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Report

The Effects of Formulation Additives on the Degradation of Freeze-Dried Ribonuclease A

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The stability of a freeze-dried model protein, ribonuclease A (RNase), was investigated under accelerated storage conditions at 45°C for time periods up to 60 days. Because RNase is a fairly stable molecule around pH 7, lyophilization was performed in phosphate buffers at pH 4.0 or 10.0 to accelerate degradation kinetics. Degradation was studied by measuring enzymatic activity, the concentrations of soluble monomeric RNase, soluble aggregated (polymerized) RNase, and insoluble aggregated RNase following reconstitution of the lyophilized material at different times. The presence of air in the vial headspace accelerated degradation in the solid state in all cases. When argon or nitrogen was employed in the headspace, degradation kinetics were reduced, implying that molecular oxygen was involved in the degradation process. This interpretation was supported by the observation that 0.05% (w/v) EDTA in the formulation prior to freeze-drying retarded RNase degradation dramatically. EDTA was believed to chelate cations which may have been introduced with the buffer salts in trace quantities sufficient to catalyze autoxidation reactions. Incorporation of antioxidants ascorbic acid (at pH 4.0) and POBN (a spin trap which could have functioned as an antioxidant at pH 10.0) accelerated the degradation of RNase and appeared, in both cases, to be involved in interactions with the protein molecules. Additionally, in the presence of the antioxidants RNase degradation appeared to be accelerated by light. Although there is strong support for the oxidative hypothesis, the possibility of other competing reactions cannot be discounted. These investigations demonstrate the importance of challenging the extrapolation of some of our well-established ideas concerning small molecule solution kinetics to macromolecules in the solid state.

KEY WORDS: proteins; formulation; lyophilization; freeze-drying; protein aggregation.

INTRODUCTION

Even though lyophilization has been used as a means of stabilizing organic compounds having pharmacological activity, inappropriate formulation and freeze-drying conditions can adversely affect the solid-state stability of the final product. In recent years the production of proteins and peptides for both therapeutic and diagnostic purposes has generated the need to extend the understanding of the effects of freeze-drying on the stability of smaller molecules to polypeptides. Lyophilization involves two physical processes, freezing and drying, that may adversely affect the structural integrity of protein molecules by causing irreversible polypeptide chain unfolding and/or molecular aggregation. A number of investigators have documented increased levels of aggregation of purified growth hormones, prolactins, and enzymes following freeze-drying (1–3). A recent study using ribonuclease A as a model showed that storage of the enzyme which had been freeze-dried in a solution containing phosphate buffer resulted in the production of covalently bonded aggregates (4). Aggregate production paralleled the decrease in ribonuclease enzymatic activity.

Many organic compounds have been found to undergo a degradative process which involved molecular oxygen in the role of an oxidizing agent (5,6). This autoxidation process may be catalyzed by metal ions (7) and sometimes results in the formation of free radical intermediates (8). Free radicals are known to be involved in polymerization reactions, which could reasonably explain the origin of covalent bonds between polypeptide chains in close juxtaposition within a freeze-dried cake.

Oxygen may be involved in the autoxidation scheme in several ways. First, regeneration of metal ion catalysts could proceed according to the following reaction (7):

$$M^{(n-1)+} + O_2 \rightarrow M^{n+} + {}^{\circ}O_2 \qquad (1)$$

The superoxide radical, $^{\circ}O_2$, readily leads to the production of hydrogen peroxide and, finally, to the extremely reactive hydroxy radical, $^{\circ}OH$, which may initiate other free radical cascades at sites on the organic molecule (9). Second, oxygen could react with a carbon-centered alkyl radical, R°, to yield an alkylperoxyl radical, ROO°, which could continue to propogate the free radical cascade as follows:

