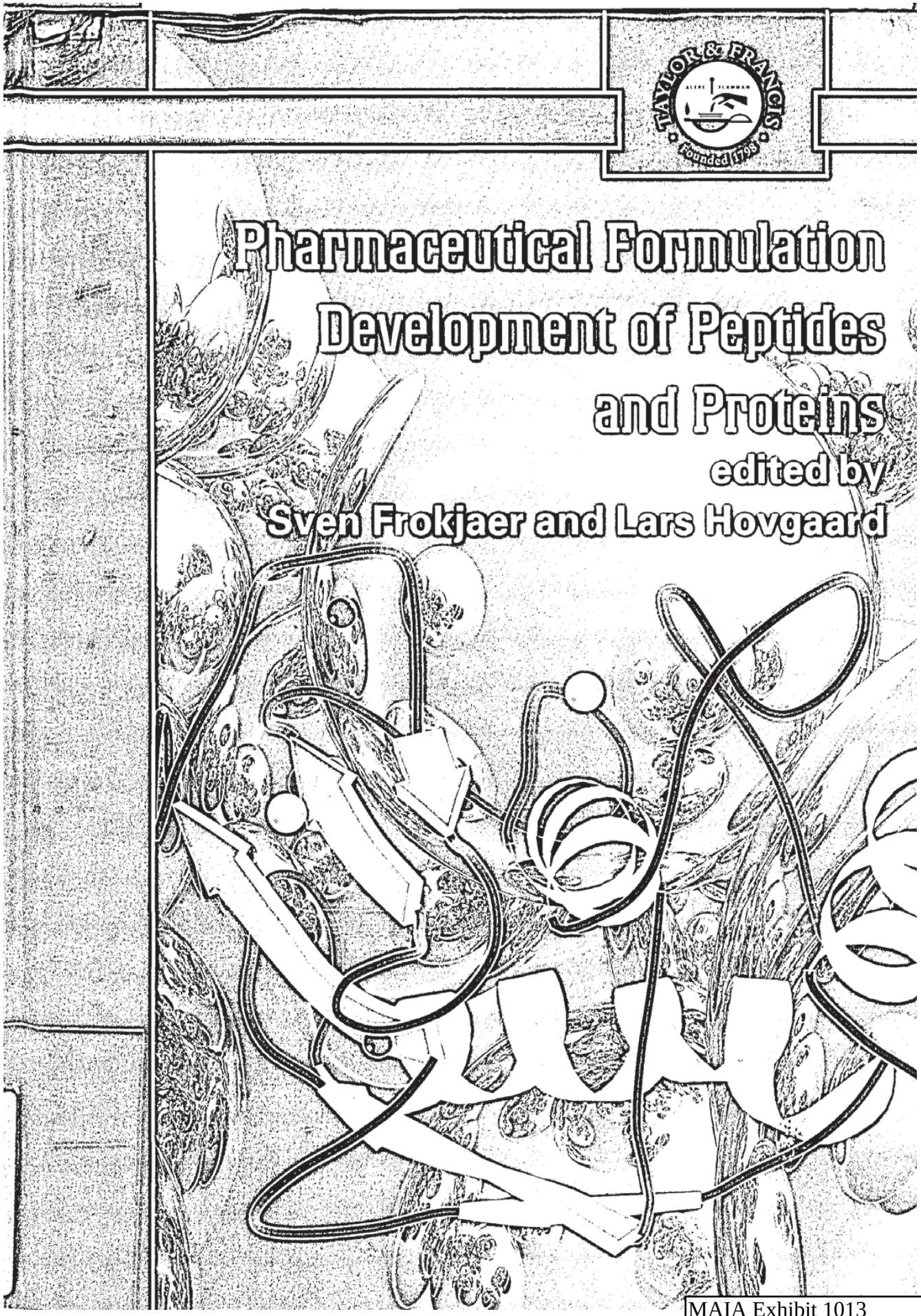

Pharmaceutical Formulation Development of Peptides and Proteins

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Peptides and Proteins as Parenteral Solutions

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8.1 Overview and introduction

The purpose of this chapter is to provide practical guidance to formulation scientists charged with the development of stable, manufacturable, and elegant solution dosage forms of peptides and proteins. The chapter will cover the basics of chemical stabilization, physical stabilization, and microbiological quality of proteins and peptides in solution. We will place more emphasis on the *approaches* used to solve protein/peptide solution

formulation problems than on discussing the nature of degradation mechanisms which are covered elsewhere in this text and in many other excellent publications. We also will have some coverage of packaging and manufacturing of protein solution dosage forms, in the spirit of emphasizing that scientists developing these dosage forms must be equally concerned with the formulation, the package, and the manufacturing process.

There are at least 22 protein products on the market, four of which are stored as ready-to-use solutions, and the rest of which are stored as freeze-dried powders, then reconstituted into solutions by adding a diluent before administration. Approximately 200 peptides and proteins are being studied in the clinic, most of which are freeze-dried products. It is reasonable to assume that nearly every one of these peptide and protein products, commercial or in clinical study, has had to overcome and control stability issues in solution. The type of stability issue and the degree of complexity of the degradation mechanism differ from protein to protein, but approaches to resolve instability issues in solution are relatively universal.

There are some basic guidelines to consider in the development of parenteral solutions of proteins and peptides. These are summarized as follows.

1 A thorough understanding of the physical and chemical properties of the protein or peptide bulk drug substance is necessary. Well-documented analytical techniques are now available for studying these properties in solution. Effects of temperature, pH, shear, oxygen, buffer type and concentration, ionic strength, and protein/peptide concentration must be understood. From preformulation studies, protein/peptide chemical and physical degradation pathways will be better understood so that the final formulation, manufacturing process, and packaging system will be rationally developed.

2 The route of administration must be known in order to select the final dosage form, vehicle, volume, and tonicity requirements for the product. For example, if the primary route of administration is intravenous, the vehicle has to be water although some water-miscible co-solvents can be used. The volume can be limitless (unless an antimicrobial preservative is part of the formulation, in which case the volume is limited to 15 ml), and the tonicity does not necessarily have to be isotonic because the injected solution will be rapidly diluted. However, if the route of administration will be subcutaneous or intramuscular, then the vehicle can be aqueous or nonaqueous, the volumes are limited (usually no more than 2 ml for subcutaneous, 3 ml for intramuscular), and the tonicity of the product needs to be more tightly controlled since the product is not quickly or readily diluted. The rate of injection is also a factor to be considered in selection of final formulation ingredients in that some ingredients, including the protein/peptide itself, can be irritating and even cause local inflammatory reactions if injected too quickly and/or at too high a concentration.

3 Careful screening and choice of solutes for solubilization, stabilization, preservation, and tonicity adjustment must take place. These aspects will be the thrust of this chapter.

4 Potential effects of the manufacturing process on the stability of the protein/peptide in the final formulation must be understood. Proteins/peptides cannot withstand terminal sterilization techniques (heat, gas, radiation) and, thus, must be sterilized by aseptic filtration. The filter used must be qualified so that it does not bind the protein/peptide. The effect of flow rate during filtration and filling on solution stability must be studied. Also, the effect of shear (mechanical stress) that is encountered during manufacturing must be known. Time limitations must be established from the time the protein solution is compounded until it is sterile-filtered in order to avoid any increase in endotoxin levels from whatever the bioburden, however small, may be in the nonsterile solution. Harwood *et al.* (1993) and Nail and Akers (2000) are excellent

references that deal thoroughly with all aspects of the manufacturing of sterile protein and peptides dosage forms.

5 Selection of the most compatible container/closure system is tremendously important. Formulation scientists must appreciate that the container and closure system is just as important as the final solution formulation in assuring long-term stability and maintenance of sterility and other quality parameters of the product. Proteins and peptides are well known to adsorb to glass, so experiments must be designed to study this possibility and, if adsorption occurs significantly, additives such as albumin must be considered to reduce the adsorption. Glass leachates and particulates are possible, and the formulator must be aware of this. Experiments must be conducted to assure elimination of this potential problem. The choice of rubber closure is particularly important because of known potential for the closure to leach some of its own ingredients into a solution, to adsorb components of the protein/peptide formulation, to core (rubber particulates) when penetrated by a needle, to generate particulates, and to leak due to problems with the fitment on the glass vial or resealability of the elastomer after needle penetration. Studies on adsorption of the protein to plastic surfaces will be necessary if the final product will be a plastic container. Even if plastic is not part of the primary container, protein–plastic compatibility studies should be done since plastic tubing such as silicone or polyvinyl chloride will be used in pharmaceutical process equipment (e.g. filling machines), and the final dosage form might be added to large-volume parenteral solutions contained in plastic bags.

6 Studies must be conducted to understand the effects of distribution and storage on the stability of the final product. Temperature excursions during shipping, mechanical stress, exposure to light, and other simulated shipping and storage conditions must be studied. From these studies, appropriate procedures for distribution and long-term storage of these relatively unstable dosage forms can be developed.

Table 8.1 provides of summary of the key steps in the development of solution dosage forms of peptides and proteins.

