Physical state of L-histidine after freeze-drying and long-term storage

Thomas Österberg[a], Tommy Wadsten[b]

[a]*Pharmaceutical and Analytical R&D, Astra Pain Control AB, S-151 85 Södertälje, Sweden*
[b]*Dev & Res Wadsten AB, S-11327 Stockholm, Sweden*

Abstract

Liquid samples of L-histidine of varying pH values and mixed with salt, metal ions, polysorbate 80 and sucrose have been analysed by differential scanning calorimetry to evaluate the influence of these additives on the glass transition temperature and crystallisation of L-histidine during freezing and thawing. L-Histidine solutions of varying pH were freeze-dried with and without a thermal cycle and the physical state of the freeze-dried cakes, following long-term storage, were studied by powder X-ray diffraction. Amorphous L-histidine crystallised when it was exposed to moisture, and the identity of the crystalline materials is reported. The crystallisation of L-histidine during freezing and thawing is dependent on the pH of the solution and is shown to be at a minimum at pH 6, which coincides with the pK_a of the imidazoline function. Sucrose inhibited the crystallisation of L-histidine during thawing, while sodium chloride or polysorbate 80 did not. The addition of metal ions (Ca^{2+} and Mg^{2+}) up to 10% (w/w) did not depress the glass transition temperature significantly, while the addition of Zn^{2+} increased it. The physical state of L-histidine after freeze-drying is shown to be dependent on both the pH of the solution and the freezing cycle. The risk of crystallisation of amorphous L-histidine is low if the freeze-dried material is protected from moisture. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Amorphous; Crystallisation; Freeze-drying; Glass transition; L-Histidine; Metal ions

1. Introduction

Protein drugs are generally chemically and physically unstable in solution and freeze-drying is frequently used to obtain an acceptable shelf life (MacKenzie, 1966, 1977; Pikal, 1990). Sugars and/or amino acids are often included in the formulation to prevent inactivation during freeze-drying and to stabilise the protein during long-term storage. Sugars and amino acids protect the protein by preferential exclusion during freezing and by glass formation and/or by functioning as a water substitute in the dried state (Carpenter and Crowe, 1989; Franks et al., 1991; Arakawa et al., 1992). In contrast to sugars, amino acids, in addition to their stabilising properties, may also function as buffers. L-Histidine has recently been shown to function as both buffer and stabiliser in freeze-dried formulations of recombinant factor VIII (Österberg et al., 1997) and recombinant factor IX (Bush et al., 1998). It is a basic amino acid often found at the active site in enzymes and in the coordination of metal ions in metalloproteins. The specific properties of L-histidine reside in the imidazoline function, which possesses both basicity and π-

electron acceptor capability (Sundberg and Martin, 1974). The imidazoline function confers good buffer capacity in the pH range 5–7 (Fig. 1), which is often a suitable pH range for many protein drugs. L-Histidine forms strong complexes with certain metal ions such as Cu^{2+}, Zn^{2+} and Fe^{2+}, but simple salts with the alkali metals (Na^+, K^+, Ca^{2+} and Ba^{2+}) (Greenstein and Winitz, 1961). The latter fact is especially important in the formulation of protein drugs that require free calcium ions in the buffer for stability reasons (e.g. factor VIII). Trace levels of metal ions such as copper and iron are often present in buffer salts. These ions can often facilitate the oxidation of proteins (Lamfrom and Nielsen, 1970; Shihong et al., 1993). Since L-histidine forms strong complexes with these ions, it may also function as an antioxidant. The solubility of L-histidine in water is 41.9 mg/ml at 25°C and is sufficient for proper buffering and to allow it to function as a non-crystallising (amorphous) stabiliser for many protein drugs. In this study the freezing/thawing behaviour of L-histidine of varying pH in the presence of sodium chloride, metal ions and sucrose was studied by means of differential scanning calorimetry (DSC). L-Histidine solutions of varying pH were freeze-dried with and without a thermal cycle. The freeze-dried cakes were examined with