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$$R^2 + O_2 \rightarrow ROO^o \quad (2)$$

$$ROO^o + RH \rightarrow ROOH + R^o \quad (3)$$

Free radicals are propogated by a chain reaction which can lead to addition, substitution, elimination, rearrangement, reduction, radical coupling, or disproportionation reactions (10). Chain termination occurs when two radical species react to generate a stable, covalently linked product. These termination reactions may be described as follows:

$$ROO^o + ROO^o \rightarrow ROOR + O_2 \quad (4)$$

$$ROO^o + R^o \rightarrow ROOR \quad (5)$$

$$R^o + R^o \rightarrow RR \quad (6)$$

RNase was chosen as a model to test the hypothesis that oxygen attack on freeze-dried proteins may be responsible for a loss of biological activity and formation of aggregates. The effects of formulation additives, headspace gas, and storage conditions on freeze-dried stability were the objective evaluation criteria to be used for this investigation. Because freeze-dried RNase degraded relatively slowly around neutral pH (20% loss of enzymatic activity after 120 days at 45°C), conditions were chosen such that under normal conditions ≥40% loss of enzymatic activity would occur over a 30- to 60-day storage period. Results from preliminary experiments indicated that RNase solutions to be freeze-dried having pH values of 4.0 and 10.0 would satisfy these requirements.

Autoxidation of organic molecules has frequently been found to be catalyzed by metal cations which are felt to be involved in the initiation of the free radical cascade using one or both of the following schemes (11):

$$M^{n+} + RH \rightarrow M^{(n-1)+} + R^o + H^+ \quad (7)$$

$$M^{n+} + ROOH \rightarrow M^{(n-1)+} + ROO^o + H^+ \quad (8)$$

The metal ion complexing agent, EDTA, was used as a tool in this study to investigate the possible involvement of metal cations in the oxidation process. Similarly, different antioxidants were used to test the autoxidation hypothesis. Ascorbic acid, a widely used antioxidant in pharmaceutical applications in the range of 3.5–7.2 (12), was chosen for study at pH 4.0 because it undergoes autoxidative degradation relatively slowly at this pH. At pH 10.0 a "spin trap" was used because of its ability to form stable free radical adducts by covalently bonding to highly reactive and short-lived free radicals. The spin trap, POBN, [α-(4-pyridyl 1-oxide)-*N*-t-butylnitrone], has been used as an antioxidant in polymer applications (13). Since the degradation mechanisms may have been photosensitive, experiments were performed under dark and light conditions.

MATERIALS AND METHODS

Preparation, Filling, and Freeze-Drying

Aliquots of ribonuclease A, Type X-A (Sigma Chemical Co., St. Louis, MO), were stored frozen at −70°C at a concentration of 3–4 mg/ml in 0.2 *M* sodium phosphate buffer, pH 6.4. At the initiation of a freeze-drying study the required number of RNase aliquots were thawed in running water at a temperature of 15–20°C. The pH of RNase solutions containing phosphate buffer was changed by dialyzing at 4°C in cellulose dialysis tubing (Spectra/Por 3, Spectrum Medical Ind., Los Angeles, CA) against a sufficient number of buffer changes to achieve the desired pH. In no case did the dialysis exceed 12 hr. The buffer salts used were mono- and dibasic sodium phosphate (J. T. Baker Chem. Co., Phillipsburg, NJ). The two pH values chosen for this study were pH 4.0 and 10.0.

