Reversible Adsorption of Soluble Hexameric Insulin onto the Surface of Insulin Crystals Cocrystallized with Protamine: An Electrostatic Interaction

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Mixing pharmaceutical preparations of soluble neutral regular insulin solution (NRI) and neutral protamine Hagedorn (NPH) crystalline insulin suspension leads to a reduction in the measurable amount of soluble insulin in the formulation supernatant. However in spite of the loss in soluble insulin, the time-actions of these components have been shown, in clinical trials, to be unaffected. The interaction between these different physical forms of insulin has been studied using reversed-phase HPLC, isothermal titrating calorimetry, and Doppler electrophoretic light scattering analysis. Sorbent surface and solution perturbation studies revealed that the NRI adsorbs to the surface of the NPH crystal with an equilibrium constant ranging from 10^4 M^{-1} to 10^7 M^{-1}, depending on the protamine concentration, pH, ionic strength, and temperature. This adsorption behavior suggests that the binding is mediated by electrostatic interactions arising between the positively-charged NPH crystal and the negatively-charged NRI hexamer. Doppler electrophoretic light scattering results, used to probe the pH-dependent surface charge of NPH and soluble insulin hexamer, support the conclusion that electrostatic interactions mediate the adsorption process. Adsorption studies under physiological conditions indicate that the elevated temperature and ionic strength, in a subcutaneous depot, are sufficient to lead to the dissociation of the NRI/NPH complex that exists in these NPH mixture formulations.

KEY WORDS: buffered regular insulin; NPH insulin; insulin mixtures; surface adsorption; electrostatic attraction.

INTRODUCTION

The importance of protein adsorption at solid-liquid interfaces and the principles that govern these interactions have been reviewed in detail in numerous articles [1]. These interactions are of particular interest in the formulation of biopharmaceutical products because of the significant impact they can have on the bioavailability and stability of the protein. In this paper we investigated the reversible protein-surface interactions that take place in mixtures of neutral regular insulin solution (NRI) and neutral protamine Hagedorn (NPH) insulin crystal suspension. The interactions have been treated as a colloidal-chemical problem and have been studied using isothermal titrating calorimetry (ITC), reversed-phase HPLC (RP-HPLC), and Doppler electrophoretic light scattering analysis (DELSA). The roles of pH, ionic strength, temperature, and protamine concentration in conjunction with DELSA measurements have been used to explore the role of electrostatic interactions in the adsorption process. The thermochemical behavior of the interactions has also been evaluated to determine the intermolecular nature of this phenomenon.

NPH insulin suspension is one of the most widely used intermediate-acting insulin preparations [2]. The insulin in this formulation is in a crystalline form that is prepared by precipitating insulin with protamine in the presence of zinc, glycerol, sodium phosphate, phenol, and m-cresol at neutral pH [3]. Protamine is a mixture of highly basic proteins, isolated from fish sperm, with a molecular weight of ~4 kD [4]. Although an excess amount of protamine is used in the precipitation of the insulin, no protamine has been detected free in solution; thus, all the protamine remains associated with the crystalline insulin.

The soluble NRI formulation contains zinc, glycerol, phenol, m-cresol, and sodium phosphate. The insulin in this formulation adopts a hexameric structure that is stabilized by the formation of two zinc binding sites and six hydrophobic phenolic preservative binding sites [5, 6].

We report in this paper studies that were developed to simulate the post-crystallization perturbations that occur to NPH/NRI mixtures in a clinical setting, *i.e.*, changes in ionic strength, pH, and temperature. The results indicate that small changes in the solution conditions can drastically effect the reversible adsorption of the negatively-charged hexamer, in the NRI formulation, onto the positively-charged surface, of the NPH crystal.

MATERIALS AND METHODS

Materials

Recombinant human insulin and protamine sulfate were obtained through Eli Lilly and Company (Indianapolis, IN). All other materials were either U.S.P. or analytical reagent grade.

Preparation of NPH and NRI

Stock solutions of NPH were prepared in a fashion analogous to the methodology previously published by Krayenbühl and Rosenberg [3] and contained insulin (3.5 mg insulin/mL or U-100), zinc (0.15 to 0.35 μg zinc/IU of insulin), protamine (0.25 to 0.30 mg protamine base/100 U of insulin), phenol (0.65 mg/mL), m-cresol (1.6 mg/mL), glycerin (16 mg/mL), and dibasic sodium phosphate (3.78 mg/mL).

NPH crystallization is extremely dependent upon ionic strength, pH, temperature, purity of excipients, protamine concentration, and zinc concentration [7]. To minimize the effects of these parameters, all crystallizations were performed at pH 7.4, 20°C, in the presence of at least 0.15 μg zinc/IU of insulin, and in the absence of NaCl. Complete NPH crystallization was achieved under these conditions

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with all the protamine concentrations studied, ranging from 0.25 to 0.30 mg/100 U of insulin. Completion of NPH crystallization was determined by analyzing the NPH supernatants by HPLC, for residual insulin in solution, and by examining the crystals using light microscopy to confirm tetragonal crystal formation. The ionic strength and pH of the formulations for the various experiments were adjusted after the completion of crystallization by adding aliquots of concentrated stock solutions of either NaCl (3 M), HCl (6 N), or NaOH (6 N). These adjustments were performed post-crystallization to avoid significant changes to the crystal size and morphology as well as the minimum protamine/insulin ratio required for complete NPH crystallization. The pH adjustments introduced a dilution error of approximately 0.2%, well within the error of the experiment. The dilution errors in the ionic strength experiments were on the order of 5%.