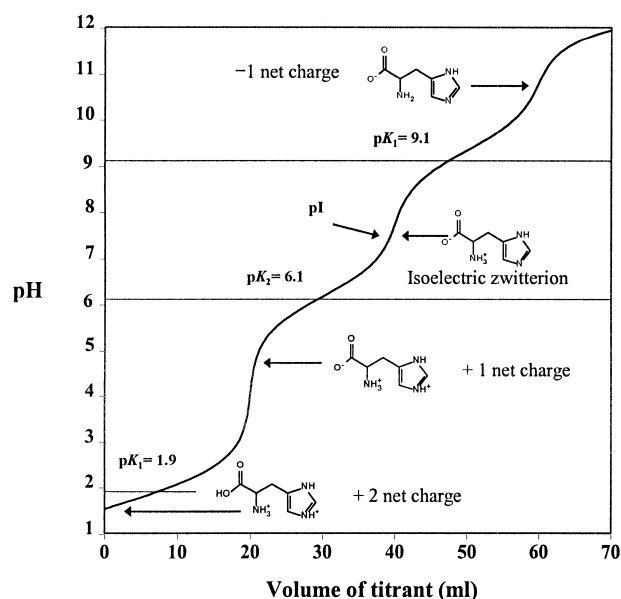*E-mail address:* thomas.osterberg@eu.pnu.com (T. Österberg)

Fig. 1. Simulated titration curve of L-histidine showing the major electrical forms at varying pH.

powder X-ray diffraction after long term-storage and after moisture exposure. The exposure to moisture induced crystallisation and the identity of the crystalline materials is reported.

2. Experimental

2.1. Materials

L-Histidine used for the freeze-drying experiments conformed to the requirements laid down in DAB and USP. L-Histidine (SigmaUltra), L-histidine monohydrochloride monohydrate, sucrose (SigmaUltra), and sodium chloride (SigmaUltra), used for DSC studies and as references for the powder X-ray experiments, were from Sigma (Sweden). Magnesium chloride (ACS reagent grade, ICN Biomedicals), calcium chloride dihydrate (ACS reagent grade, Acros) and Tween 80 (Polysorbate 80) were obtained from Chemicon (Sweden). Zinc chloride (Ph. Eur) came from KEBO Lab (Sweden). The sterile filter used was a 0.22-μm, Millex GV (Millipore, Sweden). The containers used were of type 1 (Ph. Eur). Bottles were closed with a bromobutyl rubber stopper and sealed with an aluminium seal. Water for injections, hydrochloric acid and sodium hydroxide were of pharmacopoeial grade.

2.2. Methods

2.2.1. Preparation of freeze-dried samples

L-Histidine was dissolved in water for injections (15 mg/ml) and the pH was adjusted to pH 4, 5, 6, and 7 with hydrochloric acid and to pH 8 with sodium hydroxide.

The solutions were sterile-filtered and 1.0 ml was dispensed into glass vials. About 10 vials of each formulation were prepared. A pilot freeze-dryer with a 1.2-m^2 shelf area (Edward Kniese, Marburg, Germany) was used. The samples were frozen on the shelves (from 0 to −60°C in 1 h). The thermal cycle was −60 to −35°C in 1 h, maintaining −35°C for 4 h and cooling to −60° in 1 h. Primary drying was carried out at a shelf temperature of about −20°C and the pressure was adjusted to give a product temperature of about −40°C. On completion of the primary drying, the shelf temperature was raised to 40°C over 10 h. The completion of the primary and secondary drying was determined by a pressure increase test. The vials were stoppered under a vacuum in the freeze-drier. The freeze-drying process is illustrated schematically in Fig. 2.

2.2.2. Powder X-ray diffraction

Powder X-ray diffraction data were obtained with a powder diffractometer (XPért, Philips, The Netherlands). Data were collected at room temperature from 3 to 35°, 2θ, the step size was 0.02° and the count time was 0.5 s. Powder samples were prepared as thin layers on glass or aluminium specimen holders. The samples were first analysed at ambient temperature and then exposed to moisture from 20 to 80% RH at room temperature in the diffractometer by using a humidity control unit (also Philips-made).

2.2.3. Water sorption/desorption

The water sorption/desorption profile was determined with an isothermal dynamic water uptake instrument (MB-300W, VTI Corporation, USA). The sample was dried under nitrogen at 60°C. The temperature was thereafter set to 35°C and the relative humidity was increased from 0 to 80% for sorption and decreased from 80 to 5% for desorption in steps of 5%. The initial sample mass was about 7 mg and the equilibrium criterion was less than 0.005 mg mass gain per 3 min. Two vials of each formulation (pH 5, 6 and 8) were analysed.
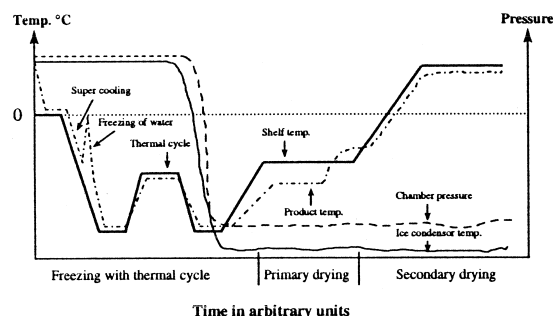

Fig. 2. Plot of process variables during freeze-drying.