Sufficient double-distilled water was added to the stock RNase solution to adjust the RNase concentration to 1.5–2.0 mg/ml in 0.1 *M* sodium phosphate. Formulation additives, L-ascorbic acid (Sigma, St. Louis, MO), EDTA (Fischer Scientific Co., Fairlawn, NJ), and POBN (Aldrich Chemical Co., Milwaukee, WI) were incorporated at concentrations of 0.01, 0.05, and 0.1% (w/v), respectively. The pH of the solutions was adjusted to the desired value using 10 *N* NaOH (Fisher Scientific, Fairlawn, NJ). The solutions were mixed by swirling, filtered through a 0.22-μm-pore size filter (Millex GV filter, Millipore Corp., Bedford, MA) and filled in 2-ml aliquots into 10-ml sterile, siliconized vials (Kimble Glass Co., Vineland, NJ). Butyl rubber freeze-dry stoppers (20-mm diameter, West Co., Phoenixville, PA) were inserted fully into the vials and the vial contents were rapidly frozen by immersing the bottoms of the vials into an acetone/dry ice bath. The frozen vials were transferred to the freeze-dryer shelves (Hull Corp., Hatboro, PA), which had been precooled to −40°C. The temperature of the vials was then maintained at −40°C for 1 hr. After raising the vial stoppers, primary drying was carried out at a vacuum of <100 mTorr, a condenser temperature of <−60°C, and a shelf temperature of −28°C for 12 hr followed by a shelf temperature of −10°C for 4 hr. Secondary drying was at +20°C for 4 hr. At the end of the freeze-dry cycle either compressed air (Zero Air, Liquid Carbonic, Chicago, IL) or argon (Zero Argon) was introduced into the chamber to return it to atmospheric pressure. The chamber pressure was reduced to 100 mTorr and the venting procedure repeated prior to stoppering the vials in the chamber. Aluminum overseals were crimped into place prior to storage testing.

Stability of Samples at 45°C

The freeze-dried samples were divided into four groups: vials containing either air or argon as the headspace gas and stored at 45°C in either the dark or the light. The illumination was provided by a fluorescent lamp (14 W, General Electric, Cleveland, OH) located 1 ft above the sample vials.

The RNase specific activity assay was determined using the Kunitz enzymatic assay (14) at zero time, 8 days, and 30 days for the pH 10.0 samples and at 0, 30, and 60 days for the pH 4.0 samples. Samples were reconstituted with double-distilled water 10 min before the start of the assay. RNase specific activities were standardized by expressing them as a fraction of non-freeze-dried RNase activity at pH 6.4. Preliminary experiments showed that none of the additives (ascorbic acid, EDTA nor POBN) affected the RNase specific activity results obtained using the Kunitz assay.

The fraction of RNase remaining in solution was determined by calculating the ratio of total protein concentration after storage at 45°C to the total protein concentration at zero time for each category of sample. The method of Lowry

et al. (15) was used to determine the total protein concentration with bovine serum albumin (Sigma, St. Louis, MO) as the standard. The fraction of insoluble RNase (aggregate) was calculated by subtracting the soluble fraction from unity.

Soluble RNase was further subdivided into monomers and soluble aggregates by gel filtration chromatography on a 1.5 × 20-cm column (Sephadex G-75; Pharmacia Fine Chemicals, Piscataway, NJ). The eluent buffer was 0.05 *M* Tris–HCl at pH 7.5 (Sigma, St. Louis, MO) containing 0.5 NaCl and pumped at a flow rate of 0.25 ml/min. RNase was detected by UV spectroscopy (Milton Roy fixed wavelength detector, LDC/Milton Roy, Riviera Beach, FL; Model 740 data module, Waters Chromatography Division, Millipore Corp., Bedford, MA) at 280 nm. The fraction of RNase in the monomeric form was calculated from the ratio of monomeric peak height after storage to the height at zero time. The extinction coefficient of the monomer was constant throughout the plots of peak height versus RNase concentration were rectilinear. Because of possible variations in the absorptivities of soluble aggregates, fractions of RNase in this form, designated f_{sa}, were determined from the following equation

$$f_{sa} = 1 - f_{ia} - f_m \quad (9)$$

where f_{ia} and f_m are fractions of insoluble aggregate and monomers, respectively.

In the freeze-dried samples containing either POBN or ascorbic acid it was not possible to resolve the monomeric and aggregate peaks because the POBN or ascorbate molecules, both of which have large absorbances at 280 nm, were apparently binding to the aggregated RNase. For this reason the fraction of soluble monomer and soluble aggregate could not be quantitated. In these samples the RNase was reported as the fraction of the initial RNase found as soluble RNase and the fraction as insoluble aggregated RNase.