NRI solutions (7.0 mg/mL or U-200) were prepared to match the experimental formulation conditions of the NPH formulation with respect to pH, ionic strength, zinc concentration, and other excipients, with the exception of protamine.

To determine if heats of ionization from the buffer were contributing to the signal response, solutions of NPH and NRI were prepared in the presence of HEPES (50 mM) instead of the standard buffer, sodium phosphate (14.1 mM). The NPH crystallizations in this alternate buffer were shown to be complete by HPLC and microscopy, as described above.

Isothermal Titration Calorimetry

The titration calorimetry experiments were performed on an Omega Titration Calorimeter (MicroCal Inc.; Northampton, MA). The methodology was based on the protocols described in the operating manual. NPH was placed in the reaction chamber and suspended by stirring. The NRI was then added to the solution, and the heat of the interaction was measured. Heats of dilution were determined in a separate run and subtracted from the experimental data.

All experiments were performed at fixed temperatures between 20°C and 40°C. The temperature dependence of the pKa (dpK_a/dT) of HEPES was taken into account in studies requiring this buffer.

Mathematical Model. A mathematical binding model was developed assuming (1) a simple one-to-one interaction between the insulin hexamer and the binding site, (2) no interactions between occupied sites, and (3) that a simple Langmuir monolayer adsorption occurs with a finite number of surface sites of equal energy [8, 9]. The binding isotherms were modeled using equation 1 where Q_c is the calculated heat, V_o is the working volume of the calorimeter cell (1.2486 mL), $\Delta_{ads}H$ is the enthalpic change for the reaction, $K_{R6,ads}$ is the association constant, $[M]_{tot}$ is the total concentration of adsorption sites on the NPH crystals in moles/liter of solution, and $[L]_{tot}$ is the total concentration of soluble insulin hexamer.

$$Q_c = \frac{V_o \Delta_{ads}H [M]_{tot}}{2}\left[1 + \frac{[L]_{tot}}{[M]_{tot}} + \frac{1}{K_{R6,ads}\,[M]_{tot}} - \sqrt{\left(1 + \frac{[L]_{tot}}{[M]_{tot}} + \frac{1}{K_{R6,ads}\,[M]_{tot}}\right)^2 - \left(\frac{4[L]_{tot}}{[M]_{tot}}\right)}\right] \quad (1)$$

The data were fit using the non-linear least-squares fitting routine in a software package for statistical visualization on the Apple Macintosh, JMP (v2.0; SAS Institute, Inc.; Cary, NC). In equation 1, V_o, and $[L]_{tot}$ are known. The remaining parameters $\Delta_{ads}H$, $K_{R6,ads}$, and $[M]_{tot}$ were treated as variables and allowed to float. Convergence was achieved when the residuals between the experimentally-determined heat, Q_{exp}, and computationally-determined heat, Q_c, were minimized. The concentration of adsorption sites on the NPH crystals, $[M]_{tot}$, was solved for as the soluble insulin hexamer concentration at which saturation was attained.

The value of $[M]_{tot}$ was converted to an adsorptivity capacity value, N_s, in moles of soluble insulin hexamer/grams of NPH crystals, as follows:

$$N_s = \frac{[M]_{tot}}{c_{[NPH]}} \quad (2)$$

The anhydrous concentration of NPH crystals, $c_{[NPH]}$, in grams/L for a 100-U formulation of NPH was calculated for the various conditions by summing the gravimetric concentrations for all components composing the crystals. An assumption was made that the six phenolic binding sites on the insulin hexamer in NPH were filled with the same ratio of phenol and m-cresol as was used to initiate the crystallization and that glycerol is not an integral component of the crystal. The resulting range was 3.83 to 3.89 grams of NPH crystals/L of solution, based on the lower and upper concentrations for each component. The value used to compute adsorptivity capacities was the average of these values, 3.86 ± 0.03 grams of NPH crystals/L of NPH solution.

Reversed-Phase HPLC

The adsorption of NRI onto NPH crystals was also quantitated by RP-HPLC for the amount of free (unbound) NRI in the mixture. The amount of unbound or free NRI was measured in the supernatant after the suspension was subjected to centrifugation. The amount of free insulin was quantitated by integrating the peak area, monitored at 214 nm, and comparing the results to a standard curve obtained using solutions of recombinant human insulin reference standard. The insulin was separated from m-cresol and phenol isocratically with a mobile phase of approximately 76% 0.2 M sodium sulfate at pH 2.3 and 24% acetonitrile. The separations were performed on a Vydac Protein & Peptide C18 column (15 cm × 4.6 mm).

Mathematical Model. The binding isotherms from the HPLC determinations were fit to the following equation:

$$[L]_B = \frac{([M]_{tot} \cdot K_{R6,ads} \cdot [L]_F)}{(1 + K_{R6,ads} \cdot [L]_F)} \quad (3)$$

where, $[L]_F$ and $[L]_B$ are the free and bound ligand, respectively. The remaining parameters have been previously described.