2.2.4. Thermal analysis

2.2.4.1. Preparation of samples for thermal analysis

L-Histidine, 30 mg/ml, was dissolved in water for injections and adjusted with hydrochloric acid or sodium hydroxide to the different pH values. Mixtures of L-histidine and sucrose were prepared by mixing L-histidine (30 mg/ml) and sucrose (30 mg/ml) in different volume ratios. The solutions were dispensed (about 30 μl) in a 50-μl aluminium pan without a lid. An empty pan was used as reference.

2.2.4.2. DSC

A differential scanning calorimeter, DSC6200, with an EXTAR6000 workstation (Seiko Instruments, Japan), was used. Cyclohexane (SigmaUltra) and indium (Laboratory of the Government Chemist, UK) were used to calibrate the instrument. The oven was cooled with liquid nitrogen. The sample was cooled to −110°C at a rate of 10°C/min. After an equilibration period of 2 min the sample was heated to −20°C at 10°C/min. The sample was cooled again to −110°C, equilibrated for 2 min and subsequently reheated to 0°C at 10°C/min. The sample was inspected under a magnifying glass or stereomicroscope directly after analysis when some ice was still melting to observe possible crystallisation. Runs were also conducted with a glass lid on the oven so that crystallisation could be observed visually. Reported glass transition temperatures are midpoint values (mean value from two runs) from the second scan, and were determined with help of the software and the derivative of the DSC signal.

3. Results and discussion

The selection of buffer for a protein formulation is very important and several factors have to be considered. The buffer must have low local and systemic toxicity and be compatible with the active protein and other essential ingredients (e.g. metal ions). It must also be chemically stable and the pK_a should preferably be close to the formulation pH in order to give good buffer capacity. The latter requirement also implies that the extent of ionisation of the buffer varies considerably around the formulation pH. This also applies to drug compounds with a pK_a close to the pH of the formulation. For example, a compound with a pK_a of 7.0 is 50% in the ionised state at pH 7.0, and a decrease by one pH unit increases the ionisation to 90%. Thus, for compounds with a pK_a near the formulation pH, we are dealing with different ratios of charged and uncharged forms depending on relatively small changes in the formulation pH. This is in contrast to non-ionisable compounds (such as sucrose and mannitol) or compounds with a pK_a a long way from the formulation pH. Histidine has three ionisable functions: the carboxyl group ($pK_1 = 1.9$), the imidazole nitrogen ($pK_2 = 6.1$) and the amino

nitrogen ($pK_3 = 9.1$) at 25°C and 0.15 ionic strength (Sundberg and Martin, 1974). A theoretical titration curve with the major electrical forms of L-histidine is shown in Fig. 1. Since the pK_2 is close to the pH range often used in the formulation of protein drugs, the net electrical charge of the imidazoline moiety as a function of pH is of fundamental importance for its physicochemical properties and its behaviour during freezing and in the dried state.