The data points plotted in Figs. 1–6 represent the mean of four sample values.

RESULTS

RNase in Phosphate Buffer, pH 10.0, Without Additives

As a baseline, the results of RNase freeze-dried in phosphate buffer alone, vented to compressed air and stored in the light and dark are shown in Figs. 1a and b, respectively. The histograms show the fraction of soluble monomer, soluble aggregates, and insoluble aggregates, while the line graphs depict the fraction of control RNase specific activity remaining for each sample. Samples stored in both the light and the dark showed an 80% decline in specific activity after 8 days and a corresponding increase in soluble aggregated protein. After 30 days at 45°C both the light and the dark samples had specific activities that were about 10% of initial activity. The major change was the large increase in insoluble, aggregated protein, which amounted to about one-half of the total protein present at zero time. The increase in insoluble aggregates paralleled a decrease in the quantity of soluble aggregates, implying that the insoluble aggregates resulted from more extensive cross-linking or polymerization of the RNase molecules as a function of storage time at 45°C.

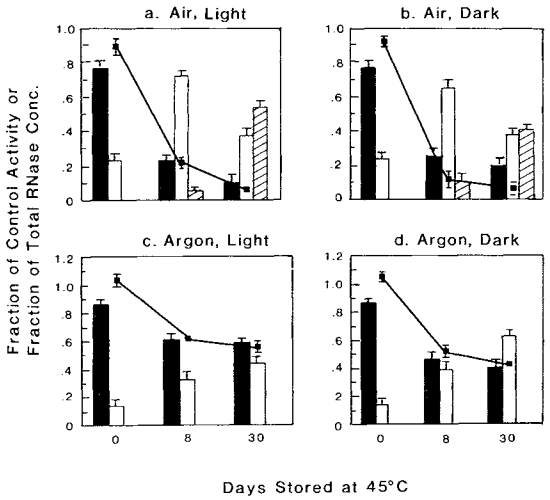

Fig. 1. RNase freeze-dried in pH 10.0 phosphate buffer without additives. The line graph depicts fraction of control RNase specific activity and the bar graphs represent the fraction of total RNase concentration. (■) Soluble monomeric RNase; (□) soluble aggregate; (▨) insoluble aggregate.

The presence or absence of light during the incubation had no apparent effect on the increase in RNase aggregate or loss of specific activity.

A marked difference in the quantity of both soluble and insoluble aggregate after 8 and 30 days of incubation at the elevated temperature can be seen in Figs. 1c and d, where samples contained argon instead of air in the vial headspace. For both the light- and the dark-incubated samples the loss of specific activity with argon was about half of that seen in the presence of air. Even though some soluble aggregate did form during the incubation period, there was no insoluble aggregate present even after 30 days at 45°C. Equivalent results were obtained (data not shown) when ultra-high-purity nitrogen was used to fill the vial headspaces at the end of the freeze-dry cycle. These data suggested that air, namely, oxygen, was necessary for the production of both soluble and insoluble aggregates and for the corresponding decline in specific activity. Soluble aggregates that formed in the argon-containing vials may have been due to the residual oxygen molecules adsorbed to the solid surfaces following freeze-drying.

RNase in Phosphate Buffer, pH 10.0, with EDTA

Figure 2 shows the results of the stability studies on RNase-containing solutions when the chelating agent EDTA was added to the formulation. In the samples exposed to air there was no insoluble aggregate formed. The loss of specific activity amounted to half that observed without EDTA. In the presence of argon there was no insoluble aggregate formed and much less soluble aggregate than in the vials filled with air. The specific activities for the argon-filled vials also remained higher than those filled with air. It is likely that oxygen as well as divalent metal cations, which would have been chelated by EDTA in these samples, played a role in the aggregation process that resulted in the production of soluble and insoluble protein aggregates. Samples stored in the dark behaved similarly to those exposed to light.