Doppler Electrophoretic Light Scattering Analyzer

Electrophoretic mobilities were determined using a Doppler electrophoretic light scattering analyzer (DELSA 440, Coulter Electronics, Inc.) [10]. Electrophoretic light scattering studies on NPH were carried out using samples from commercially available vials (Eli Lilly and Company, Humulin N, U 100) and using crystals prepared in the laboratory following procedures outlined in this manuscript. For the lab-prepared samples, the protamine level was held constant at 0.27 mg/100 U insulin and the studies were restricted to phosphate buffers. The mobilities of the NPH crystals were measured using either the concentrated suspensions or by diluting (up to 1:50) into a diluent identical to the NPH formulation. The NPH diluent consisted of 0.65 mg/mL phenol, 1.6 mg/mL m-cresol, 16 mg/mL glycerin, and 3.78 mg/mL dibasic sodium phosphate pH 7.4.

Coulter-Counting Particle Sizing

Particle size measurements were performed using a Coulter Multisizer (Coulter Electronics, Ltd., Luton, England) with a Coulter Sampling Stand II and software version 1.00. Samples were prepared in duplicate by diluting 0.200 mL into 100 mL of NPH diluent (listed in the previous paragraph) contained in a glass sampling goblet. Samples were stirred during the data collection period at a setting of 1.5, which allowed for constant gentle agitation without the generation of a vortex within the goblet. After zeroing the instrument, data collection was initiated by vacuum aspiration of the sample through a 50μm orifice. The aperture current was 400 μA, gain setting was 8 and Kd value of 505. The stated conditions allowed for the counting of 140,000-200,000 particles over a 50 second elapsed time period, with a coincidence count less than 15%. Calibration of the instrument was verified with 10 μm polystyrene spheres.

Each sample was examined by making two separate dilutions into the NPH diluent. The two resulting particle diameters ($D_{4,3}$ in μm) were averaged to obtain a mean particle diameter. The standard deviation of the mean particle diameter was less than 1 μm regardless of the sample examined.

Microscopy

The morphology of the NPH crystals was determined by microscopy on a Zeiss Axial Plane Polarized Light Microscope equipped with an oil-based objective. Samples were prepared by placing a drop of the formulation between a glass slide and a cover slip. A drop of oil emersion was then placed on top of the cover slip for use with the oil-based objective. All observations were performed at 1000× magnification using differential interface contrast methodology.

RESULTS

Assessment of Post-Crystallization Solution Perturbations

The effects solution perturbations have on crystal dissolution and re-equilibration of the protamine/insulin ratio were investigated. In particular, experiments in which the pH was increased to 8.0, the temperature was increased to 40°C, or the NaCl concentration was increased to 150 mM were of particular interest because of the known effect these extreme conditions have on NPH crystallization [7]. The results showed that these conditions did not significantly affect changes in the morphology or particle size of the crystals as measured by microscopy and Coulter-counter particle sizing, respectively (Table I). The concentration of free insulin in the supernatant as measured by RP-HPLC showed a minimal increase, 0.3 U/mL to maximum of ~6.0 U/mL, at elevated pH, temperature, and ionic strength. Changes in the insulin supernatant values were not time dependent up to 24 hours. In addition, the heats of dilution for the blank titrations into the NPH supernatants were linear under all conditions studied.

Comparison of Techniques and Buffers on the Measurement of Equilibrium Constant and Adsorptivity Capacity of NPH

The association constants, $K_{R6,ads}$, measured by either

Table I: The effects of post-crystallization solution perturbations of pH, temperature, and ionic strength on NPH morphology, particle size, and free insulin concentration in the supernatant. All NPH solutions contained insulin (3.5 mg insulin/mL or U-100), zinc (0.25 μg zinc/IU of insulin), protamine (0.27 mg protamine base/100 U of insulin), phenol (0.65 mg/mL), m-cresol (1.6 mg/mL), glycerin (16 mg/mL), and dibasic sodium phosphate (3.78 mg/mL). The pH and ionic strength adjustments were made by adding small aliquots of concentrated stock solutions

Perturbation Conditions	Time[a] (hrs)	Microscopic Morphology[b]	$D_{4,3}$[c] (μm)	$[Ins]_{supernatant}$[d] (U/mL)
pH 7.5, 25°C	Initial	tetragonal	4.4 ± 0.7	0.3 ± 0.1
pH 7.5, 30°C	2.5	tetragonal	4.3 ± 0.4	0.5 ± 0.3
	24.0	tetragonal	4.4 ± 0.4	0.4 ± 0.2
pH 7.5, 30°C, 150 mM NaCl	2.5	tetragonal	4.5 ± 0.4	5.0 ± 0.5
	24.0	tetragonal	4.7 ± 0.8	4.4 ± 0.1
pH 7.0, 40°C	2.5	tetragonal	5.0 ± 0.5	0.2 ± 0.1
	24.0	tetragonal	4.9 ± 0.5	0.2 ± 0.1
pH 8.0, 40°C	2.5	tetragonal	4.1 ± 0.4	6.2 ± 1.1
	24.0	tetragonal	4.2 ± 0.4	5.9 ± 0.9

[a] NPH crystal incubation time at a given condition.
[b] Crystallograpic morphology determined at 1000× using differential interface contrast methodology.
[c] The mean volume diameter determined on the Coulter Multisizer.
[d] The concentration of insulin in the supernatant solution, as determined by RP-HPLC, after centrifugation of a U-100 formulation.

