1988
SUPPLEMENT
Volume 42
Number 2S

Technical Report No. 10

Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers

Journal of Parenteral Science and Technology

A publication of The Parenteral Drug Association

Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers

YU-CHANG JOHN WANG* and MUSETTA A. HANSON†

* *Research Laboratories, Ortho Pharmaceutical Corporation, Raritan, New Jersey.* † *Rorer Central Research, Fort Washington, Pennsylvania*

I. Introduction

Recent developments in biotechnology will cause a tremendous increase in the number of injectable products containing proteins or peptides, and there exists a definite need for skill in formulating such products. Scientists who have no difficulty in formulating chemical compounds into injectable products often find that dealing with proteins and peptides is a completely different experience. To illustrate this point, the degradation of a chemical compound, such as penicillin or aspirin, may be readily detected by use of HPLC or other assays indicative of stability, but degradation/denaturation of a protein is not a simple one-step reaction and products of denatured protein are not readily detectable. In predicting shelf life, formulators frequently determine the degradation rate of a chemical compound at an elevated temperature (50 °C or 37 °C), then extrapolate the result to storage at low temperatures. This approach is often not applicable to proteins because some may not be stable at 50 °C for any reasonable period of time, but may be extremely stable at 5 °C. The Arrhenius equation often works only in a limited temperature range.

This report reviews the scientific literature on degradation pathways, and on methods of stabilizing proteins and peptides, particularly methods that can be employed for a parenteral product. The peptides and proteins employed in parenteral formulations vary tremendously in their properties. The following list of selected market products used parenterally is arranged in ascending order of complexity.

	Number of Amino Acids	Mol. wt.	Comments
Thymopentin*	5		
Oxytocin	9	1007	
Leuprolide	9	1209	
Gonadorelin	10	1182	
Cyclosporin	11		orally active
Glucagon	29		
Calcitonin	32		produced by chemical synthesis
Insulin	51	5808	
Interleukin-2*	133		
Interferon	165	19,000	
Erythropoietin*	166	35,000	
Growth hormone	191		
Urokinase	267		
Asparaginase			
Alteplase*	527		
Chymopapain			
RhoGAM®		150,000	
Atgam®			polyclonal antibody
Gamimune®			intravenous infusion
Orthoclone OKT® 3			monoclonal antibody
Recombivax HB®			vaccine against hepatitis B virus

* These products are not on the U.S. market as of July 1987.

Information on how to stabilize proteins and peptides in products for parenteral use is not readily available to formulation scientists. In most cases, information concerning the stabilizing of proteins is contained in journals or textbooks that are unfamiliar to formulation scientists. Scientists working in areas other than pharmaceutical chemistry have long been concerned with stabilizing proteins in a hostile environment and/or for long-term storage as, for example, proteases, carbohydrases in detergents, hydrolytic enzymes in food processing, and enzymes used in analytical and diagnostic applications. Information generated in those areas is very useful, and this review seeks to assemble that information in one place, together with knowledge from existing parenteral products containing proteins and peptides, thus providing comprehensive information to the scientist setting out to design a stable and elegant injectable formulation containing protein, peptide, or both.

II. Degradation of Proteins and Peptides

The degradation of proteins and peptides can be divided into two main categories. One involves a covalent bond and the other does not. The latter process is often referred to as denaturation.

A. Covalent Bond Reactions

Nine major reactions involve covalent bonds:

a. Hydrolysis: The peptide (amide) linkage (RNH–CO–R) is much more resistant to hydrolysis than is the ester linkage (R–O–CO–R) and amide bonds are considered stable unless hydrolysis is assisted by a neighboring group. The formulation factor that most influences the hydrolytic rate is solution pH. Examples of pH-stability studies on therapeutic peptides are: degradation of nafarelin (Johnson, 1986), thermal stability of D-Trp-LHRH

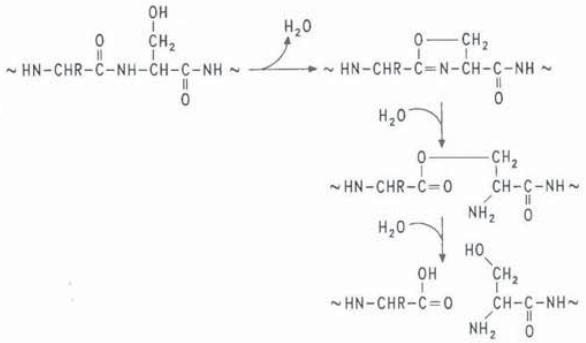
Received September 16, 1987.
This review was prepared under the auspices of The Parenteral Drug Association Research Committee.

(Winterer, 1983), acid hydrolysis of captopril, a quasi-dipeptide (Timmins, 1983), heat stability of urokinase (Miwa, 1981), and summaries presented in "Analytical Profiles" (K. Florey, Ed.) for oxytocin (**10,** 563), gramicidin (**8,** 179) and bacitracin (**9,** 1). In general, these reports demonstrated that peptides are stable for parenteral use. 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 a general trend of stability: for instance, leuprolide and oxytocin injections have a shelf life of at least two years under refrigeration and three months at room temperature. LH-RH injection (Relefact®), made by Hoechst AG, does not even require storage under refrigeration. Oxytocin injection made by Sandoz was reported to be stable at room temperature for five years (Wolfert, 1975). Protirelin, a tripeptide (PyrGlu-His-Pro) is stable for 20 hours at 80 °C at both pH 3.3 and pH 6 (Rao, 1987).

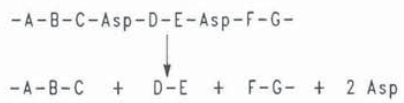
Hydrolysis of peptides can become very complex, because the side chains on certain amino acids are capable of influencing the hydrolysis. The steric effect of amino acid side chains on hydrolysis can be exemplified by valine and leucine; their side chains, isopropyl and isobutyl, provide a hindrance to the hydronium ion in its attack on the peptide bond (Hill, 1965).

The positive charge at the terminal amino group tends to repel hydronium ions, thus making the adjacent peptide bond resistant to acid hydrolysis (Hammel, 1954). However, the positive charge on ϵ-ammonium, imidazolium, or guanidinium groups on the side chains does not decrease the rate of hydrolysis of peptide bonds significantly. The charge on the side chain of lysine, histidine, or arginine is too far removed from the peptide bonds to play an important stabilizing role.

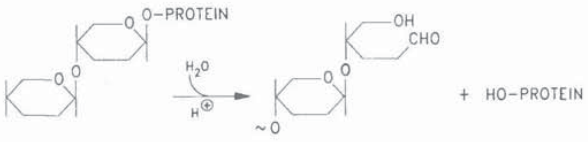
The peptide bonds formed by the amino group of serine and threonine are more labile (Harris, 1956) (Eq. 1). The bonds formed by aspartyl residues are very susceptible to hydrolysis in dilute acid (Eq. 2). It has been suggested



Equation 1—Hydrolysis promoted by serine residue.



Equation 2—Hydrolysis promoted by aspartyl residue.



Equation 3—Deglycosylation.

that the negatively charged carboxyl group of aspartic acid attracts hydrogen ions in dilute acid and thereby increases the lability of neighboring peptide bonds; these bonds are severed and aspartic acid is liberated (Schultz, 1961).

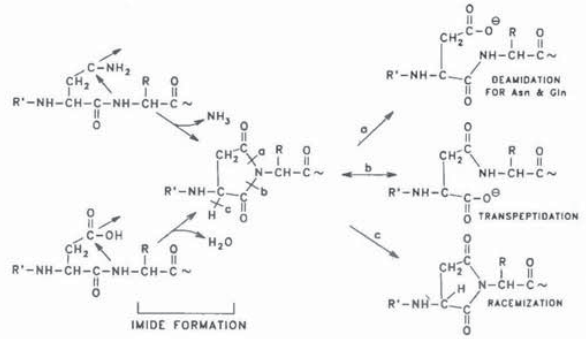
Hydrolysis other than of peptide bonds can also be responsible for protein inactivation. For example, hydrolysis of the tyrosine-O-sulfate moiety was responsible for inactivation of cholecystokinin (Wunsch, 1983). Acetal or hemi-acetal (Eq. 3) hydrolysis by acid is responsible for severing sugar moieties from glycoprotein (deglycosylation). Although reaction rates of deglycosylation have not been reported, data generated for the digitalis glycoside, digoxin (Sternson, 1978) can be useful as a reference.

b. Imide Formation: The α-amino group of asparagine, glutamine, and aspartic and glutamic acids attacks the side-chain carbonyl carbon of these amino acid residues, thus forming aspartimides or glutarimides (Eq. 4). These cyclic imides are sensitive to hydrolysis and can be opened in two ways. One is to break the newly formed bond; this is the deamidation reaction when imides are formed by asparagine and glutamine. Another is to break the original C–N bond on the peptide backbone, leading to the formation of β- (for Asn and Asp) or γ- (for Gln and Glu) carboxyl isomers, a process often referred to as isomerization. The imides can also undergo racemization at the α-carbon position.

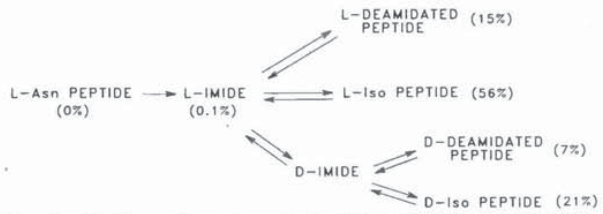
Geiger and Clarke (1987) conducted a comprehensive study on the rates of the aforementioned reactions, using a hexapeptide that is the residue 22–27 of adrenocorticotropic hormone (Val-Tyr-Pro-Asn-Gly-Ala). The amounts of peptides obtained from all possible degradation routes are shown in Eq. 5.

Because early reports on deamidation and isomerization were not analyzed with respect to other concurrent reactions, these reactions will be discussed separately in the following sections.

c. Deamidation: Deamidation is the hydrolysis of the



Equation 4—Imide formation and subsequent reactions.



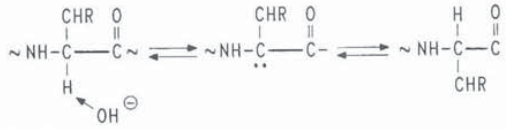
Equation 5—Proportions of reaction products of Val-Tyr-Pro-Asn-Gly-Ala (Geiger, 1977).

side chain amide on glutamine and asparagine (Eq. 3). It is a major route of degradation for insulin (Rytting, 1982) and ACTH (Graf, 1971). Amnonia, if generated in significant amount upon hydrolysis, can accelerate hydrolysis of the peptide backbone (Gilbert, 1949). The deamidation rates were determined for 42 synthetic pentapeptides containing glutaminyl or asparaginyl residues (McKerrow, 1971; Robinson, 1973). Asparagine is more labile than glutamine, and is most labile in the presence of an adjacent glycine. The half-lives of these model compounds at 37 °C in pH 7.2 phosphate buffer range from 20 days to nine years. Geiger and Clarke (1987) concluded that deamidation occurs almost exclusively through an imide pathway, and that direct solvent hydrolysis is insignificant. The heat of activation for deamidation of their L-Asn hexapeptide is approximately 21 Kcal/mole, which is higher than for unassisted hydrolytic reactions.

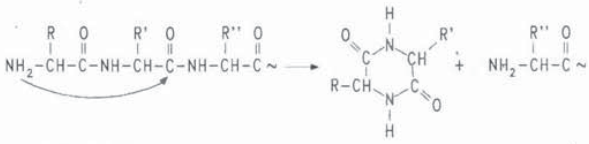
d. Isomerization: Asparagine, glutamine, aspartic acid, and glutamic acid can cyclize back onto the peptide chain (Eq. 4). Subsequent hydrolysis of such cyclic imides resulted in isomerization. Hydrolysis of asparagine and glutamine is accelerated by low pH; that of aspartic acid and glutamic acid is accelerated by high pH (Hill, 1965). Isomerization in the sequence 3–4 represents one of the mechanisms for the inactivation of secretin in solution (Wunsch, 1983). Isomerization is often referred to as transpeptidation.

e. Racemization: Racemization produces enantiomers in both acidic and alkaline solutions. Racemization of the serine residue in analogs of gonadotropin-releasing hormone was studied by Nishi (1980). A comprehensive review of racemization in alkali-treated food proteins was provided by Masters and Friedman (1980). Racemization 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 (Eq. 6). An electron-withdrawing group in the side chain will stabilize the negatively charged intermediate. Thus, asparagine, tyrosine, serine, phenylalanine, etc., all promote racemization.

In an imide-containing peptide, the nitrogen atom and the α- and β-carbonyl groups all contribute to the stable



Equation 6—Racemization.



Equation 7—Diketopiperazine formation.

Equation 8—Disulfide exchange.

resonance of a carbanion, thus racemization is facilitated through imide formation (Eq. 4). At neutral pH this pathway is principally responsible for the racemization of D and L isomers. (Geiger and Clarke, 1987)

f. Diketopiperazine Formation: Rearrangement of the *N*-terminal dipeptide will result in the splitting off of a cyclic diketopiperazine at high pH (Eq. 7). Proline and glycine in the *N*-terminal promote the reaction. Recently, it was reported (Bouvette and Digenis, 1987) that the cyclization of aspartame is minimal at pH 6 to 7, moderate at 7.0–8.5, and rapid at pH above 8.5.

g. Oxidation: Tryptophan, methionine, and cysteine are susceptible to oxidation by air. Disulfide bridging is the main course of reaction in somatostatin. Oxidation of the sulfhydryl group is promoted at both neutral and basic pH and the rate-pH profile for captopril showed an increase in oxidation rate starting at pH 4 (Fig. 1).

h. Disulfide Exchange: The reaction (Eq. 8) is base (OH^-)-catalyzed and promoted by mercaptoethanol, a sometimes employed antioxidant. The reaction is concentration dependent, and oligomers are frequently formed as a result of disulfide bonds between peptide chains. Improper linkages of disulfide bond were responsible for a

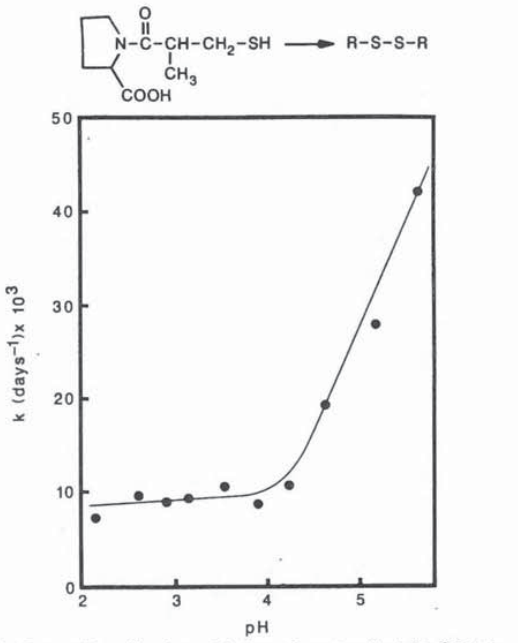


Figure 1—Rate-pH profile for oxidation of captopril at 50 °C (Timmins, 1982).

reduction in biological activity of interleukin-2 (Kenney, 1986).

i. Photodecomposition: Tryptophan is one of the most photosensitive amino acid residues in proteins (Holtz, 1977). Decomposition of tryptophan, which liberates ammonia and forms photoproducts of increased molecular weight, has been responsible for the discoloration of proteins.