8.2 Optimizing hydrolytic stability

The effect of solution pH on stability is a very important factor to study in early protein solution development. Figure 8.1 schematically depicts expected stability problems of proteins as a function of pH. Preformulation stability studies are conducted very early in the product development cycle to elucidate relative protein solubility and stability over an appropriate pH range (normally pH 3 to pH 10). The relationship of stability and solubility at various pH values usually follows a pattern of higher solubility, lower chemical stability; or lower solubility, lower physical stability. Protein solubility is minimum generally 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 the pH ranges (Brange *et al.*, 1992b). An example of the effect of pH on deamidation and polymerization of insulin is shown in Figure 8.2 (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 give-and-take relationship between solubility and stability, and it is up to the scientist to identify what pH is optimal for both.

Table 8.1 Development strategy for protein and peptide parenteral solution dosage forms

Formulation and package development studies	Process studies
Final strategy/objectives Development of final formulation • Justification of choice of excipients, pH, specifications Selection of container/closure • Extractables • Container/closure integrity • Glass leachates, particulates Stability and compatibility studies • Effects of light, oxygen, high temperature, freezing • Interaction of excipients with active components • Long-term stability studies of final container formulation in final container/closure system • Temperature/shipping excursions Microbiological characteristics • Antimicrobial properties • Preservative efficacy • Endotoxin control	Optimization studies of excipients, pH, other possible variations Process development • Process control (e.g. time, temperature during each processing step • Filter selection/validation – Microbial retention – Adsorption – Extractables • Effect of terminal sterilization • Justification of excess • Process validation – Sterilization of components – Aseptic process – Cleaning – Filling Establishment of critical process parameters Establishment of control strategy

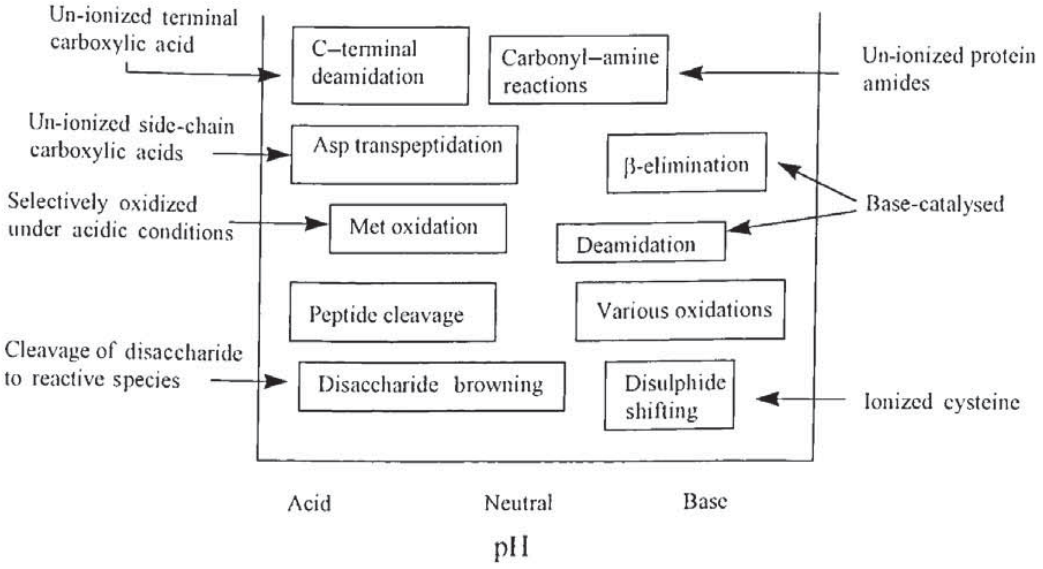

Figure 8.1 Protein reactions as a function of pH (courtesy of Dr Lee Kirsch).

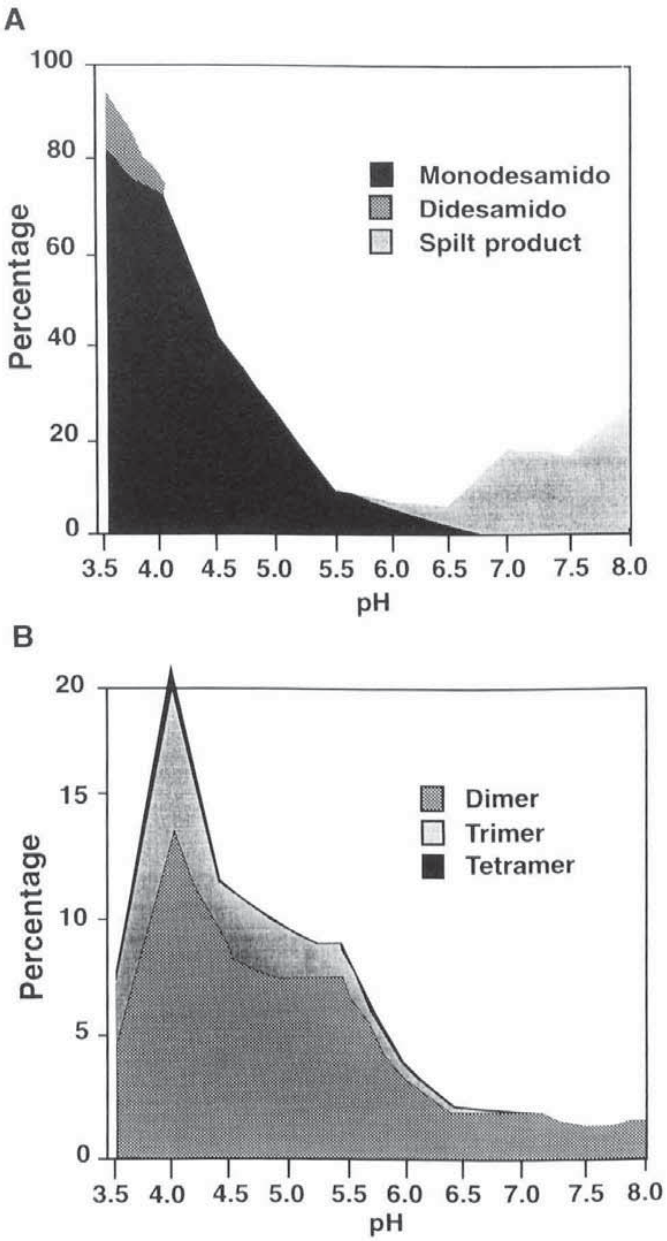

Figure 8.2 Effect of pH on deamidation and polymerization of insulin: chemical transformation during storage (25°C, 12 months) of rhombohedral bovine insulin crystals (0.7% NaCl, 0.2% phenol) as a function of pH. (A) Formation of the hydrolysis products monodesamido and didesamido insulins and the insulin split product (A8–A9). (B) Formation of covalent dimers and oligomers. Reprinted with permission from Brange and Langkjær (1993); ©Plenum Press.

Hydrolysis or deamidation occurs with peptides and proteins containing susceptible Asn and Gln amino acids, the only two amino acids that are primary amines. The side-chain amide linkage in a Gln and Asn residue may undergo deamidation to form free carboxylic acid. Deamidation can be promoted by a variety of factors including high pH, temperature and ionic strength (Manning *et al.*, 1989). The rate of deamidation is affected

by amino acid sequence, particularly the amino acid immediately following the Asn or Gln amino acids. Oliyai and Borchardt (1994) studied the influence of primary amino acid sequence on the degradation of Asp residues under both acidic and alkaline conditions. As expected, the rate of intramolecular formation of the cyclic imide, the first step in the hydrolytic degradation pathway (Patel and Borchardt, 1990), was most affected by the size of the amino acid on the C-terminal side of the Asp residue. Deamidation rates for peptides will be highest when Asn is immediately followed by a Gly amino acid since Gly has no side-chain, thus no opportunity to hinder the hydrolysis reaction sterically. C-terminal substitution of Gly with increasingly more bulky residues (Ser, Val) inhibits the amount of cyclic imide produced. However, with respect to Asp amide bond hydrolysis with adjacent amino acids either before or after Asp, such structural changes had little or no effect.

For larger protein structures, the effects of adjacent amino acid sequences on the deamidation rates of Asn and Gln are more difficult to estimate simply due to the three-dimensional complexities of these structures. However, it certainly is intuitive that adjacent amino acids and their size will have some effect on Asn and Gln deamidation regardless of the size of the total protein. Hydrolytic stability of peptides and proteins can be minimized, therefore, through one or more of the following approaches.

1 Optimization of amino acid sequence, i.e. engineering protein structures to remove unstable amino acids or insert amino acids that sterically hinder Asn or Gln deamidation, as long as this does not affect protein activity, potency, toxicity, or any other quality attribute.

2 Formulate at optimal solution pH. For example, human epidermal growth factor 1–48 demonstrates some interesting pH-dependent stability in that at pH <6 succinimide formation at Asp^{11} is favoured, while at pH >6 deamidation of Asn^{1} is favoured (Senderoff *et al.*, 1994). The optimal pH, therefore, is right at pH = 6.

3 Store at low temperatures, although this will always create difficulties during distribution and long-term storage of the product.

