods include electrophoresis, high-resolution chromatrography, and peptide mapping |

**Table XVI**
*(continued)*

| Stability-indicating profile | Guidance |
|---|---|
| D. Other product characteristics | Visual appearance, visible particulates in ready-to-use or reconstituted solutions, pH, moisture level of solid products<br>Sterility testing or alternatives (e.g., container/closure integrity) should be performed at a minimum initially and at the end of the proposed shelf life<br>Additives such as stabilizers and preservatives that may degrade during the dating period of the product and might adversely affect product quality need to be monitored during stability |
| **Storage conditions** | **Guidance** |
| A. Temperature | Storage conditions for real-time/real-temperature stability studies may be confined to the proposed storage temperature |
| B. Humidity | As long as humidity-protected containers are used, stability tests at different relative humidities can be omitted |
| C. Accelerated and stress conditions | Studies should be conducted on the drug substance and drug product under accelerated and stress conditions to provide useful support data for establishing the expiration data, be used for support of change in formulation or scaleup, assist in analytical method validation, or generate information that may help elucidate degradation profile of the drug; other benefits include data on accidental exposure to stress conditions, e.g., during transportation, determine what test parameters are best indicators of product stability, and give further insight into degradation patterns not seen under normal storage conditions |
| D. Light | Appropriate regulatory authories should be consulted on a case-by-case basis |
| E. Container/closure | Stability studies should include samples maintained in inverted or horizontal position for product to have maximum contact with closure<br>Data should be supplied for all different container/closure combinations that will be marketed<br>Data must be generated that demonstrate that the closure used for multiple-dose application is capable of withstanding repeated insertions and withdrawals so that product retains full potency, purity, and quality |
| F. Stability after reconstitution of freeze-dried product | Stability of product after reconstitution should be demonstrated for the conditions and maximum storage period specified |
| **Testing frequency** | |
| Shelf life $\leq$ 1 year | Monthly testing for first 3 months; 3-month intervals thereafter |
| Shelf life $\geq$ 1 year | Every 3 months during first year of storage, every 6 months during second year; annually thereafter |

[a]Summarized from FDA (1996). Also published in *Federal Register* on July 10, 1996 (61 FR 36466).

punctures of the closure, effect of volume change due to repeated withdrawals on potency, purity, and quality.

Stability testing intervals usually are every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

Although accelerated stability testing is not acceptable for direct assignment and support of expiration dates for finished protein products, it does have a role in developmental stability studies. ICH stability guidelines emphasize the importance of conducting stability studies under accelerated conditions, because such studies usually provide useful supporting data for establishing the expiration date. Additionally, accelerated stability studies provide information for short-term assessment of proposed formulation, packaging, or manufacturing changes, evaluate equivalence of materials from different bulk process methods, assist in the validation of analytical methods for the stability program, and can generate information which may help elucidate degradation profiles of the protein alone and in the final formulation.

Accelerated stability studies also may be useful in determining whether accidental exposures to stress conditions during transportation of the final product are harmful to the product. The guidelines generally define accelerated stability test conditions at temperatures at least 15°C above the designated long-term storage temperature for the product.

Typically, kinetic experiments are conducted at elevated temperatures. Estimates of rate constants at lower temperatures are then obtained by extrapolation of an Arrhenius plot. An assumption of this approach is that the kinetics does not change as a function of temperature. For proteins, it is important to determine the appropriate temperature range for these stability studies (Yoshioka *et al.*, 1994). Ideally, it is desirable to obtain an approximately linear relationship to permit prediction of shelf life of the drug product. However, in cases where the results cannot be extrapolated, these data are still useful for determining the effects of short-term temperature excursions on the product and for specifying any special storage precautions that may be encountered during shipping and distribution.

Shnek *et al.* (1998) described two automated physical stress tests for evaluating physical stability of insulin suspensions and solutions. One is a moderate stress test combining temperature cycling (25°C to 37°C) and resuspenion by agitation, and the other is a more considerable stress test combining high temperature (37°C) and extreme agitation (4 h daily). These tests provide more relevant physical stability predictions for using suspensions and solutions in pen-cartridge devices and determining effects of stressful conditions these protein products could encounter during shipping, distribution, and patient use.