RP-HPLC or ITC were in good agreement, within a factor of 3, with values ranging from 15.5 (±2.9) × 10^5 M^{-1} to 37.7 (±5.5) × 10^5 M^{-1} (Table II). The average adsorptivity capacities, N_s, measured by RP-HPLC, 5.75 (±0.15) × 10^{-6} mol hexamer/g NPH, were consistently threefold higher than the values determined by ITC, 1.96 (±0.06) × 10^{-6} mol hexamer/g NPH (Table II).

Studies were performed in both phosphate- and HEPES-buffered systems to (a) determine if protons were being released or taken up in the binding process and subsequently contributing to the enthalpy of binding, $\Delta_{ads}H$, through heat of ionization of the buffer and (b) determine the role of buffers as counter ions in the adsorption process. The results in Table II show that the buffering species had no measurable effect on the equilibrium constant, enthalpy, or adsorptivity capacity.

Effects of Protamine, pH, Temperature, and Ionic Strength on NRI Adsorption onto NPH Crystals

The effects of NPH surface charge, pH, temperature, and ionic strength were investigated to determine if the adsorption process was electrostatically-mediated. Under all the conditions explored, the thermodynamic parameters ($\Delta_{ads}G°$, $\Delta_{ads}H$, and $\Delta_{ads}S°$) were all less than zero. The effects of the various conditions on the equilibrium constant and surface capacity are explained in detail below.

Protamine Studies. The surface charge of NPH was modified by utilizing different concentrations of protamine sulfate in the crystallization process. The equilibrium constant, adsorptivity capacity of NPH crystals, and thermodynamic parameters for the protamine study are listed in Table III.

Analysis of the data shows that the binding is protamine-dependent and that an increase of 0.05 mg/mL in protamine concentration can cause an order of magnitude change in the equilibrium constant. The results in Table III illustrate that the equilibrium strongly favors the associated state, particularly at high protamine concentrations, where $K_{R6,ads}$ = 43.1 (±23.7) × 10^5 M^{-1}. In addition, the adsorptivity capacity of NPH crystals increases with increasing concentration of protamine in the NPH crystals (Table III).

pH Studies. The pH of the formulation was found to have a profound effect on the adsorption of NRI onto NPH (Table III). Increasing the pH from 7.0 to 8.0, at 20° C, inhibits the adsorption processes by reducing the equilibrium constant and the adsorptivity capacity of the NPH crystals. In particular, the equilibrium constant can be altered by approximately two orders of magnitude in this pH range.

DELSA measurements were performed on NPH crystals and on the NRI to determine the electrostatic charge on their respective surfaces. Preliminary studies showed sigmoidal curves for the pH dependence of the electrophoretic mobilities for both NRI and NPH, and hence the surface charges on the particles (Figure 1). While the pI for the NRI solutions under the experimental conditions is approximately 5.25, for the NPH crystals the point of zero charge (pzc) is in the pH range of 7.0 to 7.5. At pH values below the pzc, the particles carry a net positive surface charge whereas at the more alkaline pH values, the net charge on the surface is negative.

Temperature Studies. The effects of temperature on the equilibrium constants and adsorptivity capacities of NPH crystals are presented in Table III. The data show that the equilibrium constants decrease by an order of magnitude with a moderate temperature increase from 20°C to 40°C. Similar behavior was observed with the adsorptivity capacities: increasing temperature reduced the surface capacity of the NPH crystals (Table III).

Ionic Strength Studies. ITC studies have shown that increases in ionic strength can significantly affect NRI adsorption. Results in Table III show that increasing the sodium chloride concentration from 0 to 150 mM inhibits adsorption of soluble insulin onto the NPH surface by an order of magnitude. Additional studies performed under physiological conditions, [NaCl] = 150 mM, pH 7.4, and 37°C, yielded the weakest association measured in these studies, $K_{R6,ads}$ = 3 × 10^4 M^{-1} (Table III).

Under weak binding conditions, the binding isotherms generated in the ITC experiments ($K_{R6,ads} \leq 10^4$ M^{-1}) were difficult to fit with equation 1. The fits were not well defined with respect to the $\Delta_{ads}H$ and $[M]_{tot}$ parameters, *i.e.*, a global minimum could not be obtained with a unique combination of $\Delta_{ads}H$ and $[M]_{tot}$ values. It is likely that these parameters could compensate for each other in these fits, allowing for a large degree of variability in the parameters while fitting the binding isotherm within the experimental error. This inability to fit the weak binding isotherms is most likely due to the difficulty the fitting routine had in estimating the $[M]_{tot}$ value from the gradual inflection point in the binding curves. As a result, it was not possible to accurately determine N_s values for data obtained at pH 7.5 and 40°C or pH 8.0 and 40°C. However, the equilibrium constants were well-defined, despite the variability in $\Delta_{ads}H$ and $[M]_{tot}$ values, because $K_{R6,ads}$ is very sensitive to the initial slope of the binding isotherm.