B. Denaturation

a. Protein Conformation: In considering the formulation of a protein or peptide molecule for use in parenteral therapy, one must take into account conformation, i.e., the spatial three-dimensional structure of the molecule, how this structure can be disrupted (leading to loss of potency), and how this structure can be stabilized. The primary structure (amino acid sequence) and secondary structure (α-helix or β-sheet) contribute to the tertiary structure of a protein or peptide. It is this tertiary structure that must be stabilized against the forces that work to disrupt it. The tertiary structure of the protein suggests what forces can be used in stabilization. For example, the conformation of each peptide group is usually one of a stable energy state and directs the secondary and tertiary structures. Charged residues are generally on the surface, since it is energetically costly to bury them within the folded structure.

These charged residues and their arrangement on the surface of the folded molecule contribute to an overall molecular dipole and determine how the molecule interacts with solvent molecules in the solution. The nonpolar hydrophobic residues are usually found within the globular structure of these water-soluble proteins. Perhaps the exceptions to this rule are those proteins known to interact or associate with lipids. Elucidation of the structure of proteins with exposed hydrophobic areas suggests that lipid type molecules should contribute to overall stability. Finally, polar side-chain residues will also tend to be on the surface, hydrogen-bonding with each other to further stabilize the tertiary structure.

Even this brief consideration of protein structure suggests that the more a formulator knows about the structure of the protein, the better the chances are to stabilize the drug. It is also important to recognize the potentially denaturing forces that may be applied to the molecule during its processing, e.g., chemical stress from factors used in purification of the protein, such as pH, ionic strength or detergents, or physical stress from processes such as filtration and filling, in which the protein is subject to surface adsorption and shear. These factors must be considered in addition to "simple" solution stability once the product is in its final container. As biological polymers, proteins can be unfolded or denatured from their thermodynamically preferred compact tertiary globular structure, usually with a concomitant loss of potency or activity. An understanding of how the preferred conformation is determined and the mechanisms by which it can be unfolded is important in formulating the molecule as a stable drug.

b. Multistate Mechanism: The denaturation process has been described variously as a two-state or multiple process and the kinetics of these transitions have been described (references in Table VII). Three possible mechanisms can be entertained. First, the folded structure may simply unwind to a random coil without passing through an intermediate state (a real two-state process). Second, the folded molecule may pass through a series of energetically different, partially folded coils as intermediate states. (Schematic presentation of this process is shown in Fig. 2.) Third, there may be a core-organized intermediate structure that exists before complete unfolding to a random coil occurs.

c. Effect of Solvent: Hydrogen bonds are perhaps most important in determining overall protein conformation, since they are the major stabilizers of the secondary α-helix and β-sheet, as well as stabilizing the folded structure. Water, the solvent of most proteins, contributes to this hydrogen bonding. It is the nature of this water structure around a protein that further complicates studies of conformation. It is necessary to understand the solvent-protein interaction because of the strong influence the solvent exerts on protein conformation. A disrupting solvent effect is evident when even low levels of urea or guanidinium chloride are placed in solution with a protein, they completely disrupt the native structure of the protein molecule. On the other hand, some solvent changes contribute to the stability of an ordered globular state.

Because the hydrocarbon side chains are usually tucked inside the protein's globular structure, significant stabilizing effects result from their hydrophobic bonding. This conformation, too, is sensitive to solvent effects, especially if the solvent partitions the hydrocarbon sidechains be-

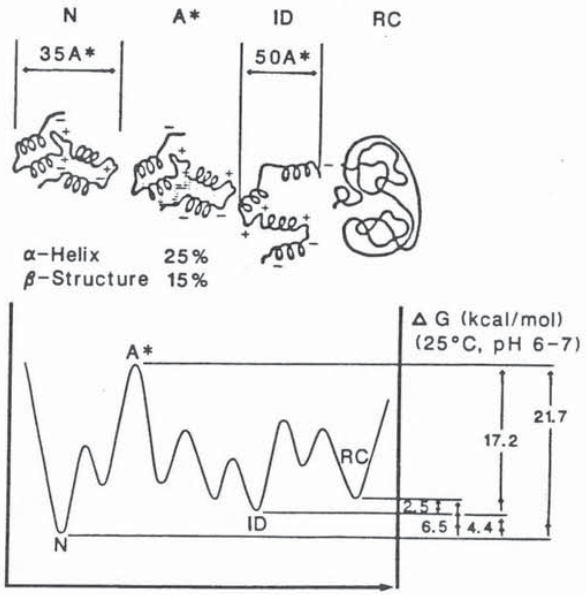


Figure 2—A schematic illustration of conformational transition for denaturation of protein (Kuwajima, 1977).

tween a hydrocarbon phase and an aqueous phase. Measuring the free energy of transfer of a side chain from the apolar interior to a solvent exterior, such as 8*M* urea, indicates that the change in stability of the globular structure caused by urea can be very great.

The ionic side chains of aspartic acid, glutamic acid, lysine, arginine, and histidine, usually on the surface of the molecule, contribute to the stability of the native structure by forming salt bridges. Again, the composition of the surrounding solvent will contribute either to stabilization or to denaturation by virtue of its effects on these bonds. The pH of the solvent will determine the charge of the side groups and the extent of ionic bonding. Further, the dielectric constant of the solvent will influence the stength of salt bridges.

d. Effect of Hydrophobic Interaction: Hydrophobic interaction is often considered the driving force for denaturation. When a protein molecule unfolds, and before it refolds to its natural conformation, hydrophobic interaction can cause either of two different things to happen. In a concentrated protein solution, these hydrophobic groups interact with such groups from other molecules, resulting in protein aggregation. In dilute solutions of protein, however, intermolecular aggregation is unlikely; much more likely is an intramolecular hydrophobic interaction that results in a conformation different from the native one. Such an incorrect structure may show partial or complete loss of biological activity.

III. Experimental Methods to Study Denaturation and Evaluate Stabilizers

A. Cloud Point as a Model

A number of techniques are available for studying the effect of such factors as heat, shear, surface phenomena, and solvent additions on the denaturation or stabilization of a protein's native globular active state. Horne (1971) describes the use of cloud points and synthetic polymers as model proteins. The cloud point is thought to arise from the dissolution of a molecule's hydration layer, exposing the hydrophobic character of its interior, and exclusion of the entire molecule (precipitation) from the aqueous solvent. He studied polyvinyl methylether as a model polymer and measured the effect of electrolytes on the stability of the polymer in solution. Magnesium sulfate lowered the cloud point markedly. The effect of alcohols, sugars, and urea was also measured. The author suggests some caution in using simple polymers as models for protein denaturation, but the effects of various factors on macromolecular stability in aqueous solution may be predictable by use of this system.

B. Critical Micelle Concentration as a Model

Gratzer (1969) studied the effect of protein denaturants on breakage of hydrophobic bonds. One measure of a reagent's ability to destroy hydrophobic interactions is its ability to affect the critical micelle concentration (CMC) of a detergent. The UV absorption spectrum of a detergent undergoes a perturbation upon formation of a micelle. Plots of absorbance against volume of detergent solution added to a volume of solution showed a clear discontinuity at the CMC. The effect of agents such as sucrose or urea on this CMC were measured. Sucrose has no effect on CMC and is known to have no denaturing effect on proteins. However, urea and guanidinium salts, known to denature proteins, disrupt the formation of micelles, and cause CMC to increase. Thus, in general, the effect of solutes on CMC seems to parallel their effect on denaturation of a protein by disruption of hydrophobic bonds.

C. Charge-Transfer Absorption

Coan (1975) described a method of looking at denaturation by measuring charge-transfer (CT) absorption of complexes between exposed tryptophan or tyrosine residues and 1-alkyl-3-carbamido-pyridinium ions. A weak complex between the indole or phenol chromophores and the acceptor ion exhibit a broad CT absorption separated from the near UV absorption bands of the chromophore themselves. The authors studied denatured proteins with oxidized or reduced disulfide bonds and found that the aromatic residues are less exposed in denatured proteins with intact disulfide bonds. This finding suggests that these residues are in more structured, less random regions of the molecule and that the aromatic residues may function to act as refolding centers for renaturation of the molecule. Fully denatured proteins with no crosslinks conform to the full random coil model of denaturation.

D. Thermal Analysis

Ross (1974, 1984) described the use of a scanning microcalorimeter to measure energies of transition in solution: the energies of transition of 25–250 mJ of heat at temperature range from ambient to 90 °C. In the case of the gel-to-liquid transformation of dipalmitoyl-L-lecithin, a sharp transition occurred over three degrees and the energy of transition was in agreement with other reported values. The transition of poly(rA.rU) helix unwinding occurred over a nine-degree range and, again, the results agreed with reported values.

Differential scanning calorimetry is gaining more widespread use as a tool in investigating transitions of proteins as a function of temperature and, more importantly, the effect of potential stabilizing excipients on a protein solution. Yu (1984) used this technique to study the effects of stabilizers on human serum albumin. In the presence of stabilizers, transition temperature increased by several degrees (Fig. 3). By repeat scanning, one can determine whether the thermal denaturation is a reversible process. A stabilizer found effective in thermal denaturation may be effective for prolonging shelf-life at refrigeration temperatures. Other research groups have also used thermal analysis as a tool in studying denaturation (Back, 1979; Gekko, 1982; Fujita, 1981; Uedaira, 1980).

E. Fluorescence Spectroscopy

A fluorescent probe can detect thermal denaturation and other conformational changes in proteins. A hydrophobic fluorescent compound, such as 8-anilino-l-naphthalenesulfonate, has the convenient property of being al-

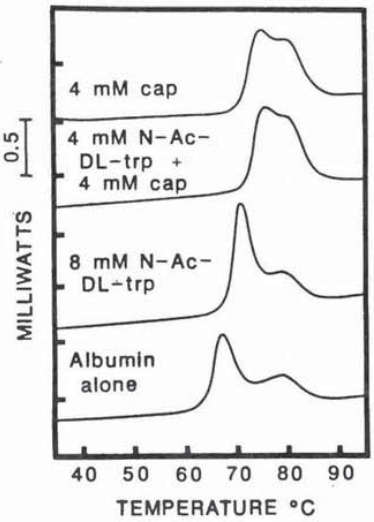


Figure 3—Thermograms of albumin with or without stabilizers: sodium caprylate and sodium *N*-acetyl-DL-tryptophanate (Shrake, 1984).

most nonfluorescent in aqueous solution, but fluorescing strongly when bound to hydrophobic sites on certain proteins. As temperature increases to the point where thermal denaturation takes place, the fluorescence intensity increases sharply, giving a well defined curve that resembles a thermogram in having a characteristic midpoint. Like thermal analysis, this technique provides a convenient method for evaluating potential stabilizers in terms of their ability to shift the mid-point to higher temperatures.

Busby utilized this technique to examine the stabilization of antithrombin III by heparin and lyotropic anions (1981), and by sugar derivatives (1984). Two irreversible thermal transitions were originated from two different domains on human complement (1987). A typical melt curve, as constructed by fluorescent intensity and temperature is shown in Figure 4.

F. Others

Other than the aforementioned methods, the following experimental techniques are useful in detecting denaturation: UV (Hagerman and Baldwin, 1976), circular dichroism (Galat, 1985), NMR (Brems, 1984), size-exclusion chromatography (Light, 1987), and gel electrophoresis (Goldenberg, 1984).

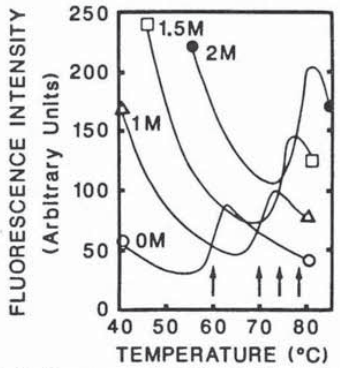


Figure 4—Effect of sodium gluconate on thermal denaturation of antithrombin III. Protein, 1 mg/mL; fluorescent probe, 66 μM; and gluconate, 0(○), 1.0(△), 1.5(□), and 2.0M(●), respectively (Busby, 1984).

IV. Use of Excipients to Stabilize Parenteral Formulations of Proteins and Peptides

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, such as sugars, amino acids, surfactants, and fatty acids, have served to stabilize protein and peptide products against degradation. In each class of stabilizer described below, an explanation of how stabilization was observed and what types of protein and peptide were stabilized is presented. Postulated mechanisms and typical concentrations used in parenteral products are also described. Only additives for parenteral formulations are of concern in this review; other means of stabilizing protein, such as modification of the primary peptide sequence, immobilization of proteins, and grafting protein to polymers, to name a few, are not discussed.

A. Serum Albumin

Serum albumin, regardless of its origin (rabbit, bovine, or human), has been extensively cited in patents (Table I) and literature (Table Ia) 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 hours. At pH 1–2, the albumin molecule expands and elongates; it can return to its native configuration reversibly (Peters, 1985). Also, albumin has good solubility; unlike many other proteins, it is soluble in concentrated salt solution; unlike globulin, it is soluble at the isoelectric point; unlike protamine, it is soluble in diluted base.