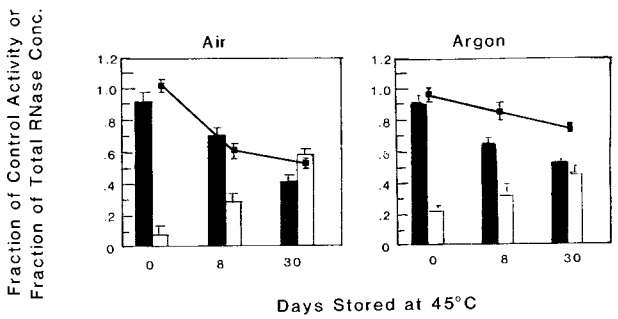

Fig. 2. RNase freeze-dried in pH 10.0 phosphate buffer with EDTA. The line graph depicts the fraction of control RNase specific activity and the bar graphs represent the fraction of total RNase concentration. (■) Soluble monomeric RNase; (□) soluble aggregate. Insoluble aggregates were not detected in these samples.

RNase in Phosphate Buffer, pH 10, with POBN

The results of incorporating the free radical "spin trap," POBN, into the freeze-dry formulation containing RNase are shown in Fig. 3. The pattern of enzymatic activity loss as a function of time when the samples were incubated in the air (Figs. 3a and b) was similar to those samples containing RNase in buffer alone. Along with the activity loss was the formation of a membranous precipitate, which after 30 days of storage, amounted to 40 to 50% of the total protein.

In the presence of argon, the specific activity of the samples stored in the dark (Fig. 3d) was ≥70% of the control RNase specific activity during the 30-day storage period, while for samples stored in the light (Fig. 3c) the activity declined to 40% of control. This finding suggests that in the POBN-mediated degradation of RNase, light leads to a greater amount of insoluble RNase and a greater reduction in specific activity. In the presence of air (Figs. 3a and b) it is likely that multiple mechanisms were operative in the RNase process.

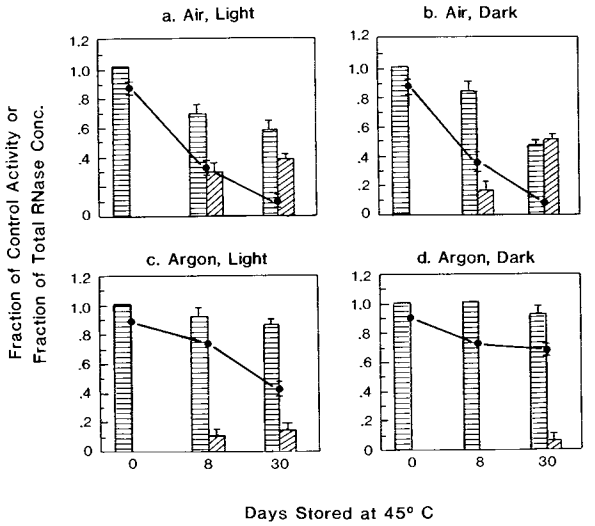

Fig. 3. RNase freeze-dried in pH 10.0 phosphate buffer with POBN added. The line graph depicts the fraction of control RNase specific activity and the bar graphs represent the fraction of total RNase concentration: (▤) the soluble RNase and (▨) the insoluble aggregated RNase. Soluble RNase was not classified into monomers and soluble aggregates in these experiments.

RNase in Phosphate Buffer, pH 4, Without Additives

At pH 4.0 the degradation reaction of freeze-dried RNase was much slower than at pH 10.0 and no insoluble aggregated RNase formed. The results for RNase in buffer alone are presented in mixed histogram and line-graph format in Fig. 4. In the presence of air, samples stored for 60 days in the light (and dark) showed a decrease in RNase specific activity to between 50 and 60% of the starting value. The specific activity assays indicated that soluble monomers, as determined by gel permeation chromatography, remained as biological active entities.