4 Optimize the effects of ionic strength (to be discussed in section 8.2.2).

8.2.1 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 8.2.

The proper selection of buffer type and concentration is determined by performing solubility and stability studies as a function of pH and buffer species. Normally, the pH of maximum solubility is not the pH of maximum stability. However, a pH range that is a good compromise between solubility and stability can be selected and maintained with the proper selection of the appropriate buffer component.

In the pH range of 7–12, buffer concentration can have a significant effect on the rate of deamidation indicating general acid–base catalysis. Generally, deamidation is much slower at acidic pH than at neutral or alkaline pH. ACTH deamidation in the pH range of 7–11 is catalysed by increasing buffer concentrations, whereas there is no buffer catalysis

Table 8.2 Buffers used in protein formulations

Buffer system	pKa	pH Range of use
Acetate	4.8	2.5–6.5
Carbonate	6.4	5.0–11.0
Citrate	3.14, 4.8, 5.2	3.0–8.0
Glutamate	2.2, 4.25, 9.67	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 (pKa 2)	3.0–8.0
Succinate	4.2, 5.64	4.8–6.3
Tartrate	2.93, 4.23	3.0–5.0
Tris	6.2 (pKb 7.8)	6.8–7.7

at pH 5–6.5 (Patel, 1993). However, insulin deamidation at the A21 position predominates at acidic pH while deamidation at B3 predominates at neutral pH (Brange *et al.*, 1992b).

Several potential problems are associated with using buffers in parenteral solutions. For example, it should not be expected that in large-scale manufacturing the compounded solution containing the buffer will always result in the exact pH specified. Dilute solutions of strong acids (hydrochloric acid) or bases (sodium hydroxide) are usually required to 'fine-tune' the final solution pH. Excessive use of the pH adjustment solutions may alter the buffer capacity and ionic strength of the buffered solution (Niebergall, 1990). Increasing buffer capacity to control pH better could significantly increase ionic strength which, in turn, may cause increased potential of pain upon injection due to the increase in solution osmolality.

General acid and/or general base buffer catalysis can accelerate the hydrolytic degradation of the protein. An example is shown in Figure 8.3 (Yoshioka *et al.*, 1993) where the inactivation rate of β-galactosidase increased with increasing concentrations of phosphate buffer up to 0.5 M, then decreased, presumably related to higher buffer components causing a reduction in water mobility. Cleland *et al.* (1993) cite several examples where the rate of protein deamidation was markedly dependent on the buffer anion. Capasso *et al.* (1991) compared the deamidation rate of a small peptide using different buffers and found that the peptide was most unstable in a phosphate buffer and most stable in Tris buffer. Wang *et al.* (1996) found that buffer type and concentration affected aggregation of basic fibroblast growth factor depending on pH. At pH 5, aggregation increased as citrate buffer concentration increased. Citrate buffer at pH 3.7 caused aggregation, whereas acetate buffer at pH 3.8 did not. At pH 5.5–5.7, phosphate, acetate and citrate buffers all showed similar aggregation rates.

8.2.2 Ionic strength

Ionic strength is a measure of the intensity of the electrical field in a solution. It depends on the total concentration of ions in solution and the valence of each ion. The ionic strength of a 0.1 M solution of sodium chloride is 0.1. The ionic strength of a 0.1 M

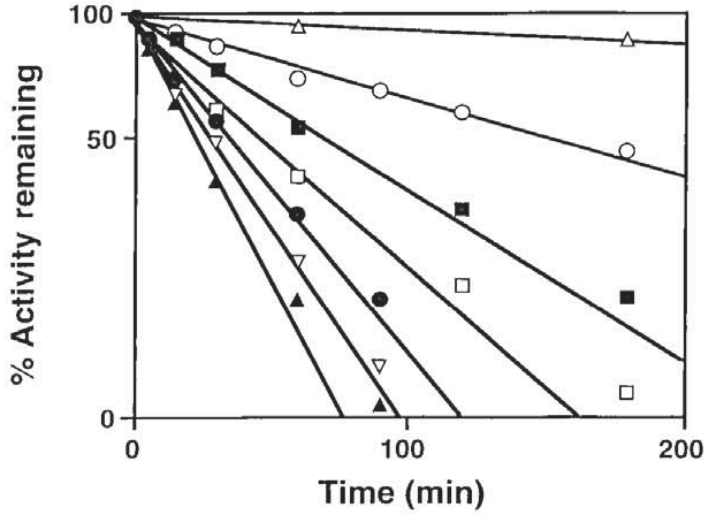

Figure 8.3 Inactivation of β-galactosidase in pH 7.4 phosphate buffer solution at 50°C, as a function of phosphate buffer concentration: (Δ) 10, (○) 50, (□) 100, (▽) 200, (▲) 500, (●) 700, (■) 900 mM. The concentration of β-galactosidase was 0.1 mg/ml. Reprinted with permission from Yoshioka *et al.* (1993); ©Plenum Press.

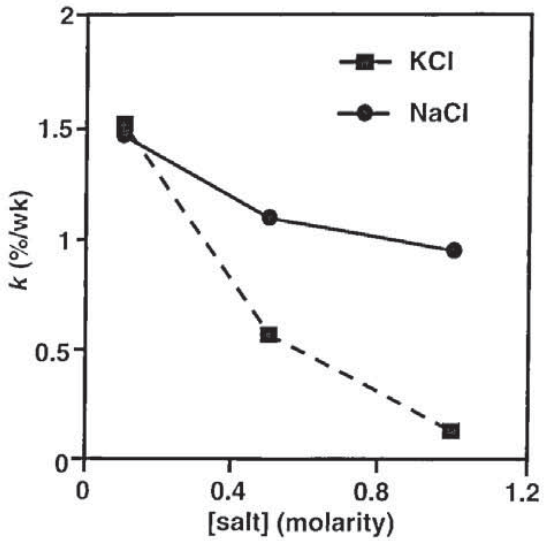

Figure 8.4 Effect of salt concentration on rAAT solution stability. Reprinted with permission from Vemuri *et al.* (1993); ©Plenum Press.

solution of sodium sulphate is 0.3, because sulphate ions have a valence of 2 added to the valence of 1 for the sodium ions. Ionic strength may have an effect on protein stability in solution. The Debye-Huckel theory predicts that increased ionic strength would be expected to decrease the rate of degradation of oppositely charged reactants, and increase the rate of degradation of similarly charged reactants.

Ionic strength will affect the stability of a protein, but in which direction (increase or decrease) differs with different proteins. For example, increasing ionic strength will increase the stability of recombinant alpha$_1$ antitrypsin (Vemuri *et al.*, 1993) (Figure 8.4).

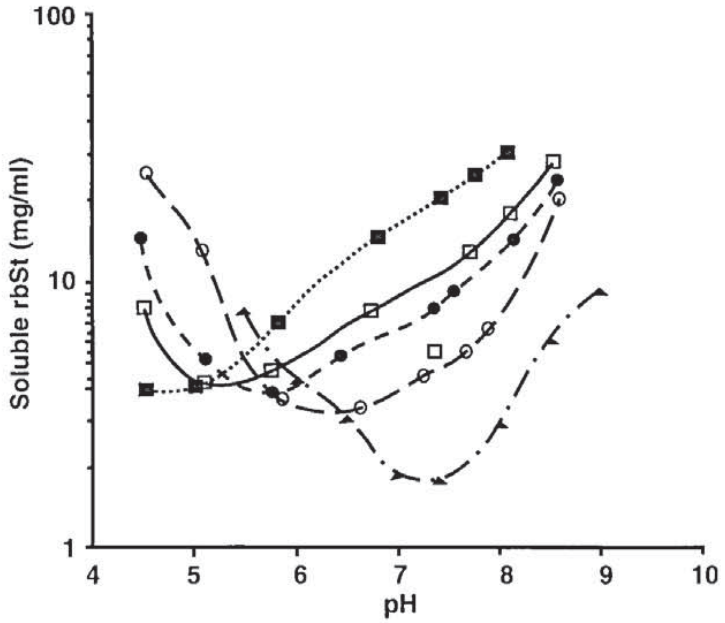

Figure 8.5 Effect of ionic strength on rbSt solubility–pH profiles. Ionic strength μ increased with increasing NaCl: (■) μ = 0.24; (□) μ = 0.12; (●) μ = 0.072; (○) μ = 0.024; (▲) μ = 0.008. Reprinted with permission from Davio and Hageman (1993); ©Plenum Press.

Conversely, increasing ionic strength will increase the rate of deamidation of human growth hormone (Pearlman and Bewley, 1993) and bovine somatotropin (Davio and Hageman, 1993) (Figure 8.5).

8.3 Optimizing oxidative stability

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 presence of oxygen, light, high temperature, metal ions, and variours free radical initiators. Oxidation of sulphydryl-containing amino acids (e.g. methionine and cysteine) will lead to disulphide bond formation and loss of biological activity. The free thiol group that is present in a Cys residue of any native biologically active protein not only may oxidize to produce an incorrect disulphide bridge, but also can result in other degradation reactions such as alkylation, addition to double bonds and complexation with heavy metals.