The mechanisms by which albumin acts as a stabilizer can be any of the following:

a. Inhibition of Surface Adsorption: Although, in a strict sense, this is not a stabilization phenomenon, in many instances it is the reason why an albumin-containing preparation shows higher recovered enzyme activity than one lacking albumin. Numerous studies have demonstrated that albumin and similar proteins effectively decrease the loss of insulin to the plastic bags or tubing used in intravenous devices and to siliconized glassware (Wang, 1984). A recent patent application showed that adsorption of erythropoietin can be retarded by albumin at concentrations as low as 0.003% (CA 104:230,446V). In contrast, an increased loss of thyroid hormone on a filter was observed at high concentrations (2.5–5%) of albumin (Law, 1983). These results should not be a surprise, because albumin binds various molecules, including peptides, while it acts as a carrier in the blood.

b. Substitution for a Nascent Complexing Protein: Wolf (1972) postulated that in the cell, the binding of adenylyltransferase to another protein has a stabilizing

TABLE I. Patents Describing Albumin as a Stabilizer

Stabilizer	Drug	Concn. (%)	Conditions	Inventor/Assignee	Reference*
HSA	interleukin-2		+ glutathione, pH 4, freeze-dried	Y. Mikura/Takeda	CA104:39703x (EPA 158, 487)
HSA	plasminogen activator	1	phosphate buffer, pH 7	A. Hasegawa/Asahi	CA104:56405u (JP 60,174,722)
BSA	uricase	0.1	+ surfactant, + arginine, freeze-dried	Y. Hayashi/Toyobo	CA104:125711e (JP 60,224,499)
Hydrolyzed ovalbumin	theophylline antibody	1	increased thermal stability	R. Dodge/Abbott	CA104:147009j (EPA 170,983)
HSA	tumor necrosis factor	0.1		H. Sakamoto/Asahi	CA104:205 522j (EPA 166,996)
HSA	erythropoietin		freeze-dried	T. Kawaguchi/Chugai	CA104:230,446v (EPA 178,665)
HSA	γ-interferon	0.1–1	+ glycine, + sugar, freeze-dried	Y. Akagi/Takeda	D86-015321/03 (EPA 168, 008) CA103:166141s
HSA	γ-interferon		freeze-dried	/Asahi	D86-039686/06
HSA	1% monoclonal antibody	2	0.1*M* phosphate buffer, freeze-dried	Y. Kamimura/Green Cross	CA103:213227m (JP 60,146,833
Albumin	γ-interferon	—	freeze-dried	M. Noda/Green Cross	CA102:154791v (EPA 133,767)
HSA	i.v. immune γ-globulin	2:1	+ NaCl, + carbohydrate	W. Curry/Armour	CA102:172619z (US 4,482,483)
Serum albumin	β-interferon		+ $MgCl_2$, freeze-dried	Y. Kato/Kyowa	CA102:12428h (EPA 123, 291)
HSA	interferon		+ glutamic acid, freeze-dried	/Sumitomo	CA102:50895r (JP 59,181,224)
Serum albumin	hematin		freeze-dried	H. Zilg/Behringwerke	CA102:67383v (DE 3,311,288)
HSA	γ-interferon, interleukin-2 and lysozyme		+ modified gelatin	/Suntory	D85-297784/48 (EPA 162,332)
HSA	hepatitis-B surface antigen		amino acid, carbohydrate	/ChemoSero	D85-013848/03 (EPA 130,619)
Albumin	cold insoluble globulin	0.25–1	+ amino acid, + carbohydrate, freeze-dried	T. Ohmura/Green Cross	CA101:28263r (EPA 106, 608)
Albumin	tissue plasminogen activator		solution and freeze-dried	H. Yoshizaki/Kowa	CA101:97631w (EPA 112,940)
HSA	urokinase	1	+ sodium citrate	/Green Cross	CA101:216406p (JP 59,139,323)
Heat treated albumin	γ-globulin		+ glucose, glycine and PEG, freeze-dried	A. Hooper/Baxter	CA100:215500g (WO 8400, 890
Human plasma albumin	leucine aminopeptidase	1–2	freeze-dried	/Green Cross	CA100:74008p (JP 58,206,530)
Albumin	aspartate amino peptidase	1	freeze-dried	/Green Cross	CA100:74009q (JP 58,206,529)
HSA	γ-interferon		+ glucose	/Sumitomo	D84-292019/47
Albumin	tissue plasminogen activator	—	for affinity chromatography	/Kowa	CA99:27999p (JP 58,65,218)
Albumin	myeloperoxidase	1	freeze-dried	/Green Cross	CA98:166911w (JP 58,13,521)
HSA	human IgG		+ gelatin derivatives	/Nippon	D83 811981/45
HSA	β-interferon	1	+ PVP, mannitol	/Inter-Yeda	D83 772523/39
Human albumin	lysozyme	0.05	+ EDTA and salt	/Green Cross	CA97:168931f (JP 82,126,422)
Serum albumin	antithrombin	10	freeze-dried	Uemura/Green Cross	CA97:150716e (US 4,340,589)
BSA	glycerophosphate oxidase	1	+ glucose, glycine and vitamin B_2	/Toyobo	CA97:106263f (JP 82,68,788)
Albumin	kallidinogenase		+ mannitol, spray-dried	/Sanwa	CA97:115,343j (JP 82,108,020)
BSA	glutamate oxalacetate transaminase	10	+ 10% sucrose, freeze-dried	H. Teranishi/Eiken	CA96:2953u (DE 3,040,005)
Albumin	thrombin	1–4	+ aprotinin, EDTA	H. Lill/Boehringer	CA96:30818x (DE 3,019,612)
BSA	dihydrofolate reductase		freeze-dried	A. Kato/	CA96:118184y (JP 81,160,991)
HSA	measles vaccine		+ sorbitol, lactose, freeze-dried	/Takeda	CA96:168711p (JP 82,07,423)
Albumin	mumps vaccine	5	+ gelatin, freeze-dried	/Green Cross	CA96:187300v (JP 82,16,838)
Albumin	antithrombin III	1	+ citrate, freeze-dried	Y. Uemura/Green Cross	CA94:162736n (US 4,340,589)

Stabilizer	Drug	Concn., %	Conditions	Inventor/Assignee	Reference*
HSA	sulfonated immunoglobulin	0.2	+ 2.25% glycine	/Teijin	CA94:109340c (JP 81,02,917)
Albumin	human granulocyte inducing glycoprotein		stable to 60 °C 10 hr	M. Nishida/Morinaga	CA94:145341n or (US 4,230,69
Albumin	secretin	0.5	+ glycine 2%	W. Koenig/Hoechst	CA91:163050j (G.O.758,578)
BSA	thymidine phosphorylase	2.5	+ uracil	/Wellcome	CA90:147794d (JP 79,08,791)
BSA	peroxidase	10:1	+ $FeSO_4$, PEG and mannitol	E. Dawson/Akzo	CA89:19229r
Albumin	dehydrogenase		+ glycine, cysteine, serine, EDTA	R. Fresenius/Fresenius	CA88:70996v
Albumin	factor III	>0.5	freeze-dried	R. Bick/Wilson	CA87:44226d (US 4,027,013)
Albumin	cancer associated polypeptide antigen	200:1	emulsified with Freund's incomplete adjuvant	K. Bjorklund/	CA85:61323j (US 3,960,827)
Bovine albumin	Sendai virus		+ sucrose, glutamate	K. Apostolov/Wellcome	CA82:7638r
HSA	lysozyme, lyase		+ polylysine, protamine freeze-dried	L. Boxendale/Biorex	CA69:80195e

* Key: CA = Chemical Abstract.
US = U.S. Patent.
EPA = European Patent Application.
D = Derwent.
JP = Japanese Patent (Jpn. Kokai Tokkyo Koho).
DE = German Patent (Ger. Offen.).

TABLE Ia. Literature Describing Albumin as a Stabilizer

Stabilizer	Drug	Concn. (%)	Conditions	Author	Reference*
HSA	β-interferon		Lyophilized with dextrose	J. Geigert	CA107:64710s
BSA	thrombin	0.5	PEG, calcium also stabilize	B. Rotoli	CA107:35563r
BSA caseinate	galactosidase			V. Kudrya	CA107:54728q
Rabbit serum	creatinine phosphokinase	—	Stabilized against inactivation at 39 °C	H. Hsu	CA96:195772r
BSA	immunoglobulin	—	Under ultracentrifugation	R. Kauffman	CA92:56624y
HSA	allergens	0.03	Six-month storage	P. Norman	CA90:37550p
Inert protein or serum albumin	horseradish peroxidase	—	Increase thermal stability	I. Berezin	CA83:174756e
Human albumin, bovine albumin	smallpox vaccine	—	Protect the vaccine from coagulation caused by heat denaturation	A. Martins	CA82:7588z
BSA	nitrate reductase	3	Retard the decay of activity	L. Schrader	CA81:34907p
BSA	colicin E2	—	Increase heat stability and resistance to surface denaturation	E. Mitusi	CA72:18529a
BSA	adenylyltransferase	0.5	Aging	D. Wolf	CA76:96261d
BSA	alcohol dehydrogenase	0.003	Thermal inactivation	A. Wiseman	CA76:1224g
Serum albumin	amylase	0.05	+ glutathione	G. Walker	See reference

* Key: See Table I.

effect. If the stabilizing protein were separated during purification, an albumin additive could act as a replacement for the nascent protein by binding at the same sites, but with a low affinity. The stabilization in this case may be attributable to serum albumin covering the SH-groups.

c. Interstitial Dispersion: Through measurement of dichroism, Hsu (1983) studied the stabilization of rabbit muscle creatine phosphokinase by rabbit serum albumin. No evidence of molecular interaction between the enzyme and the albumin was exhibited in the range of 400–200 nm. She postulated that the enzyme was just dispersed in the albumin matrices and that the much more thermoresistant albumin helped to stabilize the native conformation of the enzyme.

d. Stabilization of Conformation: Wiseman (1971) postulated that serum albumin could protect alcohol dehydrogenase from conformational change, including dissociation into subunits of the enzyme.

e. Cryoprotectant: Serum albumin acts as a cryoprotectant in the lyophilization of many proteins, such as interleukin-2, interferon, and tissue plasminogen activator (Table I).

Without elaborative research, it is difficult to delineate the true mechanism of stabilization in any particular circumstance. The concentration of serum albumin required for stabilization usually depends on the amount of protein or peptide, but the literature shows a wide range of use. Wiseman (1971) used 0.003% for general protective action, whereas concentrations cited in patents range from 0.1–1%. In the recently approved recombinant interferon, Roferon-A®, 0.5% human serum albumin is employed as a stabilizer. Another interferon, Intron-A®, contains 0.1% in its lyophilized cake. About 1–2% is present in Monoclat® (antihemophilic factor). MICRhoGAM® (an anti-Rh product) contains up to 15% serum albumin. However, caution should be exercised in using albumin as a stabilizer, because albumin may interfere with many protein assay methodologies.

Formulators unfamiliar with the biochemical properties of albumin are directed to a review chapter by Peters (1985).

B. Amino Acids

Amino acids have been used for a variety of reasons.

a. As Chelating Agents: Insulin solutions in reservoirs tend to aggregate. Bringer (1981) studied the protective effect of amino acids on this aggregation and found that, although insulin alone, when shaken over four days, formed aggregates, crystals, and fibrils, no such reaction was seen when glutamic or aspartic acids were used at their isoelectric pH's (3.3–3.5). Similar protection against aggregation by glutamic acid was seen for solutions delivered over time by an implanted minipump. Since only the dicarboxylic amino acids at their isoelectric pH are effective, the authors suggest that chelation of zinc may be the mechanism preventing aggregation. Quinn (1983) reported similar insulin solubility and aggregation phenomena and observed that lysine and EDTA or lysine alone at a higher concentration were effective in preventing the reaction. Aspartic and glutamic acids were also effective, whereas leucine was not. These authors, too, suggest that chelation of zinc may be the mechanism for preventing aggregation.

b. Reduce Surface Adsorption: Mizutani (1981) reported that proteins (bovine serum albumin, hemoglobin, serum globulin, and lysozyme) bind to silicone-coated glass surfaces. He observed that adsorption of proteins to the glass varied with the salt concentration of the solution and that the critical salt concentration for adsorption was different for different proteins. Because urea, which dissociates hydrogen bonds, did not affect adsorption, the author suggests that binding was not due to the hydrogen bonds. Also, since high salt concentration increased protein adsorption, ionic bonding to silicone was not a factor. These results suggested that hydrophobic bonding occurred between silicone and aliphatic protein residues. The author suggests that, under conditions of high salt concentration, the electric charges on the protein are weakened, allowing magnification of the hydrophobic bonding of aliphatic and aromatic amino acid residues to silicone on the glass. The effect of amino acids on this binding was studied. Basically, aliphatic and aromatic amino acids failed to inhibit the adsorption of protein, whereas acidic amino acids did inhibit, but the mechanism of inhibition was still unclear.

c. Inhibit Aggregate Formation: Gamma globulins used for the treatment of infections are isolated from human plasma. In solution, these proteins tend to aggregate and to show an increase in anticomplement activity. These reactions are thought to be responsible for the shock and other adverse effects seen when gamma globulins were administered intravenously. In an effort to stabilize the product against aggregation Coval (1979) prepared an immune globulin G solution with little aggregation and low anticomplement activity, using organic acids for pH adjustment, additional protein in the form of serum albumin, the amino acid glycine and, for those solutions to be lyophilized, mannitol. Although the author does not state specifically why glycine and albumin were added, it is recognized that amino acids and proteins tend to stabilize other proteins in solution, preventing the formation of aggregates.

Likewise, Takagi (1980), in order to formulate an intravenous immune globulin G preparation that did not increase in anticomplement activity when stored as a solution, added arginine or lysine as stabilizers; ornithine, aspartic acid, glutamic acid, alanine, and glycine had no stabilizing effects.

d. Stabilize Against Heat Denaturation: Many therapeutic blood products are heat-treated to inactivate possible viral contamination (hepatitis). Researchers have sought ways to stabilize purified components of blood in solution to such heat treatment.

The heat treatment of albumin at 60 °C for 10 hours has been shown to inactivate infective hepatitis virus and this method is being applied to other serum proteins designated for therapeutic use. Subjecting Factor XIII to these conditions destroys its activity. However, the addition of neutral amino acids (glycine or alanine) and other sub-

stances (sugars, carboxylic acids) stabilizes the protein. After heat treatment, the Factor XIII solution is dialyzed, diluted, or centrifuged to recover pure Factor XIII for further processing (Fukushima, 1981).

e. Inhibit Isomerization: The isomerization of ovalbumin at pH 4 in acetate buffer is more or less inhibited by adding glycine and proline to the medium, as shown by electrophoretic patterns (Joly, 1965, p. 46).

Glycine, the most commonly used amino acid, has been employed in a wide range of concentrations: in Atgam® (Uphohn's anti-thymocyte globulin), 0.3*M* (2.25%); in Intron® A (Schering's α-interferon), 2%; in Gamimune® (Immune globulin by Cutter), 0.015*M* (0.11%); in RhoGAM (Ortho's anti-Rh), 0.015%.

Streptokinase (by Hoechst-Roussel) utilizes sodium glutamate as a stabilizer. Patents citing amino acids as stabilizers are listed in Table II. In many of these patents, amino acids are cited as co-stabilizers with other stabilizers, such as albumin or polyhydric alcohols.

C. Fatty Acids and Phospholipids

Lipids and fatty acids or their derivatives have also been known to stabilize proteins. This is, perhaps, not surprising, since many proteins are known to bind lipids, especially those proteins that circulate in the blood, such as albumin or the coagulation factors. Therefore, in purifying these components for therapeutic use, it is natural to look to lipids and fatty acids as potential stabilizers. Boyer (1946), in his early studies, determined the effect of fatty acids on the thermal stability of serum albumin using nephelometric, viscometric, and cloud point determinations. With cloud point, maximum stabilization was provided by salts of straight-chain fatty acids of 7 to 8 carbons at 0.15*M*. The diminishing effect of higher carbon fatty acids may be caused by a denaturing effect resulting from their detergent properties. Alkyl sulfonates gave similar results. Amino acids, proteins, sugars, alcohols, and esters did not possess stabilizing properties. Nephelometric and viscometric methods confirmed the cloud point results and the view that an anion with a nonpolar group was essential. The author concluded that stabilization resulted from the association of the polar and nonpolar portions of the anion with groups on the albumin molecule. The reacting group on albumin could be the guanidine group of arginine residues. As shown by electrophoresis, the stabilization of human serum albumin against heat denaturation in the presence of sodium caprylate was inhibited by lysine, glutamine, arginine, asparagine, ammonia, etc. (Joly , 1965, p. 47).

