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# Thermal analysis of proteins of pharmaceutical interest

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**Abstract**

Four major methods for the thermal analysis of proteins are reviewed. Thermogravimetric analysis (TGA) is commonly used for moisture content measurements in raw materials and lyophilized protein products. Differential scanning calorimetry (DSC) can detect and quantitate changes in the transition temperature and enthalpy due to formulation variables such as pH, ionic strength, and stabilizers. DSC is also used to detect the glass transition temperature $T_g$ in protein solutions and lyophilized products. The determination of $T_g$ is necessary in defining a freeze-drying cycle and storage temperature for the lyophilized products. Thermomechanical analysis (TMA) is used to detect $T_g$ and is more sensitive than DSC. Electrical resistance measurement has been used to detect eutectic temperature and thermal expansion events but its application in detecting $T_g$ requires further investigation.

*Keywords:* DSC; Glass transition; Protein; Stability; TGA; TMA

## 1. Introduction

Protein pharmaceuticals have become a significant component of the world pharmaceutical market. The market value of biologics as biochemical reagents, therapeutic preparations, and diagnostic reagents is greater than $300 billion [1]. In 1992, there were 17 biotechnology drug products with $2.4 billion in US sales alone [2].

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Product development of protein drugs presents special problems to pharmaceutical scientists. A major problem is the inherent instability and loss of activity that occurs when proteins are removed from their natural environment. The mechanisms of protein degradation by chemical or physical means have been extensively reviewed [3–5]. Unlike small molecule drugs, protein drugs possess specific secondary and higher levels of structure. These higher levels of structure are maintained by relatively weak non-covalent interactions. The enthalpies of these interactions are typically in the range 5–20 kcal $mol^{-1}$ (equivalent to 3–5 hydrogen bonds). Disruption of these weak interactions can be caused by factors such as temperature (heat or cold), pH, salt, pressure, shear, surface interactions, and freeze-drying. Thermal analysis is useful for detecting the effects of these factors on proteins or protein drug products.

Analytical methods such as thermal analysis, spectroscopic methods (ultraviolet, fluorescence, circular dichroism, infrared, Raman, and light scattering), electrophoretic methods (polyacrylamide gel electrophoresis, isoelectric focusing and capillary electrophoresis), liquid chromatographic methods (size exclusion, hydrophobic interaction, ion exchange, affinity and reverse phase), liquid and solid nuclear magnetic resonance, have been applied to protein drug product development. Each method has its own limitations and advantages [3,5,6]. This review focuses on the major method of thermal analysis, their applications and significance. Detailed thermodynamics and physical chemistry are not discussed. For further information, reviews of the thermodynamics of protein conformation in aqueous solutions using calorimetry are available in the literature [7–11].

The major methods for thermal analysis of protein drugs include thermogravimetry, differential scanning calorimetry, thermomechanical analysis, and thermoelectrometry. Other methods such as dynamic thermomechanometry, thermophotometry, thermomagnetometry, and thermosonimetry require less commonly available instrumentation. Few literature citations address these techniques and they are not discussed here.

## 2. Thermogravimetric analysis (TGA)

In thermogravimetry, the change in sample mass is measured by a thermobalance as a function of temperature or time. The sample is heated in a furnace according to a time–temperature program. This method is routinely used to determine the sample moisture content, hydration level, and decomposition temperature. By considering the temperature range of the weight loss, adsorbed and bound water can be differentiated. The pattern and temperature range of sample decomposition can also serve as a qualitative tool for compound identification. In addition, the components of the evolved gases can be analyzed by GC–MS [12].

Moisture content is critical to protein stability [3,13]. Higher moisture content usually adversely affects protein stability. For example, lyophilized recombinant bovine somatotropin was shown to lose potency by an order of magnitude faster when moisture content increased from 2% to 5% [14]. The content of oxidized

hemoglobin is doubled when moisture in the lyophile increases from 2% to 8% [15]. The aggregation rate of lyophilized human growth hormone is about five-fold greater at 3% moisture than at less than 0.5% [16]. Moisture induced aggregation via disulfide scrambling was also detected in lyophilized proteins such as bovine serum albumin, ovalbumin, glucose oxidase, and $\beta$-lactoglobulin [17].