Table II: The effects of buffer and technique on the binding of soluble insulin hexamer to the surface of NPH crystals (0.27 mg protamine/100 U insulin) as studied by isothermal titration calorimetry and high performance liquid chromatography at 23°C and pH 7.5 ± 0.05

Technique	Buffer System	$K_{R6,ads}$ (×10^5) (L/mol)	$\Delta_{ads}G°$ (kcal/mol)	$\Delta_{ads}H$ (kcal/mol)	$\Delta_{ads}S°$ (cal/mol·deg)	N_s(×10^{-6}) (mol hexamer/g NPH)
ITC	PO_4	15.5 (±2.9)	−8.4 (±0.1)	−45.7 (±2.4)	−126 (±8)	1.92 (±0.09)
ITC	HEPES	37.7 (±5.5)	−8.9 (±0.1)	−46.4 (±1.0)	−127 (±3)	1.98 (±0.04)
HPLC	PO_4	21.2 (±2.1)	−8.6 (±0.1)			5.62 (±0.13)
HPLC	HEPES	26.3 (±1.5)	−8.7 (±0.1)			5.88 (±0.08)

Table III: The effects of protamine (A), pH (B), temperature (C), and ionic strength (D) on the binding of soluble insulin hexamer to the surface of NPH crystals as studied by isothermal titration calorimetry. The binding results under physiological conditions are listed in (E)

	protamine (mg/100U)	pH	Temp. (°C)	[NaCl] (mM)	$K_{R6,ads}$ ($\times 10^5$) (L/mol)	$\Delta_{ads}G°$ (kcal/mol)	$\Delta_{ads}H$ (kcal/mol)	$\Delta_{ads}S°$ (cal/mol·deg)	N_s($\times 10^{-6}$) (mol hexamer/g NPH)
A	0.30	7.4	30	0.0	43.1 (±23.7)	−9.2 (±0.2)	−47.1 (±1.5)	−125 (±5)	4.84 (±0.13)
	0.27	7.4	30	0.0	6.8 (±2.1)	−8.1 (±0.2)	−66.7 (±9.1)	−194 (±30)	2.10 (±0.25)
	0.25	7.4	30	0.0	3.1 (±0.2)	−7.6 (±0.1)	−80.4 (±8.5)	−240 (±28)	1.95 (±0.18)
B	0.27	7.0	20	0.0	180 (±32)	−9.7 (±1.0)	−12.8 (±0.3)	−10.5 (±3.7)	6.24 (±0.16)
	0.27	7.5	20	0.0	24.0 (±3.2)	−8.6 (±0.1)	−43.3 (±0.6)	−119 (±2)	3.68 (±0.05)
	0.27	8.0	20	0.0	3.1 (±0.2)	−7.4 (±0.1)	−76.4 (±3.8)	−236 (±13)	1.21 (±0.08)
C	0.27	7.0	20	0.0	180 (±32)	−9.7 (±1.0)	−12.8 (±0.3)	−10.5 (±3.7)	6.24 (±0.16)
	0.27	7.0	30	0.0	35.2 (±9.6)	−9.1 (±0.2)	−31.3 (±0.8)	−73.4 (±2.8)	3.13 (±0.07)
	0.27	7.0	40	0.0	12.2 (±3.9)	−8.7 (±0.2)	−62.3 (±5.6)	−171 (±18)	2.57 (±0.20)
D	0.27	7.5	30	0.0	4.3 (±0.6)	−7.8 (±0.1)	−87.1 (±13.7)	−262 (±45)	1.76 (±0.26)
	0.27	7.5	30	75.0	1.5 (±0.1)	−7.2 (±0.2)	−120 (±12)	−373 (±40)	1.36 (±0.80)
	0.27	7.5	30	150.0	0.4	−6.4	nd[a]	nd	nd
E	0.27	7.4	37	150.0	0.3	−6.3	nd	nd	nd

[a] nd refers to not determined.

DISCUSSION

Effects of Post-Crystallization, Solution Perturbations

Post-crystallization perturbations had no observable effect on the morphology or crystal size of the NPH. Small changes in the amount of free insulin in the supernatant were observed under conditions of elevated pH, temperature, and/or ionic strength. This insulin release and the lack of any time-dependence under these conditions suggests that, in NPH preparations containing 0.27 mg protamine base/100 U of insulin, a fraction of the insulin is only loosely associated with the crystals. However, in spite of the release of insulin into solutions, no measurable protamine appears to be concomitantly released into the solution. This was apparent from the linear heats of dilution observed in the blank runs used to correct the binding profiles, under all conditions presented in this paper. In addition, no observable precipitation (amorphous NPH) was generated in the reaction chamber, a further indication of the absence of free protamine. However, it should be noted that experimental conditions, not reported in this paper, have been identified (protamine concentrations greater than 0.34 mg protamine base/100 U of insulin and pH values less than 7.1 at 25°C) where residual protamine was present in solution. These conditions yielded nonlinear heats of dilution curves and the blank solutions contained a precipitant presumed to be amorphous NPH (data not shown).