More recently, Shrake (1984) and Ross (1974) reported on the use of differential scanning calorimetry (Fig. 3) to measure the stabilizing effects of caprylate or *N*-acetyl-DL-tryptophanate on human albumin during heating. Only acetyltryptophanate (8mM) or the combination of 4mM acetyltryptophanate and 4mM caprylate are permitted by the U.S. Code of Federal Regulations as thermal stabilizers. These authors found that caprylate (4mM) alone was more effective as a stabilizer than the combination that is currently allowed. Their second study examined enantiomers of acetyltryptophanate and tryptophanate, as well as the effect of pH on stability of heated albumin. L-tryptophanate increased stability, whereas D-tryptophanate did not, a result consistent with the reported weaker binding of the D isomer. Thermal stability was also greater at pH 6.4 than at pH 7.4. Yu et al. (1984) found similar stabilizing effects with acetyltryptophanate and caprylate by monitoring albumin polymer formation during heating.

There are examples of phospholipids (such as phosphatidyl choline, serine and ethanolamine) stabilizing protein activity, especially the various coagulation factors of plasma. Broden (1983) reported that Factor VIII, when incubated with phospholipid, showed a two- to three-fold increase in coagulant activity. Furthermore, association of Factor VIII with phospholipid was demonstrated by gel filtration. Andersson (1981) reported that Factor VIIIc can be dissociated from the von Willebrand factor with which it normally circulates as a noncovalent complex by binding to phospholipid; this Factor VIIIc:phopholipid complex is more stable than is Factor VIIIc alone. Lajmanovich (1981) reported that the stabilizing effect of phospholipid on Factor VIIIc is a function of the type of phospholipid used. Lollar (1984) reported a similar stabilization of porcine Factor VIIIc by phospholipid and a second substance, Factor IXa. Factor VIIIc, once activated by thrombin, is very labile unless phospholipid and Factor IXa are present. Other coagulation factors (V, X) have been reported to be bound to and stabilized by phospholipid.

Additionally, Moss (1984) reported that certain lysophosphatidyl cholines, but not related phospholipids, activate and stabilize NAD:arginine ADP ribosyltransferase. The structural requirements in the phospholipid to achieve activation may reflect the features needed to stabilize the active conformation of the enzyme. The increased stability supports the notion of binding of the protein to lysolecithin micelles.

The known interaction and stabilization of proteins by lipids leads one to consider the entire field of liposomal delivery of proteins and peptides as a way to protect the protein drug, attain a degree of sustained release, or to target the protein or peptide to a specific site. The field is too broad to be considered in this review.

D. Surfactants

Surfactants frequently cause denaturation of protein, both by hydrophobic disruption and by charge group separation. Relatively low concentrations of surfactant exert a high denaturing action, as compared with that of urea or guanidine, because of the strong interactions between surfactant moieties and the reactive sites on protein. However, judicious use of this interaction can stabilize protein against other denaturants. For instance, if reactive groups, such as sulfhydryl groups, can be reversibly prevented from interacting, protein should be stable to normal manipulations during purification. Gibbs (1952) demonstrated that denatured egg albumin could be stabilized against the flocculation process by the addition of aliphatic amines at a concentration above its critical mi-

TABLE II. Patents Citing Amino Acids as Stabilizers

Stabilizer	Compound	Concn.	Conditions	Inventor/Assignee	Reference*
Threonine	L-arginase		improve stabilization by Mn^{++}, Cd^{++}	K. Kimura	CA79:16949f
Glycine, serine	dehydrogenase	0.1*M*	+ EDTA	R. Fresenius/Fresenius	CA88:70996v
Glycine	secretin	4%	+ gelatin or albumin	W. Koenig	CA91:163050j
Glycine	sulfonated Ig	0.2–1%	+ albumin	—	CA94:109340c
Arginine, lysine, histidine	ascorbic acid oxidase	2%	+ sodium borate	/Toyobo	CA97:19847f
Glycine	γ-interferon	0.5%	—	Y. Agaki	D86-015321/03
Amino acids	hepatitis B vaccines		—	—	D85-013848/03
Amino acids	IgG		+ gelatin, albumin	—	D83-811981/45
Glycine, alanine	α-interferon	5–150 mg/mL	freeze-dried	Kwan/Schering	CA99:76913q (US 4,496,537)
Glycine	immunoglobulin		stabilize immunoglobulin under heat inactivation of virus	Uemura/Green Cross	CA105:59370k
Glycine	IgG	0.1*M*	stabilization under ultrafiltration	Friesen/Winnipeg	CA105:12079g
Glycine	erythropoietin	0.2%	+ albumin, mannitol	Kawaguchi/Chugai	CA104:230446v
Glycine, lysine alanine, etc.	coagulating factor VIII		stable to heat and lyophilization	Togunaga/J. Red Cross	CA104:75002b
Leucine, methionine, alanine	human placental leucine aminopeptidase	1%	resistant to heat inactivation of virus	/Green Cross	CA100:144972z
Glycine	cholinesterase	2.5%	freeze-dried	/Green Cross	CA98:166886s
Glycine	plasminogen	15%	+ saccharose	Heber/Behringwerke	CA98:95645z
Glycine	coagulating factor XIII	10–30%	+ citrate	Fukushima/Green Cross	CA96:11653y
Amino acids	urokinase	5%	for thermal inactivation of virus	Suyama/Green Cross	CA94:145333m
Polar amino acids	urokinase	1%	+ 1% HSA, freeze-dried	Yamahira/Sumitomo	CA92:135434q
γ-amino butyric acid	plasmin	0.1*M*	freeze-dried	Jenssen/Novo	US 3,950,513 (1976)

* Key: see Table I.

TABLE III. Patents Citing Surfactants as Stabilizers

Stabilizer	Compound	Concn. (%)	Conditions	Inventor/Assignee	Reference*
Polysorbate	interleukin-2	0.03	2*M* $CaCl_2$	Matsuda/Toray	CA106:162592d
Poloxamer	insulin	1	+1.6% glycerin	Massey/Lilly	CA104:193185m
Brij	uricase	0.01	+ albumin, arginine	Hayashi/Toyobo	CA104:125711e
Polysorbate 80	tissue plasminogen activator	0.05	+ gelatin	Murakami/Asahi	CA102:58409f
Polysorbate 80 and other nontoxic surfactants	tumor necrosis factor	> 0.01	+ sugars	Hayashi/Asahi	U.S. 4,457,916 CA101:12207q
Brij	insulin	0.1		Balschmidt/Nordisk	CA98:149579b
Nonionic surfactant	anti-hepatitis B surface globulin	0.04	+ amino acids	/Green Cross	CA94:145328p

* Key: see Table I.

celle concentration. Stabilization could be explained by the formation of an amine micelle in which peptide chains were solubilized and reactive groups were segregated. Similarly, a low concentration of sodium dodecylsulfate had a protective effect on human serum albumin against 6*M* urea (Markus, 1957). Takeda and Hizukuri (1971), in their elaborate studies on the stabilization of β-amylase, found that octoxynol-9 (Triton-100) or polysorbate 20, when used at concentrations above the critical micelle concentration, were effective in preventing inactivation of amylase by high dilution or by heat denaturation. Stewart (1974) employed sodium dodecyl sulfate at $3.5 \times 10^{-3}M$ to stabilize mouse L-cell interferon against inactivation by heat (100 °C, 2.5 min). Mogensen (1974) reported a similar observation with human leukocyte interferon. These interferons were completely renatured once the surfactant had been removed. For an injectable formulation, surfactant concentration can be greatly diluted in intravenous bags to facilitate renaturation prior to injection. There is another molecular species of interferon that can be stabilized by surfactant only when disulfide bonds are disrupted by strong reducing agents, such as mercaptoethanol (Stewart, 1975). Presumably, removal of reducing agent prior to injection can be accomplished by a premixing operation in an intravenous mini-bag.

Protein has a tendency to denature at an interface, i.e., air/liquid or liquid/liquid. Because of their mixture of polar and nonpolar side chains, proteins have a tendency to concentrate at interfaces. A monolayer of protein will form spontaneously at the surface of a solution of the protein, but will not redissolve when the surface area is increased; irreversible unfolding has occured (Adamson, 1960). This phenomenom could be the reason for the turbidity observed in some protein solutions that were shaken vigorously (Henson, 1970). The addition of surfactant could either reduce the interfacial tension, thus reducing the propensity for 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 is advantageous to add a surfactant, such as poloxamer 188 (Pluronic 68) or a polysorbate, to the injectable formulation (Coval, 1979). An example is an injectable gamma globulin containing 0.1% polysorbate 80. In Orthoclone OKT® 3, a therapeutic monoclonal antibody recently approved by the FDA, polysorbate 80 was used as a stabilizer at 0.02%. U.S. Patent 4,457,916 (1984) described a long list of surfactants that are suitable stabilizers for tumor necrosis factor. Ionic surfactants are more effective than nonionic surfactants by virtue of electrostatic and hydrophobic binding. However, nonionics are less structure-perturbing because of their low monomer concentration (critical micelle concentrations for nonionics are lower) and their less directional interactions (no electrostatic binding). Table III provides a list of patents in which surfactants have been claimed as stabilizers.

E. Metals

In stabilizing the conformation of some protein, calcium plays an important role. Calcium is essential to the stability of certain amylases and proteases. Numerous patents have been recorded on the stabilization of these two types of protein because of their importance in the detergent and food industries. Amylases from different species contain various numbers of disulfide bonds and calcium ions. For example, human amylase contains one calcium ion but several disulfide bridges, whereas amylase from bacilli contains four calcium ions and no disulfide bonds. It was postulated (Sigel, 1973), therefore, that calcium may take the place of disulfide bonds in proteins that lack cross-links. This bridging function of calcium within the polypeptide chain reduces the flexibility of the polypeptide backbone, thus enhancing stability. Calcium also stabilizes bovine trypsin against denaturation by urea (Gomez, 1977). The fact that calcium also inhibits trypsin autolysis supports the proposed mechanism, that is, the calcium binds the side chains of Asp 194 and Ser 190 and the carbonyl group of Ser 139 and, consequently, strengthens the tertiary structure of trypsin.

The work of Marguerie (1977) provides a good example of investigating the stabilizing effect of metal on protein substance. The protective effect of calcium on fibrinogen was demonstrated by a number of techniques: (a) the proteolysis by plasmin, which is followed by proton release, was inhibited in the presence of calcium; (b) by monitoring the changes in UV absorption, degradation of fibrinogen started 3 °C higher in the presence of calcium; and (c) aggregation following acid denaturation was monitored at 300 μm for turbidity, and this turbidity was reduced significantly by the addition of $10^{-4}M$ calcium. In contrast, magnesium ion did not produce any of the above effects, indicating that calcium-binding sites are specific and are involved in this protective effect.

Calcium ion also stabilizes human factor VIII, a coagulating enzyme circulating in blood. Calcium, as well as other divalent ions (Ni^{++}, Sr^{++}, Mn^{++}, Mg^{++}), prevents the rapid loss of Factor VIII activity (Weiss, 1965), whereas the presence of EDTA causes rapid disappearance of this activity (Mikaelsson, 1983).

Metals can also affect the stability of other proteins or peptides that require no participation of metal for their activity (Sigel, 1982). Through complexation at nitrogen or oxygen, metal can exert different influences on peptides. When coordinated to an amide oxygen, metal ions promote the hydrolysis of an amide bond. When substituting for a hydrogen at an amide nitrogen, a metal ion inhibits the hydrolysis of amide bonds. The effect of metal on hydrolysis is very much pH dependent. For instance, copper promotes hydrolysis of glycinamide at low pH, where it forms a bidentate chelate through the amino nitrogen and amide carbonyl oxygen, but inhibits hydrolysis at high pH, where it is bidentate through the amino and deprotonated amide nitrogens. Also, the hydrolysis of glycylglycine in the presence of Cu^{++} passes through a maximum at pH 4.2–4.4. The maximum is explained by the rise and fall of the concentration of the amino-carbonyl oxygen chelated complex. On the basic side of the maximum, the peptide chelates with a deprotonated peptide nitrogen donor that does not undergo hydrolysis.

Many enzymes with disulfide bonds have been dena-

tured by reduction of the disulfide during purification. Renaturation, required to store native conformation, is accomplished by air oxidation or by reaction with oxidizing agents. In trace amounts, 0.1–10 μM, metals such as Cu^{++} and Co^{++} catalyze the oxidation reaction (Ahmed, 1975). It is worth considering the inclusion of a trace amount of copper to maintain protein in its renatured state.

Although metal ions can exert pharmacologic effects, judicious use of a metal in an injectable formulation as a stabilizer may be permissible. In fact, in many total parenteral nutrient (hyperalimentation) solutions, as much as 0.3 mg of copper or 1 mg of zinc is given to the patient (Sceppa, 1979). Conceivably, one may use up to 200 mg of calcium.

F. Polyols

Use of a polyol has been found to stabilize protein solutions. A polyol is defined as any substance with multiple hydroxyl groups, including polyhydric alcohols and carbohydrates. The polyhydric alcohols include such compounds as sorbitol, mannitol, and glycerol and are usually straight-chain molecules. Carbohydrates, on the other hand, are cyclic molecules that may have a keto or aldehyde group.

a. Prevention of Aggregation: Carbohydrates have been used routinely with lyophilized proteins and peptides to provide bulk to the lyophilized cake. There is also evidence that carbohydrates will stabilize proteins even in solution. Some investigators merely observed the effects of carbohydrates on protein stability without knowing the mechanism, but others have speculated on the mechanism of stabilization.

Some gamma globulins are particularly unstable in dilute solution (<15%) at higher than refrigerated temperature, with the instability being indicated by "shedding," i.e., the formation of insoluble protein particles, probably by aggregation of the protein. Lundblad (1980) used the disaccharide maltose at 5–10% to inhibit the "shedding" of immune serum globulin from solution with time. They report that maltose has the added advantage of not browning upon storage or heating as other carbohydrates do. Stability is apparently directly proportional to the maltose concentration.

Fernandes and Lundblad (1980) described a process for preparing an intravenous gamma globulin solution in which maltose was again used to minimize precipitation of the protein. Shedding was measured by an accelerated precipitation test, in which the protein solution is heated to 57 °C for 4 hours and the percent change in degree of opalescence is measured by transmission of visible light at 580 nm. Maltose significantly improved the ability of gamma globulin to resist shedding, and this effect was proportional to the sugar concentration and was enhanced by glycine.

