Preformulation Studies Oriented toward Sustained Delivery of Recombinant Somatotropins†

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Preformulation studies supporting development of sustained-release formulations for bovine (rbSt) and porcine (rpSt) somatotropins emphasized the importance of protein stability within the delivery systems following implantation. Intermolecular dimerization reactions via covalent non-disulfide cross-links were more predominant in the solid state than in solution. Rates of dimerization in the solid increased upon exposure to increasing relative vapor pressure (rvp) and lyophilization from solutions of increasing pH. However, the fractional amount of rbSt lost via dimerization vs other pathways was independent of rvp and pH. The loss of rbSt induced by exposure of the solid to dry heat or ^{60}Co irradiation makes the use of terminal sterilization unlikely. The dissolution of rpSt was faster than that of rbSt, consistent with rpSt's greater solubility. However, the relationship between solubility and dissolution rate for somatotropins was unclear, and application of conventional dissolution models/techniques was limited.

INTRODUCTION

Recombinant DNA technology has made it possible to produce large quantities of proteins economically. One such group of proteins that have demonstrated effectiveness in various animal species is the somatotropins (Machlin, 1972; Bauman et al., 1985; Etherton et al., 1987). The pursuit of extended-duration formulations has been a key component in development of these drugs as products (Ferguson et al., 1988; Cady et al., 1989; Pitt, 1990). Developing sustained-release formulations for these proteins has presented challenges in controlling rate, duration, and efficiency of drug release.

A thorough understanding of the mechanisms of sustained release and the physicochemical properties of the protein is necessary for developing formulations with weekly to monthly durations. Fundamental mechanisms of sustained release usually rely on control of the dissolution rate, the diffusion rate, or the rate of decomposition of the polymeric systems (Baker, 1987; Pitt, 1990; Lee, 1991). The release of drugs from such delivery systems is described with various mathematical models that underscore the importance of the solubility and the diffusion coefficient of the drug. Solubility generally dictates the driving force in the establishment of the gradient necessary for diffusion, and the diffusion coefficient governs the rate of migration of the drug (Baker, 1987). However, efficiency of delivery is often limited due to the instability of the protein within the delivery system at in vivo conditions following administration (Wyse et al., 1989; Pitt, 1990; Hageman, 1992). Additionally, the development of a parenteral delivery system requires terminal sterilization of the product or, if not possible due to protein instability, the aseptic production of a final product (Ferguson, 1988). This paper examines several key preformulation activities for somatotropins. These studies include characterization of stability, solubility, dissolution, and the effect of irradiation or heat sterilization on the integrity of the proteins.

MATERIALS AND METHODS

Materials. Recombinant DNA-derived bovine and porcine somatotropins (rbSt and rpSt, respectively) were obtained by expression in *Escherichia coli* carrying a temperature-sensitive runaway plasmid which had been inserted with the respective somatotropin gene sequence and a tryptophan promoter system (Olson et al., 1987). The somatotropins were purified according to the method of Evans and Knuth (1987). Pituitary-derived porcine and human somatotropins were obtained from A. F. Parlow (UCLA, Los Angeles, CA). The homology for the primary sequences (191 amino acids) of various somatotropins has been compiled and conformational structures have been discussed (Abdel-Meguid et al., 1987; Cohen and Kuntz, 1987; Carlacci et al., 1991). All other chemicals used were of analytical reagent grade or better.

Chromatographic Methods. Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out using a 10-cm RP-300 Aquapore octyl column (Brownlee Labs). A gradient system (Varian Vista 5500) of acetonitrile and water mobile phases, which both contained 0.1% trifluoroacetic acid (TFA), was used. Samples of protein were typically dissolved into either 0.5% TFA solutions or 0.05 M carbonate buffer (pH 9.5–10.5) solutions at concentrations of 0.1–9 mg/mL, depending on the study. These solutions were then diluted with the same corresponding buffers to concentrations of 0.1–1 mg/mL for analysis. Samples of 50–500 μL (40–65-μg loading) were injected (Perkin-Elmer ISS-100) onto the column. Samples were eluted with a linear gradient of the acetonitrile phase from 48% to 62% over 14 min or from 48% to 59% over 22 min, with detection at 215 nm (Kratos 783). Size exclusion high-performance liquid chromatography (SE-HPLC) was run with either two Du Pont Zorbax GF-450 columns or one GF-250 column and a pH 7.4 glycine/phosphate/NaCl mobile phase that contained 0.1% sodium dodecyl sulfate (SDS) (Stodola et al., 1986). Detection was either by UV 280 nm or by fluorescence with excitation at 280 nm and an emission filter of 345 nm (Kratos 980).

Electrophoretic Methods. SDS-polyacrylamide gel electrophoresis was carried out using a 20% gel on Pharmacia's PhastSystem. The reducing agent used was mercaptoethanol. Bands were detected by silver staining.

Solubility Determination. Solubilities were determined by dissolving rpSt and rbSt in water, at greater than 150 mg/mL. These solutions were then dialyzed against 0.05 M Tris buffer, adjusted to ionic strengths of 0.12 with NaCl, at pH 6.4, 7.4, and 8.0. Concentrations of somatotropin were determined by RP-HPLC.