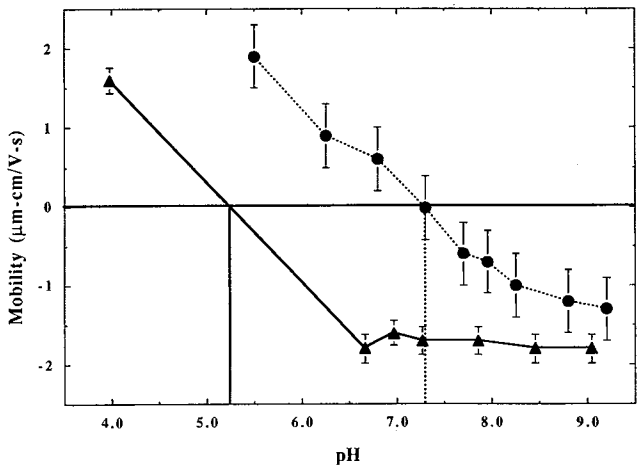

Figure 1: Plot of electrophoretic mobilities of NRI (-▲-) and NPH (··●··) as a function of pH. The error bars on the NRI curve represent a standard deviation of 10% on the measurements. The lack of data in the NRI curve from pH 4.0 to 6.5 is a result of the insolubility of NRI over this pH range. The NPH curve shown is an average of six pH curves measured using commercial and lab-prepared samples over a range of dilutions from zero to 1:50, the error bars representing the range of the mobility values over the six measurements. The vertical lines drawn from the point of intersection of the pH curves with the line at zero mobility indicate the pI at ~5.25 (for NRI) and pzc at 7.0–7.5 (for NPH).

Thus, we feel confident that these solution perturbations do not drastically affect the protamine/insulin ratio, morphology, or crystal size of the NPH and allow the exploration of solution conditions that represent perturbations known to occur in clinical settings.

Models, Techniques, and Buffers

The application of ITC for understanding soluble insulin adsorption onto NPH crystals (Figure 2) was based on the successful application of the technique to adsorption phenomenon in other colloidal systems [11]. To validate the approach, the ITC results were compared to results obtained from HPLC measurements (Figure 3), which directly monitor the amount of free insulin in solution. The excellent fits of the binding isotherms using both ITC (eqn. 1 and Figure 2) and HPLC (eqn. 3 and Figure 3) illustrate that the adsorption process can be modeled as a simple one-to-one interaction between an insulin hexamer and a single binding site on the NPH surface with no interaction between occupied binding sites, *i.e.*, assuming only simple Langmuir monolayer adsorption occurring on the NPH surface. However, this sim-

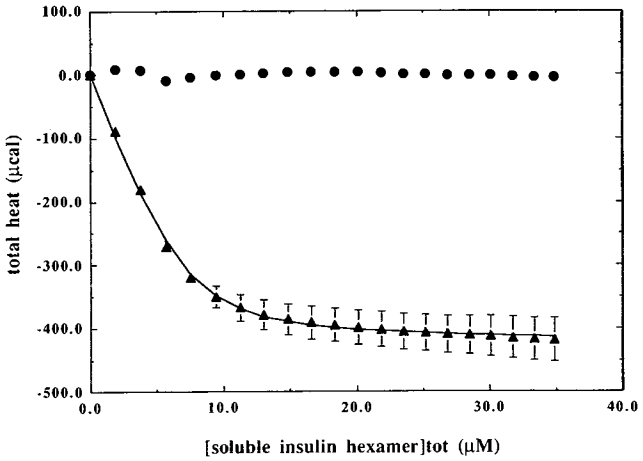

Figure 2: Plot of an integrated ITC adsorption profile (▲), the one-to-one model fit (—), and the residuals of the fit (●) of NRI (U-200) addition to NPH crystals (U-100) under formulation conditions of 0.015 mg/mL zinc/100 Units of insulin, 0.27 mg/mL protamine, pH 7.5 at 23°C in the presence of 14.1 mM Na_2HPO_4. The stir rate was 400 rpm. The fit yielded a $K_{R6,ads}$ = 1.40 (±0.18) × 10^6 L/mol, ΔH = −45.89 (±1.69) kcal/mol, and a $[M]_{tot}$ = 0.74 (±0.02) × 10^{-5} mol/L.

ple one-hexamer-to-one-binding-site model must be reconciled with the association state of soluble insulin.

In these formulation studies, the soluble insulin monomer can self-associate into a multitude of aggregated states including a monomeric, a dimeric, and two hexameric forms, T_6 and R_6, making the analysis quite complicated (Figure 4) [6,12]. The R_6 hexamer differs from the T_6 hexamer in that six phenolic preservative molecules are bound to the hexamer. Ligand binding induces a conformational change in the B1 to B8 region of each monomer in the hexamer known as