Fernandes and Lundblad (1981) also described a process to provide therapeutically active proteins while overcoming their innate thermal instability during heating to about 40–45 °C for pasteurization. These proteins include plasminogen, antithrombin III, various coagulation factors (II, VIII, and XIII), and gamma globulins. The authors used a polyol suspended or solubilized in aqueous media with the protein during heating. Lower molecular weight polyols (<5000) are preferred and include sugars such as sucrose, glucose, fructose, etc., and polyhydric alcohols such as mannitol and sorbitol. Miekka (1985) used various stabilizing molecules to protect human fibronectin, a plasma glycoprotein, during pasteurization (60 °C, 10 hr). Fibronectin binds collagenous components of tissue and has cellular opsonizing activity. The effect of heat on aggregation of the molecule was measured by dye fluorescence, light scattering, gel filtration, and electrophoresis. Biological activity was measured by its binding to gelatin and by its opsonizing effect, i.e., ability to render bacterial and other cellular debris subject to phagocytosis. When measured by dye fluorescence, unstabilized fibronectin unfolded at 58 °C and the midpoint in the transition (*Td*) was 62 °C. A mixture of citrate and lysine shifted the *Td* to 83 °C. Several other combinations of sugars, salts, and amino acids, especially lysine, stabilized fibronectin against denaturation, aggregation, and loss of biological activity.

b. Protection Against Oxidation: Labrude (1980) reported that hemoglobin can be successfully lyophilized without oxidation by the use of certain carbohydrates. The authors could find no particular structural feature, such as chain length, aldehyde function, cyclization, or reducing capability, that was correlated with protective effect. They suggest that stereospecific interaction of the protein with water in the presence of sugar may be responsible for the protective effect, since the structure of the protein would be maintained and the iron shielded from oxidation.

c. Strengthening Hydrophobic Bonds: The effects of polyhydric and monohydric alcohols on the heat-caused reversible denaturation of lysozyme and ribonuclease were reported by Gerlsma (1968) and Gerlsma and Stuur (1972). Polyhydric alcohols stabilize and monohydric alcohols destabilize the native conformation, and these effects are concentration dependent. The authors suggested that these effects of polyhydric alcohols result from the strengthening of intrahydrophobic bonds, reducing the interaction of the protein with water and the penetration of water into the hydrophobic interior, and from a lowering of the dielectric constant of the medium. Gerlsma (1970) also studied chymotrypsinogen A and derived a comparable conclusion.

Back (1979) used scanning calorimetry to study the effect of sugars and polyols on the stabilization of proteins, including lysozyme and chymotrypsinogen A, against heat denaturation. Solutions of proteins were heated and the temperature (*Tm*) at which the maximum rate of denaturation occurred was estimated. The protective effect was thought to be related to changes in solvent properties, specifically a stabilization of hydrophobic interactions. Two sugars, sucrose and glucose, and two polyhydric alcohols, sorbitol and glycerol, were used in this study. The magnitude of the stabilizing effect (ΔTm) varied with the protein and the sugar or alcohol; glucose

and sorbitol were equally effective, sucrose less so, and glycerol was the least effective.

Once more, lysozyme was also studied by other groups, Uedaira and Uedaira (1980) and Gekko (1982). Using differential scanning calorimetry and thermodynamic parameters, these authors concluded that the stabilizing effect of polyol is due to a preferential solvent interaction that intensifies the hydrophobic bonds inside the protein molecule.

A recent study by Andersson and Hahn-Hagerdal (1987) demonstrated that enzyme (penicillin acylase) stability could not be related to the water activity, and was not due to solute-solvent interactions, although polyols are known to interact strongly with water.

When the conductance of long-chain decyltrimethylammonium carboxylates was measured in the presence of sucrose and glycerol, the results suggested that hydrophobic pairs were being formed. Sucrose and glycerol strengthen hydrophobic interactions at those concentrations that stabilize proteins to heat denaturation (Oakenful and Fenwick, 1977). Measurements of the critical micelle concentration for a series of homologous detergents in glycerol or sucrose led to the conclusion that both compounds reduce the force needed to transfer a hydrophobic group from an aqueous to a non-polar environment. The authors conclude that sugars and polyols stabilize proteins to heat by affecting the water structure and, thus, the strength of the hydrophobic bonds of the protein.

d. Effect on Protein Hydration: Lee and Timasheff (1981) studied the thermal unfolding of three enzymes, α-chymotrypsin, chymotrypsinogen, and ribonuclease, as a function of sucrose concentration. When the effect of sucrose on structural integrity was measured via circular dichroism, no difference in structure of two of the proteins was detectable in the presence or absence of sucrose and a slight increase in band intensity was seen in the third. Thermal denaturation was then studied to determine whether sucrose caused any change in energy profiles; reversibility of the denaturation was measured by recovery of enzymatic activity and by return of the UV difference spectra to base line after the enzymes had been heated at 90 °C for 10 min. A linear relationship between thermal transition temperature and sucrose concentration was found for all three enzymes.

Since conformational changes in the presence of sucrose did not account for the stabilization, the interaction of protein molecules (aggregation) was measured by equilibrium sedimentation. Both the number and average molecular weight of the proteins remained unchanged in the presence of sucrose. Use of preferential solvent interaction parameters (partial specific volume changes), revealed that sucrose is excluded from the protein domain. According to the authors, sucrose added to a protein solution exerts pressure to reduce surface contact between the protein and the solvent. Denaturation or unfolding of the protein would increase the surface of the protein domain and increase the volume from which the sugar is excluded, an energetically unfavorable result. The increase in free energy required to unfold the protein in the presence of sucrose is related to the increase in free energy of the enlarging surface of solvent cavities that contain the protein molecules.

Arakawa and Timasheff (1982), also studying the interaction of proteins with solvent in the presence of lactose and glucose, used several pure enzyme solutions and measured the partial specific volumes of the proteins with a high-precision densitometer. The apparent specific volume of each protein was independent of protein concentration. With protein solutions containing lactose or glucose, however, both sugars were excluded from the protein domain. Preferential hydration was found to be independent of lactose concentration and a linear function of glucose concentration. These findings suggest that, in sugar solution, the preferential interaction of protein with the solvent is a function of the molecular dimension of the protein. The results of these two studies led the authors to conclude that preferential hydration of a protein in the presence of a third component results in stabilization of the protein; the cohesive force of the sugars increases the surface tension of the water governing the protein-solvent interactions. Gekko and Timasheff (1981, 1981a) reported that the same effect was obtained with glycerol.

Bull and Breese (1978) measured the kinetics of denaturation of several proteins in the presence of methanol, ethanol, propanol, and butanol. Denaturation was independent of pH. All the alcohols bound the protein, and such binding is probably followed by protein dehydration. In contrast, polyhydric alcohols, such as sucrose, do not denature protein and leave it hydrated. Sucrose, adonitol, and mannitol did not bind the proteins. The denaturing effect of monohydric alcohols on protein increases with chain length and suggests an interaction with the hydrophobic areas of the protein. These bound molecules seem to penetrate the proteins and destabilize them; in contrast, the polyhydric alcohols stabilize by enhancing the water structure around the protein. In general, any agent that maintains or increases hydration of a protein will stabilize it.

e. Steric Exclusion: Miller and Robyt (1984) studied a highly purified enzyme, dextran sucrase, that rapidly lost activity at 25 °C or with handling, even in the presence of 25% glycerol. An enzyme in dilute solution can be inactivated by surface denaturation, inactivator binding, and disassociation into subunits. Increasing the total protein content by adding an inert protein did not work in this case, so other stabilizers were sought. Nonionic polymers, such as polyethylene glycol or methyl cellulose, were effective and showed a molecular weight dependence, which suggests that raising the effective enzyme concentration may invoke steric exclusion as a mechanism. However, since even low concentrations were effective, stabilization may result from binding of the nonionic polymer to the enzyme. Nonionic and mild ionic detergents also stabilized the enzyme, but this mechanism is probably different from that of the nonionic polymers; both remain unknown.

Busby and Ingham (1984), trying to correlate stabilizing effects with structural features of the molecules, found that the addition of carboxylate groups to glucose im-

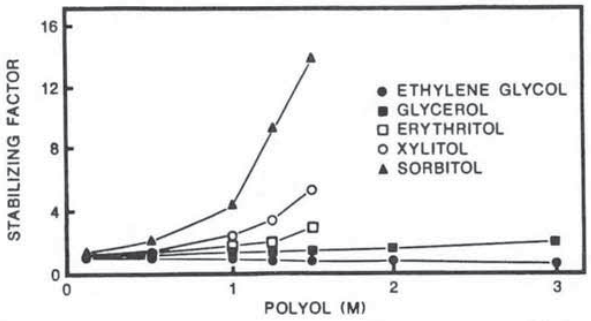


Figure 5—Influence of polyhydric alcohol chain length on stabilizing invertase. Denaturation temperature: 60 °C (Monsan, 1980).

proved its stabilizing effect. The most important common structural feature of good stabilizers appears to be negatively charged groups; the spatial arrangement of these groups does not appear to be important. Some reducing sugars, e.g., glucose, will react with amino groups of the protein, such as lysine, and should be used with caution as stabilizers.

Monsan (1984) demonstrated that the stabilizing effect of polyhydric alcohols on invertase is dependent on the chain length. Figure 5 shows that sorbitol is a much more effective stabilizer than ethylene glycol and glycerol which depress water activity slightly more than sorbitol does, but lack any marked stabilizing effect on invertase activity. Thus, the protective effect of a polyhydric alcohol is not simply related to a modification in the water activity of the medium. This brief review of the function of carbohydrates and polyols in stabilization suggests that much is still to be learned about the mechanism, but the effects of these substances on water and protein intramolecular interactions play a major role. Patents citing polyols as stabilizers are listed in Tables IV and V.

G. Reducing Agents

Many thiol compounds are capable of inhibiting disulfide bond formation, a frequent cause of decrease in protein activities. Cartwright (1977) examined the various thiols for their stabilizing effect on shear-induced inactivation of fibroblast interferon. Reduced glutathione, thioethanolamine, thiodiglycol, thioacetic acid, monothioglycerol, and cysteine all produced some stabilization at 100mM. *N*-Acetylcysteine, dithiothreitol, and DL-thioctic acid were effective at much lower concentrations. Not only did mercaptoethanol and dimercaptopropanol fail to protect interferon from shear, they actually inactivated unsheared interferon. These reducing agents offered no protection when interferon samples were heated. Interestingly, those reagents that protected most effectively against shearing had the greatest destabilizing effect during heating. This contrast was attributed to the difference in accessibility of these agents to the interferon's sulfhydryl group; mercaptoethanol may readily penetrate into the interferon molecule and disrupt all disulfide bonds, whereas larger molecules, such as dithiothreitol, may initially act only on the more accessible sulfhydryl groups involved in interferon inactivation.

Another use of the reducing agent dithiothreitol was described in a patent issued to Cutter Labs (Pappenhagen, 1975). Serum globulin for intramuscular injection was not given intravenously because of its anticomplement activity. After being treated with dithiothreitol or dithioerythritol and alkylation of the reduced interchain disulfide group, the resulting serum globulin showed increased biological half life, and an increased spectrum of antibody activity and was essentially free of anticomplement activity.

Bisulfite, which has been used as an anti-oxidant in some pharmaceutical preparations, is not a suitable agent for protecting sulfhydryl-containing enzymes. In studies of oxidation of the sulfhydryl group in captopril, sulfite was found to cause an increased oxidation rate (Timmins, 1982). Fujimoto (1983) reported that the reactive species produced during aerobic oxidation of bisulfite, inactivated papain, and that this inactivation could be partially restored by mercaptoethanol.

Enzymes can exist temporarily in a reduced and denatured state, e.g., trypsinogen can be re-oxidized in the presence of reducing agents, such as mercaptoethanol or dihydroascorbate. Rapid re-oxidation leads to disulfide exchange and inactivation. In the presence of reduced and oxidized glutathione, the incorrectly folded version can undergo disulfide interchange and continue to refold to a correct conformation (Sinha, 1975). The presence of small amounts of reducing agent can assist proteins with sulfhydryl groups to pair correctly (Saxena, 1970). Mercaptoethanol and thioglycolate have been cited in the literature for such use; mercaptoethanol is better because thioglycolate may cause thiolation of amino groups as a result of the presence of thiol ester impurities.

The cleavage of parathyroid hormone, which is presumed to be a hydrolytic process even though it is catalyzed by oxygen, was effectively prevented in the presence of 0.12% (v/v) mercaptoethanol or the combination of 0.5 mM cysteine/0.4 mM ascorbate (Barrett, 1978). Of all the reducing agents employed in pharmaceutical preparations, sulfurous acid salts have been used most frequently. These salts include sodium salts of bisulfite, metabisulfite, sulfite, formaldehyde sulfoxylate, thiosulfate and acetone bisulfite (Akers, 1982). Discase® (chymopapain) contains sodium bisulfite as one of the stabilizers. When these sulfurous salts are used, the product label must carry a warning statement, as required by law. Other reducing agents, including ascorbic acid, erythrobic acid, acetylcysteine, thioglycerol, and thioglycolic acid, have been used for aqueous parenteral products. Chymodiactin® (chymopapain) is co-lyophilized with L-cysteinate HCl used as a reducing agent. For use with oil-soluble antioxidants, the choices of reducing agents are ascorbyl palmitate, vitamin E derivatives, propyl gallate, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA), but only propyl gallate has been used in a marketed product, thiothixene HCl (Navane®).

H. Chelating Agents

The most probable use of chelating agents in parenteral formulations is for inhibiting oxidation by removing catalyzing metals, such as copper, iron, calcium, or manganese

TABLE IV. Patents Citing Alcohols as Stabilizers

Stabilizer	Compound	Concn.	Conditions	Inventor/Assignee	Reference*
Ethanol	diastase	40%	+ 10% sorbitol	Sawada/Nippon Shinyaku	CA105:221755P
Mannitol	immunoglobulin			Uemura/Green Cross	CA105:12132u
Mannitol	IgG			Friesen/Winnipeg	CA105:12079g
Sugar alcohol	peroxidase-labeled IgG	20%	+ serum	Jujita/Takeda	CA104:84901w
Mannitol	β-interferon				D83-772523/39
Mannitol	hemoglobin	5%		Ryono/Ajinomoto	CA104:39809m
PEG 8000	enzyme conjugates	3%	+ Ca	Anwis/Abbott	CA104:3135z
Mannitol, inositol	peroxidase-bound antibody		+ sugars	Tsubota/Yatoron	CA103:213222f
PEG 400	Phe-NH_3 lyase	0.19*M*		Kishor/Monsanto	CA103:212450
Polypropylene glycol	ovalbumin, myoglobin, interferon	0.1%		Thurow/Hoechst	CA102:137837x
PEG 4000	horseradish peroxidase	5%		/Toyobo	CA102:127910b
PEG 4000	IgG for IV	0.3%	+ sucrose	Rodolphe/Schweizer	CA99:146103b
Glycerol	interferon	45%	or other polyhydric alcohol	Hasegawa/Sunstar	CA99:76904n
Xylitol, sorbitol	glutathione peroxidase			Kobayashi/Toyobo	CA98:122070s
Propylene/ethylene glycol copolymer	insulin	0.001%	stabilizing against turbidity	Thurow/Hoechst	CA94:90319d

* Key: see Table I.