In the samples exposed to argon the specific activity decreased to about 80% of the initial value. The amount of soluble, aggregated RNase in these vials amounted to only about half of that found with the samples containing air, showing once more the involvement of oxygen in the decomposition of lyophilized RNase.

RNase in Phosphate Buffer, pH 4, with EDTA

Figure 5 shows the effect of adding EDTA to the RNase formulation at pH 4.0. The results were similar to those at pH 10 but the loss of activity due to the formation of soluble aggregation was much slower. The amount of soluble, aggregated RNase was overall somewhat less when argon was the headspace gas.

RNase in Phosphate Buffer, pH 4, with Ascorbic Acid

Figure 6 shows the results when ascorbic acid was incorporated into the RNase formulation at pH 4.0. The values plotted are the mean specific activities of RNase in stored samples with the designated headspace gas in the light or dark. There was no demonstrable difference in the specific activity values obtained for light and dark incubation in either gaseous environment but the samples under an argon environment retained much more of their initial specific activity than those containing air.

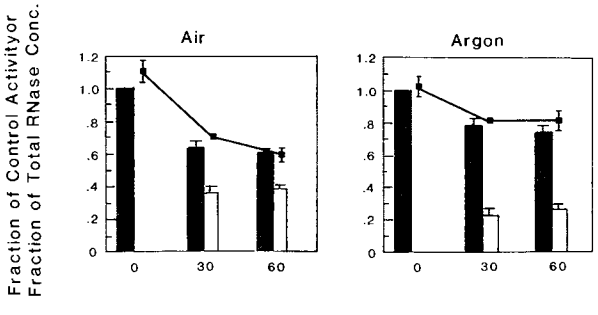

Fig. 4. RNase freeze-dried in pH 4.0 phosphate buffer without additives. The line graph depicts fraction of control RNase specific activity and the bar graphs represent the fraction of total RNase concentration. (■) Soluble monomeric RNase; (□) soluble aggregate. Insoluble aggregates were not detected in these samples.

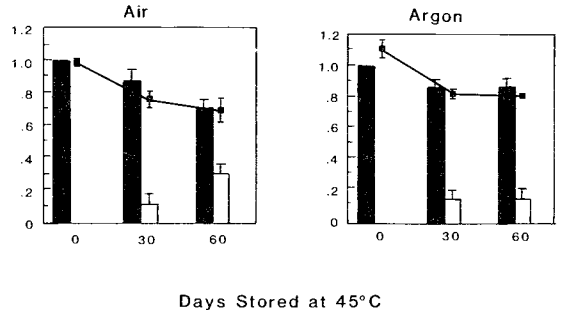

Fig. 5. RNase freeze-dried in pH 4.0 phosphate buffer with EDTA added. The line graph depicts fraction of control RNase specific activity and the bar graphs represent the fraction of total RNase concentration. (■) Soluble monomeric RNase; (□) soluble aggregate. Insoluble aggregates were not detected in these samples.

DISCUSSION

RNase samples freeze-dried in pH 10.0 buffer and stored at 45°C in the presence of argon, as compared to those stored in air, had a greater retention of specific activity as a function of time, and there was virtually no insoluble, aggregated RNase produced. This indicated that oxygen was involved in the aggregation phenomenon, which led to the concomitant loss of enzymatic activity. Although the degradation reaction proceeded more slowly at pH 4.0 than at pH 10, similar mechanisms appear to be operative. However, at the lower pH, soluble aggregates did not appear to degrade further into insoluble or nonreconstitutable material.

The aggregate-forming mechanism can be further explained by the protection afforded with the chelating agent EDTA at both pH values in the presence of air and argon. EDTA is known to chelate transition metal cations, e.g., Fe(III) and Cu(II) (16,17). Therefore, it is reasonable to postulate that metal cations played a role in the formation of RNase aggregates, although other competing mechanisms might also be involved.