3.1. DSC

The thermal behaviour of L-histidine during freezing and thawing has recently been studied by (Chang and Randall, 1992), who reported that L-histidine (10 mg/ml) remains amorphous during freezing, with a T_g' value of −32°C. The T_g' of L-histidine (30 mg/ml) measured in the present study was −31.5°C, although L-histidine crystallised during thawing at about −10°C. Since this was in contrast to the findings reported by Chang and Randall (1992), a sample of 10 mg/ml and rapid freezing was also investigated. The T_g' was −32°C, although crystallisation took place at about the same temperature as for the sample consisting of 30 mg/ml. However, since the crystallisation takes place during the softening/melting of the ice, the exotherm is obscured. An exotherm was observed at about −10°C in the sample of 30 mg/ml but not in the sample of 10 mg/ml. The crystallisation could also be observed visually if a glass lid was used on the calorimeter oven. The crystallisation takes place mainly on the surface of the sample solution and the crystals have a flat, round appearance. This type of crystals was also observed on the surface of some of the freeze-dried samples. The thermogram from the pH 4 sample showed crystallisation between −30 and −20°C. Crystallisation was also observed visually in the sample cup in this temperature range and also in the sample cup directly after thawing. No crystallisation of L-histidine during thawing was observed in samples in the pH range 5.5–6.0. The pH 6.5 samples showed one small crystal. Thus, the findings from this study indicate that the tendency for crystallisation is at a minimum between 5.5 and 6.5. Crystallisation of L-histidine was also observed in the samples containing sodium chloride. Thus, the reduced crystallisation of L-histidine between pH 5.5 and 6.5 is most likely due to the ionisation of the imidazoline function, since the addition of sodium chloride (up to 0.6 M and unadjusted pH) did not inhibit the crystallisation. The thermal analysis of L-histidine solutions of varying pH showed a sigmoidal relationship between pH and T_g' (Fig. 3). The T_g' at pH 5 was about 10°C lower than at pH 7. The effect of pH on T_g' can be explained by the plasticising effect of ions (from pH adjustment with HCl or NaOH) in the freeze-concentrated L-histidine glass and/or a lower T_g' of L-histidine with an ionised imidazole moiety. It is well known that salts decrease T_g' and the collapse temperature of freeze-concentrated amorphous materials. The T_g' and the collapse temperature of amorphous materi-
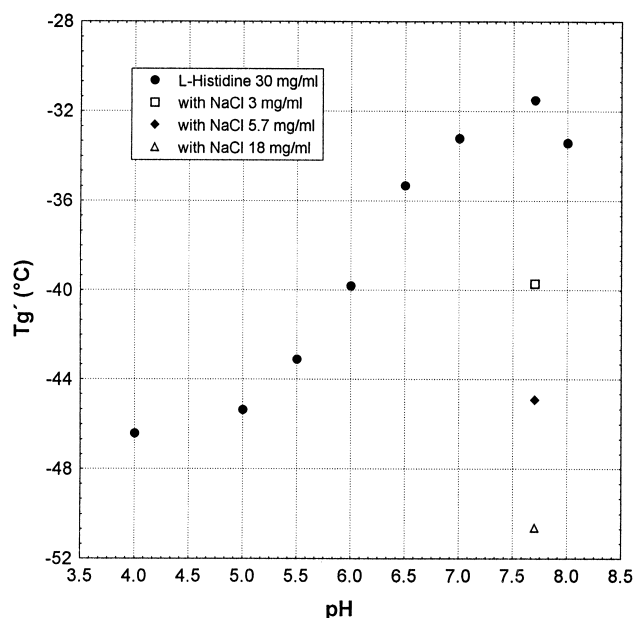
Fig. 3. Dependence of T'_g during the second heating of frozen aqueous L-histidine solutions on the pH and on the addition of sodium chloride.
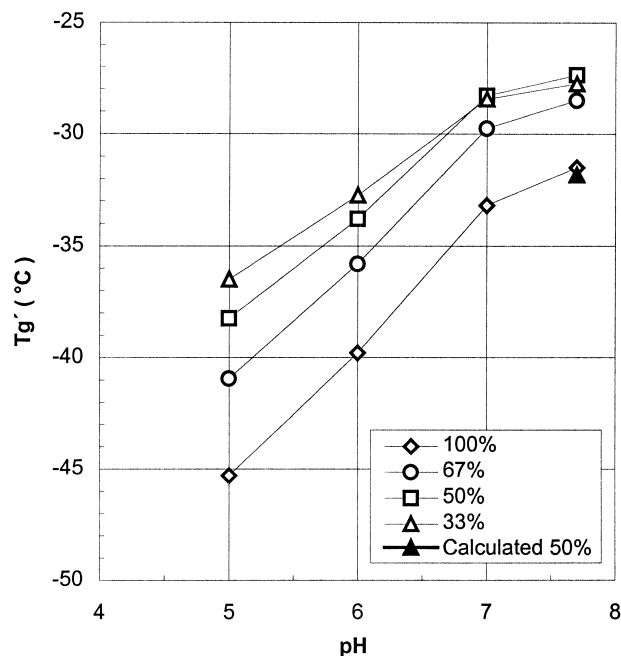

Fig. 4. Dependence of T'_g during the second heating of frozen aqueous L-histidine–sucrose solutions on the weight to weight ratio of L-histidine–sucrose and pH.