Human growth hormone, chymostrypsin, lysosyme, parathyroid hormone, human granulocyte-colony stimulating factor, insulin-like growth factor I, acidic and basic fibroblast growth factors, relaxin, the monoclonal antibody OKT3, interleukin 1β, and glucagon are examples of proteins that will degrade by this mechanism. Haemoglobin, 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 haem groups (unpublished results). Cleland *et al.*

(1993) list 61 different proteins that can be oxidized with varying degrees of loss of biological activity.

For protection against oxidation, choice of an effective antioxidant is one of several precautions that must be practised in formulation development and final product manufacture. Other factors that contribute to protein stability against oxidative degradation include:

- preparation and storage at low temperatures
- use of chelating agents to eliminate metal catalysis
- increasing ionic strength
- 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)
- replacing oxygen with nitrogen or argon during manufacturing
- removing oxygen from the headspace of the final container
- formulation at the lowest pH possible while maintaining desired protein solubility and hydrolytic stability, since 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
- assuring that phenolic or other oxidizing cleaning agent residues are minimal in the production environment, including the freeze-dry chamber (Kirsch *et al.*, 1993).

Fransson (1997) published an excellent paper on methionine oxidation and covalent aggregation in aqueous solution as studied with human insulin-like growth factor-I (hIGF). Oxidation of methionine 59 was catalysed by light and by ferric ions in combination with EDTA. In this example, Fransson suggests that EDTA actually enables ferric ions to be active by stabilizing the transfer of electrons from ferric ions to ferrous ions. Figure 8.6 shows the relationship of EDTA and ferric ion in the oxidation of methionine in hIGF-I in aqueous solution in the presence of light. Methionine in this protein is radicalized by light, then oxidized to methionine sulphoxide. Light may also trigger the generation of hydroxyl radicals by decomposition of water that may oxidize the methionine.

Formulators should be aware of the potential for polysorbate 80 to affect adversely the oxidative stability of proteins. Polysorbate 80 is a commonly used surface active agent in protein formulations to minimize surface aggregation problems. However, it has the tendency to produce peroxides which can oxidize methionine and cysteine residues. This phenomenon was reported in studies involving formulation development of Neupogen® (Herman *et al.*, 1996) and recombinant human ciliary neurotrophic factor (Knepp *et al.*, 1996).

8.3.1 Antioxidants

There are several choices of antioxidant that can be used in protein formulations (see Table 8.3). Those which have been used most frequently are ascorbic acid, salts of sulphurous acid (sodium bisulphite, sodium metabisulphite or sodium thiosulphate), and

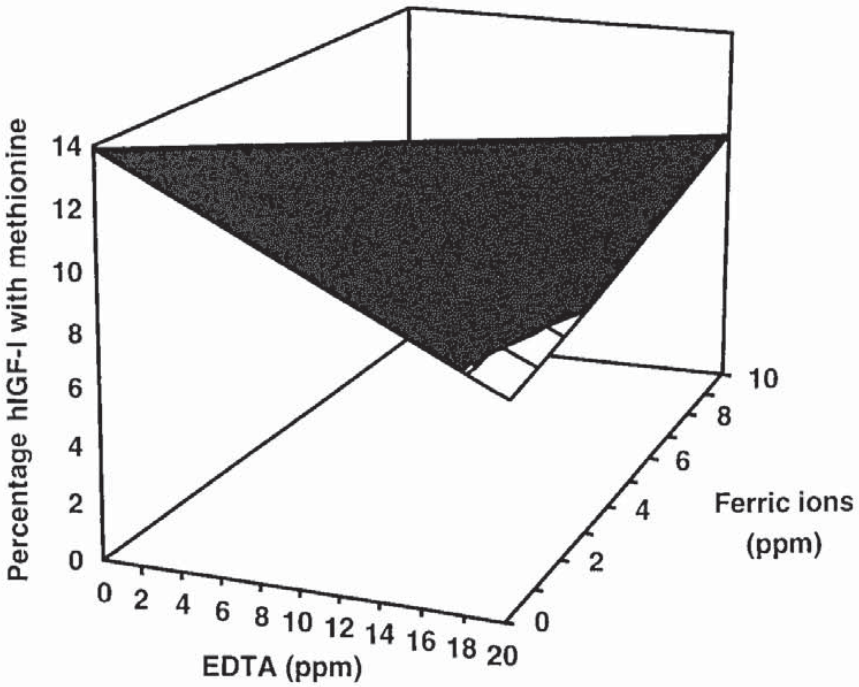

Figure 8.6 Response surface for the oxidation of methionine 59 in hIGF-I in aqueous solution at 25°C. Reprinted with permission from Fransson (1997); ©American Chemical Society and American Pharmaceutical Association.

Table 8.3 Antioxidants and chelating agents for protein formulations

Antioxidants (all water-soluble)	*Normal % used in formulation*
Ascorbic acid (including isoascorbic acid, sodium ascorbate)	0.1–1.0
Sulphurous acid salts (sodium bisulphite, sodium metabisulphite, sodium sulphite)	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 (EDTA) (usually the disodium salt)	0.05–0.1
Citric acid/sodium citrate	0.02–1

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.

Lam *et al.* (1997b) studied the inhibitory effect of various antioxidants on the oxidation of recombinant monoclonal antibody HER2. As shown in Figure 8.7, sodium thiosulphate, methionine, catalase, or platinum, as antioxidants, were effective in reducing the oxidation

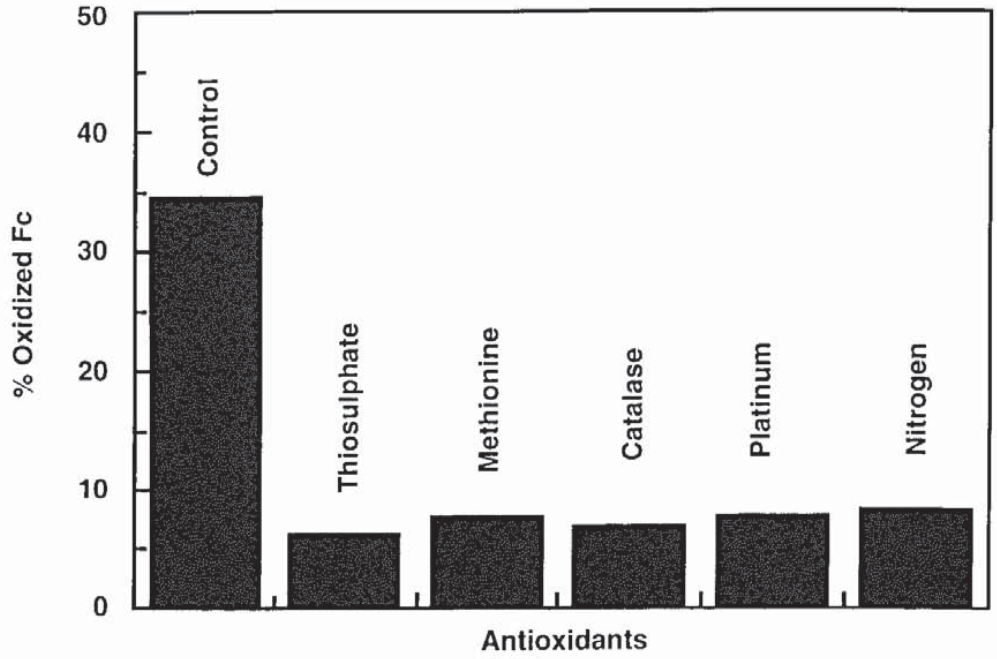

Figure 8.7 Methods to prevent temperature-induced oxidation of rhuMAb HER2 in formulation containing 5 mM sodium acetate, pH 5.0, 147 mM NaCl, and 0.01% polysorbate 20. Reprinted with permission from Lam *et al.* (1997b); ©American Chemical Society and American Pharmaceutical Association.

of the antibody. Figure 8.7 also shows that replacing air with nitrogen in sample vials was also effective in reducing antibody oxidation. The authors proposed that thiosulphate and methionine either inhibit free-radical induced oxidation by terminating the chain reaction or compete with the methionine residues in the antibody for reaction with the free hydroxyl radicals. Catalase and platinum serve as free-radical scavengers.

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 in that it 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, HEPES, and MOPS) accelerated the degradation of methionine in the presence of ascorbic acid. The addition of EDTA did not enhance stability even though ferric ion and other transition metals were components in the formulation, either purposely added or as trace components of the buffer and peptide. This prooxidant effect of ascorbate methionine oxidation was concentration-dependent and occurred most readily at pH 6–7.

It is also known that sodium bisulphite can cause stability problems with certain drugs and proteins. For example, bisulphite will rapidly destroy insulin (Asahara *et al.*, 1991). These authors studied the compatibility of human insulin in solutions containing sodium bisulphite since human insulin and sodium bisulphite were being used in some intravenous admixtures in their hospital practice. Bisulphite was found to cleave the interchain disulphide bonds of insulin. The addition of glucose to these solutions stabilized human insulin in the presence of sodium bisulphite, with the stabilization postulated to be the formation of a bisulphite–glucose adduct in solution.

