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Parenteral Products of Peptides and Proteins

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I. INTRODUCTION

Scientific advances in molecular and cell biology have resulted in the development of two new biotechnologies. The first utilizes recombinant DNA to produce protein products. Given the amino acid sequence of a protein, the gene responsible for producing such protein can be isolated, modified, and recombined with a plasmid DNA (Fig. 1). The modified plasmid can then be implanted into a host cell. The recombinant DNA will be replicated and transcribed by the host cell to produce the specific protein in large quantities. *Escherichia coli* have been modified to produce growth hormone and growth factors, yeast to produce hepatitis B vaccine, and mammalian cells to produce erythropoietin. The advantages of such production are practically unlimited supplies of protein of high purity, and consistent quality in lot-to-lot production.

The second technology involves fusion of antibody-producing B-lymphocytes and myeloma (tumor) cells to form hybridomas (Fig. 2). Hybridomas grown in vivo in ascitic fluid or in vitro in tissue culture produce a single species of antibody, known as monoclonal antibody, which recognizes a specific antigen. In contrast, conventional production methods in laboratory animals produce polyclonal antibodies that are comprised of a wide variety of antibodies with different affinity and antigenic specificities. Monoclonal antibodies, because of their unique properties of homogeneity, specificity, and affinity, can be used to: (1) isolate and purify proteins, e.g., Monoclat Factor VIII:C is produced in this fashion; (2) neutralize a specific group of cells, e.g., Orthoclone OKT-3 neutralizes T-cells; (3) target specific cells or organs, e.g., antibodies to tumor antigens allow for tumor imaging or therapy by linking imaging radioactive isotopes or cytotoxic drugs to the antibodies.

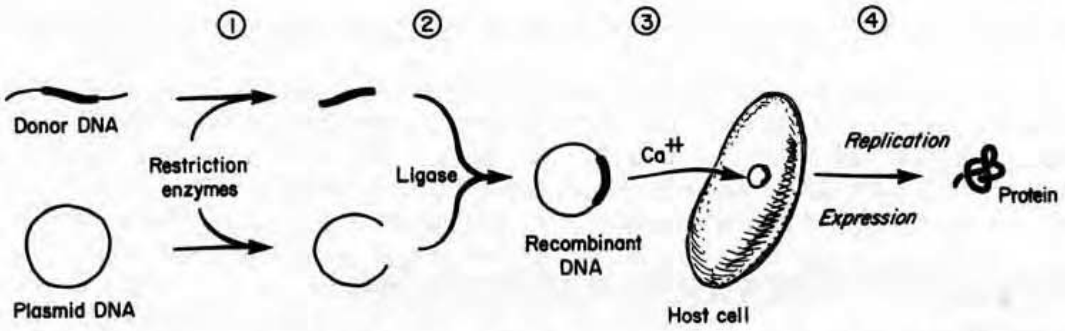

Figure 1 Recombinant DNA for protein production. The technique of recombining genes from one species with those of another. Plasmid is an extrachromosomal, independently replicating small circular DNA molecule. (1) Restriction enzymes cut DNA at specific places. The donor DNA, represented by a heavy band, contains the information to produce the protein of interest. (2) Ligase fuses donor DNA with plasmid together. (3) Calcium is used to open cell wall or cell membrane to allow recombinant DNA to enter the host cell (bacterium, yeast or mammalian cell). (4) Replication of the host cells and recombinant DNA increase its number. Expression of the DNA produces desired protein.

New products approved by the FDA between 1982 and 1989 derived from these two new biotechnologies are listed in Table 1.

Although these two new biotechnologies have greatly expanded the capability for producing large amounts of high quality proteins, not all new protein products are produced in this way. Small proteins and peptides, such as calcitonin, may be produced by chemical synthesis. Human serum albumin is sourced from human blood, urokinase from urine, and streptokinase from fungi. Most of these proteins and peptides are formulated as injectable products, although there are some exceptions. Cyclosporin is given *per os*. Oxytocin and desmopresin are available as nasal preparations. A topical ointment, Elase, contains two lytic enzymes, fibrinolysin and desoxyribonuclease, in lyophilized form. Wound-healing growth factors are being developed for use as topicals.

Among the parenteral formulations, most of them are in aqueous solution or in freeze-dried form for reconstitution. However, one of the potent luteinizing hormone release hormone (LHRH) analogues, leuprolide, and bovine somatotropin have been developed as erodable microspheres for depot injection.

II. CHARACTERISTICS OF PROTEINS AND PEPTIDES

A. Protein Structures

Proteins and peptides are made of amino acids linked by peptide bonds. The sequence of these amino acids defines the protein's structure. The sequence of amino acids in human insulin is illustrated in Figure 3. A protein may also show a secondary structure, which is formed by either intrachain or interchain hydrogen bonds. These bonds may result in either an α-helix or β-sheet

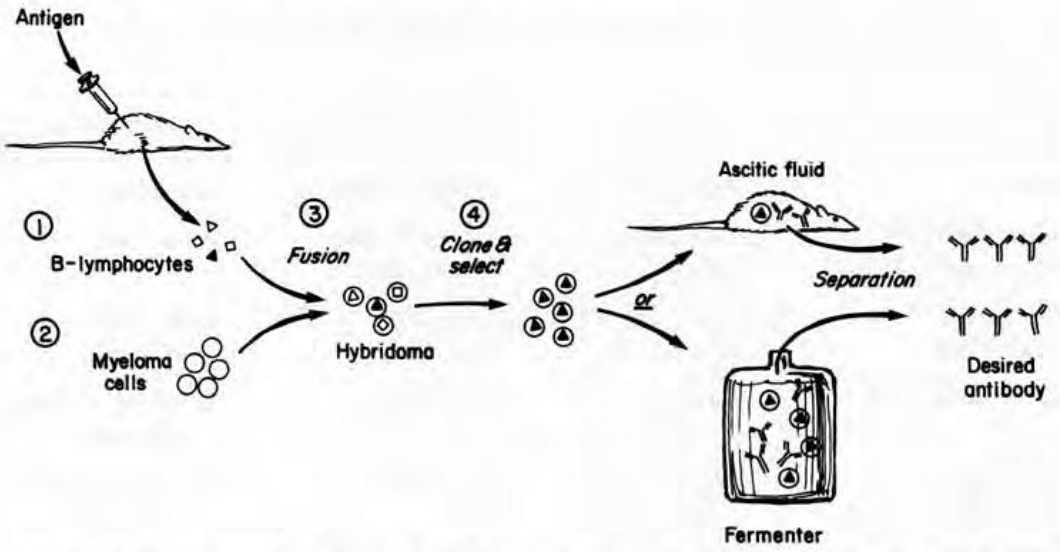

Figure 2 Monoclonal antibody production. (1) A mouse or rat is immunized with a specific antigen. The spleen is removed and antibody-producing cells (B-lymphocytes) are isolated. (2) Transformed myeloma cells from another animal are isolated. (3) B-lymphocytes are fused with myeloma cells deficient in a particular enzyme, to form hybridoma cells. (4) Only the fusion product, hybridoma cells, can grow indefinitely in culture. The unfused myeloma cells and lymphocytes die. The hybridoma cells are cloned and ones expressing the desired antibody are selected to culture in large amounts. The antibody can be produced by these hybridoma cells in either ascitic fluid or fermentation tanks. The ascitic fluid or culture media is then processed to yield purified monoclonal antibodies.

structure. Figure 4 shows a peptide chain of a protein coiled to form an α-helix.

The tertiary structure or conformation of a protein refers to the spatial, three-dimensional structure of the polypeptide chain. Given the primary and secondary structure, there are four kinds of interactions cooperating which may contribute to the tertiary structure of a protein. They are: (1) hydrogen bonds between residues in adjacent loops of the chain, (2) ionic interactions (salt bridges) between oppositely charged residues, (3) hydrophobic interactions between the aliphatic or aromatic residues, or (4) disulfide linkages (Fig. 5). Protein, in its native state, exists as a tight, compact folded structure. The folded structure of insulin is shown in Figure 6. When the tertiary or secondary structure is destroyed, the protein unfolds and is in its denatured state.

If dimers, trimers, or other oligomers are formed, the arrangement of these subunits is a quaternary structure. The best-known example of an oligomeric protein is hemoglobin that contains four chains and no disulfide linkages. The quaternary structure is essential to its oxygen-carrying activity. Insulin also forms dimers and, in the presence of zinc, hexamers. The role of the zinc insulin hexamers is to provide a storage form that is thermodynamically stable and more resistant to enzymatic degradation than the unassociated monomer.

Table 1 Biotech Products Approved in the United States Between 1982 and 1989

Drug	Company	Date approved	Indication
Insulin	Lilly	October 1982	Diabetes
Human growth hormone[a] (hGH)	Genentech Lilly	October 1985 March 1987	Dwarfism
Interferon A2[b] (IFN)	Schering Roche	June 1986 June 1986	Hairy cell leukemia
Muromonab-CD3	Ortho	June 1986	Reverse kidney rejection
Hepatitis B vaccine	Merck	July 1986	Vaccine against hepatitis B
Tissue plasminogen activator (t-PA)	Genentech	October 1987	Thrombolytic agent
Erythropoietin (EPO)	Amgen	June 1989	Anemia
Hepatitis B vaccine	SmithKline Biologics	August 1989	Vaccine against hepatitis B

[a]Growth hormone from Genentech differs from Lilly's product by a single methionine at the *N*-terminus.
[b]Interferons from Schering and Roche differ by one amino acid.

In some proteins, there are carbohydrates attached to specific amino acid residues to form glycoproteins. For example, erythropoietin is a glycoprotein hormone with a molecular weight of 44 kilodaltons, having 40% of its weight accounted for by sialic acid, an amino sugar. Carbohydrate residues stabilize the tertiary structure and may influence receptor binding, bioactivity, and pharmacokinetics of the protein in vivo.

B. Isoelectric Point

In the course of a potentiometric titration of a protein there is a pH at which the mean charge on the protein is zero. This pH is termed the isoelectric point, pI. Table 2 provides examples of pIs for a number of therapeutic proteins and peptides. The pI may be approximately calculated from the amino acid composition data, i.e., $pI = (pK_1 + pK_2 + pK_3 \ldots + pK_n)/n$ for n ionizable groups. However, because the dielectric constant in the immediate vicinity of an ionizable group depends on protein structure and, because hydrogen bonding may alter dissociation constants (K_as), the true pI can differ significantly from the calculated one.

Just like pK_as of small organic compounds, the pI of a protein plays an important role in solubility. In general, near the pH of the isoelectric point, protein solubility is at its minimum. This trend is similar to the low solubility exhibited by a zwitterion compound.

Figure 3 Amino acid sequence of human insulin. Insulin has two chains, the A chain with 21 and the B chain with 30 amino acids. The two chains are connected by two disulfide cross-linkages, and one of the chains has an internal disulfide linkage. Although insulins isolated from the pancreas of a cow or a pig are used in treatment of diabetic patients, they are not identical with human insulin. The highly conserved amino acid residues, i.e., invariant among species, are boxed. Human insulin can be produced by *E. coli* or yeast with recombinant DNA or from pork insulin by an enzymatic transpeptidation process which selectively substitutes a threonine for the alanine B_{30}, thus forming the human-insulin molecules.