TABLE V. Patents Citing Carbohydrates as Stabilizers

Stabilizer	Drug	Concn. (%)	Conditions	Inventor/Assignee	Reference*
Sucrose	fibrinogens	4.5	60 °C, 72 hr	Nishimaki/Green Cross	CA107:28382
Sucrose/mannitol and others	hepatitis B vaccines			K. Mizuno	D85-013848/
Glucose	α-interferon			—	D84-292019/
Sugars	IgG			—	D83-811981/
Sugar (maltose)	peroxidase labeled IgG	20		Fujita/Takeda	CA104:84901
Glucose	modified hemoglobin	5		Ryono/Ajinomoto	CA104:39809
Sucrose, trehalose, raffinose	peroxidase-bound antibody			Tsubota/Yatoron	CA103:21322
Sucrose	coagulating factor			Heimburger/Behringwerke	CA103:59289
Glucose, maltose sucrose	immune γ-globulin			Curry/Armour	CA102:17261 US 4,482,483
Glucose	interferon	5		/Sumitomo	CA102:50896
Mono, disaccharides	γ-interferon			Noda/Green Cross	CA102:15479
Galactose	β-galactosidase	0.5		/Mitsui	CA99:154412
Sucrose	IgG for IV	7		Rodolphe/Schweizer	CA99:146103
Arabinose, glucose, sucrose	glutathione peroxidase			Kobayashi/Toyobo	CA98:122070
Saccharides	cold-insoluble globulins	10	amino acid, sugar alcohol, + citrate	Ohmura/Green Cross	CA97:188259
Lactose	sarcosine oxidase	0.2	+ Na glutamate 0.5%	/Noda	CA93:68683a

* Key: See Table I.

that are ubiquitous in trace amounts. In studies of the oxidation rate of the sulfhydryl group in captopril, a quasidipeptide, disodium edetate was the most effective additive in inhibiting the oxidative reaction (Timmins, 1982). Metals have also been reported to catalyze the hydrolytic reaction of chemical bonds of pharmaceutical interest, such as amide and ester hydrolysis (Jencks, 1969), and deiodination (Wang, 1980). Edetate salts (disodium, calcium disodium, or tetrasodium) have been used in injectable products, typically at a concentration of 0.04%. Wydase® (hyaluronidase) contains disodium edetate.

The presence of zinc promotes the polymerization of insulin into dimer, hexamers, etc. (Goldman 1974). Although insulin activity does not depend on the ability of the hormone to bind zinc, the formation of aggregates is an obstacle in the development of long-term insulin delivery system. Chelation of the metal by either tromethamine (TRIS) buffer or edetates (EDTA) delayed the onset of insulin aggregation (Quinn, 1983).

I. Miscellaneous

Numerous publications were cited to illustrate the utility of PVP (polyvinyl pyrrolidone) in biotechnology in a 1985 product brochure prepared by the manufacturer of PVP, GAF. Its use in the stabilization of interferon-β was cited in a European Patent Application (No. 79245) filed in 1983. Enzyme stabilization was categorized as involving three mechanisms: immobilization, complexation, and shielding. PVP may be more useful as a stabilizer in a crude extract, in which PVP may complex with unwanted inactivating compounds, than in a highly purified parenteral formulation. One should be concerned about foreign body granuloma when high molecular weight PVP is used in intramuscular preparations (Farraj, 1987).

Hydrolyzed gelatin is used in a lyophilized corticotropin for injecting (Acthar®), as a bulking substance during lyophilization, or as a matrix to hold minute quantity of protein upon reconstitution. Reports (Wang, 1984) have shown that hydrolyzed gelatin can reduce surface adsorption of insulin.

Ammonium sulfate is routinely used to stabilize protein preparations and there are many reports of its effectiveness, although the mechanism of action is unclear (Wiseman, 1978).

V. Special Considerations

Three topics of particular interest to manufacturers of protein and peptide products are: cryoprotectants, which relates to stability under freezing conditions and lyophilization; aggregate formation, which relates to quality control; and pH, which relates to the overall stability of the product.

A. Cryoprotectants

For several decades, scientists have been searching for vehicles to preserve biological products under freezing conditions. Cryoprotectants are compounds like carbohydrates, alcohols, polyvinylpyrrolidone, and glutamic acid, that lessen the denaturation of proteins when the surrounding water becomes frozen. They have been studied for effectiveness as stabilizers in frozen storage of microorganisms, enzymes, vaccines, organs, erythrocytes, semen, foods, etc.

A protein or peptide product for parenteral use may be frozen during its entire shelf-life to keep it stable or during a freeze-drying cycle if it is to be a lyophilized product, or during transportation and inadvertent exposure to freezing temperatures. A suitable cryoprotectant would undoubtedly be beneficial to the overall stability of a protein or peptide product.

Of many reasons why freezing is detrimental to the stability of a protein, the most widely applicable are (Matsumoto, 1980):

a. Effect of Concentrated Salt: After most of the water becomes frozen, salt concentration in the interstitial unfrozen liquid increases to a detrimental level that will accelerate denaturation of the protein. Soliman (1971a) demonstrated that, at −10 °C, the concentration of sodium chloride (liquid phase) in equilibrium with ice is 3*M*. Loss of enzyme activity was proportional to salt concentration at subzero temperatures. If buffer species are present in the formulation, the pH of the unfrozen liquid may shift away from its optimal value; as a result, denaturation or precipitation of protein, and accelerated hydrolysis of peptide bonds may occur. Larsen (1973) provided extensive data on the effect of freezing upon pH for a number of pharmaceutical buffer systems. For example, the pH for a 0.1*M* sodium phosphate buffer is 7.4 at 22 °C, but only 3.7 at −10 °C. Addition of sodium chloride can reduce the magnitude of change, i.e., for the same system with 0.4*M* sodium chloride the pH is 4.7 at −10 °C.

b. Aggregation of Protein: When a solution is frozen and water molecules freeze out, the protein molecules come closer to each other, resulting in intermolecular crosslinkages. The α-helical structure remains intact and undergoes little or no unfolding. Rehydration of these aggregates cannot achieve complete renaturation. A cryoprotectant molecule may bind or associate with protein molecules at one or more functional sites. Thus, protein molecules are partially "coated" with cryoprotectant and this coating prevents protein from reacting and aggregating.

c. Unfolding of Protein: The native conformation is held together by a balance between two forces, hydrophobic bonding of hydrophobic side chains, which does not require the presence of water, and hydrophilic bonding, such as hydrogen bonds and salt bridges, which does require water as a medium. When water is removed during the freezing process, hydrophobic bonding becomes predominant, hydrophilic bonding is unable to hold the native conformation, and the protein becomes unfolded. It is generally accepted that, after lyophilization, an optimal amount of residual moisture must be left associated with the freeze-dried protein, that amount should be sufficient to maintain the conformation of protein, but not enough to support chemical reactions that might modify the protein structure (Greiff, 1971). Freeze-drying is generally thought to be more disruptive to enzyme function than

freeze-thawing or thermally induced perturbations (Crowe, 1987). Unless stability is known, it is prudent to store a concentrated protein drug at 5 °C or at freezing temperature, instead of in its freeze-dried form. If the protein concentrate is to be frozen and stored for a long period until further processing, proper selection of a buffer system is extremely important.

For a freeze-dried product, one may consider using lactose, mannitol, sodium glutamate, PVP, glycine, and albumin as cryoprotectants. Zinc and sugars (trehalose, maltose and sucrose) synergetically stabilized the freeze-drying destruction of phosphofructokinase, the most dehydration-sensitive enzyme tested to date (Crowe, 1987). Much wider selections are available for a liquid product: to the list just cited for freeze-dried products one may add polyethylene glycols (Soliman, 1971) and glycerol (Greaves, 1965).

B. Aggregate Formation

Aggregation in a protein solution is a phenomenom in which the protein molecules form aggregates ranging from 0.1 to 100 micrometers or greater and precipitate out of solution. In package inserts for asparaginase, Atgam®, and Orthoclone OKT® 3, the aggregates are described as "gelatinous fiber-like particles," "granular or flaky deposits," and "fine translucent particles," respectively. In some cases, aggregation is merely a nuisance because the potency of the product is not affected; the aggregates 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, however, 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 (Ochs, 1980).

Aggregation is typically caused either by shaking or by heat denaturation. The mechanism of heat-induced aggregation is straightforward; according to Habeeb (1977), aggregation is caused by oxidation of sulfhydryl groups to form intermolecular disulfide bridges. Aggregation was effectively prevented by blocking the sulfhydryl groups with iodoacetamide before heating. To avoid heat-induced aggregation, one can simply keep the protein solution refrigerated. Shaking-induced aggregation, however, remains a constant challenge to a formulator, because shaking is unavoidable during manufacturing and shipping.

Aggregaion of insulin has received the most attention, because the tendency of insulin solutions to form aggregates is a fundamental obstacle to the development of long-term insulin delivery systems. Blockage by aggregates necessitates that any device containing the solution be flushed frequently. This inconvenience, as well as a desire to characterize the aggregation and adsorption of insulin, prompted researchers to seek a physiologically acceptable solvent and additives that would stabilize the insulin solutions. Quinn (1983) examined a number of amino acids and other additives and found that aspartic acid, glutamic acid, EDTA, lysine, and tromethamine improved the solubility of insulin and, perhaps, even inhibited the aggregation of the dissolved insulin. Albisser (1980) described a suspension of crystalline insulin and amorphous aggregates, 1-100 micrometers in diameter, that blocked catheters. They promoted the use of 1.5% autologous serum in the aqueous diluent for insulin to minimize aggregation and to achieve continuous uninterrupted intravenous insulin infusion. The same group (Lougheed, 1983) later examined a large number of surfactants and various injectable additives, such as benzyl alcohol, glycerol, and polyethylene glycol. It is noteworthy that in the motion test they employed, crystalline zinc insulin, 5 μg/mL formed turbid gels in 5 days. Surfactants at or near their critical micelle concentration were most effective in stabilizing insulin against the effect of continuous rotation at 37 °C. Glycerol at 5-50% and isopropanol at 10-50% were moderately effective, but the degree of stabilization was less than that seen with the surfactant group as a whole.

Most recently, Massey (1986) reported the development of aggregation-resistant insulin formulations. They reported that: (a) human insulin aggregates more readily than does pork or beef insulin; (b) ionic ingredients and phenolic preservatives accelerate aggregation; (c) zinc insulin is more stable than are zinc-free formulations; and (d) nonionic surfactants appear to be promising stabilizers.

The ease of coagulation under shaking for a number of proteins was studied by Henson (1970). Ovalbumin is especially susceptible to shaking-induced aggregation and β-lactoglobulin, globulin, and hemoglobin also aggregate readily at the air-water interface.

Many mechanisms can contribute to the occurrence of aggregation, probably in the following order of likelihood:

(a) Conformation changes as protein molecules are adsorbed to the air-water interface, consequently, denatured protein molecules aggregate at the interface (MacRitchie, 1978).
(b) 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 completed the process of irreversible denaturation.
(c) Disulfide bonds are exchanged intermolecularly.
(d) Protein molecules associate among themselves in bulk.

Knowledge of the mechanisms for aggregation makes it easier to understand the stabilization observed. Molecules of the surfactants composed of a hydrophilic end group and long hydrocarbon moieties, form a hydrophobic core of micelles in which the protein molecule can be sheltered from interaction with another protein molecule. The presence of surfactant also reduces the concentration of protein at the interface.

Denaturation by mechanical stress is a phenomenon closely related to aggregation. Inactivation of interferon by mechanical stress was demonstrated by shearing the product for different amounts of time or between different widths of a homogenizer gap (Cartwright, 1977). Shear-induced inactivation was prevented by the addition of 0.1% polysorbate 80 or of a reducing agent, DL-thioctic acid (Sedmak, 1977). These observations demonstrated the similarity of shear inactivation and surface aggregation.

Methodologies to study aggregation vary widely. Henson (1970) employed a nephelometer to measure the relative degree of turbidity and electron microscopy (80,000×) to examine the morphology of the aggregates. Lougheed (1983) used visual examination and transmittance at 540 nm by a spectrophotometer. Busby used light scattering to demonstrate that irreversible aggregation resulted from thermal transition of human complement C1 (Busby 1987). It would be interesting to see in the future, methods developed that use the light-obscuring principle, which is currently the compendial method described in the US Pharmacopeia, or a laser beam to measure the number and sizes of particulate matter.

C. 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 overall degradation reactions are minimal. Many peptides are formulated at a slightly acidic pH: the pH of oxytocin injection is in the range of 2.5–4.5; that of desmopressin injection is 3.5; and nafrelin is most stable at pH 5 (Johnson, 1986). At a low pH, one must be concerned about deamidation of asparagine and glutamine and general hydrolysis of the peptide backbone and glycosides. On the other hand, at high pH, one must be concerned about a number of potential degradations, e.g., oxidation of cysteine, methionine, and tryptophan, 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. The complexity of degradation mechanisms at neutral or high pH probably explains why a degradation rate-pH profile does not have a simple slope of unity, as seen in the case of nafrelin (Fig. 6).

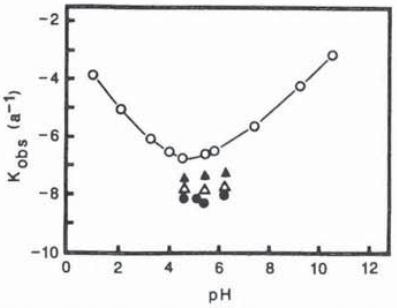


Figure 6—Rate (K_{obs})-pH profile for the apparent degradation of nafarelin solution at 80 °C (●) 60 °C (△), 50 °C (▲), and 40 °C (○) (Johnson, 1984).

For a more complex protein, where conformation stability is a primary concern, product pH is often set at the isoelectric point. Presumably, at the isoelectric point, zero net charge should allow maximum interaction between salt bridges (ion pairs) and exert least interaction between protein molecules. The pH of globulin products is close to neutrality, 7.4 for Orthoclone OKT® 3 and 6.6 for Sandoglobulin®. However, a new immunoglobin for intravenous use (Gamimune® N) was formulated at pH 4.25 for reasons of stability. As described in a patent (Tenold, 1983), immunoglobin was rendered more stable and with less anticomplement activity simply by adjusting pH to 4.25 with hydrochloric acid.

VI. Recommendations

At the onset of formulation development, formulators frequently come to a crossroad, facing the choice between a lyophilized dosage form and a solution. If a protein or peptide is sufficiently stable in solution, obviously, the solution form is preferable. However, lacking long-term stability data and having to rely on untrustworthy stability predictions from stressed samples, undertaking the development of a solution form can be a risky decision. A lyophilized form, in general, offers the advantages of being a robust product in terms of storage and transportation. However, developing a reliable freeze-drying cycle and creating a favorable condition for renaturation of protein are not easy tasks. In addition to these technical concerns, formulations also have to be based on the preference in dosage forms of practitioners, i.e., physician, pharmacist, or nurse. Perhaps scientists' attention should compare the data on unit sales (IMS, 1986) for a specific protein or peptide that appears in both lyophilized and solution forms. For example, α-interferon in both Intron®-A (lyophilized) and Roferon®-A (solution and lyophilized) and Wydase (hyaluronidase) are sold in both lyophilized and "stabilized solution" forms. This issue of market preference should be treated with caution, because pricing can easily swing the direction of preference.