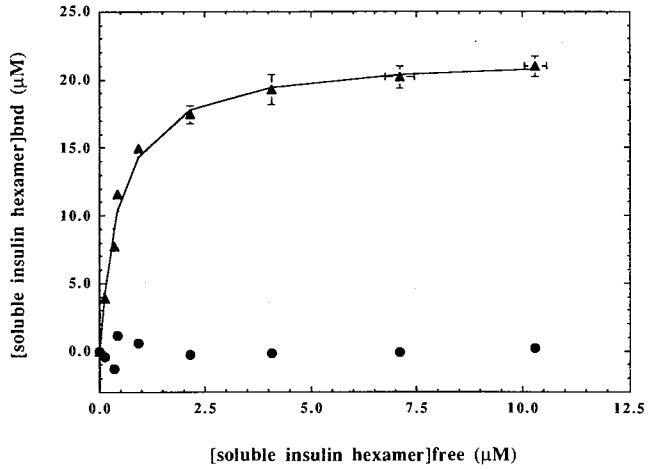

Figure 3: A plot of the binding isotherm determined by HPLC (▲), the one-to-one model fit (—), and the residuals of the fit (●) of NRI (U-200) addition to NPH crystals (U-100) under formulation conditions of 0.015 mg/mL zinc/100 Units of insulin, 0.27 mg/mL protamine, pH 7.5 at 23°C in the presence of 14.1 mM Na_2HPO_4. The fit yielded a $K_{R6,ads}$ = 2.12 (±0.21) × 10^6 L/mol and a $[M]_{tot}$ = 2.17 (±0.05) × 10^{-5} mol/L. The errors are the standard deviations for each point determined from multiple observations.

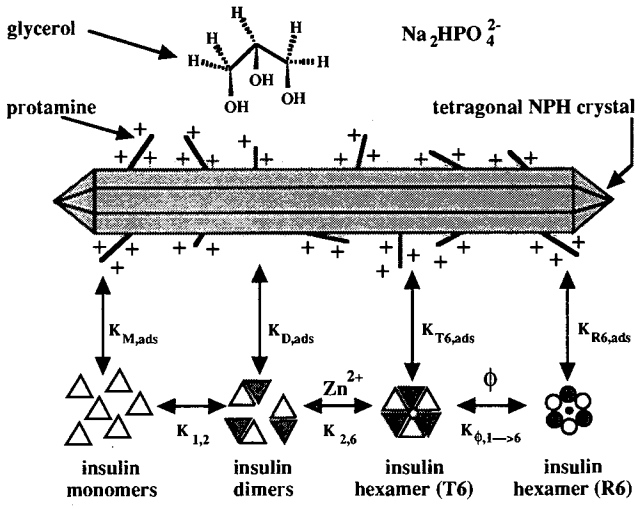

Figure 4: Schematic diagram of components and interactions in premixed formulations. The formulations contain (1) NPH tetragonal crystals with accessible protamine ($_+$—$^+$), (2) soluble insulin that can exist in monomeric (Δ or O), dimeric, T_6-hexameric and R_6-hexameric state, (3) phenolic preservative (ϕ), (4) zinc (Zn^{2+}), (5) sodium phosphate ($Na_2HPO_4^{2-}$), and (6) glycerol. The conversion of T_6-hexameric insulin to R_6-hexameric insulin involves the binding of six molecules of phenolic preservative per hexamer. The R_6-hexameric state of soluble insulin is proposed as the species that is adsorbed onto the surface of tetragonal NPH crystal.

the T→R transition [6]. In the T→R transition, the B1 to B8 region is converted from an extended structure into an α-helix and stabilizes the hexamer. It is important in these studies that the physical state of the hexamer remain constant through the course of the experiment because the R_6 or T_6 states are conformationally quite different [13] and this change in conformation could subsequently alter the surface of the hexamer and affect the adsorption process.

The association state of insulin under formulation conditions has been studied in our laboratory (unpublished data) and by Hvidt [5]. The results from these studies indicate that insulin remains associated in a hexameric state in the presence of phenolic preservative and elevated levels of zinc across the titration range used in this study. Thus, the predominant species interacting with the NPH crystal is presumed to be the hexameric-R_6 state, and therefore the equilibrium constant being measured is referred to as $K_{R6,ads}$ (Figure 4).

Overall, the two methods used to study the adsorption process, ITC and HPLC, provided results that were in close agreement. However, the data indicate a systematic discrepancy between the calorimetric-derived and the HPLC-derived adsorptivity capacity values, with the latter being threefold higher. It is possible that two different binding sites exist on NPH with the same $\Delta_{ads}G°$ but different $\Delta_{ads}H$, and consequently the calorimetric technique can only detect the heat generated by binding to one of the sites in the deposition of the monolayer; however, the multi-site binding would be detected by HPLC. This hypothesis is consistent with the possible existence of a non-uniform positively charged surface on the NPH generated by the ill-defined binding behavior of protamine in NPH [14]. Despite the difference, the

adsorptivity capacities derived from the ITC studies should be useful in making relative comparisons between different formulation conditions.

The buffering system used in these studies had no apparent effect on the equilibrium constants or the adsorptivity capacity of the NPH crystals, indicating that buffer ions do not significantly alter the sorbent surface and protein surface electrical layers. In addition, the $\Delta_{ads}H$ of binding is not affected by the type of buffer present in the formulation, indicating that there are no complications due to enthalpy contributions associated with heats of ionization from the buffer, the heats of ionization for HEPES and phosphate are +5.01 kcal mol^{-1} and +1.13 kcal mol^{-1}, respectively [15].