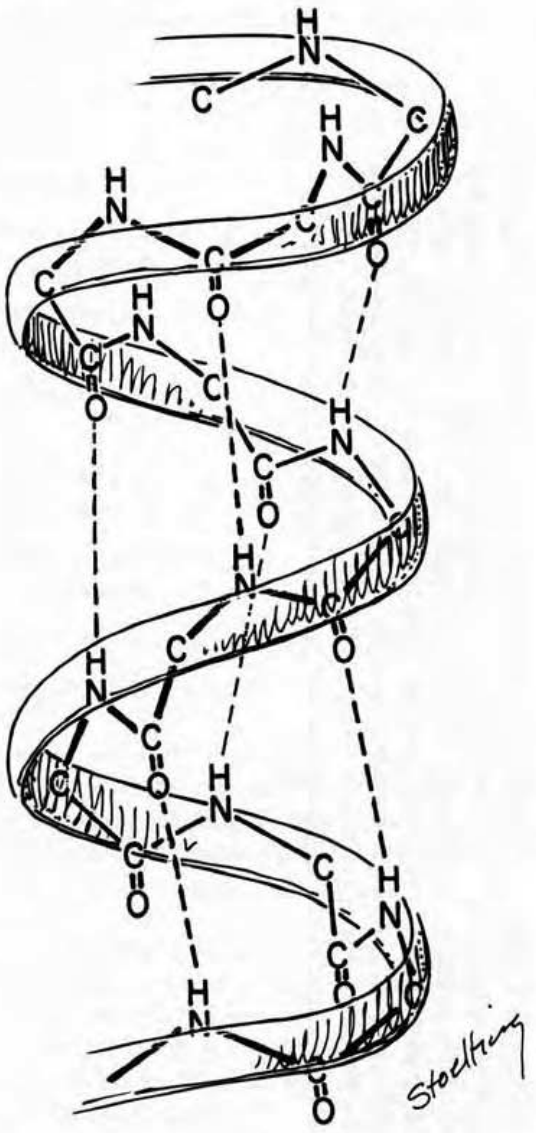

Figure 4 Peptide chain of a protein coiled to form an α-helix. Configuration of the helix is maintained by hydrogen bonds shown as vertical dotted lines.

C. Degradation Through Covalent Bonds

The degradation of proteins and peptides can be divided into two main categories: those that involve a covalent bond and those involving a conformational change. The latter process is often referred to as denaturation.

Numerous types of chemical reactions leading to modification of the covalent structure are possible in a protein. The following describes only those that are likely to be seen in a protein or peptide formulation.

Peptide Fragmentation

The peptide bond (RNH—CO—R) is much more resistant to hydrolysis than is the ester linkage (R—O—CO—R) and peptide bonds are considered stable un-

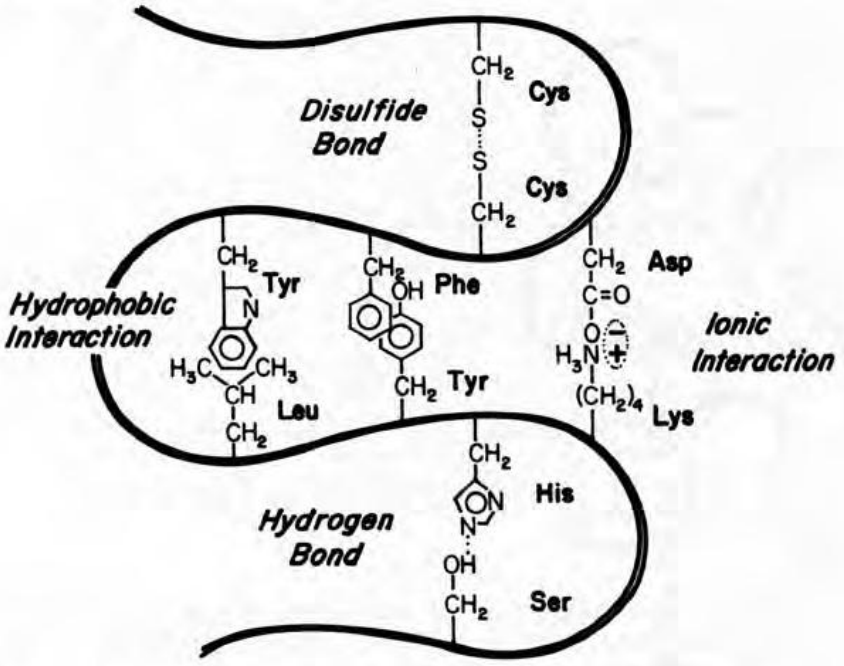

Figure 5 Interactions that stabilize folded conformation.

less hydrolysis is assisted by a neighboring group. The formulation factor that most influences the hydrolytic rate is solution pH. The rate of hydrolysis is in direct proportion to the activity of hydronium, or hydroxide ions, when in acidic or alkaline pHs, respectively. A rate-pH profile for hydrolysis of tetraglycine is shown in Figure 7. The reaction minimum is in the region of pH 3 to 5 [2]. Published examples of stability studies on therapeutic peptides are: degradation of nafarelin [3], thermal stability and degradation mechanism of secretin [4], acid hydrolysis of captopril, a quasidipeptide [5], and heat stability of urokinase [6].

Many peptides are stable enough to formulate as a ready-to-use parenteral formulation. For instance, D-Trp-LHRH was stable at 60°C for 5 days and seven cycles of freezing and thawing; gramicidin, although used only for dermatological indications when dissolved in glycols, can be autoclaved. Information available on package inserts also shows it is possible to have good long-term stability of peptides. Leuprolide injection has a shelf-life of at least 2 years under refrigeration and 3 months at room temperature. LH-RH injection (Relefact) does not even require storage under refrigeration. Oxytocin injection was reported to be stable at room temperature for 5 years [7]. Protirelin, a tripeptide (PyrGlu-His-Pro), is stable for 20 hr at 80°C at both pH 3.3 and pH 6 [8].

Certain amino acids form the weak link of the chain. The bond between aspartic acid and proline or tyrosine is sensitive to acid hydrolysis [9]. The resultant products are peptides with aspartic acid at the C-terminus. The C-terminal peptide bond adjacent to serine is also a reactive one due to the neighboring group effect of the alcohol on serine [10].

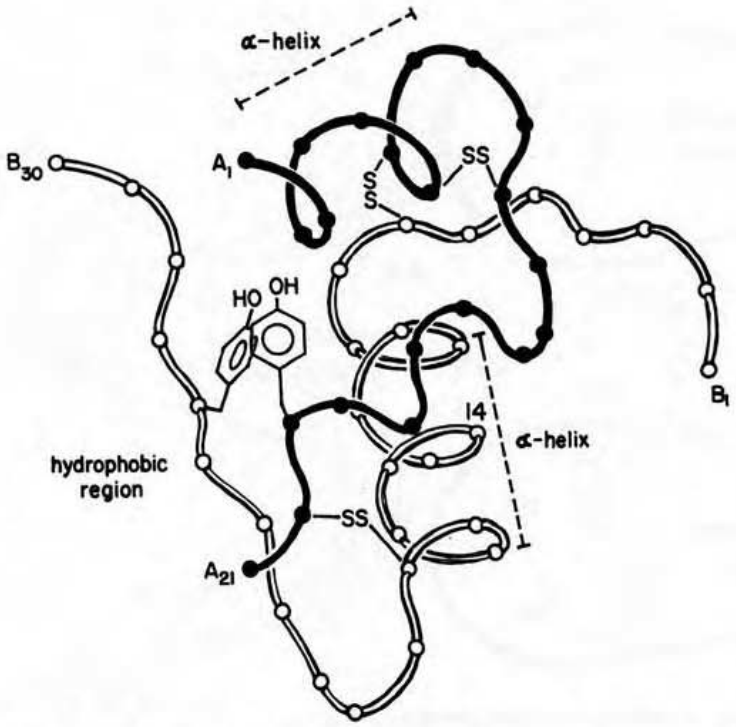

Figure 6 Folded three-dimensional structure of insulin. The A-chain is arranged between the two terminal arms of the B-chain and lies on top of the central α-helix (B_9-B_{19}). The hydrophobic regions are responsible for the association of monomer into dimers and hexamers. (Adapted from Ref. 1.)

Table 2 pIs of Selected Proteins

Proteins	pI
Pepsin	1.0
Erythropoietin	3.5-4.0
Serum albumin	4.8
Growth hormone	5.0
Insulin	5.4
Interleukin-2	7.8
Calcitonin (salmon)	7.8
Chymotrypsin	9.1
Basic fibroblast growth factor	9.8
Interferon-γ	10.0
Lysozyme	11.0
Nafarelin	11.3
Protamines	12.0

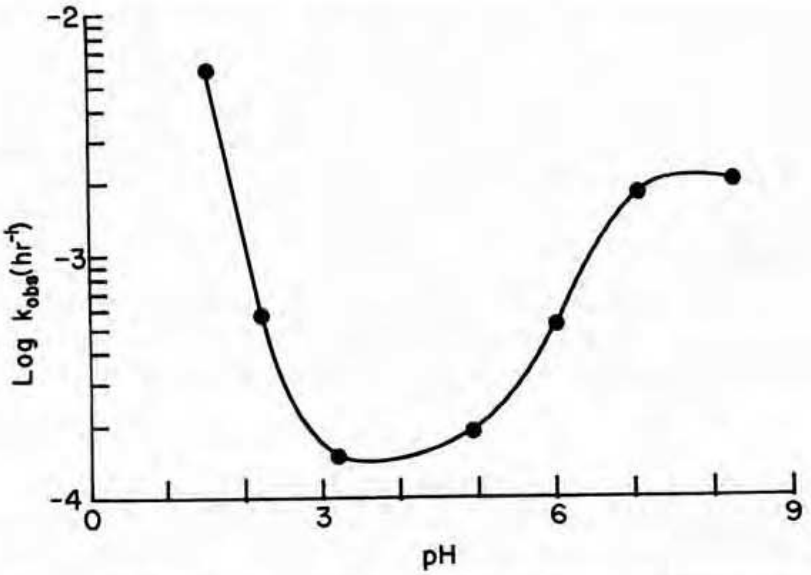

Figure 7 Rate-pH profile for the hydrolysis of tetraglycine. Peptide solutions containing phosphate buffer, 0.03 M, and sodium chloride, 0.1 M, were incubated at 60°C. Samples were analyzed by HPLC assay. At pHs 1 to 3, the slope is -1, indicating an acid catalyzed reaction. In the pH region 4 to 7, the reaction is hydroxide ion catalyzed. The plateau at pH 8 is caused by change in ionization state, zwitter ion to negatively charged ion, which is less susceptible to hydroxide-ion attack [2].

Deamidation

Deamidation refers to the removal of ammonia from the amide ($RCONH_2$) moiety, whether it is at the end of a C-terminus or at asparagine (Asn) or glutamine (Gln) residues. Stability studies of insulin [11], ACTH [12], human growth hormone [13], and RS-26306 [10], an LH-RH analogue, have shown this route to be the major factor resulting in instability of these proteins and peptides in aqueous solution.