In general, for nonprotein drugs, formulators tend to keep the number of excipients in an injectable formulation to a minimum. Different approaches may be required for a protein drug. In view of the stability of protein, a diluted aqueous solution may be a hostile environment. An enzyme in diluted state may be subject to dissociation into subunits that are inactive or, even if active, prone to proteolytic denaturation. One should always create a native environment for a purified enzyme or proteinaceous material. The essential features of a native environment 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. NMR studies on the water of cells indicate that much of the water may not be freely mobile (Scopes, 1982). It is only rational to expect that proteins have evolved to be stable in these conditions, rather than in the artificial solution commonly used for parenteral formulations. Indeed, many scientists have taken this ap-

TABLE VI. Patents Employing Multiple Stabilizers

Protein	List of Stabilizers	Inventor/Assignee	Reference*
γ-globulin	pyrrolidone, glycol, sugar, polyvinyl alcohol	Dickinson/Preco	CA105:85180t
Immunoglobulin	glycine, NaCl, NaOAc, PEG albumin, mannitol	Uemura/Green Cross	CA105:121320
Erythropoietin	glycine + albumin + mannitol, dextran + phosphate salts, glutathione + albumin + glucose	Kawaguchi/Chugai	CA104:230446
Antibody to human chorionic gonadotropin	glycine 1% + BSA 1% + mannitol 10% sucrose 5%	/Takeda	CA101:128737s
γ-globulin	albumin + glucose + glycine + PEG 4000 + NaCl	Hooper/Baxter	CA100:215500g
Plasminogen-activating enzyme	PEG-3-sorbitan oleate + hydrolyzed gelatin + dextrin + mannitol + glycine	/Toryo	CA100:197765r

* Key: see Table I.

proach in their effort to stabilize protein products. Table VI lists several patents that utilize numerous ingredients in parenteral products. However, as the mechanisms of denaturation and of stabilization become better understood, and methodologies for studying these phenomena improve, it should become possible to selectively limit the number of excipients needed in a formulation.

If there are any concerns about using a high concentration of stabilizer in the formulation, they would be hemolysis when injected intravenously (Fort, 1984; Yalkowsky, 1987), and potential irritation at the injection site. Glycerol and other solvents, or a hypertonic vehicle, cause pain when injected subcutaneously or intramuscularly. This shortcoming can be overcome by diluting the product with water for injection or with physiological saline at the time of use. The diluted solution, in greater volume, can be administered by intravenous intermittent therapy or pumped subcutaneously over an extended period of time. Other drawbacks of a parenteral product that contains too many stabilizers are the difficulty in developing lyophilization cycles and interference by albumin or surfactants with quality control testing.

All the injectable vehicles discussed thus far are aqueous in nature, but there are other avenues to the creation of stable, elegant parenteral products. Oleaginous vehicles, e.g., sesame oil or medium-chain triglycerides, would be feasible for hydrophobic peptides; these vehicles offer stability (no hydrolysis), solubility, and a depot effect for slow release. An example is vasopressin tannate in peanut oil manufactured by Parke-Davis. Peptides impregnated in biodegradable polymers for intramuscular injection are being tested in clinical studies (Sanders, 1982). Potent peptides and proteins are ideal for liposome systems that usually carry a limited payload.

TABLE VII. Books and Chapters on Stability of Proteins

Author	Title	Source
M. Joly	A Physico-chemical Approach to the Denaturation of Proteins	Academic Press (1965)
C. Tanford	Protein Denaturation	Adv. Protein Chem., **23,** 122–281 (1968); **24,** 2–96 (1969)
S. Timasheff and G. Fasman, eds.	Structure and Stability of Biological Macromolecules	Marcel Dekker (1969)
C. Pace	The Stability of Globular Proteins	Crit. Rev. Biochem., **3,** 1–43 (1975)
A. Habeeb	Influence of Conformation on Immunochemical Properties of Proteins	Chapt. 2 in "Immunochemistry of Proteins," **1,** 174–183, M. Atassi, ed., Plenum Press (1977)
S. Lapanje	Physicochemical Aspects of Protein Denaturation	Wiley-Interscience (1978)
A. Wiseman	Stabilization of Enzymes	Chapt. 6 in "Topics in Enzyme and Fermentation," Biotechnol., **2,** 280–303 (1978)
R. Schmid	Stabilized Soluble Enzymes	Adv. Biochem. Engineering **12,** 41–118 (1979)
P. Privalov	Stability of Proteins, Small Globular Proteins	Adv. Protein Chem., **33,** 167–241 (1979)
C. Ghelis and J. Yon	Protein Folding	Academic Press (1982)
A. Klibanov	Stabilization of Enzymes against Thermal Inactivation	Adv. Appl. Microbiol., **29,** 1–28 (1983)
A. Laskin, G. Tsao, and L. Wingard, eds.	Stability and Stabilization of Enzymes	Ann. NY Acad. Sci., **434,** 1–63 (1984)

In closing, this article was intended to provide formulators of parenteral product with an overview of the stability aspects of proteins and peptides and a general discussion of how stabilizers may be utilized. Although the list of original articles is certainly not complete, it is hoped that the citation of pertinent examples of stabilizers for parenteral products has been adequate. In-depth discussions of protein stability can be found in the review articles and textbooks listed in Table VII.

VII. References

Information related to marketed products, such as concentration of excipients, pH, storage condition, was obtained from the 1987 editions of "Drug Information™ " (American Society of Hospital Pharmacists, Inc., Bethesda, MD) and of "Physicians' Desk Reference® " (Medical Economics Co., Oradell, NJ) both published annually. For some products, information was listed only in early editions of these two sources.

Adamson, A. W., *Physical Chemistry of Surfaces*, 2nd ed., Interscience Publishers, NY, 1960, p. 174.

Ahmed, A. K., Schaffer, S. W., and Wetlaufer, D. B., "Nonenzymatic reaction of reduced bovine pancreatic ribonuclease by air oxidation and by glutathione oxidoreduction buffers," *J. Biol. Chem.*, **250,** 8477 (1975).

Akers, M. J., "Antioxidants in pharmaceutical products," *J. Parenter. Sci. Tech.*, **36,** 222 (1982).

Albisser, A. M., Lougheed, W. D., Perlman, K., and Bahoric, A., "Non-aggregating insulin solutions for long-term glucose control in experimental and human diabetes," *Diabetes*, **29,** 241 (1980).

Andersson, E., and Hahn-Hagerdal, B., "Enzyme action in polymer and salt solutions. I. Stability of penicillin acylase in poly(ethylene glycol) and potassium phosphate solutions in relation to water activity," *Biochim. Biophys. Acta*, **912,** 317 (1987).

Andersson, L., and Brown, J. E., "Interaction of Factor VIII–von Willebrand Factor with phospholipid vesicles," *Biochem. J.*, **200,** 161 (1981).

Arakawa, T., and Timasheff, S. N., "Stabilization of protein structure by sugars," *Biochem.*, **21,** 6536 (1982).

Back, J., Oakenfull, D., and Smith, M. B., "Increased thermal stability of proteins with the presence of sugars and polyols," *Biochem.*, **18,** 5191 (1979).

Ballowitz, L., Schmid, J., Siegert, M., and Steffen, B., "Effects of stabilizers in preparations of human serum albumin on the serum bilirubin in Gunn rats," *Acta Paediatr. Scand.*, **67,** 505 (1978).

Barrett, P. Q. and Neuman W. F., "The cleavage and absorption of parathyroid hormone at high dilution," *Biochim. Biophys. Acta*, **541,** 223 (1978).

Bouvette, R. E., and Digenis, G. A., "The pH dependent hydrolysis and intramolecular aminolysis of L-aspartyl-L-phenylalanine methyl ester," *Pharm. Res.*, **4,** 25S (1987).

Boyer, P. D., Lum, F. G., Ballow, G. A., Luck, J. M., and Rice, R. G., "The combination of fatty acids and related compounds with serum albumin. I. Stabilization against heat denaturation," *J. Biol. Chem.*, **162,** 181, (1946).

Brems, D. N., and Baldwin, R. L., "Amide proton exchange used to monitor the formulation of a stable α-helix by residue 3 to 13 during folding of ribonuclease S.," *J. Mol. Biol.*, **180,** 1141 (1984).

Bringer, J., Heldt, A., and Grodsky, G. M., "Prevention of insulin aggregation by dicarboxylic amino acids during prolonged infusion," *Diabetes*, **30,** 83 (1981).

Broden, K., Brown, J. E., Carton, C., and Andersson, L., "Effect of phospholipid on Factor VIII coagulant activity and coagulant antigen," *Thromb. Res.*, **30,** 651 (1983).

Bull, H. B., and Breese, K., "Interaction of alcohols with proteins," *Biopolymers*, **17,** 2121 (1978).

Busby, T. F., Atha, D. H., and Ingham, K. C., "Thermal denaturation of Antithrombin III, stabilization by heparin and lyotropic anions," *J. Biol. Chem.*, **256,** 12140 (1981).

Busby, T. F., and Ingham, K. C., "Thermal stabilization of Antithrombin III by sugars and sugar derivatives and the effects of nonenzymatic glycosylation," *Biochim. Biophys. Acta*, **799,** 80 (1984).

Busby, T. F., and Ingham, K. C., "Calcium sensitive thermal transitions and domain structure of human complement subcomponent C1r," *Biochem.*, **26,** 5564 (1987).

Cartwright, T., Senussi, O., and Grady, M. D., "The mechanism of the inactivation of human fibroblast interferon by mechanical stress," *J. Gen. Virol.*, **36,** 317 (1977).

Coan, C. R., Hinman, L. M., and Deranleau, D. A., "Charge-transfer studies on the availability of aromatic side chains of proteins in guanidine hydrochloride," *Biochem.*, **14,** 4421 (1975).

Coan, M. H., and Mitra, G., "Stabilization of alpha-1-proteinase inhibitor by citrate," *Vox Sang.*, **46,** 142 (1984).

Coval, M. L., "Injectable gamma globulin," U.S. Patent, 4,165,370 (1979).

Coval, M. L., "Method of producing intravenously injectable gamma globulin," U.S. Patent, 4,124,576 (1978).

Crowe, J. H., Crowe, L. M., Carpenter, J. F., and Aurell-Wistrom, C., "Stabilization of dry phospholipid bilayers and proteins by sugars," *Biochem. J.*, **242,** 1 (1987).

Farraj, N. F., Davis, S. S., Parr, G. D., and Withington, R., "A new intramuscular formulation of rifamycin SV," *Int. J. Pharm.*, **38,** 39 (1987).

Fernandes, P. M., and Lundblad, J. L., "Preparation of a stable intravenous gamma globulin: Process design and scale-up," *Vox Sang.*, **39,** 101 (1980).

Fernandes, P. M., and Lundblad, J. L., "Pasteurized therapeutically active protein compositions," European Patent 35204 (1981).

Fort, F. L., Heyman, I. A., and Kesterson, J. W., "Hemolysis study of aqueous ployethylene glycol 400, propylene glycol and ethanol combinations in vivo and in vitro," *J. Parenter. Sci Technol.*, **38,** 82 (1984).

Fujimoto, S., Nakagawa, T., Ishimitsu, S., and Ohara, A. "On the mechanism of inactivation of papain by bisulfite," *Chem. Pharm. Bull.*, **31,** 992 (1983).

Fujita, Y., and Noda, Y., "Effect of hydration on the thermal stability of protein as measured by DSC; chymotrypsinogen A," *Int. J. Pept. Protein Res.*, **18,** 12 (1981).

Fukushima, T., Matsunaga, T., and Funakoshi, S., "Process for heat treatment of aqueous solution containing human blood coagulation factor XIII," European Patent 37078 (1981).

GAF Corporation, "Biotechnology, applications of polyvinylpyrrolidone and polyvinylpolypyrrolidone," Wayne, NJ, 1985.

Galat, A., Yang, C-C, and Blout, E. R., "Circular dichroism study of the unfolding refolding of a cardiotoxin from Taiwan cobra venom," *Biochem.*, **24,** 5678 (1985).

Geiger, T., and Clarke, S., "Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides," *J. Biol. Chem.*, **262,** 785 (1987).

Gekko, K., "Calorimetric study of thermal denaturation of lysozyme in polyol-water mixtures," *J. Biochem.*, **91,** 1197 (1982).

Gekko, K., and Timasheff, S. N., "Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures," *Biochem.*, **20,** 4667 (1981).

Gekko, K., and Timasheff, S. N., "Thermodynamic and kinetic examination of protein stabilization by glycerol," *Biochem.*, **20,** 4677 (1981).

Gerlsma, S. Y., "Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and some other additives," *J. Biol. Chem.*, **243,** 957 (1968).

Gerlsma, S. Y., "The effect of polyhydric and monohydric alcohols on the heat induced reversible denaturation of chymotrypsinogen A," *Eur. J. Biochem.*, **14,** 150 (1970).

Gerlsma, S. Y., and Sturr, E. R., "The effect of polyhydric and monohydric alcohols on the heat-induced reversible denaturation of lysozyme and ribonuclease," *Int. J. Pept. Protein Res.*, **4,** 377 (1972).

Gibbs, R. J., Timasheff, S. N., and Nord, F. F., "Investigations on proteins and polymers, IX. Denaturation studies of egg albumin with aliphatic amines," *Arch. Biochem. Biophys.*, **40,** 85 (1952).

Gilbert, J. B., Price, V. E., and Greenstein, J. P., "Effect of anions on the non-enzymatic desamidation of glutamine," *J. Biol. Chem.*, **80,** 209 (1949).

Goldenberg, D. P., and Creighton, T. E., "Review, gel electrophoresis in studies of protein conformation and folding," *Anal. Biochem.*, **138,** 1 (1984).

Goldman, J., and Carpenter, F. H., "Zinc binding, CD and equilibrium sedimentation studies on insulin and several of its derivatives," *Biochem.*, **13,** 4566 (1974).

Gomez, J. E., Birnbaum, E. R., Royer, G. P., and Darnal, D. W., "The effect of calcium ion on the urea denaturation of immobilized bovine trypsin," *Biochim. Biophys. Acta*, **495,** 177 (1977).

Graf, L., Bajusz, S., Patthy, A., Barat, E., and Cseh, G., "Revised amide location for porcine and human adrenocorticotropic hormone," *Acta Biochim. Biophys. Acad. Sci. Hung.*, **6,** 415 (1971).

Gratzer, W. B., and Beaven, G. H., "Effect of protein denaturation on micelle stability," *J. Phys. Chem.*, **73,** 2270 (1969).