For some proteins, the popular concept of "the drier the better" may not be appropriate. When tissue type plasminogen activator (tPA) was dried to a moisture content below the calculated monolayer water level, some denaturation occurred during lyophilization [18]. Without stabilizer, the aggregation of human growth hormone increases from 4% to 6% when moisture decreased from 6%–10% down to 3% [16].

In lyophilized products, moisture can come from residual water in the product or from the stopper. Moisture from the stopper can affect the physical (collapse of plug) or chemical (degradation or/and aggregation) stability of the product. Pikal and Shah [19] showed that the significance of this moisture depends on product hygroscopicity and storage temperature. In their study, the time required to double the moisture content in freeze-dried vancomycin and lactose was less than one month at 25°C and 40°C and about 10 months at 5°C. Although PedvaxHIB™ can tolerate moisture level of 5% without loss of physical and chemical stability, the moisture content was 1.53% for the lot with dried stoppers and 3.87% for the lot with non-dried stoppers after storage at 2–8°C for 8 months. After one year, the moisture content was 1.61% and 5.35% respectively [20].

The moisture content of lyophilized products is commonly determined by gravimetric, Karl Fischer, or thermogravimetric methods. Table 1 shows the published moisture data for six freeze-dried viral vaccines using these three methods [21].

In summary, the Karl Fischer and TGA methods were in good agreement while the gravimetric method was consistently lower. One of the reasons for the discrepancy is that the gravimetric method is measuring only some of the bound water; one water of hydration only in the case of sodium tartrate dihydrate. However, the routine application of TGA for moisture level determination in a raw material or product is cautioned due to the possible coevolution of other volatile components. It should be noted that water pick-up during sample preparation and handling can contribute to variability in data, especially for hygroscopic materials.

## 3. Differential scanning calorimetry (DSC)

In DSC, the difference in the energy input required to maintain a test sample and a reference cell at the same temperature is measured while being scanned across a controlled range of time and temperature. In a related method, differential thermal analysis (DTA), the temperature difference between a sample and a reference material is measured. DSC and DTA curves are very similar in appearance except for the ordinate axis units. In each method, the area under a transition peak is proportional to enthalpy. Due to simplified calculations to obtain thermodynamic parameters and advances in technology, DSC has largely replaced DTA in most applications.

Table 1
Results of the replicate determinations of residual moisture by gravimetric, Karl Fischer, TG (profile), and TG (60°C hold) methods in a sodium tartrate dihydrate control sample and six types of viral vaccines freeze-dried in buffered sorbitol–gelatin (from Ref. [21])

| Test method | Percentage residual moisture ± s.d. | | | | | | |
|---|---|---|---|---|---|---|---|
| | Measles virus vaccine Lot A | Rubella virus vaccine Lot B | Rubella and mumps virus Lot C | Mumps virus vaccine Lot D | Measles, mumps, and rubella virus vaccine Lot E | Measles and rubella virus Lot F | Sodium tartrate dihydrate |
| Gravimetric | 0.79 ± 0.32 | 0.42 ± 0.18 | 0.41 ± 0.26 | 1.10 ± 0.40 | 0.18 ± 0.14 | 1.15 ± 0.14 | 7.93 ± 0.02 |
| Karl Fischer | 1.18 ± 0.14 | 1.03 ± 0.14 | 0.72 ± 0.16 | 1.54 ± 0.20 | 0.80 ± 0.14 | 2.26 ± 0.35 | – |
| TG (profile) | 1.25 ± 0.16 | 0.99 ± 0.12 | 0.76 ± 0.12 | 1.54 ± 0.15 | 0.76 ± 0.11 | 2.35 ± 0.11 | 15.68 ± 0.21 |
| TG (60°C hold) | 1.17 ± 0.20 | 1.17 ± 0.19 | 0.74 ± 0.13 | 1.53 ± 0.17 | 0.70 ± 0.08 | 2.20 ± 0.40 | – |