8.3.2 Chelating agents

Chelating agents (see Table 8.3) 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. While 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 disodium ethylenediaminetetraacetic acid (DSEDTA). The concentration of DSEDTA is usually very small, e.g. ≤0.04%. DSEDTA tends to dissolve slowly and is usually among the first formulation ingredients to be dissolved during compounding before adding other ingredients, including the protein.

EDTA should not be used in formulations of metalloproteins such as insulin and fibrolase (Pretzer *et al.*, 1993), as the chelating agent will attack the metal that is part of the stable conformation of the protein.

As already discussed, EDTA can also accelerate the oxidative degradation of methionine in hIGF-I solutions (Fransson, 1997). Thus, the formulator must not indiscriminately include EDTA in protein formulations, but must carefully determine that its presence aids in oxidative stabilization of the protein.

8.3.3 Inert gases

Inert gases are frequently used in production of protein dosage forms. The most commonly used inert gas is nitrogen. Other inert gases which can be used, although not very often primarily because of expense, include argon and helium. Argon, however, has been shown to be more efficient in displacing oxygen because it is heavier than air and will more readily stay in the vial compared to nitrogen, which is lighter than air (Harwood *et al.*, 1993). Inert gases are normally used in protein formulation and production in two ways.

1 Added to water and compounding solutions prior to aseptic filtration to saturate the solution and minimize the level of dissolved oxygen. However, oxygen is never completely displaced with an inert gas when the solution is sparged. Many manufacturers use a dual needle which permits filling of a liquid and purge gas at the same time.

2 Introduced into the headspace of a filled vial right before the vial is stoppered with a rubber closure, thereby, theoretically, displacing oxygen in the headspace. Again, a dual needle can be used to fill solution and purge gas into the final container at the same time.

The inert gas must be high-quality grade and must be sterilized, usually with a 0.45 μm hydrophobic membrane filter. The integrity of the gas filter is tested before and after use by diffusion flow methods.

8.3.4 Packaging and oxidation

All the appropriate formulation and processing procedures can be in place for stabilizing protein solutions against oxidation, but if the packaging system is inadequate from an integrity standpoint, the product will readily degrade. Most protein products are packaged in glass vials with rubber closures. The rubber–glass interface and the oxygen transmission coefficient of the rubber closure will dictate the quality of the container/closure system. The integrity of the rubber–glass interface can be tested by a variety of techniques including helium mass spectometry (Kirsch *et al.*, 1997a, 1997b, 1997c). This technique

is used to validate the integrity of the specific type of rubber closure with the specific type of glass vial and, then, can be used to check integrity of a representative number of product vials per lot.

Oxygen transmission coefficients are determined for a particular rubber closure formulation by the rubber closure manufacturer. Rubber formulations having the lowest oxygen transmission coefficients are the synthetic butyl and halobutyl types. The formulator should determine from the rubber manufacturer how the halobutyl rubber is cured (shaped, moulded) since common curing agents are zinc oxide, aluminium, and peroxide, which potentially can leach out of the rubber formulation with time and catalyse oxidative degradation (Boyett and Avis, 1976, Milano *et al.*, 1982, Danielson *et al.*, 1984, Liebe, 1995).

8.3.5 Other chemical stabilizers

Sugars and polyols, such as ethylene glycol, glycerol, glucose, and dextran, at high concentrations, can inhibit the metal-catalysed oxidation of human relaxin (Li *et al.*, 1996). All but dextran act as chelating agents in complexing transition metal ions whereas dextran, which has a higher binding affinity to metal ions and undergoes depolymerization in a metal-catalysed oxidation, protects relaxin by a radical scavenging mechanism.

Mannitol has been shown to inhibit the iron-catalysed oxidation of Met-containing peptides (Li *et al.*, 1995). Mannitol is the most commonly used excipient in freeze-dried formulations, often serving a dual role as a bulking agent and a stabilizer.

Fibroblast growth factors, both acidic and basic, possess nearly identical three-dimensional structures of 12 antiparallel β-strands arranged with approximate three-fold internal symmetry (Tsai *et al.*, 1993). Acidic fibroblast growth factor formulation design was studied by Tsai *et al.* (1993), who found that its tendency to aggregate in solution was inhibited by a variety of polyanionic additives such as inositol hexasulphate or sulphated β-cyclodextrin and by a number of commonly used exicipients such as sucrose, dextrose, trehalose, glycerol, and glycine. The polyanionic additives interacted with the polyanion binding site of the protein while the non-specific agents were thought to be preferentially hydrated. In all cases, these interactions between acidic fibroblast growth factor and various excipients resulted in an increase in the protein's T_m, the midpoint of the temperature of the transition from the folded to unfolded protein. Basic fibroblast growth factor formulation design was studied by Shahrokh *et al.* (1994a), who found that its major degradation pathway involves not only aggregation and precipitation, but also a succinimide replacement of aspartate at position 15 of the protein sequence. Adjusting solution pH from 5 to 6.5 and storage at low temperatures will help to avoid this reaction.

A variety of co-solvents can stabilize proteins in solution because the co-solvent is preferentially excluded from surface interaction with the protein (Arakawa *et al.*, 1991). Co-solvents behaving this way include glycerol and sorbital. Polyethylene glycol is also preferentially excluded from the protein, yet will still denature or destabilize proteins in solution.

8.4 Optimizing physical stability

Unlike small molecules, where physical instability is rarely encountered except for poorly water-soluble compounds, proteins, because of their unique ability to adopt three-dimensional forms, tend to undergo a number of structural changes, independent of chemical modifications. Physical instability of proteins is sometimes a greater cause for concern

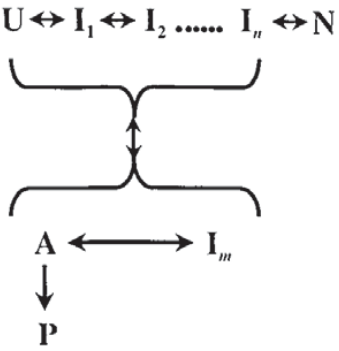

Figure 8.8 Aggregation scheme. The potential folding pathways for an unfolded protein (U) are shown assuming that it is refolded *in vitro* by dilution with a simple buffer (no additives). The unfolded protein will fold to form an intermediate structure (I_1) which has some secondary structure. This intermediate and others in the folding pathway ($I_2 \ldots I_n$) often associate to form soluble aggregates (A). These soluble aggregates can agglomerate to form large irreversible precipitates (P) that must be resolubilized in denaturants. All intermediates as well as the unfolded protein can form misfolded or off-pathway intermediates (I_m) that can reduce the yield of native protein by becoming a kinetic trap for the preceding species. Some intermediates in the later portion of the pathway obtain a native-like conformation (I_n) and eventually assume the native state (N). Reprinted with permission from Cleland (1993); ©American Chemical Society.

and is more difficult to control than chemical instability. All protein structures are hydrophobic to some extent. Many proteins, particularly when exposed to stressful conditions, e.g. extremes in temperature, will unfold such that the hydrophobic portions become exposed to the aqueous environment. Such exposure will promote aggregation or self-association, possibly leading to physical instability and loss of biological activity since the interaction with the receptor site requires folded structures with correct conformation. The relationship of the different pathways of physical destabilization of proteins is shown in Figure 8.8. The physical stability of proteins is also dealt with in Chapter 6.

8.4.1 Denaturation

Denaturation is unique to proteins and occurs when their native quarternary, 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 chaotropic agents (urea, guanidine hydrochloride) where, if the denaturing condition is removed, the protein will regain its native state and maintain 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 its lost activity can be restored by renaturation with guanidine hydrochloride (Wetzel *et al.*, 1988). When a protein recovers its activity by the addition of denaturants such as guanidine hydrochloride or urea and by subsequent dialysis, such a process may indicate the influence of events such as aggregation, precipitation, and adsorption. Regardless of the observed phenomenon, the approach is of little use in solution formulation design.

For stabilizing proteins against denaturation in solution, Arakawa and Timasheff (1982, 1984) and Arakawa *et al.* (1993) have shown that reversible denaturation can be decreased by the use of additives such as salts that bind to non-specific 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) showed the ability of Zn $(NO_3)_2$ to increase significantly 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 0.1M NaCl, $MgCl_2$ and Na_2HPO_4, respectively. Through genetic engineering they were able to introduce appropriate amino acid substitution in ribonuclease T_1 creating specific cation/anion binding sites on the protein. The stability profile of ribonuclease T_1 was enhanced considerably with this approach. Pantoliano *et al.* (1988) successfully introduced negatively charged side-chains such as Asp in the vicinity of the weak Ca^{2+} binding site of subtilisin. Such modifications caused an increase in binding affinity for Ca^{2+}, thereby increasing the thermal stability of subtilisin. These engineering approaches are acceptable provided that protein activity and other quality aspects are not adversely affected.