In acidic media, the peptides deamidate by direct hydrolysis. Thus, an Asn or Gln residue yields an Asp or Glu peptide, respectively. The neighboring amino acid does not affect the deamidation rate. Hydrolytic mechanisms in neutral or alkaline pHs are more complex, however. Under these conditions, the side chain carbonyl group on the Asn or Gln residue attaches to the nitrogen atom on the peptide backbone to form a cyclic imide intermediate. Depending on which bond in the cyclic imide breaks (Fig. 8), the reaction product can be: (1) the des-amido peptide, (2) the isopeptide, or (3) D-isomers. The formation of isopeptide is sometimes referred to as transpeptidation because an extra methylene group is inserted to the peptide backbone. At neutral to alkaline pHs, the rate of deamidation is significantly affected by the size of the amino acid on the C-terminal side of the Asn or Gln residue. In general, Asn is more labile than Gln, and is most labile when adjacent to glycine, which is the least obstructive to the formation of a cyclic imide.

Deamidation rate-pH profile is V-shaped [14], usually with a minimal rate at a pH of about 4 to 5 (Fig. 9). In a number of synthetic peptides the half-

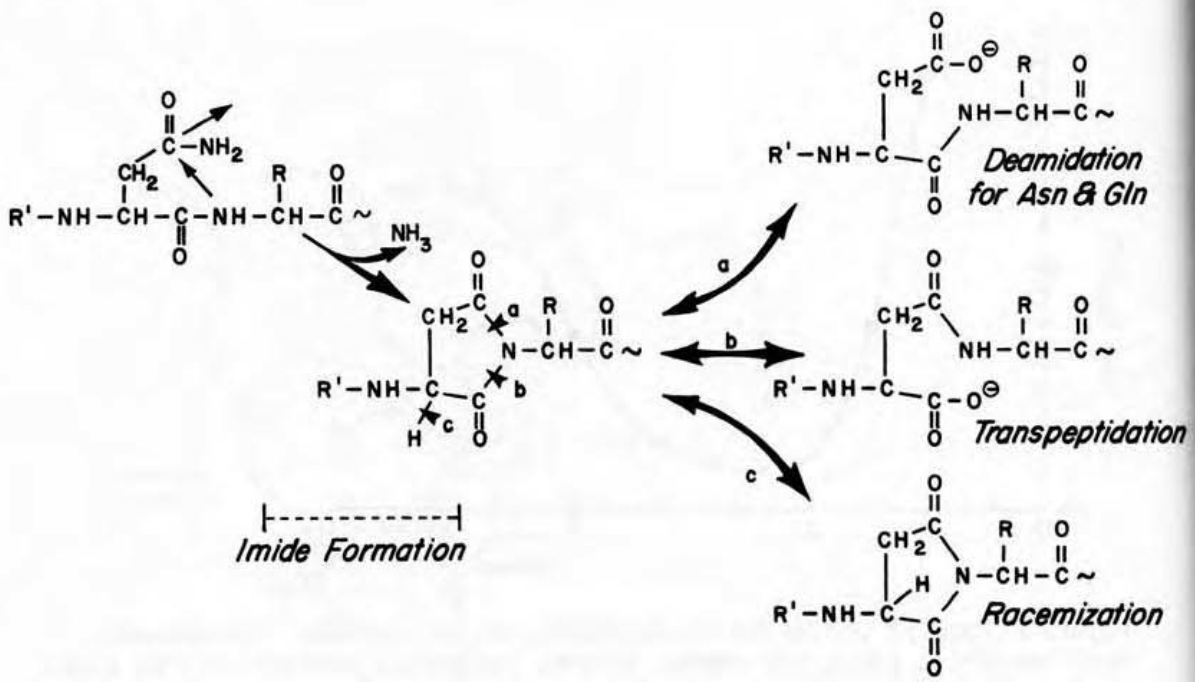

Figure 8 Reaction pathways in deamidation of asparagine. Deamidation of asparagine or glutamine goes through an intermediate, a cyclic imide. Three routes of degradation can take place, which leads to products of deamidation (removal of the amino group), transpeptidation (insertion of an extra methylene group), and racemization (D aspartic or glutamic acids).

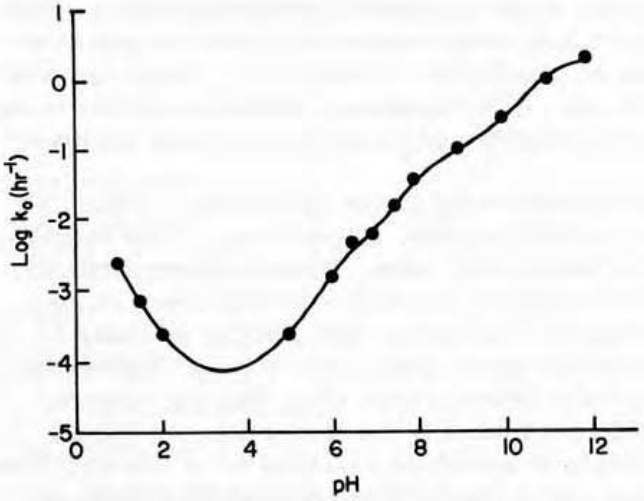

Figure 9 Deamidation rate-pH profile of an active segment of adrenocorticotropic hormone, Val-Tyr-Pro-Asn-Gly-Ala, in aqueous solution at 37°C and ionic strength of 0.5. The apparent rate is followed by the disappearance of the parent peak in HPLC. (From Ref. 14.)

lives of deamidation reactions of Asns at 37°C in pH 7.2 phosphate buffer range from 2 days to nine years [15]. Not all Asns are equally labile; those buried within the interior portion of a protein are inaccessible to water and thus less reactive. In a large protein, secondary and tertiary structures play an important role in determining the site and the rate of deamidation.

There are three asparagines in insulin (Fig. 3), the monodesamido-(A21)-insulin is the prevailing degradant formed in acid solution. In neutral solution, deamidation is slow at 5°C. However, when accelerated at higher storage temperatures, deamidation of Asn at the B3 position can be detected [11]. Figure 10 shows deamidation of insulin in neutral formulations at three different temperatures. With growth hormone, there are nine asparagines among 191 amino acids, and deamidation occurs primarily at the Asn-149 position [13].

Oxidation of Cysteine

Under neutral or basic conditions, the free thiol (—SH) group of a cysteine is the most reactive moiety of all amino acid components. The disulfide (—S—S—) bond formed from the oxidation of two thiol groups results in significant changes in conformation both intramolecularly and intermolecularly.

Oxidation of the thiol group is promoted at both neutral and basic pH. The rate-pH profile for captopril, a quasi dipeptide, showed an increase in oxidation rate, starting at pH 5 (Fig. 11). This reaction can be effectively retarded by the addition of a metal chelating agent such as EDTA [5].

A strategy to stabilize proteins that have reactive thiol groups is to replace the cysteine with serine. Serine, a stable amino acid with a hydroxy (—OH) group, mimicks the size and polarity of cysteine. In many cases, these serine mutants retain full biological activities.

Oxidation of Methionine

The methiol ($-S-CH_3$) moiety on methionine is susceptible to oxidation to form methylsulfoxide ($-SO-CH_3$) derivatives. Of the three methionines in human growth hormone, Met-125 is most reactive, Met-14 is less so and Met-170 is not reactive at all [16]. The reactive methionine is likely to be the one exposed on a protein surface, and the unreactive one buried within the core. Air in the headspace of formulated and freeze-dried growth hormone can cause 40% of the growth hormone molecules to be oxidized during a 6 month storage period [17].

Disulfide Exchange

Disulfide exchange takes place when a cystine (disulfide) bond is reduced to two cysteines; one of them then reacts with another cysteine to form a new disulfide (Fig. 12). The reaction is base (hydroxide ion) catalyzed and promoted by mercaptoethanol, which is sometimes used as an antioxidant. The reaction is concentration-dependent, and oligomers are frequently formed as a result of disulfide scrambling of bonds between peptide chains. Improper linkages of disulfide bonds were responsible for a reduction in biological activity of interleukin-2 (IL-2) (Fig. 12). There are three cysteines in IL-2 at positions 58, 105, and 125. The native protein forms a disulfide linkage between the two cysteines at 58 and 105. Cleavage of this disulfide in IL-2 and the subsequent formation of two less-active isomers with disulfide bonds

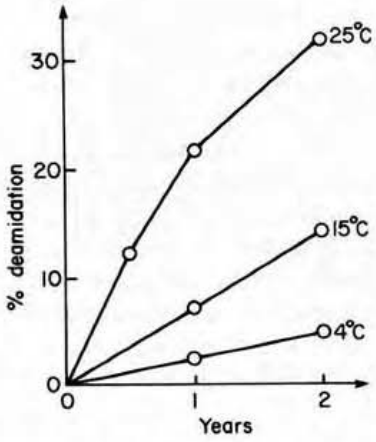

Figure 10 Deamidation of insulin during storage of Actrapid MC (BP-formulation) at different temperatures. Each point represents the mean of analyses of 4-6 different batches. The desamido insulin content was determined by basic disc electrophoresis followed by densitometric scanning of the stained gels. (From Ref. 11.)

at incorrect positions are promoted by high pH and copper ions [18]. The number of disulfide bonds in therapeutic proteins can be shown in the following examples: IL-2:1; salmon calcitonin:1; interferon-α:2; human growth hormone:2; insulin:3; urokinase:12; t-plasminogen activator:17; albumin:17.

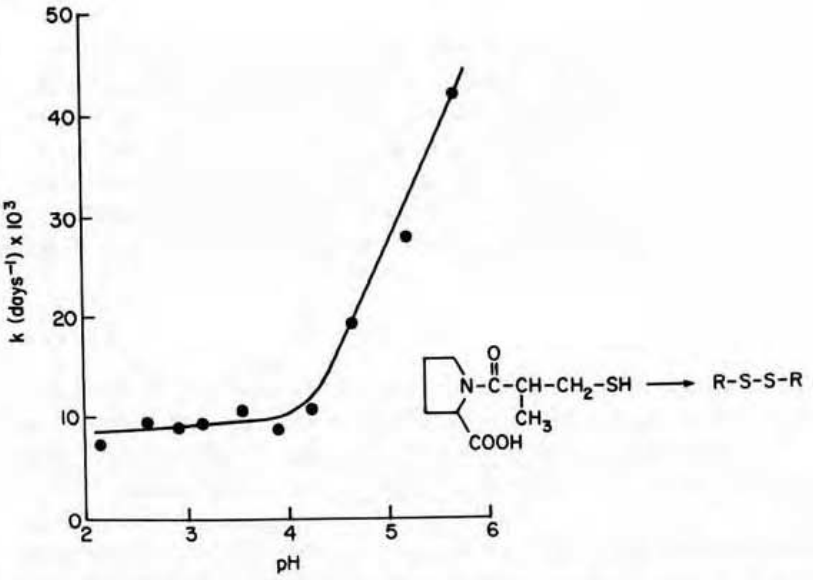

Figure 11 Oxidation of the thiol moiety in captopril. Reaction rates at 50°C is followed by a peak on the HPLC chromatogram. All solutions contain disodium edetate. Without the chelating agent, reaction rates are much faster. (From Ref. 5.)