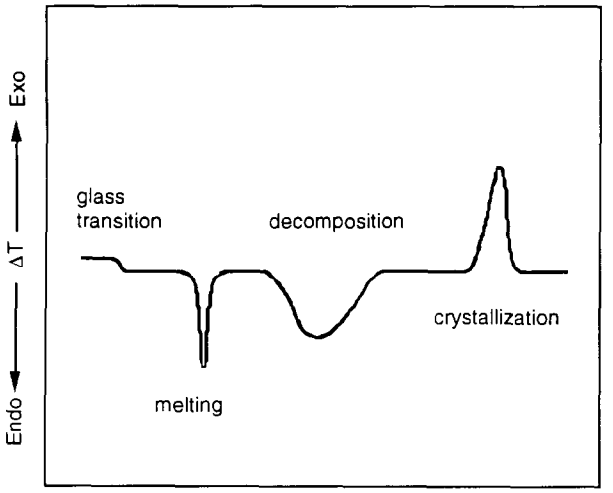


Fig. 1. Typical thermal events of differential scanning calorimetry.

DSC can be used to characterize a drug component by measurement of its specific heat, glass transition temperature, melting point, sublimation, decomposition by heat, isomerization, or polymorphic transitions. Examples of the four most common thermal events detected by DSC are illustrated in Fig. 1. These events include: (i) a second order glass transition $T_g$ in which a change in the horizontal baseline is seen; (ii) first order transitions such as an endothermic peak caused by a melting transition $T_m$; or (iii) an endothermic peak due to a decomposition or dissociation reaction; and (iv) an exothermic peak due to crystallization. Thermal transitions of proteins that are often studied include the first order transitions $T_m$ associated with conformation changes and the second order glass transition $T_g$ seen in frozen protein solutions or lyophiles.

### *3.1. DSC and protein stability*

The earliest application of calorimetry to the study of protein stability was conducted to determine the melting/transition temperature $T_m$, and partial heat capacity $C_p$ of protein solution as a function of temperature [7–11]. Use of DSC is increasing for investigating protein conformational changes as a function of temperature and, more importantly, the effect of potential stabilizing excipients in a protein formulation.

A limitation to the application of DSC is that protein solutions are often dilute (by weight and especially on molar basis) and the change in enthalpy or heat capacity is small. However, practical application of equipment commonly found in research laboratories is possible. Uedaira and Uedaira [22] used a Perkin-Elmer DSC 2 to report that sugars increased the transition temperature ($\Delta T_m = 5°C$, $\Delta H = 32$ kJ mol$^{-1}$) of lysozyme solution and stabilized its native conformation. Eynard et al. [23] reported good correlation between transition temperature mea-

sured by a Perkin-Elmer DSC 7 and those by fluorescence methods ($\Delta T_m = 22°C$ between native and denatured states). Using a Perkin-Elmer DSC 2, Yoo and Lee [24] demonstrated improved stability ($\Delta T_m = 0.4–0.7°C$, $\Delta H = 0.11–0.26$ mcal $mg^{-1}$) in spray-dried egg white when sorbitol was used as a stabilizer. Advances in technical features and a historical perspective of the development of scanning calorimeters have been reviewed by Privalov and Plotnikov [25].

In microcalorimetry, both reference and sample cells are fixed in place (not disposable), enclosed, and suspended in adiabatic shields. Thermopile systems detect temperature differences between the cells and also between the shields and the cells, and activate heating mechanisms to ensure that very little, if any, temperature difference exists inside the adiabatic assembly. Thus, there is near identity of cell geometry, cell volume, rate of heating, physical properties (e.g. thermal conductivity) and environment. This ensures that the calorimetric output reflects only the thermally induced event(s) in the protein and not factors such as non-identical heating between the reference and sample cells, enthalpy of the solvents, etc. In conducting measurements, the most apparent differences between a regular DSC instrument and a microcalorimeter are larger sample size, slower scanning rate and suspended cells. Chowdhry and Cole [26] summarized the differences between a regular DSC instrument (DSC 7) and a microcalorimeter (DASM-4, MC-2, Bio DSC); their results are shown in Table 2.

Due to the sensitivity of microcalorimetry, it has been used by biochemists to identify the appropriate sites for amino acid replacement and for cross linking. Using microcalorimetry, lysozyme cross-linked between Glu 35 and Trp 108 was found to be more stable ($\Delta T_m = 17–19°C$) than the wild type [27]. For T4 lysozyme, the R96H mutant increased $T_m$ by 15°C when compared with the wild type [28]. Ladbury et al. [29] identified the most stable mutant form of T4 lysozyme by the largest difference in $T_m$ (2.8°C). This application of microcalorimetry is attracting much attention in protein engineering.