### 13.4. Stability Studies Supporting Distribution of Protein Products

Formulation development typically focuses on protein dosage form stability under well-controlled conditions. However, studies must be done to demonstrate that the final protein formulation in the final package produced by a validated process will retain its stability and other quality properties during distribution of the product throughout the world. These studies can be accomplished both by simulation in the laboratory and actual distribution of the product. During distribution, even if the product is to be held in controlled (e.g., refrigerated) conditions, aberrant situations can occur, such as due to transportation breakdown, mechanical failure, dropping or otherwise mishandling packages, and even overt violation of required handling procedures. Data should be available to aid in knowing what these extreme situations will do to the quality of the product. The effect of shear (i.e., agitation, mixing, and other mechanical forces experienced during processing and handling) and temperature extremes on protein stability during simulated and actual conditions should be evaluated.

## 14. EXAMPLES OF FORMULATION PROBLEMS

### 14.1. Aggregation

Protein aggregation, as already emphasized in this chapter, is a major problem in developing stable protein solutions.* Acidic fibroblast growth factor (aFGF) is an example of a protein that aggregates readily when exposed to temperatures above refrigeration (Tsai *et al.*, 1993). To solve this problem, the authors described a four-part approach:

1. Use a rapid screening procedure, based on turbidimetric measurement, to identify solution conditions, polyanions, and common excipients that stabilize aFGF against heat-induced aggregation.
2. Combine the most promising agents with aFGF and use circular dichroism and differential scanning calorimetry to quantitate stabilization effects against aggregation.
3. Formulate the most promising agents with aFGF in solution and monitor protein stability at room temperature.

*For more thorough treatment of protein formulation problems, the reader is referred to the two books edited by Wang and Pearlman (1993; Pearlman and Wang, 1996) on case histories involving stability and characterization of protein and peptide drugs.

4. Test *in vivo* efficacy of the best formulations of aFGF using a wound healing animal model.

This approach enabled the authors to learn that in addition to the well-known protection of aFGF by heparin, a surprisingly wide variety of polyanions (e.g., ATP, dextran sulfate, pentosan polysulfate, sulodexide, phytic acid, inositol hexasulfate, inorganic phosphate salts) stabilize aFGF against aggregation by increasing the temperature at which the protein unfolds by 15–30°C. Stabilization by polyanionic compounds occurs because these compounds enhance the structural integrity by binding to the protein. Common excipients also were studied for their stabilizing effects. Many were effective (e.g., glycine, glycerol, sucrose, dextrose, trehalose), but at relatively high concentrations. Their final formulation choices, all of which were equally capable of accelerating wound healing, contained either heparin, inositol hexasulfate, or sulfated $\beta$-cyclodextrin (with the latter two formulations also containing 1% hydroxyethyl cellulose in phosphate buffer with 0.2 m*M* EDTA). This is an excellent paper from which to learn approaches for solving protein aggregation problems.

### 14.2. Oxidation and Deamidation

Human growth hormone is a protein that both in aqueous solution and in the solid state can undergo chemical decomposition both by oxidation (methionine 14 to methionine sulfoxide) and deamidation (asparagine at position 149) (Becker *et al.*, 1987). This protein is also prone to aggregation. Pikal *et al.* (1991a, b) studied how to overcome these stability problems. Variables that they studied included pH, levels of salts, type of lyoprotectant excipient, residual water content, and oxygen level in the vial headspace. They found that a combination of glycine and mannitol enhanced stability, largely because glycine in this combination can remain amorphous. In the amorphous state glycine serves as a lyoprotectant and as a "sink" for residual moisture, both effects serving to stabilize the protein against deamidation and aggregation. The level of phosphate buffer is important as an aid in the minimization of deamidation, as a pH value on either side of pH 7 increased chemical decomposition. Increases in levels of sodium chloride increased the rate of decomposition.

Oxidation of human growth hormone is due to methionine oxidation. Oxidation occurred even when the air in the vial headspace was replaced with nitrogen (headspace <1% oxygen). Phosphate-buffered formulations containing glycine and mannitol as stabilizing excipients, although found to

be overall the most stable of all hGH formulations studied (Pikal *et al.*, 1991a), were also found to be the most sensitive to changes in the oxygen level of the vial headspace. Because these formulations are amorphous due to the effect of glycine in the presence of mannitol, any dissolution of headspace oxygen in the amorphous phase can significantly affect protein oxidation. It was assumed by the authors that most of the oxygen supply in the formulated vial with nitrogen headspace came from oxygen "trapped" in the amorphous phase of the product during freeze-drying, the trapped oxygen originally being dissolved in the solution filled into the vial.