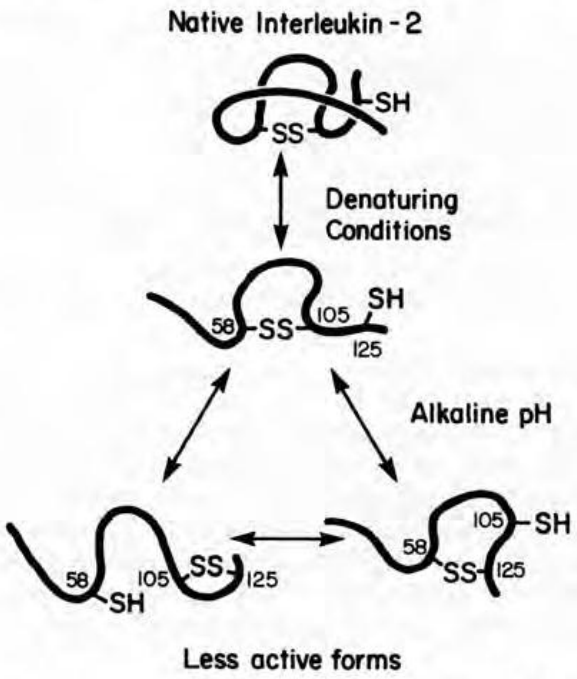

Figure 12 Schematic representation of the various disulfide-linked isomers of interleukin-2 [18]. Under denaturing conditions, i.e., high temperature or alkaline pH, a free sulfhydryl group forms a disulfide bond with the sulfhydryl group from an existing disulfide. The results are proteins with incorrect disulfide linkages and "non-native" conformation.

With N-disulfide bonds, there exist $(2N)!/2^N \times N!$ possible isomers. Caution must be exercised to preserve the correct disulfide linkage throughout the production process for therapeutic proteins.

Racemization

The racemization reaction is catalyzed by both acid and base. Racemization of peptides and proteins results in the formation of diastereomers. As an example, racemization of the serine residue in a gonadotropin-releasing hormone analogue was one of the main degradation reactions when treated with 0.1 N NaOH at 5°C for 48 hr [19]. Racemization under basic conditions is thought to proceed by abstraction of the α-proton from an amino acid in a peptide to give a negatively charged planar carbanion. A proton can then be returned to this optically inactive intermediate, thus producing a mixture of D- and L-enantiomers for the individual amino acid (Fig. 13). Since a peptide is composed of multiple chiral centers, the product formed is a diastereomer. Racemization is biologically significant since a peptide comprised of D-amino acids is generally metabolized much slower than a naturally occurring peptide made of only L-amino acids. For this reason, many new synthetic peptides, agonists or antagonists, incorporate D-amino acids. A pH dependency on racemization was demonstrated in an aqueous degradation study of a decapeptide, RS-26306 [10]. At neutral and alkaline pHs, racemization contributed to more degradation than deamidation (Fig. 14).

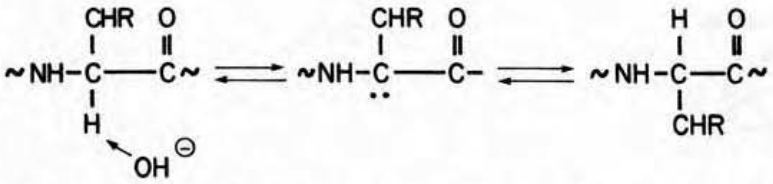

Figure 13 Racemization of an amino acid resulting in a mixture of enantiomers.

Maillard Reaction

In the Maillard reaction, the carbonyl group (RCH=0) from glucose can react with the free amino group (e.g., $R'NH_2$ in lysine) in a peptide to form a Schiff base (RCH=NR'). This reaction is acid catalyzed. During pasteurization (60°C, 10 hr), the loss of antithrombin III biological activity seen in the presence of reducing sugars, especially glucose, was attributed to this reaction [20]. An alternative assessment of poor blood-glucose control in diabetic patients is the measurement of glycosylated hemoglobin, which is the product of the Maillard reaction. The hospital pharmacist should thus be concerned about storing protein in dextrose solution for a prolonged period of time.

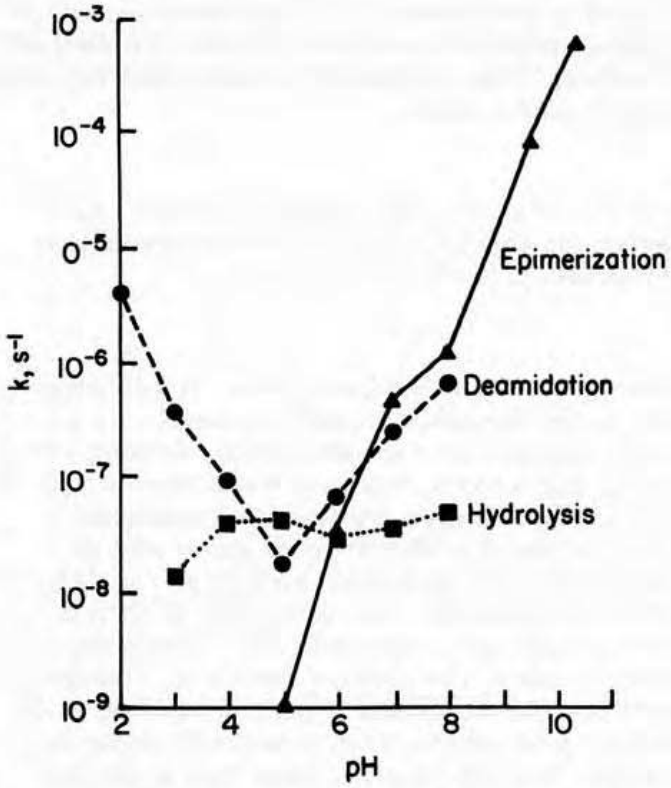

Figure 14 Rate-pH profiles for degradation of RS-26306, *N*-acetyl-3-(2-naphthyl)-D-Ala-4-Chloro-D-Phe-3-(3-pyridinyl)-D-Ala-Ser-Tyr-*N*,*N*-diethyl-D-Arg-Leu-*N*,*N*-diethyl-Arg-Pro-D-Alanylamide, a decapeptide [10].

Dimerization and Polymerization

Insulin forms a small amount (about 1%) of covalent dimer and polymer during 2 years storage at 4°C. Production of these species increases dramatically with increasing temperature. The dimerization is mainly due to a reaction between an *N*-terminal amino group in one insulin with a carboxamide group of a glutamine or an asparagine in another insulin molecule [11].

The covalent bond reactions aforementioned are examples of key reactions that may cause protein or peptide instability during storage. A complex protein may undergo many reactions simultaneously.

D. Denaturation

Specific conformation is required for proteins to exert physiological and pharmacological activities. Denaturation is a process of altering protein conformation. The denaturation process is illustrated in Figure 15 [21]. The folded structure (N) unwinds, passes through a critical activated state (A*), to an incompletely disordered conformation (ID). Finally, some of the ID forms uncoil to become random boils (RC). Heat, extreme pHs, organic solvents, high salt concentration, lyophilization, or mechanical stress can denature proteins.

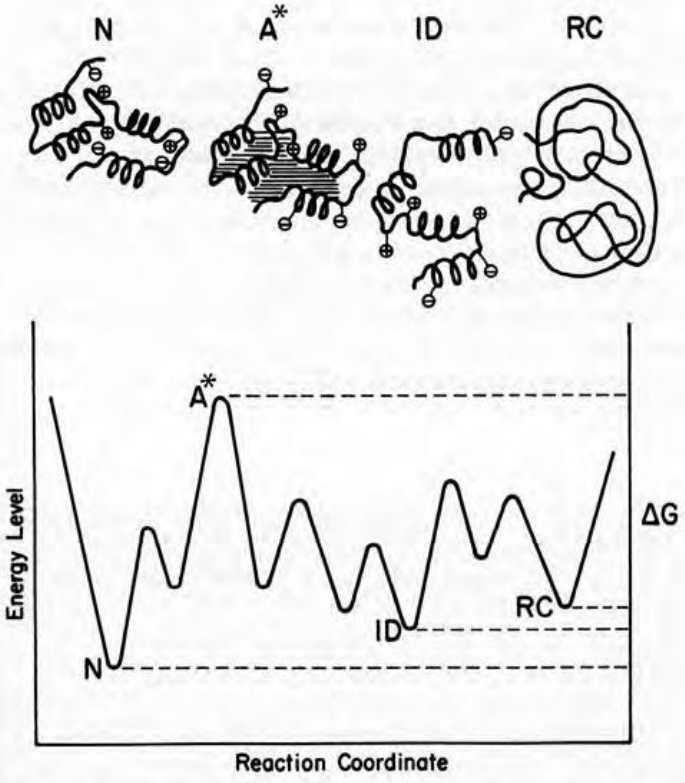

Figure 15 Schematic illustration of reversible conformational transitions of α-lactalbumin [21]. N, Native conformation; A*, critical activated state; ID, incompletely disordered conformation; and RC, random coil (fully denatured state).

Protein conformation refers to the specific tertiary structure, which is determined by the primary and secondary structures and the disulfide bonds, and is held together by three forces: hydrogen bonding, salt bridges, and hydrophobic interactions (Fig. 5).

Hydrogen bonds are the most important in determining overall protein conformation, since they are the major forces that stabilize the secondary α-helices and β-sheets, as well as the overall folded structure. Water, the ubiquitous medium for most proteins, contributes to this hydrogen bonding. Thus, to avoid denaturation in protein during a freeze-drying process, a small amount of residual moisture is critical for recovering biological activity of the reconstituted protein. Cosolvents such as ethanol and acetone, and chaotropic agents such as urea and guanidinium chloride disrupt the hydrogen bonds thus readily denature proteins.

The ionic side-chains of aspartic acid, glutamic acid, lysine, arginine, and histidine, normally found on the surface of the protein, contribute to the stability of the native conformation by forming salt bridges. The pH of the solvent will determine the charge of the side-chains on these amino acids and the extent of ionic bonding. Thus, an extreme pH shift can disrupt these salt bridges and lead to denaturation. Further, organic solvents weaken the streangth of salt bridges, thus, inappropriate exposure to organic solvent can also result in denaturation.

Because hydrophobic side-chains, i.e., phenyl and hydrocarbon chains, are usually tucked inside the protein's globular structure, significant stabilizing effects result from their hydrophobic interactions. This interaction, too, is sensitive to the effects of solvents. Disruption of hydrophobic interactions is often considered the mechanism of denaturation by surfactants, heat, mechanical stress, or storage. When a protein molecule unfolds under thermal stress, and before it refolds to its natural conformation, hydrophobic interaction can result in one of two different types of unnatural conformations. In a concentrated protein solution, the hydrophobic groups may interact between molecules, resulting in protein aggregation. In dilute solutions of protein, however, intramolecular interactions are much more likely, and may result in a conformation different from the native one. Such unnatural conformations may show partial or complete loss of biological activity.