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Dissolution Rate Methods. The sodium salts of rbSt were prepared by dialyzing against NaOH and lyophilizing the resultant solution. Dissolution studies were conducted using a rotating disk system (Mooney et al., 1981), containing rbSt or rpSt pellets and rotating at 600 rpm. The dissolution media (same Tris buffers as used for solubility determinations) was maintained at a constant temperature and hooked up to a flow-through UV spectrophotometer (Milton Roy Spectronic 1001). UV absorbance was monitored at 215 nm as a function of time. Approximate extinction coefficients at 215 nm for rpSt and rbSt, at the various pH values, were determined by weighing samples (adjusted for water content) into water and diluting into the appropriate Tris buffer.

Solution Stability Studies. Solutions of the somatotropins (10 mg/mL) were prepared in sodium bicarbonate buffer (0.05 M, pH 9.8) and then filtered through a 0.22-μm Millipore GV filter and stored in septum-sealed vials at 30 °C. Samples were aseptically withdrawn, centrifuged (Eppendorf 5414), and diluted to 0.5 mg/mL with 0.1% v/v concentrated NH_4OH for assay. Denaturation/aggregation studies were conducted by preparing rbSt solutions of the indicated concentration at 5 °C, in citrate (pH 7.0) and 0.05 M phosphate (pH 7.0) buffers of 0.15 ionic strengths, adjusted with NaCl. These solutions were placed in tubes, which were then placed in a water bath at the designated temperatures. Samples were obtained at various time points and centrifuged (Eppendorf 5414). UV absorbance of the supernatants of these samples was read directly or after dilution in their respective buffers. The concentration of rbSt remaining in solution was determined by comparison to the time zero value. The amount remaining as measured by UV was verified by RP-HPLC on selected samples (not reported here).

Solid-State Stability Studies. Desiccators containing saturated solutions of K_2SO_4, KNO_3, NaCl, and NaBr were used to obtain 30 °C/96%, 30 °C/90%, 30 °C/75%, and 47 °C/50% relative vapor pressure (rvp), respectively (Dean, 1979). For the 30 °C/90% rvp studies, lyophilized solids were obtained by dialyzing 10 mg/mL somatotropin solutions against water adjusted to pH 9.7 with sodium hydroxide. The apparent pH in the lyophilized solid was obtained by measuring the pH following reconstitution to 6.25 mg/mL with water. After exposure to 30 °C/96% rvp, samples were reconstituted with 0.05 M sodium bicarbonate, pH 9.8, to 0.8 mg/mL for assay. The percent somatotropin remaining was determined by comparison to time zero values.

For the 47 °C/50% rvp studies, lyophilized solids were obtained by dialyzing 20 mg/mL rbSt solutions against varying buffer-free solutions of pH 4.5–10.5. The apparent pH in the lyophilized solid was determined by measuring the pH following reconstitution to 20 mg/mL with a 0.01 M NaCl solution. Samples below pH 7 were reconstituted to 1.5 mg/mL with 0.01 M sodium acetate buffer (pH 4.0). Samples above pH 7 were reconstituted using 0.025 M Tris buffer (pH 9.5). All samples were centrifuged after reconstitution using an Eppendorf 5414. Supernatants were assayed without dilution in the 30 °C/96% rvp studies. Supernatants from the 47 °C studies were diluted for SE-HPLC to 1.0 mg/mL using 0.33% v/v concentrated NH_4OH that contained 0.33% w/v SDS. Samples were diluted to 0.5 mg/mL with 0.5% TFA for RP-HPLC. For both solid-state studies, the lyophilized cakes were less than 4 mm in depth and were demonstrated to equilibrate to a constant moisture level very rapidly, <24 h, when exposed to a given humidity. Water content of samples was determined by coulometric Karl Fischer titration.

Irradiation Studies. Samples of lyophilized rbSt, sodium salt, were weighed into vials under atmospheric conditions and shipped to Isomedix (Morton Grove, IL) for ^{60}Co γ irradiation at room temperature. Levels of irradiation ranged from 0.5 to 4.0 Mrad dosed at approximately 0.5 Mrad/h. Irradiated samples were at ambient conditions for 3–4 days and then stored at 4 °C for 2–3 weeks prior to analysis. Samples were dissolved with 0.5% TFA for assay by RP-HPLC. These results were compared to results from nonirradiated samples stored under similar conditions.

Samples of lyophilized rbSt, sodium salt, were distributed very finely (<0.3 mg/cm^2) and exposed to UV irradiation at 254 nm and room temperature (UVG-54 Mineralight lamp). Irradiation intensity at the target site was 425–465 μW/cm^2 as measured by a UVX digital radiometer using a UVX-25 sensor. Samples were then dissolved in 0.05 M carbonate buffer (pH 10.5) and assayed by RP-HPLC with comparison to unexposed samples.

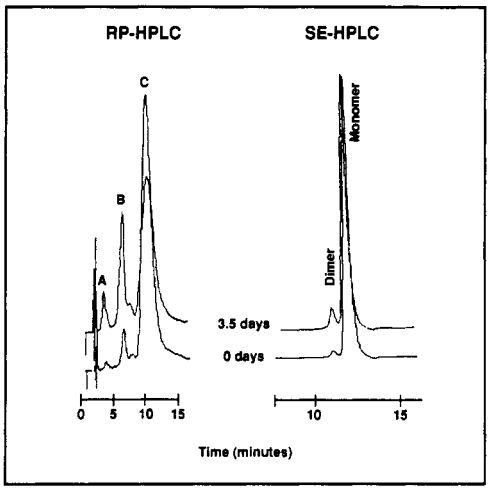

Figure 1. Sample chromatogram of rbSt before and after incubation at 10 mg/mL in pH 9.8 bicarbonate buffer at 30 °C. Peaks A, B, and C refer to chain cleaved, deamidated isopeptide, and intact rbSt, respectively.