8.4.2 Protein aggregation

Aggregation of peptides and proteins is caused mainly by hydrophobic interactions that eventually lead to denaturation. When the hydrophobic region of a partially or fully unfolded protein is exposed to water, this creates a thermodynamically unfavourable situation due to the fact that the normally buried hydrophobic interior is now exposed to a hydrophilic aqueous environment. Consequently, the decrease in entropy from structuring water molecules around the hydrophobic region forces the denatured protein to aggregate, mainly through the exposed hydrophobic regions. Thus, solubility of the protein may also be compromised. 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 (Mitraki and King, 1989; Shahrokh *et al.*, 1994b; Brange *et al.*, 1992b; Brange and Langkjaer, 1993; Silvestri *et al.*, 1993). The protein, antithrombin, aggregates when denaturated with guanidine hydrochloride which proceeds through an intermediate partially-unfolded state. It is possible to return antithrombin to its native state by dialysing 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 which occurs due to denaturation can be prevented by the use of surfactants, polyols, or sugars. The use of surfactants is elaborated in section 8.4.5.

Factors that affect protein aggregation in solution generally include protein concentration, pH, temperature, other excipients, and mechanical stress. Some factors (e.g. temperature) can be more easily controlled during compounding, manufacturing, storage and use than others (e.g. mechanical stress). Formulation studies will dictate appropriate choice(s) of pH and excipients that will not induce aggregation and/or, in fact, will aid in the prevention of aggregation. Protein concentration is dictated by the required therapeutic dose and, depending on what this concentration is, will determine whether the potential for higher associated states (dimers, tetramers, etc.) exists, which can then lead to aggregation in solution. Careful studies must be done during formulation development to determine

what factors influence protein aggregation and then how these factors can be eliminated or controlled.

The desire to identify stable solution preparations of insulin for use in novel delivery systems such as continuous infusion pumps has led to the development of test methodology for assessing the impact of various additives on physical stability. Based on the known factors influencing protein aggregation and the requirements of such applications, physical stability has been evaluated using thermomechanical procedures involving agitation or rotation of insulin solutions at elevated temperature. In some cases, Teflon beads have been added to the solutions to introduce hydrophobic surfaces in order to avoid complications with air headspace (Sluzky *et al.*, 1991). Turbidity resulting from aggregation is usually determined as a function of time by visual inspection or light scattering analysis. Alternatively, reductions in the soluble protein content due to precipitation can be quantitated by HPLC assay as a function of time. Relative stability is defined by the length of time a preparation remains on the test without showing a change in either parameter. It should be noted that the greatest difficulty in applying such testing strategies is in interpreting the experimental data and correlating them in a practical way to 'real life' conditions that the formulation may actually experience. Nevertheless, regulatory agencies may request data from such testing to support dating periods or other product claims. Physical stress testing, however, is more appropriately used as a development screening tool to identify the capability of various additives to prevent aggregation, as we shall discuss. Since the principles involved in physical stress testing studies are fairly similar, we will only summarize some of the methodology and formulation strategies described for insulin.

Brange and Havelund (1983) report the effect of carbohydrate additives on the physical stability of neutral insulin formulations in vials subjected to shaking (amplitude 5 cm, frequency 100 rev/min) at 41°C. While some of the carbohydrates improved physical stability, formulations containing these additives also demonstrated a reduction in both chemical and biological stability. However, the addition of dissociable calcium salts improved physical stability without impacting other quality attributes. A variety of additives to insulin solutions including bacteriostatic agents, non-ionic detergents, anionic detergents, physiologic compounds and extracts, salts, buffers, and alcohols were examined in other work (Lougheed *et al.*, 1983). The physical stability of solutions prepared with the various additives and stored in vials with an air headspace was assessed at defined time intervals after exposure to 37°C and either shaking (130 cpm in the horizontal plane) or rotation (20 cm from the axis of a wheel rotating at 60 rev/min in the vertical plane). The formulation compatibility with certain device materials was also examined by inserting samples directly into the solution being tested. Additives that reduced solvent polarity (e.g. anionic and non-ionic surfactants with long hydrophobic chains or alcohols) were most effective in preventing insulin aggregation. However, the concentrations of these additives necessary to achieve the beneficial effect on physical stability may not be appropriate for pharmaceutical preparations. In similar, but somewhat more sophisticated stress-testing experiments, Sluzky *et al.* (1992) reported on the mechanism of additive stabilization. It was concluded that the presence of surfactants enhanced physical stability by reducing unfavourable interfacial interactions between insulin and hydrophobic surfaces rather than by lowering surface tension. Thurow and Geisen (1984) examined the physical stability of insulin and other protein solutions containing polypropylene glycol/polyethylene glycol block polymers. Samples were filled into ampoules with an air headspace and subjected to rotation (20 cm from the axle, 60 rev/min) at 37°C. The additive, Genapol PF-10, was shown to have a stabilizing effect.

8.4.3 Adsorption

Proteins exhibit a certain degree of surface activity, i.e. they adsorb to surfaces due to their innate nature as 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. The process of adsorption in the case of proteins depends on protein–protein interactions, time, temperature, pH and ionic strength of the medium and the nature of the surface (Absolom *et al.*, 1987). 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 liquid–air interfaces has been shown by Lenk *et al.* (1989) to involve conformational changes such as loss of α-helices to ß-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, or chemical denaturants. In the case of proteins, sources such as the polymer of the membrane filter, the administration set, agitation that occurs during the purification process, and the method of manufacture are known or at least suspected to cause surface denaturation. Strategies often used to overcome protein denaturation due to adsorption are:

1 increase protein concentration during filtration and/or using extra volume to saturate the filter with protein solution
2 modify (e.g. siliconize) the surface of the glass containers, providing a resistant barrier to protein–surface interaction
3 decrease the rate of mixing when it is known that shear will affect protein adsorption
4 add excipients such as surfactants that have higher surface activity
5 add macromolecules such as albumin and gelatin (although one must realize the increased concern regarding these natural materials because of their potential for pyrogenic and/or BSE[1] contamination).

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

There are several approaches to minimize or overcome protein adsorption. 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

[1] BSE is bovine spongiform encephalopathy, also known as 'mad cow disease', a chronic degenerative disease affecting the central nervous system of cattle. First diagnosed in Great Britain in 1986, this transmissible disease could be a contaminant in bovine-sourced pharmaceutical excipients such as gelatin, bovine serum albumin, and polysorbate 80.

the surface of the container with either lecithin or BSA. Adsorption of urokinase to glass can be prevented by the addition of 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). Johnston (1996) reported that adsorption of recombinant human granulocyte to glass, polyvinyl chloride, and polypropylene surfaces can be minimized by the addition of certain additives such as polysorbate 20, polysorbate 80, or Pluronic F-127.

8.4.4 Precipitation

Precipitation of proteins 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) for more detailed information. Brennan *et al.* (1985) have established the tendency of insulin to precipitate when loaded into a long-term infusion device, making it cumbersome for delivery.

8.4.5 Surfactants

Surfactants are surface active agents that can exert their effect at surfaces of solid–solid, solid–liquid, liquid–liquid, and liquid–air because of their chemical composition, containing both hydrophilic and hydrophobic groups. These materials reduce the concentration of proteins in dilute solutions at the air–water and/or water–solid interfaces where proteins can be adsorbed and potentially aggregated. Surfactants can bind to hydrophobic interfaces in protein formulations and packaging. Glass, rubber, or plastic adsorption of proteins is well documented (Christensen *et al.*, 1978; Hirsch *et al.*, 1977, 1981; Anik and Hwang, 1983; Suelter and DeLuca, 1983; Wang and Chien, 1984; Chawla *et al.*, 1985; Dong *et al.*, 1987; Seres, 1990; Johnston, 1996). 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 can also stabilize them against surface denaturation. Generally, ionic surfactants can denature proteins. However, non-ionic surfactants usually do not denature proteins even at relatively high concentrations (1% w/v) (Cleland *et al.*, 1993). Most parenterally acceptable non-ionic surfactants come from either the polysorbate (sorbitol–polyethylene oxide polymers) or polyether (polyethylene oxide–polypropylene oxide block co-polymers) groups. Polysorbate 20 and 80 and sodium dodecyl sulphate are effective and acceptable surfactant stabilizers in marketed protein formulations (see Table 8.4). However, other surfactants used in protein formulations for clinical studies and/or found in the patent literature include Pluronic F-68 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 (Lougheed *et al.*, 1983; Sato *et al.*, 1984; Chawla *et al.*, 1985). However, the choice of surfactant and the final concentration optimal for stabilization is quite dependent on a variety of factors including other formulation ingredients, protein concentration, headspace in the container, the type of container, and test methodology.

Table 8.4 Examples of commercial protein solution formulations containing surface active agents

Generic name	Brand name	Manufacturer	Surface active agent in formulation
Aldesleukin	Proleukin	Chiron	Sodium dodecyl sulphate, 0.018% (following reconstitution)
Filgrastim	Neupogen	Amgen	Polysorbate 80, 0.004%
Interferon gamma 1B	Actimmune	Genentech	Polysorbate 20, 0.01%
Muromonab CD3	Orthoclone	Ortho Biotech	Polysorbate 80, 0.1%

Recombinant human growth hormone 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). 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 mechanical stress. Pluronic F-68 and Brij 35 will stabilize hGH at their critical micelle concentrations (CMCs) (0.1% and 0.013%, respectively), while stabilization with polysorbate 80 requires a concentration of 0.1%, higher than the CMC value for polysorbate 80 of 0.0013%. The reasons for these differences in stabilizing concentrations are not clear, but simply reflect differences in interactions between different surfactants and proteins. It is interesting to note that these surfactants do not stabilize hGH from 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). They 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-10, 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 be needed to protect proteins from denaturation during the freezing step only, and that other stabilizers, e.g. sucrose, may be needed to protect the protein further during freeze-drying.