E. Analytical Methods

For protein products a battery of tests are required to assure identity, purity, potency, and stability of the formulation. Due to complexity of proteins, as of yet no chemical or physical assay can substitute for a bioassay for assessing the potency. There are in vitro and in vivo bioassays. The in vitro bioassay monitors the response of cells or excised tissue to the stimulation of hormones, growth factors, or antibodies. For example, the activity of IL-2 is determined by measuring [^{3}H]-thymidine incorporated into an IL-2 dependent murine T-cell line. The in vivo bioassay monitors the pharmacological responses of animals to the proteins. For example, the activities of insulin and glucagon are determined by measuring post-injection blood sugar in rabbits and cats, respectively. Often, in vitro and in vivo bioassays are not sensitive enough to detect subtle changes in protein characteristics. Thus, consistency in product performance relies heavily on validated manufacturing

processes as well as end product testing. When the manufacturing process or formulation is significantly changed, the only assured way to prove bioequivalency is to conduct clinical studies in patients.

The multiplicity of degradation routes for proteins requires combined information from various assays to assess the stability of the product. The following sections describe common analytical methods employed in protein formulations.

UV Spectrometry

Any protein with at least one tryptophan residue can be detected, at about the 0.1 mg/ml level, by ultraviolet (UV) spectroscopy at 270 to 280 nm. Phenylalanine and tyrosine contribute about one-sixth the molar absorptivity to that of tryptophan. One may use UV for batching assay because of its precision, reproducibility, and simplicity. The absorptivity can be determined from literature values or derived from the absorbance and mass obtained from amino-acid composition. For example, the generally accepted value for the absorbance at 275 nm of a 1 mg/ml solution of immune globulin is 1.5. The UV method can be a rapid assay for in-process quality control. Also, because protein aggregates scatter UV light with increased absorbance over the range 210 to 350 nm, UV spectrometry can be used to monitor protein aggregation.

Proteins Assay

Bradford, Lowry and bicinchoninic acid (BCA) assays are three commonly employed colorimetric assays for determination of protein content.

The Bradford assay is based on a change in the absorption maximum, from 465 to 595 nm, of an aromatic sulfonate dye, Coomassie Brilliant Blue G-250, in the presence of protein.

Biuret, with a structure of $NH_2CONHCONH_2$, and peptides, having a similar structure, reduce copper to cuprous ion in alkaline solution, and concomittently a color complex is developed. The Lowry assay utilizes Folin-Ciocalteau reagent (phosphomolybdotungstic acid) to enhance the response of this color complex. The BCA assay utilizes bicinchoninic acid (2,2,bi-4-quinolinecarboxylic acid) to form a stable, purple complex.

Thermal Analysis

Differential scanning calorimetry (DSC) is gaining widespread use as a tool for investigating transitions of conformation as a function of temperature and, more importantly, the effect of potential stabilizing excipients in a protein solution. When protein is heated, it behaves like the melting of a crystal. The apex of the endothermic peak is the transition temperature between native and partially unfolded conformations. Figure 16 illustrates the thermograms and shows the increase in transition temperature for lysozyme with stabilizers of increasing chain length [22]. In the absence of carbohydrates, lysozyme denatures at about 78°C. In the presence of 1 M sucrose, the onset of denaturation was raised to 83°C. By repeat scanning, one can determine whether or not the thermal denaturation is a reversible process. A stabilizer found to be effective in preventing thermal denaturation may be effective in prolonging shelf life.

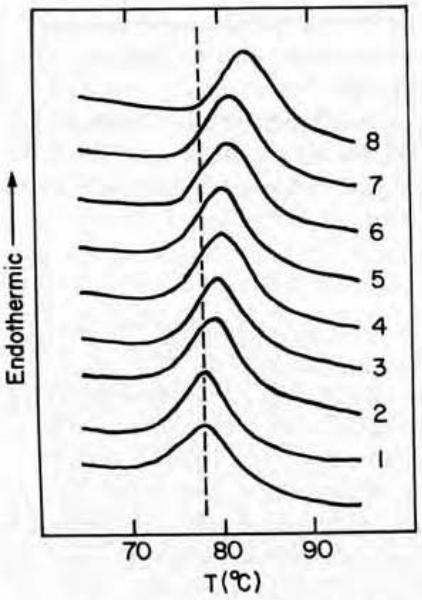

Figure 16 DSC curves for the thermal denaturation of lysozyme (7%) in 1 M sugar solutions at pH 5 and ionic strength of 0.1. The lowest curve is for a solution without a sugar. Curves 1, with deoxyribose; 2, ribose; 3, lyxose; 4, arabinose; 5, xylose, 6 fructose; 7, glucose; and 8, sucrose. (From Ref. 22.)

Because serum albumin or other blood-derived proteins need to be pasteurized, stability against heat denaturation is essential for these proteins. For years, sodium caprylate and acetyltryptophanate have been used as stabilizers in the preparation of human serum albumin. In the presence of these stabilizers, the transition temperature of human serum albumin is increased from 67 to 73°C [23].

Electrophoresis

Proteins, having multiple ionizing moieties, will migrate as a function of both charge and size in an immobilized liquid medium under the influence of an applied electric field.

When a pH gradient is set up across a liquid medium, the isoelectric focusing (IEF) will determine the pI of a protein, regardless of the size of the molecule. It is quite sensitive for detection of a degradant resulting from deamidation because of the addition of a negative charge from the carboxylate group.

The most-often-used electrophoretic technique for protein products is SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis). Proteins are first denatured by boiling in a surfactant (SDS) solution. All inherent charges of the protein are masked by the negative charge of dodecylsulfate; thus, the protein moves on the polyacrylamide gel based strictly on the size of protein molecule. This technique is ideal for assessing the molecular weight of the protein in question, particularly in detecting dimerization and oligomerization when these species are formed by covalent bonds.

Other variations of SDS-PAGE have been utilized for special cases. The use of a native gel for electrophoresis refers to electrophoresis on PAGE without using the denaturing surfactant, SDS. Under these conditions, the net charge of protein as well as its size will influence its mobility in the gel. Also, protein aggregates held together through hydrophobic interactions will move as multimers. Thus, this technique can be used to verify aggregation of this kind. Another variation, the reduced gel, refers to the use of dithiothreitol or mercaptoethanol, both strong reducing agents, which sever disulfide linkages. Disulfide linkages make proteins compact and make them appear as molecules of smaller molecular weight on a gel. Reducing agents will break disulfides and correct this artifact. Another use of reducing agents in SDS-PAGE is to dissociate disulfide-linked dimers. The nonreducible dimers (amide bond developed intermolecularly) will not be affected by the addition of the reducing agent, whereas, the reducible dimers resulting from a disulfide bond developed intermolecularly will exhibit a molecular weight identical to the monomer.

For visualization of proteins on a gel, two staining reagents, silver nitrate and Coomassie Blue R-250, are frequently used. In general, the former is more sensitive for low quantity of protein, however, the latter develops a stable, quantifiable stain.

Liquid Chromatography

The most versatile tool that gives reasonably precise results regarding the stability of proteins and peptides is high performance liquid chromatography (HPLC). Different kinds of packing material in the column are used for the detection of various changes in the protein molecule (Table 3).

If a precise method can be developed and various degradants can be separated, liquid chromatography is the most suitable method for determination of product shelf-life. Chromatographic assays for biological products have begun to appear in pharmacopeias. For instance, a reversed phase liquid chromatography (RPLC) is being used for the assay of insulin and quantitation of desamido insulin and other insulin-related substances.

Table 3 Various HPLC Columns for Use in Stability Tests

Test method	Mobile phase	Changes can be detected
Size exclusion column	Buffer solution	Fragmentation, aggregation, oligomerization
Ion exchange column	Buffer solution	Deamidation
Reversed phase column	Acetonitrile (or methanol): trifluoroacetic: acid: water	Oxidation at methionine or cysteine, disulfide exchange, deamidation, cross-linking, racemization
Affinity column, such as heparin, or metal ion column	Salt solution	Fragmentation, oligomerization, conformational change at binding site

III. FORMULATION PRINCIPLES

A. pH

Solution pH often plays an important role in the stability of a protein or peptide product. A proper selection of pH is key to having a stable product. For simple peptides, consideration should be given to identifying a pH at which the sum of the various degradation reactions is at a minimum. Many peptides are formulated at a slightly acidic pH: the pH of oxytocin injection is in the range of 2.5 to 4.5; that of salmon calcitonin is 3.9 to 4.5; that of desmopressin injection is 3.5; and nafarelin is most stable at about pH 5 [3]. At a slightly acidic pH (pH 3-5), the major concerns are deamidation of asparagine, glutamine, and C-terminal amide, and general hydrolysis of the peptide backbone and the glycosides. At neutral pH (pH 6-7), one must be concerned about oxidation of cysteine and methionine in addition to deamidation and peptide hydrolysis. At pH above 8, the following reactions occur readily: disulfide exchange of cystine, transpeptidation of aspartic acid and glutamic acid, deamidation of asparagine and glutamine, and racemization of amino acids with an electron-withdrawing sidechain. For more complex proteins, especially where aggregation is a major concern, the product pH is often set away from the isoelectric point. Presumably, at the isoelectric point, minimal water solubility promotes the formation of aggregates (see Sec. III.D). A subtle change in pH can result in a significant change in aggregation. An example is the observation of increased aggregation of γ-interferon (pI = 10) when the pH is raised from 5 to 6 [24]. The pH of marketed immunoglobulin products is close to neutrality, for example, 7.4 for Orthoclone OKT-3 and 6.6 for Sandoglobin. However, a new immunoglobin for intravenous use (Gamimune N) was formulated at pH 4.25 for reasons of stability. A patent claims immunoglobin to be more stable and with less anticomplement activity at pH 4.25 [25].

Protein solubility is at its minimum when pH is at its isoelectric point (pI). Insulin is a clear example that the pH of products is dictated by the solubility. Although insulin (pI = 5.4) is more stable to deamidation at pH 5 than at pH 3 or pH 7.5, pHs of insulin injection are either 2.5 to 3.5 or 7.0 to 7.8 for solubility reasons. The insulin identification test in the USP is showing precipitation when pH is adjusted to 5.1 to 5.3.

B. Effects of Salts

The effects of organic or inorganic salts can be divided into two areas. At low concentrations of divalent ions, such as calcium, magnesium, and zinc, the thermal stability of a protein may be enhanced. However, this effect is very protein-specific. At high concentrations, 1 M or higher, salts may salt-in, increase protein solubility, or salt-out, decrease protein solubility. Salting-out can stabilize proteins. When hydrophobic residues from the protein surface are "salted-out," they are compressed into the interior space of the protein molecule, thus the protein becomes more resistant to thermal unfolding and demonstrates an increased thermal stability. The stabilizing effect of cations and anions decreases in the following order:

$$(CH_3)_4N^+ > NH_4^+ > K^+, Na^+ > Mg^{+2} > Ca^{+2} > Ba^{+2}$$

and

$$SO_4^{-2} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

Therefore, $(NH_4)_2SO_4$ is a strong stabilizer. For this reason, a saturated solution of ammoniuum sulfate is often used in protein purification processes.

C. Common Stabilizers

A search of the literature for methods to prepare formulations of therapeutically useful proteins and peptides and, specifically, how proteins and peptides can be stabilized, makes it apparent that this is an area in which trial and error plays a major role. Various types of molecules, including sugars, amino acids, surfactants, and fatty acids, used singly or in combination, have served to stabilize protein and peptide products against degradation. A detailed reivew of stability and stabilizers for proteins and peptides can be found in the literature [26,27].