Greaves, R. I. N., and Davies, J. D., "Separate effects of freezing,

thawing and drying living cells," *Ann. NY Acad. Sci.*, **125,** 548 (1965).
Greiff, D., "Protein structure and freeze-drying: The effects of residual moisture and gases," *Cryobiol.*, **8,** 145 (1971).
Habeeb, A. (1977), see Table VII.
Hagerman, P. J., and Baldwin, R. L., "A quantitative treatment of the kinetics of the folding transition of ribonuclease A," *Biochem.*, **15,** 1462 (1976).
Hammel, E. F., and Glasstone, S., "Physicochemical studies of the simpler polypeptides. III. The acid and base catalyzed hydrolysis of di-, tri-, tetra-, penta-, and hexaglycine," *J. Am. Chem. Soc.*, **76,** 3741 (1954).
Harris, J. I., Cole, R. D., and Pon, N. G., "The kinetics of acid hydrolysis of dipeptides," *Biochem. J.*, **62,** 154 (1956).
Henson, A. F., Mitchell, J. R., and Musselwhite, P. R., "The surface coagulation of proteins during shaking," *J. Colloid. Interface Sci.*, **32,** 162 (1970).
Hill, R. L., "Hydrolysis of protein," *Adv. Protein Chem.*, **20,** 37 (1965).
Holt, L. A., Milligan, B., Rivett, D. E., and Steward, F. H. C., "The photodecomposition of tryptophan peptides," *Biochim. Biophys. Acta.*, **499,** 131 (1977).
Horne, R. A., Almeida, J. P., Day, A. F., and Yu, N., "Macromolecule hydration and the effect of solutes on the cloud point of aqueous solutions of polyvinyl methylether: A possible model for protein denaturation and temperature control in homeothermic animals," *J. Colloid Interface, Sci.*, **35,** 77 (1971).
Hsu, H., Ozeki, S., and Watanabe, J., "Effects of pH, albumin and urate on inactivation profile of rabbit muscle creatine phosphokinase," *Chem. Pharm. Bull.*, **30,** 1002 (1982).
IMS "U.S. Pharmaceutical Market," a periodical published by IMS America Ltd., Ambler, PA, 1986.
Jencks, W. P., *Catalysis in Chemistry and Enzymology*, McGraw-Hill, NY, 1969.
Johnson, D. M., Pritchard, R. A., Taylor, W. F., Conley, D., Zyniga, G., and McGreevy, K. G., "Degradation of the LH-RH analog nafarelin acetate in aqueous solution," *Int. J. Pharm.*, **31,** 125 (1986).
Joly, M., (1965), see Table VII.
Kenney, W. C., Watson, E., Bartley, T., Boone, T., and Altrock, B. W., "Parameters for the evaluation of IL-2 stability," *Lymph. Res.*, **5,** 23 (1986).
Labrude, P., Chaillot, B., Bonneaux, F., and Vigneron, C., "Freeze-drying haemoglobin in the presence of carbohydrates," *J. Pharm. Pharmacol.*, **32,** 588 (1980).
Lajmanovich, A., Hudry-Clergeon, G., Freyssinet, J., and Marguerie, G., "Human factor VIII procoagulant activity and phospholipid interaction," *Biochim. Biophys. Acta*, **678,** 132 (1981).
Larsen, S. S., "Studies on stability of drugs in frozen systems," *Arch. Pharm. Chem. Sci. Ed.*, **1,** 43 (1973).
Law, W. M., Nissenson, R. A., and Heath, H., "Preparation of synthetic bovine parathyroid hormone fragment 1-34 for parenteral use in human studies," *J. Clin. Endocrinol. Metab.*, **56,** 1335 (1983).
Lee, J. C., and Timasheff, S. N., "The stabilization of proteins by sucrose," *J. Biol. Chem.*, **256,** 7193 (1981).
Light, A., and Higaki, J. N., "Detection of intermediate species in the refolding of bovine trypsinogen," *Biochem.*, **26,** 5556 (1987).
Lollar, P., Knutson, G. J., and Fass, D. N., "Stabilization of thrombin-activated porcine Factor VIII:C by Factor IXa and phospholipid," *Blood*, **63,** 1303 (1984).
Lougheed, W. D., Albisser, A. M., Martindale, H. M., Chow, J. C., and Clement, J. R., "Physical stability of insulin formulations," *Diabetes*, **32,** 424 (1983).
Lundblad, J. L., Warner, W. L., and Fernandes, P. M., "Stabilized Immune Serum Globulin," U.S. Patent 4,186,192 (1980).
MacRitchie, F., "Proteins at interfaces," *Adv. Protein Chem.*, **32,** 283 (1978).
Marguerie, G., "The binding of calcium to fibrinogen: Some structural features," *Biochim. Biophys. Acta*, **494,** 172 (1977).
Markus, G., and Karush, F., "Structural effects of the interaction of human serum albumin with sodium decyl sulfate," *J. Am. Chem. Soc.*, **79,** 3264 (1957).
Matsumoto, J. J., "Chemical deterioration of muscle proteins during frozen storage," in *Chemical Deterioration of Proteins*, ACS Symposium Series 123, American Chemical Society (1980).
Massey, E. H., and Sheliga, T. A., "Development of aggregation resistant insulin formulation," *Chem. Abstr.*, **104,** 193185m (1986).
Masters, P. A., and Friedman, M., "Amino acid racemization in alkali-treated food proteins," in *Chemical Deterioration of Proteins*, ACS Symposium Series 123, American Chemical Society (1980).
McKerrow, J. H., and Robinson, A. B., "De-amidation of asparaginyl residues as a hazard in experimental protein and peptide procedures," *Anal. Biochem.*, **42,** 565 (1971).
Miekka, S. I., Busby, T. F., Tarshis, L., Forastieri, H., and Ingham, K. C., "Biological and physical properties of fibronectin pasteurized in the presence of stabilizers," *Vox Sang.*, **48,** 284 (1985).
Mikaelsson, M. E., Forsman, N., and Oswaldsson, U. M., "Human Factor VIII: A calcium-linked protein complex," *Blood*, **62,** 1006 (1983).
Miller, A. W., and Robyt, J. F., "Stabilization of Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by nonionic detergents, poly(ethylene glycol) and high-molecular-weight dextran," *Biochim. Biophys. Acta*, **785,** 89 (1984).
Mizutani, T., "Adsorption of protein on silicone-coated glass surface," *J. Colloid Interface Sci.*, **82,** 162 (1981).
Miwa, N., Obata, Y., and Suzuki, A., "Comparative studies on two active enzyme forms of human urinary urokinase II: pH- and heat-stabilities of plasminogen activator activity," *Chem. Pharm. Bull.*, **29,** 472 (1981).
Mogensen, K. E., and Cantell, K., "Human leukocyte interferon, role for disulfide bonds," *J. Gen. Virol.*, **22,** 95 (1974).
Monsan, P., and Combes, D., "Effect of water activity on enzyme action and stability," in *Enzyme Engineering 7*, Laskin, A. I., et al. eds., *Annal NY Acad. Sci.*, **434,** 48 (1984).
Moss, J., Osborne, J. C., and Stanley, S. J., "Activation of an erythrocyte NAD: arginine ADP-ribosyltransferase by lysolecithin and nonionic and zwitterionic detergents," *Biochem.*, **23,** 1353 (1984).
Nishi, K., Ito, H., and Shinagawa, S., "Racemization of LH-RH analogs in alkaline solution," in *Peptide Chemistry*, H. Yonehara, ed., Protein Research Foundation, Osaka, Japan, 1980, p. 175.
Oakenfull, D., and Fenwick, D. E., "Thermodynamics and mechanism of hydrophobic interaction," *Aust. J. Chem.*, **30,** 741 (1977).
Ochs, H. D., et al., "Safety and patient acceptability of intravenous immune globulin in 10% maltose," *Lancet*, 1158 (1980).
Pappenhagen, A. R., Lundblad, J. L., and Schroeder, D. D., "Pharmaceutical Compositions Comprising Intravenously Injectable Modified Serum Globulin, Its Production and Use," U.S. Patent, 3,903,262 (1975).
Peters, T., "Serum albumin," *Adv. Protein Chem.*, **37,** 161 (1985).
Quinn, R., and Andrade, J. D., "Minimizing the aggregation of neutral insulin solutions," *J. Pharm. Sci.*, **72,** 1472 (1983).
Rao, G. N., Sutherland, J. W., and Menon, G. N., "HPLC assay for thyrotropin release hormone and benzyl alcohol in injection formulation," *Pharm. Res.*, **4,** 38(1977).
Robinson, A. B., Scotchler, J. W., and McKerrow, J. H., "Rates of nonenzymatic deamidation of glutaminyl and asparaginyl residues in pentapeptides," *J. Am. Chem. Soc.*, **95,** 8156(1973).
Ross, P. D., "A scanning microcalorimeter for thermally induced transitions in solution," *Thermochimica Acta*, **10,** 143 (1974).
Ross, P. D., Finlayson, J. S., and Shrake, A., "Thermal stability of human albumin measured by differential scanning calorimetry. II. Effects of isomers of N-acetyltryptophanate and tryptophanate, pH, reheating, and dimerization," *Vox Sang.*, **47,** 19 (1984).
Ruwart, M. J., and Suelter, C. H., "Activation of yeast pyruvate kinase by natural and artificial cryoprotectants," *J. Biol. Chem.*, **246,** 5990 (1971).
Rytting, J. H., "Insulin," in *Chemical Stability of Pharmaceuticals*, 2nd ed., Connors, K. A., Amidon, G. L., and Stella, V. J., eds., John Wiley, NY, 1986.
Sanders, L. M., et al., "Controlled release of an LHRH analog from injected polymeric microcapsules: formulation characteristics," *Contracep. Delivery Syst.*, **3,** 60 (1982).
Saxena, V. O., and Wetlaufer, D. B., "Formation of three-dimension structure in proteins. I, Rapid nonenzymatic reactivation of reduced lysozyme," *Biochem.*, **9,** 5015 (1970).
Sceppa, J. M., "Total parenteral nutrition," in *Handbook of Drug Therapy*, Miller, R. R., and Greenblatt, D. J., eds., Elsevier, NY, 1979.
Schultz, J., Allison, H., and Grice, M., "Specificity of the cleavage of proteins by dilute acid. I. Release of aspartic acid from insulin, ribonuclease and glucagon," *Biochem.*, **1,** 694 (1961).
Scopes, R. K., *Protein Purification*, Springer-Verlag, NY, 1982, p. 199.
Sedmak, J. J., and Grossberg, S. E., "Stabilization of interferons," Texas Reports on Biology and Medicine, **35,** 198 (1977).
Shrake, A., Finlayson, J. S., and Ross, P. D., "Thermal stability of human albumin measured by differential scanning calorimetry. I. Effects of caprylate and N-acetyltryptophanate," *Vox Sang.*, **47,** 7 (1984).
Siegel, F. L., "Calcium-binding proteins," in *Structure and Bonding*, Dunitz, J., et al., eds. Vol. 17, Springer, NY, 1973, p. 221-268.
Sigel, H., and Martin, B., "Coordinating properties of the amide bond. Stability and structure of metal ion complexes of peptides and related

ligands," *Chem. Rev.*, **82,** 385 (1982).
Sinha, N. K., and Light, A., "Refolding of reduced, denatured trypsinogen and trypsin immobilized on agarose beads," *J. Biol. Chem.*, **250,** 8624 (1975).
Soliman, F. S., and Van den Berg, L., "Factors affecting freezing damage of lactic dehydrogenase," *Cryobiol.*, **8,** 73 (1971a).
Soliman, F. S., and Van den Berg, L., "Factors affecting freeze aggregation of lipoprotein," *Cryobiol.*, **8,** 265 (1971b).
Sternson, L. A., and Shaffer, R. D., "Kinetics of digoxin stability in aqueous solution," *J. Pharm. Sci.*, **67,** 327 (1978).
Stewart, W. E., Somer, P. D., and Clercq, E. D., "Protective effect of anionic detergents on interferons: reversible denaturation," *Biochim. Biophys. Acta*, **359,** 364 (1974).
Stewart, W. E., et al., "Distinct molecular species of human interferons: requirements for stabilization and reactivation of human leukocyte and fibroblast interferons," *J. Gen. Virol.*, **26,** 327 (1975).
Takeda, Y., and Hizukuri, S., "Effect of Triton X-100 on sweet potato β-amylase," *Biochim. Biophys. Acta*, **268,** 175 (1972).
Takagi, T., and Matsuo, T., "Gamma-globulin preparation for intravenous administration, process for production thereof and process for preparation of gamma-globulin low anticomplementary activity," European Patent 25719 (1980).
Tenold, R. A., "Intravenously injectable immune serum globulin," U.S. Patent 4,396,608 (1983).
Timmins, P., Jackson, I. M., and Wang, Y. J., "Factors affecting captopril stability in aqueous solution," *Int. J. Pharm.*, **11,** 329 (1982).
Uedaira, H., and Uedaira, H., "The effect of sugars on the thermal denaturation of lysozyme," *Bull. Chem. Soc. Jpn.*, **53,** 2451 (1980).
Walker, G. J., and Whelan, W. J., "The mechanisms of carbohydrate action: 8. Structures of the muscle-phosphorylase limit dextrins of glycogen and amylopectin," *Biochem. J.*, **76,** 264 (1960).
Wang, Y. J., "Deiodination kinetics of water-soluble radiopaques," *J. Pharm. Sci.*, **69,** 671 (1980).
Wang, Y. J., and Kowal, R. B., "Review of excipients and pH's for parenteral products used in the United States," *J. Parenter. Drug Assn.*, **34,** 452 (1980).
Wang, Y. J., and Chien, Y. W., "Sterile Pharmaceutical Packaging: Compatibility and Stability," Parenteral Drug Association, Inc., Philadelphia, 1984.
Weiss, H. J., "A study of the cation- and pH-dependent stability of Factors V and VIII in plasma," *Thromb. Diath. Haemorrh.*, **14,** 32 (1965).
Winterer, J., Chatterji, D., Comite, F., Decker, M., Loriaux, D., Gallelli, J., and Cutler, G., "Thermal stability of a long-acting analogue of luteinizing hormone releasing hormone (D-Trp^6-Pro^9-NEt-LHRH)," *Contraception*, **27,** 195 (1983).
Wiseman, A., and Williams, N. J., "Thermal inactivation of alcohol dehydrogenases in the presence of NDA or NADP," *Biochim. Biophys. Acta.*, **250,** 1 (1971).
Wiseman, A., (1978). see Table VII.
Wolf, D., Ebner, E., and Hinze, H., "Inactivation, stabilization and some properties of ATP: glutamine synthetase adenylyltransferase from E. Coli," *Eur. J. Biochem.*, **25,** 239 (1972).
Wolfert, R. R., and Cox, R. M., "Room temperature stability of drug products labeled for refrigerated storage," *Am. J. Hosp. Pharm.*, **32,** 585 (1975).
Wunsch, E., "Peptide factors as pharmaceuticals: criteria for application," *Biopolymer*, **22,** 493 (1983).
Yalkowsky, S. H., and Reed, K. W., "The lysis of human red blood cells in the presence of various cosolvents. III. The relationship between hemolytic potential and structure," *J. Parenter. Sci. Technol.*, **41,** 37 (1987).
Yu, M. W., and Finlayson, J. S., "Stabilization of human albumin by caprylate and acetyltryptophanate," *Vox Sang.*, **47,** 28 (1984).

Acknowledgment

Drs. J. DeYoung, K. Iqbal, and Z. Yim assisted in gathering literature; Dr. David Frost, consultant editor, improved the readability of the manuscript. Comments received from Dr. S. Levine are appreciated.