Bovine somatotropin (bSt) presents an example where surfactants are not effective in preventing protein aggregation and precipitation in solution at elevated temperature,[2] whereas other stabilizers such as sucrose are effective. Figure 8.9 shows the effect of polysorbate 80 on bSt precipitation at 54°C, where bSt is more stable without the presence of polysorbate 80 and increasing the amount of polysorbate 80 increases the extent of bSt precipitation. Interestingly, for bSt, increasing concentrations of sucrose have a positive effect on stabilizing bSt against aggregation and precipitation.

[2] While polysorbate 80 was not effective in stabilizing bSt at elevated temperature, it was effective when the applied stress was agitation. Also, the authors noted that polysorbate 80 destabilization of bSt was not observed at ambient or refrigerated temperatures as other decomposition pathways, e.g., deamidation, became more predominant at lower temperatures.

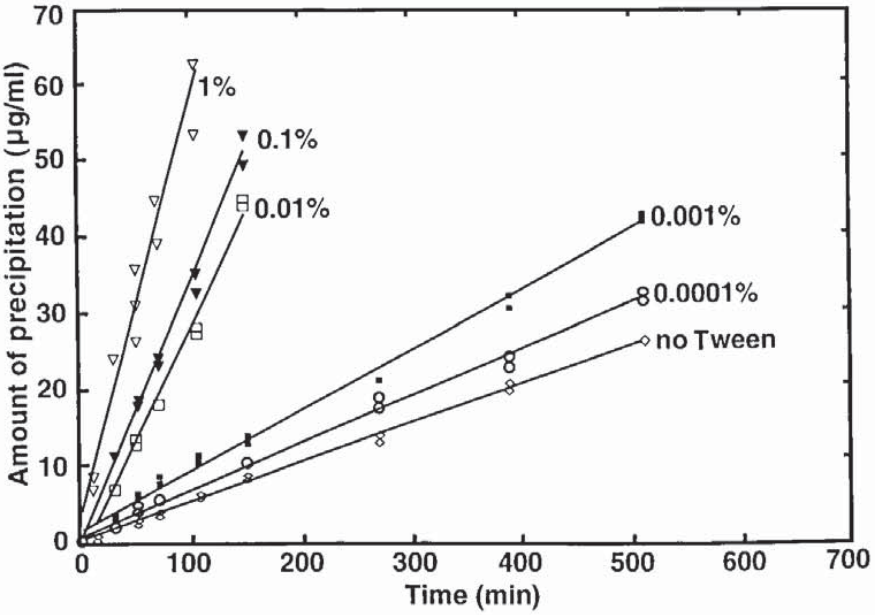

Figure 8.9 Effect of Tween 80 concentration on precipation of rbSt as a function of thermal stress at 54°C. Reprinted with permission from Hageman *et al.* (1993); ©Plenum Press.

Peroxides are known contaminants of non-ionic surfactants. Knepp *et al.* (1996) reported on the peroxide levels of polysorbate 80 obtained from different manufacturers using a colorimetric titration method. Levels ranged from less than 1 mEq/kg 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. 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 non-ionic surfactants to serve as oxidative catalysts for oxygen-sensitive proteins.

Studies on protein–surfactant interactions are beginning to be published (Bam *et al.*, 1995) where electron paramagnetic resonance (EPR) spectroscopy is used to determine the binding stoichiometry of the surfactant to the protein and, thus, what potentially is the optimal amount of surfactant to use to stabilize the protein against surface denaturation and other physical instability reactions.

8.4.6 *Cyclodextrins*

Cyclodextrins are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a relatively hydrophobic central cavity and hydrophilic outer surface (Loftsson and Brewster, 1996). Cyclodextrins come in a wide variety of structural derivatives, the most common being α-, β-, and γ-cyclodextrins, which consist of six, seven, and eight glucopyranose units, respectively. Two parenteral cyclodextrins are Encapsin™, a hydroxylpropyl-β-cyclodextrin, and Captisol™, a sulphobutyletherβ-cyclodextrin. They have been used widely for increasing the solubility stability and bioavailability of small

drug molecules (Thompson, 1997). Peptides and proteins can also be stabilized in cyclodextrin complexes. Irwin *et al.* (1994) used β-cyclodextrins at a 25-fold excess to stabilize leucine enkephalin against enzymatic degradation in sheep nasal mucosa. Hydroxypropyl-β-cyclodextrin at a 1% concentration was shown to enhance the reconstituted solution stability of keratinocyte growth factor (Zhang *et al.*, 1995). Brewster *et al.* (1991) presented several examples (ovine growth hormone, interleukin-2, bovine insulin) where 2-hydroxypropyl-β-cyclodextrin enhanced the solubility and physical stability of proteins in solution. Johnson *et al.* (1994) used chemically modified cyclodextrins to solubilize a tripeptide.

8.4.7 Albumin

Serum albumin is a widely used stabilizer in protein formulations for minimizing protein adsorption to glass and other surfaces (Wang and Hanson, 1988, Edwards and Huber, 1992). Albumin preferentially competes with other proteins for binding sites on surfaces, but why this is so is not clear. It is also used as a protectant in several lyophilized formulations and has stabilizing effects on other proteins (Wang and Hanson, 1988), yet the mechanism whereby it is an effective stabilizer is not understood. Examples of commercial protein formulations containing albumin are given in Table 8.5.

Because albumin is a natural protein, concerns have been raised about its potential contamination with human prion protein, which is thought to be the infectious agent in bovine spongiform encephalopathy (BSE) (Pharmaceutical Research and Manufacturers of America BSE Committee, 1998). 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 CNS disease, in humans in the UK. 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 not only albumin but also gelatin, glycerol and polysorbate 80. 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 or processing at high temperatures and pressure to achieve inactivation of TSE agents. Several regulatory

Table 8.5 Examples of commercial protein solution formulations containing albumin

Generic name	Brand name	Manufacturer	Human serum albumin in product
Alglucerase	Cerdase	Genzyme	1.0%
Erythropoietin	Epogen	Amgen	0.25%
Interferon Alpha-2a	Roferon-A	Roche	0.5%
Interferon Alpha-2b	Intron A	Schering	0.1% (following reconstitution)
Urokinase	Abbokinase	Abbott	5.0% (following reconstitution)

agencies such as the EU, FDA, and TGA (Australia) are critically reviewing the need to establish guidelines in this area (Federal Register, 1997).

8.4.8 *Other physical complexing/stabilizing agents*

Polyethylene glycol (PEG) is a common co-solvent for solubilizing small non-proteinaceous molecules, yet it also has been reported to minimize the aggregation of several peptides and proteins (Lee and Lee, 1987; Arakawa and Timasheff, 1985; Powell *et al.*, 1991; Bhat and Timasheff, 1992). PEG modification of proteins for sustained-release purposes is beyond the scope of this chapter (see Francis *et al.*, 1992). The concentration of PEG needs to be fairly low (<1% w/v) to serve as a stabilizer; at higher concentrations (>10% w/v) it can cause precipitation (Cleland and Randolph, 1992).

Poly(vinylpyrrolidone) (PVP) is like PEG in that at low concentrations it can stabilize proteins while at high concentrations it may help lead to protein aggregation and precipitation. Gombotz *et al.* (1994) reported that PVP at low concentrations (≤2.0%) effectively stabilizes human IgM monoclonal antibody against heat-induced aggregation, while PVP concentrations of ≥5.0% will cause aggregation.

Fibroblast growth factors, acidic and basic, are prone to acid and thermal inactivation and can be stabilized by a number of heparin and heparin-like molecules (Tsai *et al.*, 1993). Human keratinocyte growth factor, also prone to aggregation at high temperature, is stabilized by heparin, sulphated polysaccharides, anionic polymers, and citrate ion (Chen *et al.*, 1994).

8.5 Optimizing microbiological activity: antimicrobial preservatives (APs)

Preservatives are required for parenteral products intended for multiple-dose use. Many protein products, because of their expense and, more importantly, because of their ability to support the growth of microorganisms, are packaged as multiple-dose products, the AP being either formulated with the protein or, more commonly, formulated in a special diluent to be combined with the protein before use. The most common APs used in protein dosage forms are phenol, meta-cresol, benzyl alcohol, methylparaben and propylparaben. Examples of use of these preservatives are listed in Table 8.6.