Serum Albumin

Serum albumin, regardless of its origin (human, porcine, or bovine) has been extensively cited in patents and the literature as a stabilizer for enzymes and other proteinaceous material. One of many reasons for the choice of albumin over other proteins is its stability. Albumin, even without stabilizers, can withstand heating to 60°C for 10 hr. At pH 1 to 2, the albumin molecule expands and elongates; however, it can return to its native configuration reversibly [28]. Furthermore, albumin has good solubility; unlike many other proteins, it is soluble in concentrated salt solution; unlike globulin, it is soluble at its isoelectric point; overall, it is soluble in a wide range of pH.

The mechanisms by which albumin acts as a stabilizer can be any of the following: (1) inhibition of surface adsorption; (2) substitution for a nascent complex protein, i.e., the inert protein that forms a complex with hormones in the cellular matrix; (3) dispersion of a small protein in the interstitial space of thermally resistant albumin; and (4) cryoprotection.

It is difficult to delineate the true mechanism of stabilization by albumin in any particular circumstance. The concentration of serum albumin required for stabilization usually depends on the amount of protein or peptide, and the literature shows a wide range of use. Albumin at 0.003% was used for protective action with the enzyme alcohol dehydrogenase [29], whereas, concentrations cited in many patents are higher and range from 0.1 to 1%. In the recently approved recombinant interferon injection, Roferon-A, 0.5% human serum albumin is employed as a stabilizer. Another interferon, Intron-A, contains 0.1% in its lyophilized cake. About 1 to 2% is present in Monoclat (antihemophilic factor). MICRhoGAM, an anti-Rh product, contains up to 15% serum albumin. However, one disadvantage to using albumin as a stabilizer is that it will interfere with many protein assay methodologies.

Human serum albumin is a monomeric polypeptide chain of over 580 amino acids, with a molecular weight of about 66 kilodaltons. The peptide chain is comprised of a series of double loops (double loop resembles the shape of an 8 having the backbone going through the center) formed by disulfide bonds

between its 34 half-cystine residues. If this chain were in a fully extended α- or β-conformation, it would be almost 90 or 200 nm long, respectively. However, physicochemical measurement indicates that albumin is a tightly folded globular protein of 13 × 3 nm in size. Formulators unfamiliar with the biochemical properties of albumin are directed to a review chapter by Peters [28].

Amino Acids

Amino acids have been used for a variety of reasons, and found to:

1. Reduce surface adsorption. Acidic amino acids inhibit the adsorption of protein to silicone-coated glass surfaces [30].
2. Inhibit aggregate formation. Glutamic acid or aspartic acid when used at pH 3.4, stabilize insulin aggregation in a similar fashion to EDTA [31,32].
3. Stabilize protein against heat denaturation. Many therapeutic blood products are heat-treated to inactivate possible viral (hepatitis, AIDS) contamination. Glycine and alanine were found to be useful in the processing of Factor XIII [33].

Glycine is the most commonly used amino acid. It has been employed in a wide range of concentrations: in Atgam (antithymocyte globulin), 0.3 M (2.25%); in Intron A (α-interferon), 2%; in Gamimune (immune globulin), 0.015 M (0.11%); in RhoGAM (anti-Rh), 0.015%. Sodium glutamate is used in the freeze-dried product of streptokinase and lysine in Eminase.

Numerous patents and literature citations report the unique stabilizing effects of some amino acids. Exmaples include the use of lysine for plasmin solution [34] and a mixture of amino acids for Factor VIII [35]. Arginine is claimed to increase the solubility of tissue plasminogen activator, the first human recombinant thrombolytic agent, by 300 fold and also to stabilize the drug against hydrolytic cleavage [36].

Surfactants

Surfactants frequently cause denaturation of protein, both by hydrophobic disruption and by salt bridge separation. Relatively low concentrations of surfactant exert a potent denaturing activity, because of the strong interactions between surfactant moieties and the reactive sites on proteins. However, judicious use of this interaction can stabilize proteins against other denaturants. For instance, if reactive groups, such as sulfhydryl groups, can be reversibly prevented from interacting, a protein may be more stable to the manipulations required during the purification process.

Because of their mixture of polar and nonpolar side chains, proteins, like surfactants, have a tendency to concentrate at liquid/liquid or air/liquid interfaces. A monolayer of protein may form spontaneously at the surface of a solution. The close proximity among these protein molecules encourages hydrophobic interaction and, possibly, disulfide exchange. Thus proteins unfold and adopt a non-native conformation that may have lower water-solubility than the native form. Consequently, proteins precipitate out of the solution. This phenomenon could be the reason for the turbidity observed in some protein

solutions when shaken vigorously. The addition of surfactant could either reduce the interfacial tension, thus reducing the propensity for protein unfolding, or solubilize the protein to reduce the amount of protein at the interface. Therefore, to prevent or reduce any denaturation at a liquid/air or liquid/solid interface of the product in solution, it may be advantageous to add a surfactant. For example, poloxamer 188 (Pluronic 68) or polysorbate, is added to the injectable gamma globulin formulation [37]. Additional examples are Orthoclone OKT 3, a therapeutic monoclonal antibody that contains 0.02% polysorbate 80, and patents each describing IL-2 [38], insulin [39], and tissue necrosis factor [40] stabilized by nonionic surfactants.

The optimal concentration of a surfactant for stabilization purposes should be greater than its critical micelle concentration. Figure 17 shows the surface tension and monoclonal-antibody stability when stressed by shaking as a function of polysorbate concentration. The stability of the monoclonal antibody increases dramatically at a concentration where surface tension begins to plateau [41]. This concentration generally coincides with the critical micelle concentration.

Ionic surfactants are more effective stabilizers than nonionic ones by virtue of their electrostatic and hydrophobic binding. However, nonionics are less structure-perturbing because of their low monomer concentration (critical micelle concentrations for nonionics are lower) and absence of electrostatic binding.

Polyhydric Alcohols and Carbohydrates

These two classes of compounds share the same feature in their backbones, i.e., —CHOH—CHOH—, which is responsible for stabilizing proteins. The polyhydric alcohols include such compounds as sorbitol, mannitol, glycerol, and polyethylene glycols (PEGs). They are straight-chain molecules. The carbohydrates, such as sucrose, glucose and lactose, on the other hand, are cyclic molecules that may contain a keto or aldehyde group. These two classes of compounds have been demonstrated to be effective in stabilizing protein against denaturation caused by elevated temperature and by freeze-thaw or freeze-drying processes.

Many important therapeutic proteins are derived from human blood. In the United States, the only practical, clinically acceptable method of destroying viral contamination is pasteurization at 60°C for 10 hr. Increasing the thermal stability of the blood-derived therapeutic proteins such as coagulation factors, fibronectin, and immune globulin is very important to developing a successful manufacturing process. The use of 54% sucrose stabilized fibronectin [42] and 10% maltose stabilized immune globulin [43], during pasteurization. Figure 18 shows a direct correlation between chain length of polyhydric alcohols and stabilizing factors for invertase at 60°C. As demonstrated, polyols of 4 or 5 carbons are effective stabilizers, whereas, those of 2 or 3 carbons are not [44]. When ribonuclease, lysozyme, etc., are subjected to elevated temperatures in a differential scanning calorimeter, the denaturation temperature of these proteins increased in the presence of carbohydrates and polyhydric alcohols (Fig. 16). The mechanism for stabilizing native conformation against thermal denaturation by these compounds is through the effect on the structure of surrounding water molecules, which, in turn, strengthens hydrophobic interactions in the protein molecule. Hydrophobic inter-

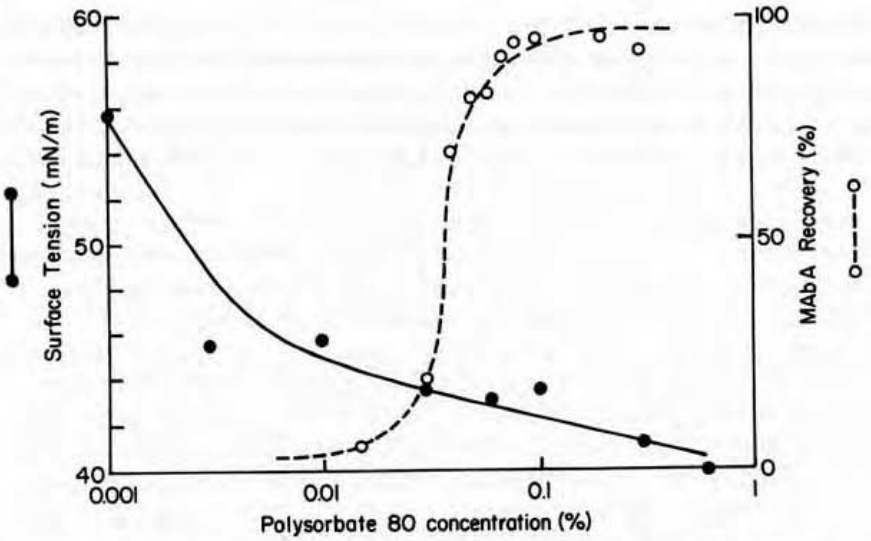

Figure 17 Stabilization of monoclonal antibody (MAb) by polysorbate 80. MAb was subjected to a simulated shipping test that involved shaking a half-filled vial horizontally at 150 rpm on an orbital shaker for 12 to 18 hr at room temperature. The recovery in % of initial MAb as measured by absorbance at 280 nm post-filtration is plotted as a dotted line. The surface tension, measured by Wilhelmy plate tensiometer, is plotted as a solid line. (From Ref. 41.)

actions (Fig. 5) are generally considered to be the major force stabilizing the three-dimensional structure of proteins.

Freeze-thawing and freeze-drying also create stress on protein structure that leads to denaturation. In a very general sense, freeze-drying is thought to be more disruptive to protein function than freeze-thawing or thermally induced perturbation. For example, asparaginase, which is fully active after freeze-thawing, was inactivated by more than 80% following freeze-drying [45].

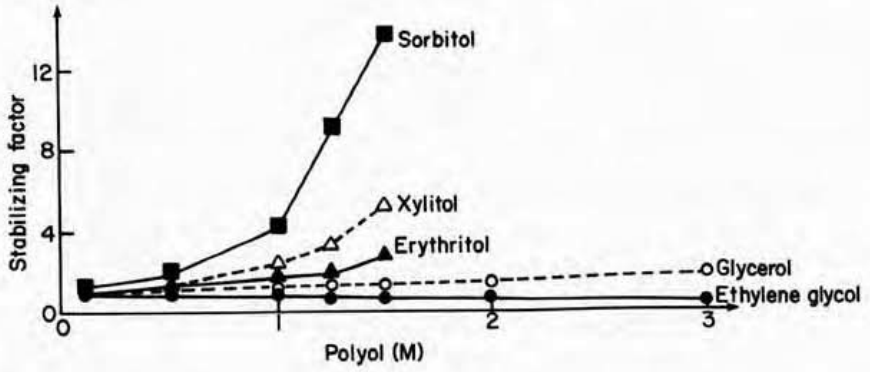

Figure 18 Influence of polyhydric alcohol chain length and concentration on stabilizing factor of invertase. Stabilizing factor is the ratio of invertase half-life at 40°C in the presence of polyol relative to that in its absence. (From Ref. 44.)

Despite the stress to which proteins are subjected during dehydration, a high degree of protection may be provided by polyhydric alcohols and carbohydrates.