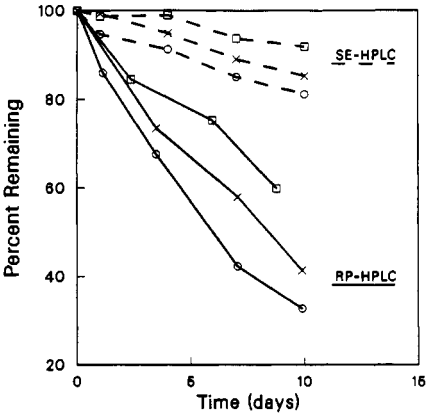

Figure 2. Amount of human (□), bovine (×), and porcine (○) somatotropin remaining following incubation at 10 mg/mL in pH 9.8 bicarbonate buffer at 30 °C, as measured by RP-HPLC (—) or SE-HPLC (- - -).

RESULTS

The rate of loss of bovine, porcine, and human somatotropin at 30 °C in aqueous solution was determined by RP-HPLC and SE-HPLC (Figures 1 and 2). The RP-HPLC method detected peaks eluting prior to the somatotropin, consistent with the losses by deamidation and chain clipping at position 99 (Figure 1). These results coincide with elution patterns for solutions of decomposed rbSt previously reported for a similar chromatographic method (Violand et al., 1990). The RP-HPLC chromatograms in Figure 1 differ from those of Violand et al. (1990) by the absence of the peak eluting just after the rbSt peak assigned to *N*-formylmethionyl-bSt. This is consistent with the non-methionyl form of rbSt used in these studies (Langley et al., 1987) vs the previously reported studies (Violand et al., 1990). Unfortunately, this RP-HPLC method will resolve only the deamidated isopeptide and not the deamidated normal peptide. Dimeric and oligomeric somatotropins do not elute from the column under these conditions. The total rate of loss, as measured by RP-HPLC, was taken to be the sum of losses by deamidation to the isopeptide, clipping, and covalent dimerization. Although not observed in these samples, other

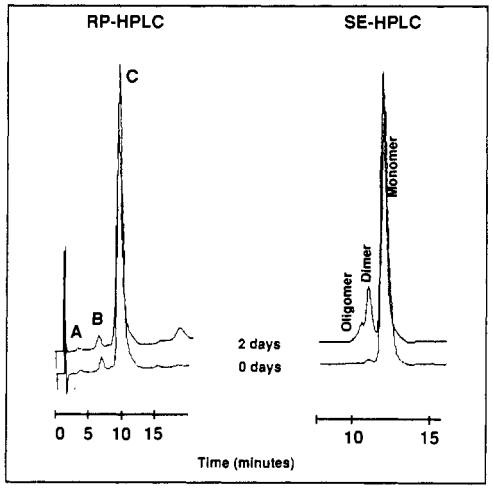

Figure 3. Sample chromatograms of rbSt before and after incubation of the lyophilized sodium salt at 96% rvp and 30 °C. Peaks are defined as in Figure 1.

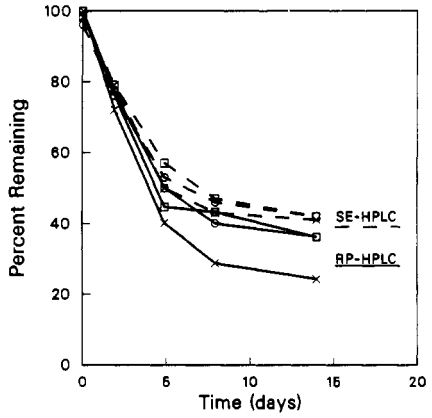

Figure 4. Amount of human (□), bovine (×), and porcine (○) somatotropin remaining following incubation of their lyophilized sodium salts at 96% rvp and 30 °C, as measured by RP-HPLC (—) or SE-HPLC (- - -).

minor (<10% total) uncharacterized products that elute after the somatotropin were occasionally observed during solution decomposition and frequently during solid-state stability studies (Figure 3).

The rate of loss of monomeric somatotropin was determined by SE-HPLC. The presence of SDS in the mobile phase limits this method to detect only covalently dimerized or cross-linked somatotropins. Soluble hydrophobic aggregates, if present, would dissociate and coelute with monomer. However, such hydrophobic aggregates of rbSt and rpSt have generally been observed to be quite insoluble and to precipitate out of solution, thus resulting in a loss of rbSt main peak. There was no observable precipitation in the samples during the study. The fractional amount of the total loss due to covalent cross-linking was then calculated by dividing the SE-HPLC percentage remaining by the RP-HPLC percentage remaining. Although the stabilities of the somatotropins in solution decreased in the order human > bovine > porcine, the fractional amount of cross-linking was relatively constant at approximately 23–26%.