als are generally closely related (MacKenzie, 1977). The T'_g of the sucrose/sodium chloride system has been described by (MacKenzie, 1985). The addition of sodium chloride to sucrose in 1:9 and 2:8 (w/w) ratios depressed the T'_g by about 10 and 21°C, respectively. If one assumes that sodium and chloride ions depress the collapse temperature equally, the chloride ions alone would depress the T'_g by about 5 and 10°C, respectively, in the sucrose/ sodium chloride system above. L-Histidine (30 mg/ml) adjusted to pH 6 with hydrochloric acid contains about 3.4 mg of chloride ions. The L-histidine/chloride ion ratio is thus approximately 10:1 (w/w). If one further assumes that sodium chloride depresses the T'_g of sucrose and L-histidine equally, the chloride ions would depress the T'_g of L-histidine (pH 6) by about 10°C. The measured T'_g was about 8°C lower at pH 6 compared to the unadjusted state, which is quite close to the value estimated from the sucrose/sodium chloride system. However, it is not possible to establish the T'_g of L-histidine at pH 6 per se due to the presence of the pH adjuster, although the reasoning above indicates that the ionisation of the imidazoline function does not change the T'_g dramatically. Since L-histidine was shown to crystallise during thawing in the important pH range 6.5–8 it was important to investigate whether the addition of a non-crystallising excipient could function as an inhibitor. Sucrose was selected since it is often used as a non-crystallising excipient in freeze-dried formulations of protein drugs. The T'_g of L-histidine/sucrose mixtures of varying pH is shown in Fig. 4. The T'_g of mixtures of two non-crystallising compounds can generally be approximated using the Fox equation (Fox, 1950),

$$1/T_{g\,12} = W_1/T_{g\,1} + W_2/T_{g\,2}$$

where $T_{g\,12}$ is the new T_g and W_1, $T_{g\,1}$, W_2 and $T_{g\,2}$ are the weight fractions and the T_g of the individual compounds. The T'_g values measured in this study were -31.5°C for L-histidine (30 mg/ml) and -32.3°C for sucrose (30 mg/ml). Thus, the estimated T'_g of any L-histidine–sucrose mixture will not deviate much from -32°C. It was, therefore, interesting to note that the T'_g of all the mixtures (unadjusted pH) was about 3–4°C higher compared to the T'_g values estimated from the Fox equation. The T'_g at pH 7 was essentially unchanged for the samples with 33 and 50% (w/w) L-histidine. The samples with 33 and 50% (w/w) L-histidine of pH 6 showed a T'_g above -35°C which is a reasonable T'_g from a practical point of view. The T'_g of pure L-histidine at pH 6 was -40°C. A T'_g at or below -40°C is generally considered to be too low since the sublimation of ice is very slow at this temperature. As a rule of thumb, the time for the primary drying is halved if the product temperature is increased by 6–7°C. Thus, the increased T'_g of the L-histidine/sucrose mixtures is of economic and practical importance. Another important observation was that the addition of sucrose abolished the crystallisation of L-histidine. The reduced tendency for crystallisation of L-histidine is very important in the formulation design, since even if the product temperature never reaches the devitrification temperature during normal operating conditions, accidental over-heating might occur in the freeze-dryer due to technical failures.

3.2. Addition of metal ions and polysorbate 80

Some proteins like factor VIII require divalent metal

ions (e.g. Ca^{2+}) in the formulation buffer for stability reasons. Proteins that are therapeutically active at very low concentrations usually require the addition of non-ionic surfactants (e.g. polysorbate 80) in order to retard the surface adsorption of the protein during manufacture and in the final packaging. It was, therefore, of interest to investigate whether the divalent metal ions, Ca^{2+}, Mg^{2+} and Zn^{2+} (added as chloride salts) or polysorbate 80, could retard the crystallisation and how these ingredients influenced the T'_g of L-histidine. Sucrose was included in order to study how an uncharged compound interacts with these metal ions. The crystallisation of L-histidine (30 mg/ml unadjusted pH) was largely unaffected by the presence of calcium chloride (up to 10 mg/ml), but was completely retarded at 20 mg/ml. The addition of polysorbate 80, 0.2 mg/ml, did not inhibit the crystallisation. The effect of Ca^{2+}, Mg^{2+} and Zn^{2+} on the T'_g of L-histidine and sucrose is shown in Fig. 5. The addition of metal ions to sucrose showed an expected concentration-dependent depression of the T'_g. Mg^{2+} depressed the T'_g of sucrose most, followed by Ca^{2+} and Zn^{2+}. However, in contrast to sucrose, the addition of Ca^{2+} and Mg^{2+} to L-histidine showed a surprisingly small depression of T'_g of up to 10% (w/w) ratio. Another unexpected finding was that the addition of Zn^{2+} increased the T'_g considerably. The small depression of T'_g by Ca^{2+} and Mg^{2+} up to 10% (w/w) ratio indicates that L-histidine interacts with these metal ions in the freeze-concentrated phase. It is well known that L-histidine forms a strong complex with Zn^{2+}, and this probably explains the increase in the T'_g. The

small depression by Ca^{2+} and Mg^{2+} of the T'_g of L-histidine is of considerable importance in the formulation of proteins that require metal ions for stability. If L-histidine is used as buffer/stabiliser in formulations containing Ca^{2+} and Mg^{2+}, the T'_g is essentially unchanged, whereas if sucrose is used, the T'_g can be depressed considerably.