Glucose, mannose, sucrose, or ribose protect asparaginase through freeze-drying [46]. Trehalose, maltose, glycerol, and inositol stabilize a very dehydration-sensitive enzyme, phosphofructokinase [47]. Haemoglobin freeze-dried with sucrose forms a dry network that provides a significant support to the native structure [48]. This freeze-dried cake can withstand a temperature of 55°C for several hours. A general guide in choosing an effective concentration of these stabilizers is that, for the freeze-drying process, stabilizers are about 0.1 M; whereas, for thermal denaturation, they are about 1 or 2 M.

Antioxidants and Metal Chelants

Many thiol compounds are capable of inhibiting disulfide bond formation, i.e., two cysteines oxidize to one cystine. Reduced glutathione, thioethanolamine, thioacetic acid, etc., produce some stabilizing for fibroblast interferon [49]. Two chymopapain parenteral formulations employ antioxidants: bisulfite is used in Discase and L-cysteinate HCl in Chymodiactin.

Besides antioxidants, a very effective method to control oxidation of cysteine is through the use of a metal chelant. EDTA was most effective in preventing the oxidation of the thiol group in captopril [5].

Miscellaneous

Proteins having specific binding sites for a substrate can be stabilized by compounds resembling their substrate. Examples are: glucose, which stabilizes glucoamylase; asparagine or aspartate, which stabilize asparaginase. Compounds, which form a stable complex with protein through ionic or hydrophobic interactions, can also stabilize the protein. Examples are: heparin sulfate or dextran sulfate, which stabilize basic fibroblast growth factor; fatty acid or tryptophan, which stabilize serum albumin.

Among divalent cations, calcium is essential to the thermal stability of certain amylases and proteases. The bridging function of calcium within the polypeptide chain reduces the flexibility of the polypeptide backbone, thus enhancing stability. Zinc was found to stabilize phosphofructokinase synergistically with glucose and maltose (Fig. 19).

In general, for nonprotein drugs, formulators tend to keep the number of excipients in an injectable formulation to a minimum. Different approaches have been taken to formulate stable protein drugs. Table 4 lists several patents that utilize multiple ingredients in parenteral products. The rationale for these kinds of formulations is to create an environment that may resemble that in a cell. The essential features of the environment in a cell are: (1) a concentrated soup of protein, at least 100 mg/ml, and other carbohydrates, salts, etc.; (2) low oxygen tension, with a variety of reducing compounds such as glutathione, used to maintain a high reducing potential; (3) immobilized water.

As the mechanisms of denaturation and of stabilization become better understood, and methodologies for studying these phenomena improve, formulators will be capable of limiting selectively the number of excipients needed in a formulation.

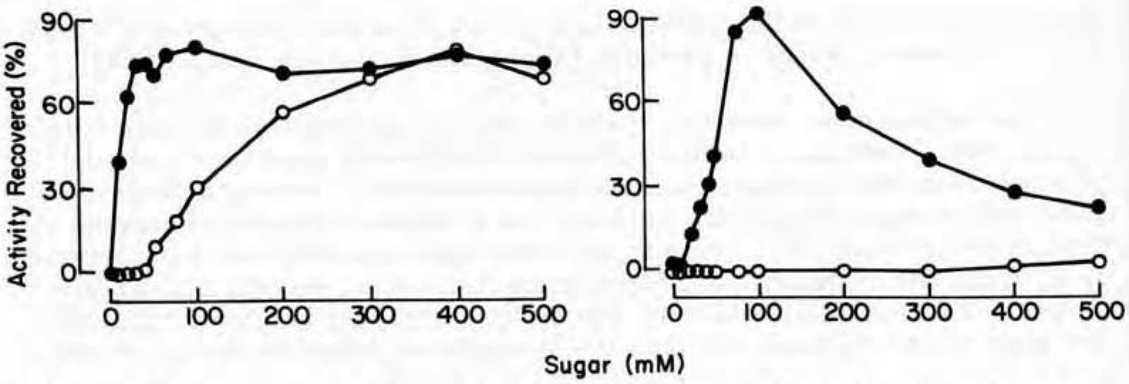

Figure 19 Recovery of phosphofructokinase activity after freeze-drying. On the left panel, maltose, a disaccharide, afforded cryoprotection to the protein (open circle). The protection is enhanced by the presence of zinc (closed circle). On the right panel, glucose, a monosaccharide, alone afforded no protection (open circle). When combined with 0.9 mM zinc, the protein was greately stabilized (closed circle). (From Ref. 50.)

D. Aggregation

Aggregation, unique to protein solutions, is a phenomenon in which the protein molecules form aggregates ranging from 0.1 to 100 μm or greater and precipitate out of solution. In package inserts for Elspar, Atgam, and Orthoclone OKT 3, the aggregates are described as "gelatinous fiberlike particles," "granular or flaky deposites," and "fine translucent particles," respectively. Other words such as lumps, frost, fibril, particle shedding, etc., all describe aggregation. In some cases, aggregation is merely a nuisance because the potency of the product is only slightly affected; nonetheless, the aggregates

Table 4 Patents[a] Employing Multiple Stabilizers

Protein	List of stabilizers
γ-Globulin	Polyvinyl pyrrolidone, glycol, sugar, polyvinyl alcohol
Immunoglobulin	Glycine, NaCl, NaOAc, PEG, albumin, mannitol
Erythropoietin	Glycine, albumin, mannitol, dextran, phosphate salts, glutathion, albumin, glucose
Antibody to human chorionic gonadotropin	Glycine 1%, BSA 1%, mannitol 10%, sucrose 5%
γ-Globulin	Albumin, glucose, glycine, PEG 4000, NaCl
Plasminogen-activating enzyme	PEG-3-sorbitan oleate, hydrolyzed gelatin, dextrin, mannitol, glycine

[a]Origins of these patents can be found in Ref. 26.

interfere with inspection for particulate matter and give the product an undesirable appearance. In these cases, the manufacturer may simply recommend that the injectable product be filtered prior to its use. In other cases, aggregation can elicit serious problems, such as reduction of biological potency or the blockage of tubing, membranes, or pumps in an infusion set. Aggregates of immune serum globulin have been alleged to cause the anticomplement reaction, which gives rise to symptoms resembling an anaphylactic reaction.

Aggregation is prominent when caused either by heat, shaking, freezing, or by freeze-drying. The mechanisms of heat-induced aggregation are oxidation of sulfhydryl groups to form intermolecular disulfide bridges or unfolding of tertiary structures to form a completely random coil. Shaking-induced aggregation is a constant challenge to a formulator, because shaking is unavoidable during manufacturing and shipping. Many mechanisms can contribute to the occurrence of shaking-induced aggregation, some of these in the following order of likelihood are:

1. Conformational changes as proteins molecules are adsorbed to the air-water interface. The denatured protein molecules then aggregate at the interface.
2. When proteins are selectively adsorbed to an interface, a high concentration of protein at the interface results in polymerization typically seen in a solution with high protein concentration. Hydrophobic bonding and crosslinking by salt bridges complete the process of irreversible denaturation.
3. Disulfide bonds are exchanged intermolecularly.

In quite a different manner, mechanisms of aggreation caused by freezing can be as follows:

1. Oxidative or hydrolytic reaction rates are enhacned when concentrations of proteins, oxygen, and salt increase due to ice formation. After the reaction, proteins denature, and aggregate. Upon thawing or reconstitution after freeze-drying, insoluble material is present in the formulation.
2. Partial or complete precipitation of buffer salts results in a pH shift by 2 or 3 units. Solubility of the protein can be reduced by several hundred fold.
3. Denaturation due to adsorption at the ice/solution interface can occur.

Aggregation can be analyzed quantitatively by size exclusion HPLC (Table 3). Using this method, aggregates will appear on the chromatogram as a species of a higher molecular weight or they may not pass through a HPLC column at all. Protein assays, by UV, before and after filtering the sample through a 0.1 μm filter can also determine the amount of protein aggregated. In cases with α-antitrypsin [51], α-interferon and interleukin-2 [52], proteins stressed at elevated temperature showed a first-order decline in monomer concentration (Fig. 20). Insulin aggregation did not follow first-order kinetics when stressed by agitation. The aggregation was effectively prevented by the addition of a mild surfactant, sodium taurodihydrofusidate (Fig. 21).

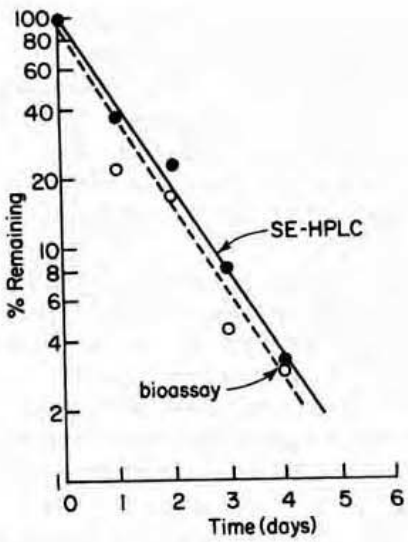

Figure 20 Loss of monomeric IL-2 (ala 125) concentration follows first-order kinetics. Percent eremaining over time at 80°C was determined by size exclusion HPLC (closed circle) and bioassay (open circle). (From Ref. 52.)

IV. COMPATIBILITY WITH PACKAGING COMPONENTS AND INFUSION SETS

Because of rapid enzymatic degradation by nonspecific proteases in blood, most proteins are not administered by bolus intravenous injection. When an infusion set is indicated, one must be extremely cautious about the adsorption of protein to the containers, in-line filters, tubing, and catheters. Examples of IL-2 and tissue necrosis factor lost to the giving sets are in Table 5. In general, protein concentration of less than 10 μg/ml will exhibit detectable loss when passing through the giving set.

Of the factors affecting adsorption to glass surfaces, solution pH is the most critical. Protein adsorption is maximal at the pH of its isoelectric point [30]. Contrary to general belief, siliconization of the glass surface does not necessarily reduce adsorption to glass surfaces. In siliconized plastic containers, proteins exhibit rapid loss due to surface adsorption. The kinetics of protein adsorption to plastic containers show maximal loss attained in a matter of hours. This is a different pattern than that exhibited by small molecules, such as preservatives, which gradually lose potency over an extended period of time. During that time, small molecules penetrate the gellike matrix of the plastic container. Proteins are thought incapable of penetrating the plastic matrix, thus only surface adsorption is operative.