The free radical-producing reactions that are catalyzed by metal cations involve the transfer of electrons by redox cycling between two different valence states of the cation. EDTA has been shown to reduce the facileness of these electron transfer reactions between valence states (11). This activity has a dampening effect on autoxidation reactions, which would explain EDTA's effectiveness in reducing aggregate formation in these experiments. It has been shown that RNase freeze-dried in distilled water without buffer salts had a degradation rate that was much slower than that of RNase freeze-dried in the presence of phosphate buffer (4). This evidence points to the buffer salts as being the most likely source of the metal cations. Since these act as catalysts in autoxidation, only trace amounts would be necessary to initiate and contribute to the propagation of the reaction.

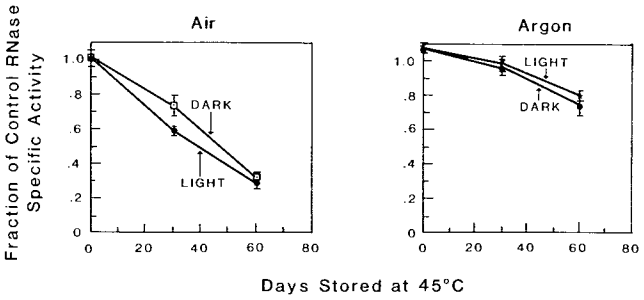

Fig. 6. RNase freeze-dried in pH 4.0 phosphate buffer with ascorbic acid added. The line graphs represent the fraction of control RNase specific activity.

It is well known that both anions and cations bind to proteins (18–21). In general, there are specific sites on each protein molecule where metal cations will preferentially bind (22–24). Since these metal cations are instrumental in at least the initiation of free radical chain reactions, it has been suggested that the generation and attack of free radicals may occur within localized environments near binding sites on the protein molecule (25). The hypothesis seems even more plausible when the limited mobility of both large and small molecules in a freeze-dried cake is considered.

The rate and extent of RNase aggregation were greater at alkaline pH than at acidic pH values. This may have been in part due to the greater tendency of the divalent cations that were responsible for catalyzing the aggregation reactions to bind to a protein in an alkaline medium. As the pH of the protein-containing medium increased, the overall charge on the protein would have become more negative, which would have allowed for greater binding of divalent cations. The increased cation binding may have acted in concert with the effects of an alkaline environment in facilitating the removal of a proton during the initial generation of carbon-centered alkyl free radicals.

The addition of antioxidants, in general, was unsuccessful as a means of stabilizing these formulations. Ascorbic acid failed to protect the RNase from loss of specific activity and may even have accelerated the rate at which the insoluble aggregate was formed in the presence of air. Uri found that even though ascorbic acid has been termed an antioxidant, it is not very effective, because in the presence of certain metal catalysts it behaves as a prooxidant (26) and in the absence of metal catalysts it has the tendency to undergo autoxidation itself, yielding highly reactive radicals and hydrogen peroxide (27). Both of these byproducts could propagate the free radical chain reaction in which RNase was involved, thereby accelerating the rate of decomposition. Similarly, POBN at pH 10.0 accelerated decomposition on the freeze-dried RNase.

Light had little discernible effect on the loss of enzymatic activity and the production of RNase aggregates except in the case of the use of POBN as an antioxidant at pH 10.0. Therefore light played no significant role in the production of aggregated RNase when only buffer salts were present in the lyophilized cake. However, when another organic compound which may have been sensitive to photochemical degradation was present in the cake, such as the nitrone POBN, and the redox microenvironment was suitable (i.e., an alkaline environment), the lytic products of its degradation may have exacerbated the background level of RNase enzymatic activity loss as a result of oxidation and any other competing reactions.

The ineffectiveness of both antioxidants emphasizes (a) the importance of screening for accelerating effects during formulation development and (b) the need to challenge the hypothesis that molecules that are preferentially oxidized in solution necessarily undergo the same reaction in the solid

state. It seems likely that molecules such as POBN and ascorbic acid are more likely to function as reactants with proteins when they are held in close proximity in formulations of this nature.

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