The rates of loss for the somatotropins in the solid state, when incubated at 30 °C, 96% rvp, and monitored by RP-HPLC and SE-HPLC, were as fast as, and sometimes faster than, the rates of those in solution (Figures 1–4). However, the rates of loss in the solid state were quite similar for the three somatotropins, especially the rate of loss by dimerization. Most importantly, the fractional

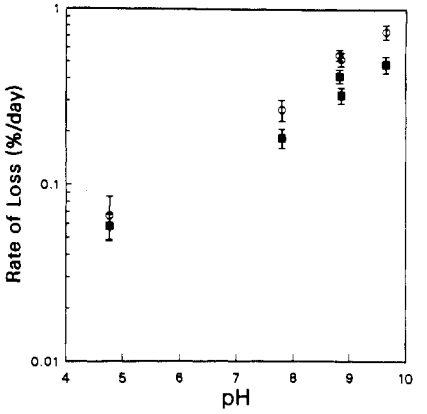

Figure 5. Effect of pH, measured upon reconstitution, on the solid-state stability of rbSt when incubated at 30 °C and 75% rvp. Initial rates were determined from loss of rbSt by RP-HPLC (○) or monomeric rbSt loss by SE-HPLC (■). Error bars are 95% confidence limits.

amount of somatotropin lost to covalent cross-linking in the solid state increased to 80–90% of the total loss, as compared to the 23–26% noted in solution. In the solid state, other minor decomposition pathways, such as deamidation, chain clipping, and an unidentified pathway resulting in the appearance of a late eluting peak (Figure 3), comprised the remaining 10–20% of the total loss.

Lyophilized rbSt samples, stored at 47 °C and 50% rvp for 10 days, were analyzed using reduced and nonreduced SDS–PAGE gels. The gels demonstrated an increase in covalently cross-linked dimers. Under these solid-state conditions, the reduced gels indicated that the dimer was not disulfide cross-linked.

The kinetics for the decomposition of the somatotropins did not fit a simple zero-, first-, or second-order kinetic model. Therefore, initial rates of loss were used to conduct further studies with rbSt and evaluate the impact of several variables on the rate of decomposition. The initial rates were typically obtained using no more than 10% loss of rbSt monomer as measured by SE-HPLC or of rbSt as measured by RP-HPLC. Increasing the rvp from 75% to 90% at 30 °C resulted in a doubling of the initial rate of monomer loss from 0.66 ± 0.06% day^{-1} to 1.25 ± 0.28% day^{-1} for samples lyophilized at pH 8.8. However, the fractional loss due to cross-linking did not change, 0.81 ± 0.11 at 75% rvp and 0.70 ± 0.20 at 90% rvp.

The effect of pH on the initial rate of loss of rbSt was determined by both SE- and RP-HPLC (Figure 5). The pH had a minor impact on the fractional amount of rbSt lost due to covalent cross-linking (Figure 6). The very large error bars in the fractional amount cross-linked at lower pH are due to the large error associated with the determination of the initial rate constants. This error was due to the very slow rates of loss noted at pH 4.8. The water content of the samples did not vary significantly with pH.

The denaturation/aggregation of rbSt in solution at pH 7.0 was studied by monitoring the loss due to precipitation. The rates were highly dependent upon the initial rbSt concentration (Figure 7). Similar, less extensive studies demonstrated that the rates of aggregation/precipitation increased when the pH was reduced from pH 7 to pH 5. The rate of loss due to precipitation was 3%/day at 37 °C, 2 mg/mL rbSt, as measured by UV absorbance loss. This was also confirmed by loss of rbSt from solution as measured by RP-HPLC.

The solubilities of bovine and porcine somatotropin were determined using dialysis at three different pHs. The

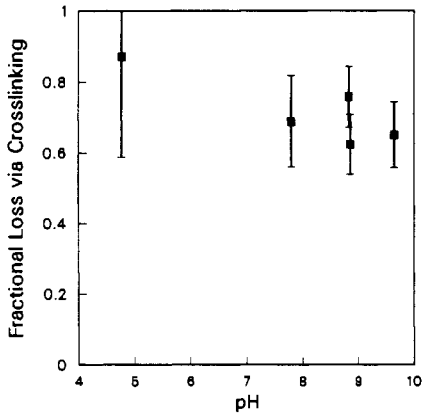

Figure 6. Effect of pH on the fractional loss of rbSt in the solid state occurring by covalent cross-linking. Error bars are 95% confidence limits.

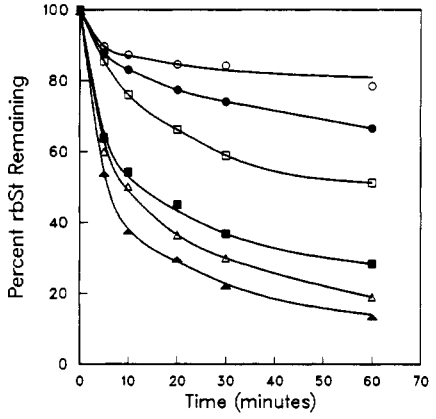

Figure 7. Percent of rbSt remaining in solution following incubation at 55 °C in pH 7.0 citrate buffer and at rbSt concentrations of 0.3 (○), 0.6 (●), 1.0 (□), 3.0 (■), 6.0 (△), and 9.0 (▲) mg/mL.

solubility of the rpSt was found to be consistently higher at all pH values (Figure 8). A decrease in temperature from 37 to 25 °C had little impact on the apparent solubilities of either rbSt or rpSt at pH 7.4 (Figure 8).