As proteins are being produced in a purer form than was available in previous decades, there will be less adjunct material present, thus more significant adsorption loss is expected. A simple solution to this problem is to flush a sufficient amount of drug solution through the apparatus so that all binding sites are nearly saturated, although this method can be wasteful and inadequate for drugs that are used at very low concentrations. The most effective means of reducing adsorption is the addition of human serum albumin, typical-

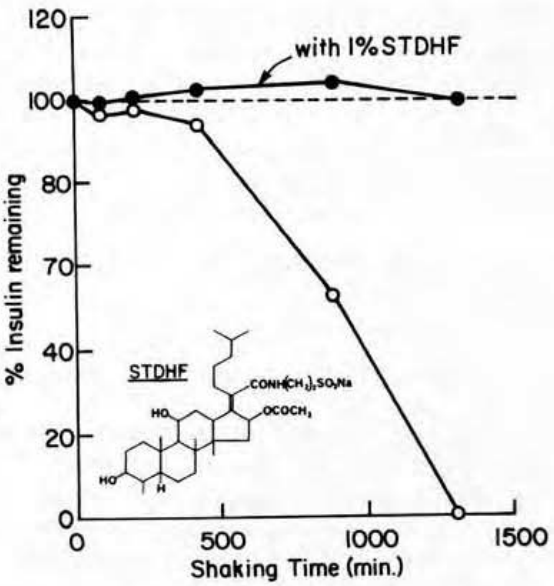

Figure 21 Stabilization against denaturation by shear. Insulin (1%) solutions, 200 µl in 300 µl glass vials were vortexed at 25°C with (closed circle) and without (open circle) sodium taurodihydrofusidate. Samples were analyzed by size exclusion HPLC after filtration through a 0.22 µ filter. (From Ref. 53.)

Table 5 Recovery of Tissue Necrosis Factor (TNF) and Interleukin-2 (IL-2) After Passing Through a Giving Set

Recovery of recombinant TNF when using a buret mixing set to deliver the drug	
Diluent	*Measured TNF activity*
5% Dextrose	340 ± 140 LU/ml
5% Dextrose with albumin 0.25%	5300 ± 1400 LU/ml
Expected activity:	5400 LU/ml
(5000 LU = 0.25 µg)	
Recovery of recombinant IL-2 when using an i.v. administration set to deliver the drug	
i.v. administration set	*Measured IL-2 activity*
Without in-line filter	26,000 ± 2000 IL/ml
With in-line filter	<400 IU/ml
Expected activity	30,000 IU/ml
(30,000 IU = 1.7 µg)	

Source: Ref. 54.

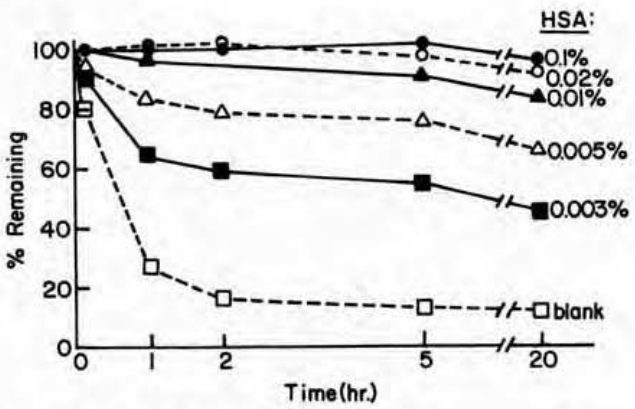

Figure 22 Adsorption of erythropoietin prevented by addition of human serum albumin (HSA). In polyethylene test tube, 210 µl of ^{14}C labeled erythropoietin at final concentration of 0.5 µg/ml with various concentrations of albumin (HSA) was allowed to stand at room temperature and sampled at various intervals. Residual protein concentration was determined by measuring radioactivity. (From Ref. 55.)

ly at 10 times the amount of the protein to be protected. Erythropoietin adsorption to polyethylene test tubes and the effect of adding human serum albumin is illustrated in Figure 22. Materials other than albumin have also been reported to be effective. Lecithin, PEG 6000, dextran and methylcellulose reduced loss of erythropoietin due to adsorption to polyethylene surfaces [55].

V. FORMULATION OF MARKET PRODUCTS

Quantitative formulas of parenteral products marketed in the United States can be readily acquired through package inserts, the *Physicians' Desk Reference*, or by requesting information from the FDA through the Freedom of Information Act. This accessibility reveals the exact formula employed in parenteral products of proteins and peptides.

Protein products are typically in a freeze-dried form using a suitable bulking agent (Table 6), whereas, peptide products are a ready-to-use injectable solution (Table 7). In these solutions, peptides are sufficiently stable for at least 2 years at refrigeration temperature. Preservatives such as phenol, benzyl alcohol, and chlorobutanol are used.

The biological half-life of most proteins and peptides in the systemic circulation is a matter of a few min. Sustained release of a protein or peptide can be a desirable feature of a formulation. Extensive efforts have gone into the development of insulin products that can protract the systemic availability of insulin. Early attempts included combination with gum arabic, lecithin and oil in a suspension; however, these were unsuccessful due to either instability, pain on injection, or variable release rates. A stable protamine zinc insulin formulation, NPH (Neutral Protamine Hegedorn) insulin also called "Isophane Suspension," was developed in 1946. At pH 7.3, insulin and protamine co-precipitate in a 5:1 molar ratio in the presence of small amounts of zinc and phenol, and later gradually transform into crystals that are then sus-

Table 6 Marketed Protein in Freeze-Dried Formulations

Product	Formulation	Route	Indication
Metrodin	Lyophilized follicle-stimulating hormone (75 IU) and lactose (10 mg)	i.m.	Induction of ovulation
Pergonal	Lyophilized follicle-stimulating hormone and leuteinizing hormone and lactose (10 mg)	i.m.	Infertility
Profasi	Lyophilized chorionic gonadotropin with mannitol (100 mg) and phosphate to adjust pH. To be reconstituted with benzyl alcohol (0.9%)	i.m.	Infertility
Elspar	Lyophilized asparaginase with mannitol (80 mg)	i.v. or i.m.	Leukemia
Glucagon	Lyophilized glucagon (1 mg) with lactose (49 mg) to be reconstituted with 1 ml of diluent-containing glycerin (1.6%) and phenol (0.2%)	i.v., i.m., or s.c.	Hypoglycemia
Acthar	Lyophilized corticotropin (25 or 40 units) and hydrolyzed gelatin (9 or 14 mg), respectively	i.v., i.m., or s.c.	Hormone deficiency

pended in an injectable vehicle. Different brands of NPH insulin preparations exhibit marked differences in duration of action, due to variation in crystal size and shape. Another approach to protract systemic insulin absorption is by crystallization of insulin in the presence of zinc ions. Up to two zinc ions per hexameric insulin can be incorporated in the crystal structure. The crystalline suspension is the basis for the ultralente formulation. Mixing the amorphous and crystalline material results in Lente or intermediate-acting insulin formulations. Other sustained release dosage forms are illustrated in Table 8.

Details of four products derived from biotechnology are shown in Table 9. These four products are formulated as injectable solutions, indicating the fact that proteins do not always require lyophilization. Glycoproteins generally are known to be more stable than their nonglycosylated counterpart. Epogen (erythropoietin) is an example of a glycoprotein. The less stable proteins, i.e., growth hormones and a number of fibrinolytic agents, are listed in Table 10 to illustrate the freeze-dried formulations of these protein products.

Much of the knowledge in formulating proteins is still evolving. As new products are approved by the FDA, information on characterization of proteins, protein stability, the use of stabilizers etc., are becoming available.

Table 7 Marketed Peptide in Ready-To-Use Formulations

Product	Formulation	Route	Indication
Pitressin	8-Arginine vasopressin (20 pressor units) and chlorobutanol (5 mg) per ml; acetic acid to adjust pH	s.c., i.m.	Postoperative abdominal distention
Lupron	Leuprolide (LHRH analogue) acetate (5 mg) and sodium chloride; benzyl alcohol (0.9 mg) per ml	s.c.	Prostatic cancer
Syntocinon	Oxytocin (10 USP units and sodium acetate (1 mg), sodium choride (0.017 mg), chlorobutanol (0.5%), alcohol (0.61% by volume), per ml, pH 4.0	i.v., i.m.	Labor induction
Sandostatin	Octreotide, a somatostatin analogue (0.05-0.5 mg) in acetate buffer (4 mg), sodium chloride (7 mg) per 1 ml, pH 4.2	s.c.	Intestinal tumor
Calcimar	Salmon calcitonin (200 IU), phenol (5 mg) sodium chloride and acetate buffer	s.c.	Paget's disease and hypercalcemia

Table 8 Sustained Release Dosage Forms

Product	Formulation	Route	Indication
Lupron	Sterile lyophilized microspheres packaged in each vial that contains leuprolide acetate (7.5 mg), purified gelatin (1.3 mg), DL-lactic acid and glycolic acids copolymer (66.2 mg), and D-mannitol (13.2 mg). The diluent contains sodium carboxymethylcellulose (7.5 mg), D-mannitol (75 mg), and polysorbate 80 (1.5 mg) per ml of water	i.m.	Prostatic cancer
Pitressin tannate in oil	Vasopressin tannate (5 pressor units) per ml in peanut oil	i.m.	Antidiuretic
H.P. Acthar gel	Highly purified adrenocorticotropic hormone in gelatin (16%), phenol (0.5%)	i.m., s.c.	Endocrine cancer

Table 9 Ready-To-Use Formulations of Products Derived from Biotechnology

Product	Active	Concentration (mg/ml)		Routes
		Buffer/salt	Stabilizer/preservatives	
Humulin[R]	Huamn insulin	Unknown[a]	m-Cresol: 2.5	s.c.
Epogen[b] (1 ml vial)	2000, 4000, 10,000 units epoetin alpha	Sodium citrate 5.8; citric acid 0.06; sodium chloride 5.8; pH 6.9 ± 0.3	Albumin: 2.5	s.c., i.v.
Orthoclone OKT-3[c] (5 ml ampule)	1 mg/ml Muromonab-CD3	Sodium phosphate dibasic 1.4; monobasic 0.45; sodium chloride 8.6; pH 7.0 ± 0.5	Polysorbate 80: 0.2	i.v.
Roferon[b] (1 ml vial)	3, 18, 36 million IU (2×10^8 IU/mg) interferon A-2a	Sodium cloride 9	Albumin: 5; phenol: 3	s.c., i.m., intralession

[a]Insulin is an over-the-counter product, quantitative composition is not required on the label.
[b]Produced by recombinant DNA technology (Fig. 1).
[c]Monoclonal antibody produced by hybridoma (Fig. 2).

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Table 10 Lyophilized Formulations of Growth Hormones and Fibrinolytic Agents

Product	Active	Composition	Diluent	Routes
Protropin	Somatrem 5 mg	Mannitol 40 mg; sodium phosphate 1.7 mg per vial pH 4.5-7.0	Benzyl alcohol 0.9%, 5 ml	s.c., i.m.
Humatrope	Somatropin 5 mg	Mannitol 25 mg/ glycine 5 mg; sodium phosphate dibasic 1.13 mg; pH 7.5	m-Cresol 0.3% and glycerine 1.7%, 5 ml	s.c., i.m.
Abbokinase	Urokinase 250,000 IU	Mannitol 25 mg; albumin 250; and sodium chloride 50 mg per vial	Water 5 ml	i.v., i.a., i.c. infusion
Streptase	Streptokinase 250,000, 750,000, 1,500,000 IU	Sodium glutamate 25 mg; cross-linked gelatin 25 mg; and albumin 100 mg per vial	Sodium chloride or dextrose solution 5 ml	i.v., i.c. infusion
Activase	Alteplase 20 mg, 50 mg (580,000 IU/mg)	Arginine 700 mg; phosphoric acid 200 mg and less than 1.6 mg polysorbate 80 per 20 mg of protein	Water 20 or 50 ml	i.v. infusion

These data are often published in journals outside the pharmaceutical field. Before embarking on a new formulation task, a thorough literature search is recommended.

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