### 14.3. Binding to Glass

Early identification of the properties of surface adsorption to solid surfaces such as glass is critical to the development of packaging and delivery systems for proteins. As discussed in earlier sections, various formulation additives can be added to minimize or inhibit adsorption. Johnston (1996) examined the effects of solvent additives which would minimize the degree of adsorption to various surfaces for a model protein, recombinant human granulocyte colony stimulating factor (rGH-CSF). For glass vials, he examined solutions containing the following additives: 0.5% w/w Pluronic® F-127, 0.05% and 0.5% w/w Pluronic® F-68, 0.5% v/v glycerin USP, and 0.005%, 0.05%, and 0.5% Tween 20. A concentration of

**Table XVII**
Adsorption of Bovine Serum Albumin (BSA) and Bovine Immunoglobulin G (IgG) to Commercial 0.2-μm Microfiltration Capsules[a]

| Membrane type and capsule manufacturer | Protein concentration μg/ml; (wt%) | BSA adsorption μg/cm$^2$ | IgG adsorption μg/cm$^2$ |
|---|---|---|---|
| Polyvinylidene difluoride, millipore | 50 (0.0050) | — | 1 |
| | 250 (0.025) | 2 | 3 |
| | 1000 (0.10) | 11 | — |
| | 5000 (0.50) | 56 | — |
| Nylon, Pall | 50 (0.0050) | 6 | 108 |
| | 250 (0.025) | 31 | 126 |
| | 1000 (0.10) | 31 | — |
| | 5000 (0.50) | 102 | — |
| Cellulose acetate, sartorius | 50 (0.0050) | — | 1 |
| | 250 (0.025) | 5 | 5 |
| | 1000 (0.10) | 17 | — |
| | 5000 (0.50) | 56 | — |

[a]Reproduced with permission from Brose and Waibel (1996). Copyright 1996 Aster Publishing.

2 ng/ml rGH-CSF in D5W was used as a control. Each solvent additive was examined in triplicate at each concentration tested, and solutions were sampled at times 0, 0.5, 1, 2, and 3 hr and assayed for rGH-CSF. A rapid adsorption was observed up to 1 hr, after which time a slower rate approaching steady state was observed, similar to adsorption reported for insulins and other proteins. Furthermore, viscometry was applied to estimate that the thickness of the adsorbed layer to glass was about 1 μm. Tween 20 at a concentration of 0.5% showed the most potential for inhibiting surface adsorption to parenteral glass vials.

### 14.4. Binding to Filter Surfaces

Proteins are known to bind to filter surfaces. Brose and Waibel (1996) reported on the adsorption of BSA and sheep IgG to three types of commercial filter surfaces, polyvinylidene difluoride (PVDF, Millipore), nylon (Ultipor N66, Pall), and cellulose acetate (CA, Sartorius). They filtered 5-mg/ml solutions of proteins at pressures <1 psi and measured protein concentration in the filtrate as a function of filtrate mass. The mass of adsorbed protein was determined by measuring the difference between theoretical (5 mg/ml = 0.5 wt%) and actual protein weight percent. This mass was divided by the membrane surface area (500 $cm^2$) to obtain the amount of protein adsorbed per unit surface area. They found that both proteins adsorb to all filters, but the extent of adsorption is markedly affected by protein concentration and the type of filter (see Table XVII). Nylon filters adsorbed the most protein. BSA adsorption was linearly related to its concentration, with adsorption to nylon being twice as high as its adsorption to PVDF and CA membranes. IgG adsorption was linear, but very low to PVDF and CA filters, whereas its adsorption to nylon was extensive and nonlinear (it leveled off at higher concentrations). Other authors (Brose and Waibel, 1996) have also reported on protein adsorption on filter surfaces, so the phenomenon will occur, but the question is how much and how can the adsorption be minimized? The choice of filter, the concentration and type of protein being filtered, and the filtration conditions (pressure, rate, size of filter, etc.) all will affect the extent of protein adsorption to filters.

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