For conventional organic molecules, the relationship of solubility to dissolution rate, defined in terms of flux, J (amount per time per cross-sectional surface area), is usually described using the Noyes–Whitney relationship (eq 1) (Ritschel, 1973). In this equation, D is the apparent

$$J = DA(C_s - C)/h \quad (1)$$

$$J = DAC_s/h \quad (2)$$

diffusion coefficient, A is the surface area from which dissolution is occurring, C_s is the saturation solubility of the drug, C is drug concentration in the bulk solution, and h is the hydrodynamic diffusion layer thickness. Under sink conditions, $C_s \gg C$, eq 1 reduces to eq 2. In the case of the rotating disk system, h can be described by the Levich equation (eq 3), where ν is the kinematic viscosity and ω is the angular rotation speed of the disk (Levich, 1962). Substituting eq 3 into eq 2 yields eq 4, where the

$$h = 1.612D^{1/3}\nu^{1/6}\omega^{-1/2} \quad (3)$$

$$J = 0.62D^{2/3}\nu^{-1/6}C_s\omega^{1/2} \quad (4)$$

flux should be directly proportional to C_s when the other variables are constant.

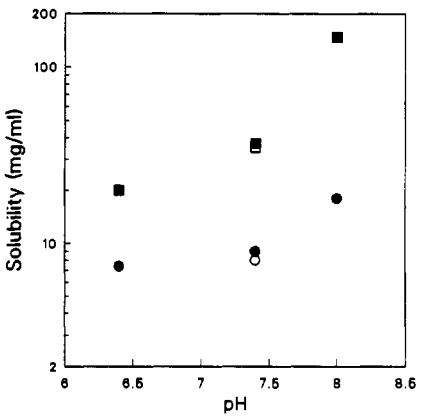

Figure 8. Solubility of rbSt (●) and rpSt (■) at 37 °C (solid symbols) as a function of pH in 0.05 M Tris buffer with ionic strength of 0.12. Solubility at pH 7.4, 25 °C, is shown by open symbols.

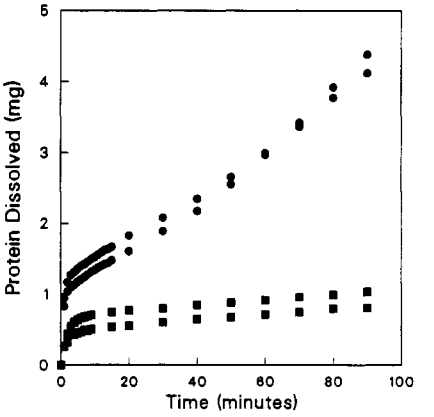

Figure 9. Dissolution of rbSt (■) and rpSt (●) sodium salts as monitored by appearance of protein in dissolution media at 37 °C and pH 7.4.

Preliminary dissolution profiles of rpSt and rbSt both showed a similar initial rapid rate of dissolution in the first 5 min which then leveled to a constant rate by 10 min. Over the next hour, the rates remained relatively constant (Figure 9). These later rates were used to calculate a flux for comparison to the solubilities (Table I). At pH 7.4, the rate of rpSt dissolution was almost 10 times faster than that of rbSt, whereas solubility was only 4 times greater (Figure 8; Table I). Dissolution studies at pH 6.4 and 8.0 further demonstrated not only the lack of a constant proportionality between dissolution and solubility but also a change in the proportionality constant depending on condition (Table I). The maintenance of sink conditions was verified in that the solution concentration of the rbSt and rpSt runs never exceeded 50 or 100 μg/mL, respectively; both were always less than 100-fold below their respective solubilities.

Provided the diffusion coefficient, kinematic viscosity, and angular rotation were constant, J/C_s should be constant, which was obviously not the case (Table I). Furthermore, the dissolution rates and the shape of the dissolution profile changed as a function of angular velocity (unpublished data). Consequently, it appeared that this dissolution rate model was inadequate for rbSt and rpSt.

The exposure of lyophilized rbSt to varying temperatures for a period of 30 min resulted in significant losses in protein as assayed by RP-HPLC (Figure 10). This was especially true at the higher temperatures which are required for dry-heat sterilization.

The effect of γ irradiation by ^{60}Co on lyophilized rbSt was monitored by RP-HPLC. A log–linear decrease in

Table I. Dissolution Rates of rbSt and rpSt Sodium Salts at 37 °C As Obtained by Rotating Disk (600 rpm) Apparatus, with 95% Confidence Limits

protein	media pH	flux, μg/(min cm²)	J/C_s,[a] cm/min
rbSt	6.4	1.63–2.07	$2.20–2.80 \times 10^{-4}$
	7.4	4.22–5.22	$4.69–5.80 \times 10^{-4}$
	8.0	39.8–47.5	$2.21–2.64 \times 10^{-3}$
rpSt	6.4	2.33–2.95	$1.17–1.47 \times 10^{-4}$
	7.4	41.5–51.9	$1.15–1.44 \times 10^{-3}$
	8.0	3400–7590	$2.30–5.13 \times 10^{-2}$

[a] C_s was obtained from Figure 6.

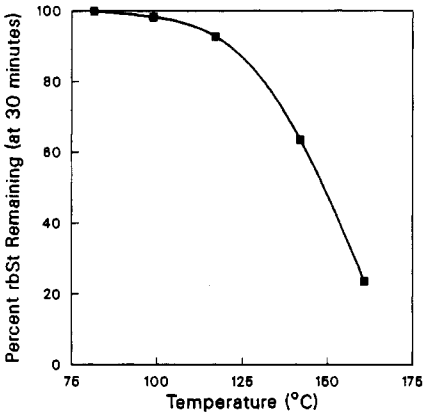

Figure 10. Fractional percent of rbSt remaining, as measured by RP-HPLC, following incubation of lyophilized rbSt at the indicated temperatures for 30 min.

the amount of rbSt remaining, as a function of the irradiation dose, was observed (Figure 11). On the basis of solid-state stability of rbSt (Hageman, 1988) and a dosing rate of 0.5 Mrad/h, there should not be any significant loss via the normal decomposition process over the irradiation time necessary for sterilization, even if slight increases in temperature occur. However, this possibility was accounted for by comparison to nonirradiated controls exposed to similar temperature conditions.