3.3. Freeze-drying

The freezing step is very important since the internal structure of the product is determined by this step. A thermal cycle (Fig. 2) is sometimes necessary to maximise the crystallisation of water and/or to promote the crystallisation of bulking agents such as sodium chloride or mannitol. Since a thermal cycle might promote crystallisation of ingredients (e.g. L-histidine) that are to remain amorphous, the L-histidine samples were freeze-dried with and without a thermal cycle. Freeze-drying of L-histidine from solutions having a pH in the range 4–8 showed that L-histidine has a rather low tendency to crystallise during freeze-drying (Table 1). The samples freeze-dried at pH 8 crystallised readily, but only when they were freeze-dried with a thermal cycle. The samples of pH 7 and 8, freeze-dried without a thermal cycle, and the sample with pH 7, freeze-dried with a thermal cycle, showed very thin crystal clusters (1–2 mm in diameter) on the surface of the cakes. All samples showed good cake structures, with the exception of the pH 4 sample, which collapsed to a transparent skin during freeze-drying. Examination of the samples with a polarising microscope showed that the pH 8 sample freeze-dried with a thermal cycle had a crystalline structure, while the other samples had an amorphous appearance. The crystallisation of L-histidine at pH 8 during freeze-drying might be explained by the fact that the imidazoline moiety is almost completely un-ionised at this pH and that crystallisation is generally facilitated if only one molecular species is present. At pH 6 there is a 1:1 molar ratio of the charged and the uncharged imidazole moiety and it is suggested that this ratio is optimum for the inhibition of crystallisation during freeze-drying. However, a complicating factor in the interpretation of the results is that both the ion product of water (pK_w) and the dissociation constant pK_a of the imidazole moiety change with temperature. In addition, when the water is frozen out, the concentration effect has also to be considered. The mean-
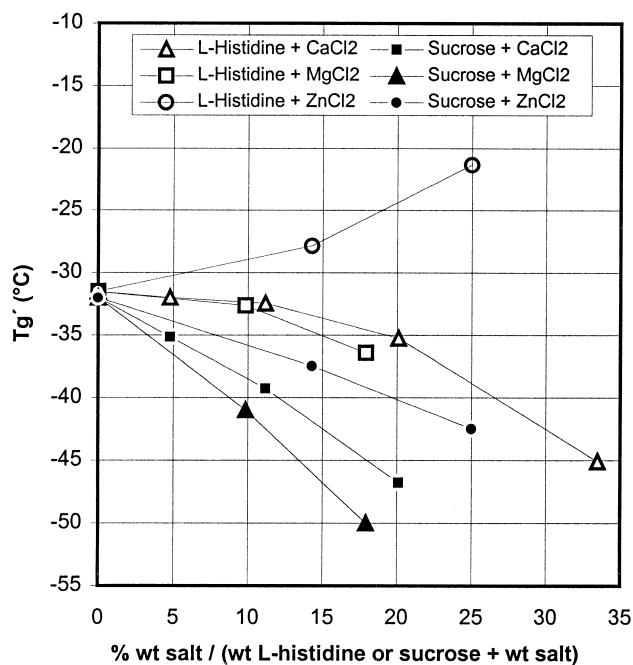

Fig. 5. Dependence of T'_g during the second heating of frozen aqueous L-histidine and sucrose solutions on the weight to weight ratio of L-histidine or sucrose/salt.

Table 1
Physical state of L-histidine after freeze-drying with and without a thermal cycle from solutions of varying pH[a]

Freezing process	pH 4	pH 5	pH 6	pH 7	pH 8
No Thermal cycle	A[b]	A	A	A[c]	A[c]
Thermal cycle	nt	nt	A	A[c]	C

[a] A, X-ray amorphous; C, Crystalline; nt, not tested.
[b] The cake collapsed.
[c] Thin crystal clusters on the surface of the cakes.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.