A shortcoming of both techniques is that the source of a thermal event cannot be unequivocally stated without supportive methodology. For example, a DSC scan cannot distinguish between destabilization of the native form of a protein and stabilization of the denatured form. It is often useful to enlist an optical method to differentiate between the two states. In addition, the observed scan is affected by the temperature scanning rate. The effects of heating rate on DSC scans have been described in detail [30].

However, this feature can be used to validate a given kinetic model to describe the unfolding processes of a protein molecule. By varying scanning rates, DSC has been used to calculate the fraction of irreversibly denatured $\beta$-lactamase as a function of temperature [31]. The denaturation of glucosamine-6-phosphate deaminase was adequately explained by a kinetic model which includes six two-state sequential transitions [32]. Using both circular dichroism and DSC, Dudich et al. [33] established that $\alpha$-interferon corresponds to the "two-state" model and can be described by a first order reversible reaction. Other calorimetric studies described the denaturation of small, globular proteins (10–25 kDa) by the "two-state" model [8,9,26]. However, larger proteins are not well described by the "two-state" model

Table 2
Characteristics of some commercial DSC instruments (from Ref. [26])

| Instrument | Sample loading by volume or weight | Effective cell volume/ml | Temperature range/K | Heating rate/K $min^{-1}$ | Calibration | Reduced noise level/ μW at 1 K $min^{-1}$ | Relative error in heat capacity determinate/% |
|---|---|---|---|---|---|---|---|
| DSC-7 (Perkin Elmer, USA) | Weight | 0.003–0.075 | 100–1000 | 0.1–1.50 | Standard sample | >2 | 0.8 |
| DASM-4 (Acad. Sci. USSR) | Volume | 0.5 | 250–403 | 0.125–2.0 | Electrical | 0.4 | 0.005 |
| MC-2 (Microcal, USA) | Volume | 1.3 | 253–383 | 0.17–1.5 | Electrical | 0.2 | 0.002 |
| Bio DSC (Setaram, France) | Volume | 1.2 | 263–373 | 0.02–100 | Electrical | 0.2–1.8 | 0.005 |

because their conformation is determined by multiple discrete associations or domains within the protein [9].

Back et al. [34] investigated the different stabilizing effects of sugars and polyhydric alcohols (polyols) on different proteins. They concluded that the magnitude of the stabilizing effect as defined by $\Delta T_m$ varies with the protein and with the sugar or polyol. Glycerol and sorbitol as well as other polyols are proposed to increase the denaturation temperature $T_m$ of proteins due to increased hydrophobic interactions [22]. A similar study was also published by Gekko [35] and supported the hypothesis that the thermal stabilization of proteins by polyols is due to the preferential exclusion mechanism. The most stabilizing polyol formulation for thrombin, 25% glycerol and 25% sorbitol, was identified using DSC data [36]. DSC also detected the stabilizing effect of calcium ions to some proteins [37,38]. Surface denaturation of seven proteins by silica was detected by DSC data. With the additional analyses by spectroscopic methods, this study indicated that increasing surface apolarity produced decreasing stability and increasing structural alteration of the adsorbed protein [39].

DSC can also measure the effects of variables such as pH, ionic strength, metal ion (e.g. Ca, Mg, etc.) and stabilizer on protein stability in solution [40–49]. Consequently, the formulation for a given protein can be optimized. For example, denaturation mechanisms of $\beta$-lactoglobulin [50] and *Streptomyces* subtilisin inhibitor [51] were studied at 0–20°C. DSC studies of $T_m$ at temperatures below 0°C will not be informative since ice melting will be the major event. DSC can also be applied to solid samples such as lyophilized products. Izutsu et al. [1] have demonstrated a good correlation between enyzme activity and the observed denaturation enthalpy of $\beta$-galactosidase powder formulation.

### *3.2. DSC, glass transition temperature, and protein stability*

Lyophilization is often used to stabilize protein products with limited shelf-lives in solution. It is therefore not surprising that more than one quarter of the therapeutic protein products on the market are lyophiles. The determination of the lyophilization cycle is important because of physical changes that occur in the solution during the freezing and drying phases of the process. Due to the amorphous nature of protein and stabilizer (most commonly sugars or polyols), lyophilized formulations often exhibit a glass–rubber transition that is an important parameter in developing the freeze-drying cycle. Therefore, the glass transition of a lyophilized product can be studied and applied to improve processability, quality, and stability of the product.