According to radiation target theory, ionizing radiation results in a random transfer of high energy throughout a given spatial volume. Consequently, the likelihood of a primary ionization or electron excitation occurring as a result of the incident energy is a function of the volume that the target occupies. Because energy disposition is large, every hit is assumed to result in destruction of that target. From this basis, eq 5 has been derived as part of

$$A_D = A_0 e^{KD} \tag{5}$$

the target volume or single hit theory (Nugent, 1986; Bradbury and Zammit, 1990). In this equation, A_0 is the activity at zero irradiation dose, A_D is the remaining activity after dose D (Mrad), and K is a constant related to the molecular weight of the protein. Empirical equations, based on data from many proteins, have been derived to relate the molecular weight, M_w, to the constant K (Nugent, 1986). At room temperature, eq 6, was obtained through rearrangements of equations presented by Nugent (1986).

$$\log (A_D/A_0) = -[(3.76 \times 10^{-6})M_w]D \tag{6}$$

The derived relationship for the decomposition of a 22 kDa somatotropin, as a function of irradiation dose, overestimated the amount of decomposition (Figure 11).

DISCUSSION

The inherent instability of proteins in aqueous solution greatly limits the potential use of aqueous-based delivery

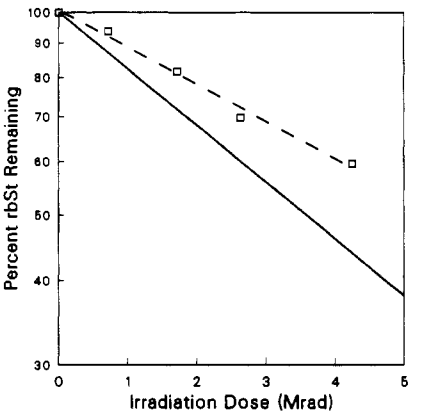

Figure 11. Semilograthmic plot of the amount of rbSt remaining, as measured by RP-HPLC, following ^{60}Co γ irradiation at the designated doses. The solid line refers to the predicted decomposition from eq 6 (r = 0.995).

systems for sustained-release delivery. The somatotropins were highly susceptible to decomposition in aqueous solution at 30 °C with greater than 50% loss of intact protein in less than 2 weeks (Figure 2). This instability can limit the use of a formulation where the protein is dissolved in an aqueous media within the delivery system and exposed to 37 °C following administration.

To avoid the instability of proteins, induced by the presence of water, typical sustained-release formulations can be prepared using dehydrated protein. However, upon administration, the protein and delivery system are exposed to an aqueous environment of 37 °C. The exposure of lyophilized somatotropins to 96% rvp and only 30 °C results in greater than 50% loss of protein in less than 2 weeks (Figure 4). Consequently, the release of somatotropins from delivery systems can be impacted by competing factors such as the rate of device hydration, protein hydration, and protein decomposition within the system. The decomposed protein may or may not be subsequently released for absorption.

The instability of the somatotropins in the presence of water greatly limits the consideration of hydrogel-type delivery systems, although adequate stability of the dehydrated protein in the device prior to administration may be possible. When exposed to an aqueous environment, the hydrogels become highly hydrated. A concentrated aqueous solution of the somatotropins forms within the polymer, permitting rapid decomposition of the somatotropin (Pitt, 1990).

Similar difficulties in release of somatotropins have been encountered with other less hydrophilic polymers such as the poly(glycolic acid) and ethylvinyl acetate polymer systems (Sivaramakrishnan et al., 1989; Wyse et al., 1989). The drug release profiles generally plateau at approximately 50% release of somatotropin when incubated in aqueous media at 37 °C. Preliminary investigations of the unreleased porcine somatotropin from poly(glycolic acid) microspheres indicate that there is a significant amount of decomposition of the somatotropin in the device (Wyse et al., 1989). Consequently, an understanding of protein hydration and the stability of the protein at high levels of hydration and concentration are especially relevant to final formulation strategies (Hageman, 1988, 1992; Hageman et al., 1992).

There was a significant change in the predominance of various decomposition pathways in solution vs those in the highly hydrated solid state (Figures 1 and 2 vs Figures 3 and 4). The importance of bimolecular reactions, such as covalent dimerization leading to oligomerization, greatly

increased in the solid state. Even though the clipping and deamidation reactions still occurred in the solid state, and also increased in rate with increasing water content, the dimerization reactions accounted for up to 80% of the total loss of protein. This was presumably due to the much higher "effective concentration" that existed in the solid state, thus preferentially favoring the intermolecular reactions. Alternatively, the protein molecules may have become preferentially oriented during the dehydration process through some type of specific intermolecular interaction that allowed for facilitated cross-linking. Certainly, bovine somatotropin can associate in solution at high concentrations as a native protein (Dellacha et al., 1968; Bewley and Li, 1972) and as a partially unfolded intermediate (Brems et al., 1986; Havel et al., 1986). The occurrence of intramolecular cross-links was not investigated in these studies but has been proposed with other proteins (Weder and Scharf, 1981).