Table 8.6 Antimicrobial preservative agents for protein products

Type	Concentration	Antimicrobial Activity*				pH	Other comment
		Gram+	Gram−	Fungi	Yeasts		
Benzyl alcohol	0.1–3.0%	+++	+++	−	−	3–6	Not effective pH>7
m-Cresol	0.1–0.3%	++	++	++	++	4–10	Most effective
Methylparaben	0.08–0.1%	++	++	++	++	3–9	Slowly soluble
Propylparaben	0.001–0.02%	+++	+++	+++	+++	3–9	Very slowly soluble
Phenol	0.2–0.5%	++	++	++	++	4–10	Most effective
Thimerosal	0.1–0.4%	++	++	++	++	4–8	Japan won't allow

* +++, most effective; ++, moderately effective; −, poor.

Table 8.7 Comparison of USP 24 and EP 2 requirements for preservative efficacy testing

Time after inoculation with microorganisms	Log reduction in microorganism count		
	USP 24	EP 'A' criteria	EP 'B' criteria
6 hours	Not required	3 (bacteria)	Not required
24 hours	Not required	No recovery (bac)	1 (bacteria)
2 days	Not required	No recovery (bac)	Not required
7 days	1 (bacteria)	No recovery (bac) 2 (fungi)	3 (bacteria)
14 days	3 (bacteria) No increase (fungi)	No recovery (bac) No increase (fungi)	1 (fungi)
21 days	No increase	No increase	Not required
28 days	No increase	No recovery (bac) No increase (fungi)	No increase (bac) No increase (fungi)

Antimicrobial agents must pass a preservative efficacy test (PET). Unfortunately, the USP and the British and/or European Pharmacopeial (BP/EP) tests for PET are different in their requirements. Table 8.7 summarizes the differences between the tests. The USP basically requires a bacteriostatic preservative system, while the BP/EP requires a bacteriocidal system. For example, the USP requires a 3 log reduction in the bacterial challenge by the 14th day after inoculation, while criteria A of the BP/EP test requires that same 3 log reduction within 24 hours. This great difference in compendial requirements for preservative efficacy has caused many problems in the formulation of protein dosage forms for various markets. Passing the BP/EP preservative 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. Increasing AP concentrations also increases the potential for toxicological hazards.

It is well known that APs not only protect insulin formulations against inadvertent contamination, but also may have a significant effect on protein stability. For example, phenolic preservatives have a profound effect on the conformation of insulin in solution (Wollmer *et al.*, 1987) and the assembly of the specific type of LysPro insulin hexamer (Birnbaum *et al.*, 1997). Furthermore, phenol and/or m-cresol in insulin solutions will have a tendency to be adsorbed by and permeate rubber closures (Brange, 1987). Therefore, rubber formulations must be designed to minimize these potential problems.

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). Phenol will produce a significant decrease in the α-helix content of insulinotropin resulting in aggregation of β-sheet structures (Kim *et al.*, 1994). Benzyl alcohol, above certain concentrations and depending on other formulation factors, will interact with recombinant human interferon-

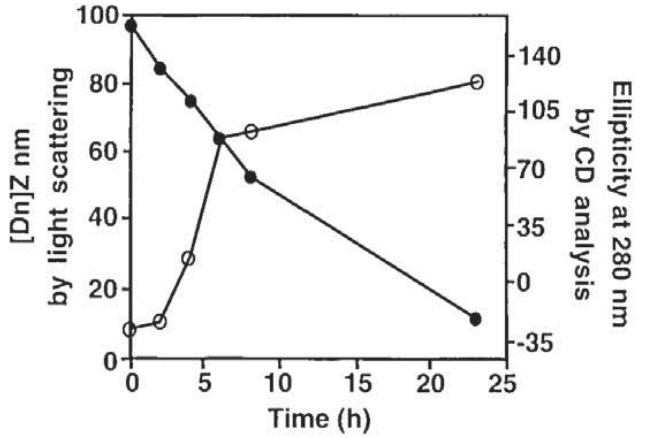

Figure 8.10 Time course of aggregate formation of 1.0 mg/ml rhIFN-γ in 5 mM succinate, pH 5.0 in the presence of 0.9% benzyl alcohol as determined by dynamic light scattering (○) and circular dichroism analysis (●). Reprinted with permission from Lam *et al.* (1997a); ©Plenum Press.

γ, causing aggregation of the protein (Figure 8.10) (Lam *et al.*, 1997a). 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 while balancing the needs for antimicrobial efficacy.

In determining the appropriate AP agent or agents, the model described by Akers *et al.* (1984) might be suitable. 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 preservative efficacy test organisms, and *D* values[3] were 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 8.8, with the AP systems listed in order of effectivess (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 include an AP agent in the protein formulation even though it is intended only for a single-dose injection. This is a very controversial practice. Regulatory agencies worldwide object to this approach if, in their opinion, APs are used in a single-dose injectable product in order to 'cover up' for inadequate aseptic manufacturing practices and controls.

Many countries require preservative effectiveness tests (PETs) be performed for routine stability protocols and for special stability studies. Also, there may be requests from agencies to do PETs 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 contamination and cross-contamination, the use of injectable products in multi-dose packages is discouraged. In order to support the use of a multi-dose

[3] *D* value = time required for a 1 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.

Table 8.8 *D* values against *Staphylococcus aureus* for different antimicrobial preservative systems in insulin solutions

Antimicrobial preservative system	*D* value (hours)
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

product and the shelf-life once a package has been reconstituted or opened for use, antimicrobial preservative efficacy data are required for approval.

8.6 Osmolality (tonicity) agents

Salts or non-electrolytes (e.g. glycerine) are added to protein formulations in order to achieve an isotonic solution. Non-electrolytes are often preferred to salts as tonicity adjusters because of the potential problems salts cause in precipitating proteins (Pikal, 1990). Generally, solutions containing proteins administered IV, IM, or SC do not have to be precisely isotonic because of immediate effects from dilution by the blood. 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 cells cannot be reproduced or replaced (Cradock *et al.*, 1977).

8.7 Packaging

Packaging issues are addressed in section 8.1 and in discussion of protein adsorption. Some additional information and guidance are summarized here. Solution dosage forms of peptides and proteins most commonly are packaged in glass vials sealed with rubber closures and aluminium seals. Other packaging systems used are cartridges, syringes, and plastic vials and bottles. In all these packaging systems, the formulator needs to be very concerned about potential reactivity of the peptide/protein and other ingredients in the formulation (e.g. antimicrobial preservatives) with the packaging components.

Selection of the packaging system not only depends on compatibility with the product formulation and the convenience to the consumer, but also depends on the integrity of the container–closure interface to assure maintenance of sterility throughout the shelf-life of the product. Container–closure integrity testing has received significant attention recently,

and usually is an integral part of the regulatory submission and subsequent regulatory GMP inspections. While it is beyond the scope of this chapter to discuss the various aspects of container–closure integrity, it is emphasized that formulation scientists developing the final product, including the final package, must appreciate the need to develop appropriate testing methods to ensure that the selected packaging system indeed has the proper seal integrity to protect the product during its shelf-life from any ingress of microbiological contamination. There are excellent review articles on this subject by Morton *et al.* (1989), Chrai *et al.* (1994), Guazzo (1994) and Kirsch *et al.* (1997a, 1997b, 1997c).

8.8 Processing

Likewise, the basic concerns to be aware of in the manufacturing of proteins and peptides have been addressed in section 8.1. Unit processes involved in the manufacturing of solution sterile dosage forms include compounding and mixing, filtration, filling, terminal sterilization (although almost always not possible for proteins and peptides), closing and sealing, sorting and inspection, labelling, and final packaging for distribution. Since this chapter deals only with solution dosage forms, one of the most complex of processes – lyophilization or freeze-drying – will not be covered.

The pharmaceutical scientist must be aware of the various issues involved in the manufacturing arena that can impact the stability and quality of the protein or peptide formulation. Among the more relevant areas of concern are shear rate and stress during compounding, filtration, and filling (Charm and Wong, 1976; Thurow and Geisen, 1984), adsorption onto process tubing and filter surfaces (Hawker and Hawker, 1975; Brophy and Lambert, 1994; Brose and Waibel, 1996), and the effects of time and temperature during each step of the manufacturing process (Hsu *et al.*, 1988). Formulation scientists and process engineers should work together to design and implement experiments to determine processing effects on protein stability and establish an appropriate control strategy. In most cases, e.g. protein adsorption onto filter surfaces, the potential problems can be avoided or minimized once understood through experimentation by alternative choices of filter material or predicting the amount of solution to be passed through the filter to saturate the binding sites.

The surge of potential heat-labile products from biotechnology and the inability to sterilize these molecules terminally has accelerated the development of barrier/isolator technology. This technology, when perfected, will enable the processing of protein and peptide solutions to occur under a much higher degree of sterility assurance than is now achievable with conventional aseptic processing. The main features of barrier/isolator technology are the ability to sterilize, not just sanitize, the environment under which sterile solution is exposed during filling and stoppering, and the removal of humans from direct contact with the exposed sterile solution.

8.9 Conclusion

The formulation of stable, manufacturable, elegant, and high-quality protein and peptide solution preparations offers significant challenges to the development scientist. However, these challenges can be overcome through sound, rational formulation approaches with manufacturing processes and packaging systems designed to maintain stability and other quality features of the formulation. This chapter has outlined current thinking on formulation science in developing commercially viable protein and peptide solution dosage forms.

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