The theory of the glass–rubber transition originates from polymer science. Proteins, being polymeric, can exhibit a glass transition and their solutions have been found to have a specific glass transition temperature $T'_g$, generally around −10°C. For example $T'_g$ is −11°C for ovalbumin, −13°C for lysozyme, −9°C for lactic dehydrogenase, and −11°C for bovine serum albumin [52–54].

Proteins can rarely be lyophilized without loss of activities (i.e. conformation) and a stabilizer such as a sugar or polyol is usually required. The addition of a

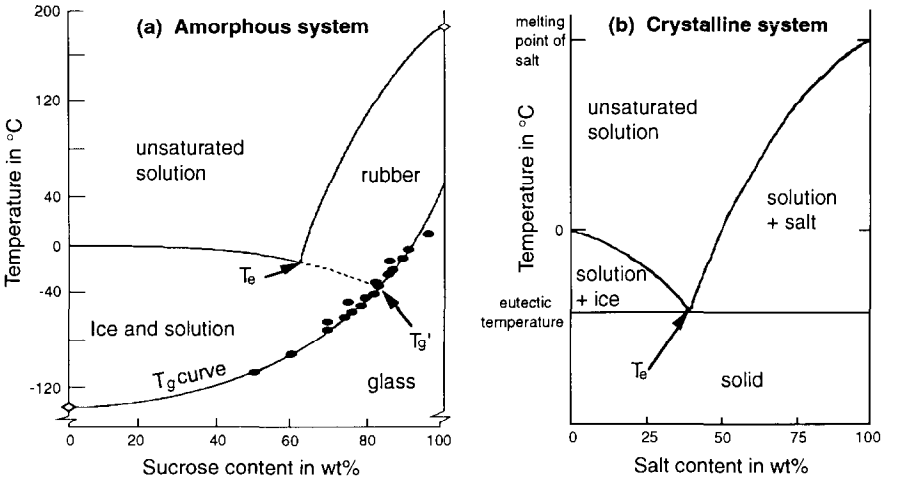


Fig. 2. Phase diagrams of (a) an amorphous system and (b) a crystalline system ( ● represents observed $T_g$ values) [54,55,76].

stabilizer, usually at a much higher concentration than the protein itself, results in a predominant glass transition which is seen on cooling or dehydration [55,56]. The protein in a formulation for lyophilization does not contribute to the resulting $T'_g$ due to the lower concentration, usually $<10^{-3}$ M. Fig. 2(a) shows a generalized phase diagram of such a transition in an amorphous protein–sugar or protein–polyol system. In comparison, Fig. 2(b) is a phase diagram of a crystalline system in which a eutectic temperature $T_e$ exists.

According to the phase diagram in Fig. 2(a), upon freezing, a dilute protein formulation solution does not nucleate and form a solid eutectic mixture. Instead, the solution becomes more concentrated as pure water freezes. At sufficiently high solute concentrations, the remaining solution will undergo a glass transition. Thus, dilute solutions usually exhibit a glass transition temperature $T'_g$ upon freezing. For highly concentrated solutions (usually >50%, w/w), an individual glass transition temperature $T_g$ is seen as shown in the phase diagram. In this region of the phase diagram, $T_g$ becomes concentration dependent. Therefore, $T_g$ is concentration dependent for concentrated systems while $T'_g$ is concentration independent for dilute systems.

At temperatures below the glass transition temperature, the solute matrix is a glass and behaves like a solid. Lyophilization is best conducted below this temperature. If the temperature of the frozen zone rises above the $T'_g$, the concentrated amorphous solution becomes less viscous and lyophilization under such a condition may cause product collapse. During lyophilization, water is removed and the solute concentration of the matrix increases. Due to water loss, the matrix becomes more rigid and the $T_g$ increases as Fig. 2(a) indicates. The product can then tolerate high lyophilization temperatures without undergoing collapse. Further differentiation of these two terms $T'_g$ and $T_g$ will not be addressed in this text and a generalized $T_g$ will be used.