Even though the rate of covalent cross-linking increased with an increase in rvp, the proportion of cross-linking to overall loss remained relatively constant. This indicated that all of the reactions involved, i.e., primarily covalent cross-linking, deamidation, and peptide cleavage, were similarly affected by increased water content. The impact of water on various chemical decomposition pathways has been discussed elsewhere (Levine and Slade, 1987; Hageman, 1988, 1992).

The covalently linked dimers did not appear to involve disulfide cross-linking, as observed by reduced and nonreduced SDS–PAGE electrophoreses. This may be in contrast to observations of somatotropin dimerization in solution (Shieh et al., 1991). The nonreducible cross-links noted for rbSt may be similar to the formation of isopeptide bonds in other proteins when incubated under stress conditions in the solid state (Otterburn, 1983; Volkin et al., 1991). The reactions of nucleophilic lysines either with naturally occurring carbonyls of glutamine and asparagine or with dehydroalanine derivatives formed from β-elimination reactions of disulfide groups are the most common cross-links (Carpenter et al., 1962; Otterburn, 1983; Volkin et al., 1991). Studies with ribonuclease have demonstrated that isopeptide linkages, aspartyllysine and glutamyllysine, occur in a somewhat random fashion, with the only preference being the involvement of the N-terminal portion of the protein (Weder and Scharf, 1981). This lack of homogeneity, coupled with the likelihood of intramolecular cross-linking, makes characterization difficult (Weder and Scharf, 1981).

The effect of pH on the rate of loss of rbSt (Figure 5) is consistent with that expected for cross-linking reactions which involve the action of lysine as a nucleophile; the nonprotonated lysine is expected to be more nucleophilic. However, the definition of pH and the degree of ionization of various functional groups in the solid state are very poorly understood (Hageman, 1992). In this particular study, the pH upon reconstitution was utilized as a measure of the apparent pH in the solid, assuming it was more representative of the apparent pH in the powder than the pH of the solution prior to lyophilization. The rates of rbSt loss can be viewed, with some risk, to be a log–linear function of pH with an approximate slope of 0.2 (Figure 5). However, given the lack of data between pH 5 and pH 8, it is inappropriate to mechanistically interpret this slope. Nonunity slopes have been noted in the pH profiles of protein decomposition in solution as well (Patel and Borchardt, 1990).

The fraction of total rbSt lost via cross-linking was relatively independent of pH (Figure 6). This indicates that either the pH dependence of the mechanisms for cross-linking, deamidation, and clipping may be similar or that they may all involve a mutual intermediate. The succinimide intermediate known to be present in deamidation (Patel and Borchardt, 1990) may provide an activated carbonyl which can greatly facilitate the nucleophilic attack of lysine to form isopeptide bonds. This is under further investigation.

The sample prepared at pH 4.8 appeared to be the most stable toward chemical modifications detected by RP-HPLC. However, the conformational stability of rbSt decreases with decreasing pH, and at pH <6, rbSt begins to unfold at room temperature (Holzman et al., 1990). During the multistep unfolding of rbSt, the presence of aggregates between partially unfolded species has been noted (Brems et al., 1986). Such aggregates may be of significant concern in sustained-delivery systems, due to their poor solubility in the absence of solubilizers (Brems, 1988). Likewise, the decreased diffusion coefficients of the aggregates (Havel et al., 1986) may impact the rates of release, depending on the type of delivery system being pursued. Therefore, while a decrease in pH can stabilize toward chemical decomposition (Figure 5), it does so at the cost of a decrease in conformational stability (Holzman et al., 1991) and a decrease in solubility (Figure 8).

A further consequence of the decreased conformational stability with decreasing pH is the greater tendency for irreversible aggregation/precipitation from solutions of the dissolved drug. The irreversible precipitation of rbSt was concentration dependent (Figure 7). The importance of this aggregation problem may lie in the fact that all solids incorporated into a device will undergo dissolution and diffusion out of the system. At the point of dissolution, the protein will be at saturation solubility and highly susceptible to this apparent hydrophobic aggregation.

The controlled dissolution of drugs and alteration of dissolution rates, based on the fundamental relationship previously defined (eq 1), has been a common approach to controlling the release of drugs from depot injections and implants (Ritschel, 1973; Baker, 1987). The rotating disk model is typically used to study dissolution phenomena because extensive work has been done in the development of theoretical models and an understanding of the hydrodynamics of the system (Levich, 1962; Mooney et al., 1981).

The more rapid dissolution of rpSt was consistent with its higher solubility (Figures 8 and 9). However, the preliminary studies on dissolution of rbSt and rpSt indicated that the model, as presently derived, was inappropriate for dissolution of these proteins. While there appeared to be rank order correlation between solubility and the rate of dissolution, it was not one of constant proportionality (Table I).

The changes in shape of the dissolution curves with changes in angular rotation indicated the inadequacy of the Levich model applied directly to the dissolution of the proteins. Potential changes in the diffusion coefficient through the boundary layer, due to either a viscosity gradient (Kuu et al., 1989) or a chemical reaction such as association–dissociation, may be important. Models typically used for describing the dissolution of macromolecular polymers may be more appropriate for describing the dissolution of proteins (Kuneida and Shinoda, 1975; Ouano et al., 1977). Regardless, the rotating disk system can be used to obtain some practical information about apparent dissolution rates under a specified set of conditions, but care should be taken when this information is extrapolated to other systems, as is typically done with intrinsic

dissolution rates. Whether these problems with dissolution of rpSt and rbSt are generally applicable to all proteins is under investigation.