$T_g$ is a second-order transition and characterized by a discontinuity in the relationship between temperature and heat capacity. As shown in Fig. 1, the DSC scan of an amorphous sample exhibits this transition as a baseline shift and $T_g$ is defined as the midpoint of such a shift. $T_g$ is also defined in kinetic terms as the temperature below which the viscosity of a liquid is at least $10^{13}$–$10^{14}$ Pa s. To define $T_g$ using DSC, three criteria must be met: (i) the scan must show a true discontinuity in heat flow and not return to the baseline; (ii) it must be possible to scan in and out of this transition, i.e. reversibly between the glass and rubbery states; (iii) when holding a sample isothermally above $T_g$, crystallization should occur [57]. The third criterion is sometimes hard to meet, because crystallization can take as little as 10 min for amorphous lactose and sucrose [58–60], 24 h for phenobarbital, or more than 2 years for indomethacin [16]. Freeze-dried sucrose, however, crystallized after storage at 60°C for only one month [62].

$T_g$ also depends on the composition of the protein solution. As previously mentioned, $T_g$ is concentration independent for dilute solutions but is dependent on the ratio of the components. A technique to increase the sensitivity of the detection is to increase the concentration of each component while maintaining a constant ratio among the components [63]. $T_g'$ increases along with molecular weight within a homologous series of polymeric materials [64–66]. Also, an increase in the moisture content lowers the $T_g$ of an amorphous solid [62,67]. To and Flink [67] reported that a 1% increase in moisture decreased $T_g$ by 5°C in most samples. The thermal history of a sample is important and will affect the measured $T_g$. To compensate for thermal history, it has been recommended that samples be heated to a high temperature, usually more than 100°C, in order to erase any residual structure in the glass remaining from any previous thermal treatment [30,68,69]. Of course, the stability of the sample at this temperature must be assured. The possibility of residual structure in the sample is likely to be the reason why some authors have suggested that a second scan be used as the representative $T_g$ [16,70–72]. There is no standardized method by which to calibrate scanning calorimeters for $T_g$ transitions of proteins. It has been suggested that the unfolding of RNAase and lysozyme be used as calorimetric standards [73,74].

Other important factors which can influence $T_g$ include the cooling rate, heating rate, anneal temperature, and anneal time. These phenomena are well documented [60–65,75–77]. To avoid the confusion due to an overlapping endothermic peak and glass transition, it is best to quench cool and then warm the sample at a fast heating rate, at least 10–40°C $min^{-1}$. Annealing is necessary for some samples in order to achieve maximum freeze-concentration and eliminate the devitrification which is typical of non-annealed carbohydrate solutions. It is suggested that annealing eliminates the microheterogeneity which occurs during quenched cooling [68,78].

Comparable $T_g$ values obtained by DSC were reported when related to those literature values of collapse temperature $T_c$ measured by cryomicroscopy [54,78,79]. Chang and Randall [54] observed a close relationship between $T_g$ and collapse phenomenon. However, Pikal and Shah [80] contended that the distinction between $T_g$ and $T_c$ is subtle but possibly important. They reported that $T_g$ is slightly lower than $T_c$ when measured at low warming rates. Pikal later agreed that $T_g$ and $T_c$ are

identical for most practical purposes [81,82]. In summary, the choice of lyophilization conditions is formulation dependent and DSC is a convenient method for assessing these effects.

The stability of proteins in the fluid or glass states is described by the Arrhenius equation

$$\frac{\mathrm{d}\ln k}{\mathrm{d}T} = \frac{\Delta E^{a}}{RT^{2}} \tag{1}$$

and the rubber state by the Williams–Landel–Ferry (WLF) equation

$$\ln k = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)} \tag{2}$$

where $k$ is the rate constant, $T$ is the temperature in kelvin, $\Delta E^{a}$ is the activation energy, $R$ is the universal gas constant, $C_1$ and $C_2$ are the coefficients that describe the temperature dependence of the relaxation process, and $T_g$ is the glass transition temperature.

It follows that the rate constant $k$ is much more sensitive to temperature when the material is in the proximity of $T_g$ as indicated by the WLF equation than would be predicted by the Arrhenius equation. The differences in the degradation rate predicted by these two kinetic models can be described by the computer simulated data of Karel and Saguy [83] shown in Fig. 3.