The preparation of sustained-release parenteral delivery systems presents some special problems from a processing perspective. One particular problem is the need to either aseptically produce or, preferably, terminally sterilize the formulation (Ferguson, 1988). Terminal sterilization relies on the ability of the sterilant, such as heat or irradiation, to elicit a bactericidal effect via the destruction of integral proteins and nucleic acids of the bacteria (Ferguson, 1988). Consequently, the feasibility of successfully implementing terminal sterilization for proteins such as the somatotropins is minimal and was only briefly addressed.

Typical conditions for dry heat sterilization suggested by the *United States Pharmacopeia* (1980) are not less than 2 h at 160–170 °C. Such conditions resulted in significantly greater than 80% loss of the rbSt (Figure 10). Therefore, as expected, dry heat sterilization was not a viable option for sterilization of protein delivery systems.

Ultraviolet irradiation, usually 254 nm, has been used to provide sterilization of equipment surfaces under carefully controlled situations (Phillips and Miller, 1980). Studies on rbSt exposed to UV irradiation at 254 nm indicated losses by RP-HPLC of 7–10% at doses of approximately 5000 $\mu W/cm^2$ (425–465 $\mu W/(cm^2\ min)$) as might be necessary to destroy the more resilient bacterial spores (Phillips and Miller, 1980). Regardless of the impact on stability, the utility of UV irradiation for sterilization will be limited due to poor penetration properties (Ferguson, 1988; Phillips and Miller, 1980).

One of the more common methods of terminal sterilization is the use of γ irradiation, where 2.5 Mrad is generally accepted as a sterilizing dose (Ferguson, 1988). Hromadova (1979) found the decomposition of bovine somatotropin to be minimal, according to radioimmunological and biological assay methods, following irradiation of samples with up to 2.5 Mrad. In our studies, irradiation doses of 2.5 Mrad resulted in approximately a 30% loss of rbSt as measured by RP-HPLC (Figure 11). Clearly, irradiation has a significant impact on rbSt in the solid state and results in significant decomposition, either from direct ionization or indirect effects due to free radicals formed from O_2 or H_2O upon primary ionization. Although no data were collected on rpSt or human somatotropin, comparable results would be expected due to their similarity in molecular weight and the general principles of irradiation and proteins as discussed below.

The amount of energy in a single photon is regarded as being sufficient to destroy the biological activity of any target it hits, which is a function of the volume the target occupies. Therefore, radiation target theory as described under Results has been used for years to estimate the molecular volume occupied by unknown proteins with measurable activities (Nugent, 1986; Bradbury and Zammit, 1990). The observed degree of decomposition was slightly less than that predicted for a protein of 22 000 molecular weight. This overestimate of decomposition may be due to the inability of the RP-HPLC method to detect all potential modifications of the protein. Although there is no evidence for a protective mechanism via residual impurities, such a phenomenon cannot be ruled out. Similarly, the impact of the physical nature of the amorphous solid resulting from lyophilization on susceptibility to irradiation is unknown. Consequently, a more valid approach for predicting susceptibility to irradiation can be obtained by concurrently running calibration samples of other proteins with known molecular weights which were prepared identically to the protein of interest (Nugent, 1986).

These empirical approaches based on target volume theory were used to provide a crude estimate of what the limiting molecular weight might be for successful application of irradiation in terminal sterilization. The appropriate substitutions were made into eq 5 to determine the molecular weight at which the protein would experience only 5% decomposition when exposed to a sterilizing irradation dose of 2.5 Mrad at room temperature. On the basis of those calculations, any protein over 2.4 kDa may experience greater than 5% decomposition following 2.5 Mrad of irradiation. Improved stability can be obtained by decreasing the temperature during irradiation. Similar treatment using empirical equations describing the temperature effect (Nugent, 1986) predicts that any protein over 4.5 kDa would experience greater than 5% decomposition following 2.5 Mrad of irradiation at −78 °C, the temperature of solid carbon dioxide. Even though the decreased temperature improves protein stability, it is likely that the bactericidal activity of the irradiation would also be diminished at lower temperatures and validation of 2.5 Mrad at the lower temperatures would be required. Similarly, the use of radio protective agents to stabilize the protein would have to be considered in light of their effect on bactericidal activity as well; any excipient or condition modification employed would have to stabilize the protein preferentially over that of bacterial integrity. Furthermore, these predictions based on target volume alone are conservative, because they do not account for any decomposition via indirect mechanisms such radicals formed from ionization of residual moisture and oxygen. Consequently, the irradiation of delivery systems containing proteins the size of somatotropins is probably not feasible.

The development of sustained-release dosage forms for the efficient delivery of somatotropins is complicated by the instability of the proteins upon exposure to water, especially at physiological conditions of pH 7.4 and 37 °C. Hydration of the system will also result in hydration of the protein, and subsequent decomposition is expected to be rather rapid. Even in the case of devices composed of very hydrophobic polymers, where diffusion through the porous matrices is rate limiting, the protein will be hydrated and will have to dissolve prior to its diffusion out of the device. At the point of dissolution the drug will be present at saturation solubility and be expected to undergo decomposition via the intermolecular reactions described in this paper. Unfortunately, the lack of knowledge on dissolution of proteins makes it difficult to systematically modify variables to obtain specific results, thus limiting options to pursue. The understanding of protein dissolution is a potential area of research that could greatly expand the formulation options now available. Lastly, the instability of proteins exposed to typical methods of terminal sterilization may limit manufacturing options exclusively to aseptic processing.

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