The WLF relationship was not observed due to the lack of $T_g$ detection in lyophilized human growth hormone [16]. However, the degradation kinetics of freeze-dried monoclonal antibody–Vinca conjugate obeyed the WLF relationship [70]. The crystallization rate of amorphous sugars is also well approximated by the WLF equation [78]. Based on these studies, many researchers support the concept that, "the higher the $T_g$, the more stable is the formulation". The development of Permazyme® technology is based on this concept and has been applied to the preparation of some commercial molecular biology reagents [84,85]. In a study by Levine and Slade [86], solutions containing glucose oxidase of a wide range of $T_g$ were prepared and observed to follow WLF kinetics. The enzyme, glucose oxidase, was only active when the solution mixture was stored at temperatures below $T_g$.

For some systems, protein degradation does not follow either model alone and more complicated mechanisms are involved. Rate constants of non-enzymatic browning in food systems were less than those predicted by WLF kinetics [87]. Using three model systems (enzyme activity, protein aggregation, and chemical reaction) Lim and Reid [88] observed that a maltodextrin system was fairly well described by the WLF model while sucrose and carboxymethylcellulose (CMC) were not. Simatos and Blond [89] also observed that chemical or biochemical reactions in frozen food systems are generally slower than those expected from the WLF theory. These deviations may come from the following: (1) reaction rate may depend on diffusivity; (2) attainment of equilibrium at a given temperature may not be instantaneous; (3) effect of solute concentration and the resulting pH changes upon freezing; and (4) interactions among solutes, particularly those between sugar

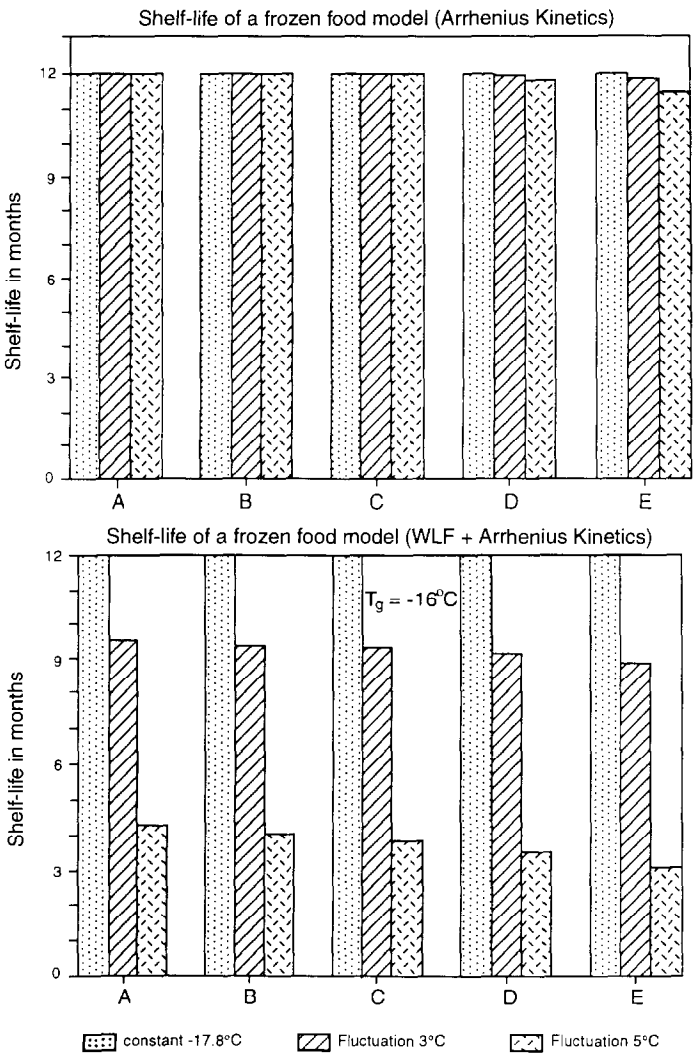


Fig. 3. Simulation of shelf-life changes when temperatures fluctuate around $T'_g$. A, B, C, D, and E are model systems of different activation energy (reproduced from Ref. [83] with permission).

and protein [78,83]. Although $T_g$ is an important parameter in evaluating protein stability at various temperatures, other mechanisms may override the effects of $T_g$.

## 4. Thermomechanical analysis (TMA)

In TMA, a stress is applied to a sample while deformation is measured as a function of temperature. In this technique, information on viscoelastic response as

well as dimensional changes are obtained. Almost all the applications of TMA have been to measure the thermal expansion characteristics of polymeric systems and hence to determine $T_g$. Actually, TMA is recommended to confirm a "suspected" $T_g$ detected by DSC [30,57,69,83].

The measurement of $T_g$ by TMA can be influenced by the shape of sample (diameter and thickness), the heating rate, the load, and annealing [90]. When compared with DSC, TMA is a more sensitive method for detecting $T_g$ [91,92].

Using TMA, Williams and Guglielmo [93] confirmed that the vital breakage by frozen mannitol solution during lyophilization was due to thermal expansion. An elegant study by Chang and Randall [54] fully illustrated the usefulness of $T_g$ determination by TMA and its application in developing a freeze-drying cycle. Buffer, tonicity modifier, protein, stabilizer, and bulking agent all contributed to the resulting $T_g$ of the formulation. Of the two $T_g$ values they routinely detected, the higher $T_g$ (i.e. the second $T_g$) corresponded to the collapse temperature. Successful freeze-drying could only be achieved when the primary drying temperature was maintained below this $T_g$. They emphasized that protein stability was not totally dependent on $T_g$. The concentration–crystallization of buffering salts and the resulting pH changes upon freezing may be more important.

## 5. Thermoelectrometry

This technique is exclusively used for samples in liquid form. In this method, the electrical properties of a substance are measured as a function of temperature. The most common application in pharmaceutical development is the measurement of the electrical resistance of a product for the purpose of developing a lyophilization cycle.

Inorganic salt solutions are frequently used as buffering solutions in formulations for lyophilization. In crystalline systems of inorganic salt solutions, the eutectic temperature $T_e$ can be detected by this technique and the information is used to avoid melt-down during lyophilization. The eutectic melt process (when temperature is above $T_e$) is different from the collapse phenomenon (when temperature is greater than $T_g$ or $T_e$). Melting is associated with the eutectic temperature of crystalline solutes and takes place throughout the frozen fraction. Collapse is related to the glass transition temperature of an amorphous material and takes place only at the drying front. When it occurs during lyophilization, melting is sudden and irreversible while collapse is gradual and reversible [54]. Due to the importance of eutectic detection, resistance measurement during lyophilization is available from some freeze drier manufacturers [94].

Resistance analysis has also been used to identify the appropriate thermal treatment to obtain a more stable and crystalline lyophile. Successful applications were seen in products such as cefazolin sodium [95], cephalothin sodium and other antibiotics [96,97]. Resistance measurement also has been used to detect the thermal event and recrystallization process that caused vital breakage of a lyophilized product [93,98]. The thermal expansion of mannitol solution can be simultaneously detected by DSC, TMA, and resistance analysis with adequate sensitivity [93].

The application of $T_c$ detection for crystalline systems by thermoelectrometry in the developing lyophilization cycle is well documented. However, references regarding its use in defining $T_g$ in amorphous systems are few. MacKenzie detected $T_g$ of aqueous saline sucrose solution (24% sucrose, 6% NaCl) [99] and 10% polyvinylpyrrolidone solution [100] by both DSC and resistance measurement. However, $T_g$ was identified in thermoelectrometry by a subtle change in slope which is barely visible upon slow freezing. Further research is needed to define the applicability of determining $T_g$ in amorphous protein solutions using electrical resistance measurement.

## 6. Conclusions

Thermal analysis of protein drugs and drug products has seen limited applications in the pharmaceutical industry in comparison with other analytical methods. This may be due to perceived lack of specificity, experimental conditions being complicated to define, and special instrument requirement such as microcalorimetry. A large portion of the references in this review are the research results from biochemists and food scientists. However, information regarding moisture content, melting transition temperature, and glass transition temperature are important parameters in evaluating protein stability and in protein formulation development. The ability of microcalorimetric DSC to detect differences in $T_m$ in complex protein formulations is extremely valuable in formation screening. The role of $T_g$ in evaluating protein stability and in process development is gaining recognition. In summary, thermal analysis techniques can quickly provide insights into the physical behavior and stability of protein drugs and can be a guide in the development of optimized formulations and processes. When used with other analytical techniques, thermal analysis is an important tool in protein drug characterization and product development.

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