Sorbent Surface and Solution Perturbations

Calculations based on the experimentally determined ionization behavior of insulin [16] and the calculated ionization behavior of protamine at pH 7.0 indicate that protamine-containing NPH crystals should have a net positive charge and that soluble insulin hexamers would have a net negative charge. The net positive charge of NPH crystals is due to the highly-basic nature, ~20 arginine residues, of protamine. The observed protamine-dependent adsorption supports the hypothesis that binding is mediated by electrostatic interactions, because the net positive charge associated with the NPH crystal would increase with increasing protamine concentration. This increase in net positive charge would drive the electrostatically-mediated process and increase the potential number of surface sites available for binding.

This conclusion is consistent with crystallographic data on NPH crystals that have identified that only a portion of protamine binds at the dimer-dimer interface of the insulin hexameric unit [14] and that the remainder of the protamine extends into the interstices between the insulin hexamers of the NPH crystal composing the crystal contact region [14, 17, 18]. Therefore, the protamine would be available to interact with the negatively-charged insulin hexamer in solution. No free protamine has been detected in the supernatant of NPH preparations, eliminating the possibility of heat due to protamine/insulin precipitation.

Analysis of the enthalpy values over the range of protamine concentrations investigated (Table III) indicates that the $\Delta_{ads}H$ per site is not constant and appears to contradict the Langmuir model, composed of independent sites, developed to analyze the data. However, it should be noted that binding sites on the NPH surface are most likely dependent on the protamine concentration, consequently affecting the relative contributions of electrostatic and van der Waals interactions that mediate the adsorption, which in turn modulate the thermodynamic response.

The perturbation of association/dissociation reactions by pH (Table III) is indicative of electrostatic contributions to the binding [19] and is borne out by the electrophoretic mobilities measured at these pH values (Figure 1). At pH < 7.0, the adsorption of NRI onto NPH is facilitated by the electrostatic attractive forces between the negatively charged NRI and the positively charged NPH, (NRI(−) and NPH(+)), while at pH > 7.5 the two species carry like charges, (NRI(−)), and (NPH(−)), leading to electrostatic repulsion and hence a decrease in adsorption. The fact that binding can still occur between similarly charged species is explained below.

The increase in net negative charge of the NPH crystal surface with increasing pH is most likely due to deprotonation of His^{B5}, pK_a = 7.0 [20], the α-amino group of Gly^{A1}, pKa = 8.0 [21], and the α-amino group of Phe^{B1}, pKa = 6.7 [21]. The deprotonation of the arginine groups in protamine does not play an important role in increasing the net negative charge on the NPH crystals due to the high pK_a (12.5) of this amino acid. However, the high pK_a of arginine ensures that protamine-containing regions of the NPH crystal surface remain positively charged within the pH range studied and therefore capable of supporting weak, localized adsorption despite the increased net negative charge of the NPH crystals. Adsorption driven by patch-controlled attractive electrostatic interactions has been observed in chromatographic systems where the protein and adsorbents have similar net charges [22]. This may partially explain why the binding is not completely inhibited at pH 8.0.

The inhibition of the adsorption process with increasing ionic strength is also consistent with the screening of electrostatic interactions (Table III) [19]. The adsorption is not completely inhibited by ionic strength because of the contributions van der Waals interactions make to the adsorption process.

The adsorption process is inversely affected by temperature. This is attributable to two factors: (1) the inherent temperature-dependent nature of equilibrium processes and (2) the temperature effect on the entropy of association, $\Delta_{ads}S°$. This decrease in entropy is only partially compensated for by an increase in the exothermic enthalpic response, $\Delta_{ads}H$. Thus, the entropy term becomes increasingly more negative and more ordered, and is affected to a greater extent than the enthalpy term at higher temperatures. The negative entropic values result from the damping of the low-frequency vibrational modes in the insulin hexamer after adsorption [23, 24]. This tightening of the hexameric structure coupled with a diminished hydrophobic effect at higher temperatures, consequently makes the $\Delta_{ads}S°$ increasingly more negative [24, 25]. This combination of events, coupled with the inverse temperature dependence of the exponential term in equation 4, results in a shift of the equilibrium to the dissociated species. These relationships are illustrated in the van't Hoff equation:

$$K_{R6,ads} = e^{\left(-\frac{1}{RT}\Delta_{ads}H + \frac{1}{R}\Delta_{ads}S°\right)} \quad (4)$$

An explanation of how increased temperatures reduce the adsorptivity capacity of the crystals is elusive but is presumed to be due to perturbations in the hydration layer and/or the NPH surface that subsequently alter the protamine/hexamer complex in NPH. More investigation is necessary to confirm these hypotheses.

Thermodynamics

Reviewing the thermodynamic data under all the conditions studied, we find that $\Delta_{ads}G°$, $\Delta_{ads}H$, and $\Delta_{ads}S°$ are all negative. The adsorption process is clearly spontaneous with $\Delta_{ads}G° < 0$. The large magnitudes of the enthalpy and en-

tropy values reflect the multiplicity of interactions that must take place in this protein/surface interaction.

Protein association processes are believed to proceed through a two-step binding process [24]. In this experimental system, the first step consists of a diffusion-controlled hydrophobic association of the insulin hexamer onto the surface of the NPH crystals resulting in partial immobilization of the soluble species. The second step involves the formation of all other intermolecular interactions, including electrostatic, van der Waals, and hydrogen bonding interactions. The thermodynamic pattern observed in this study, negative enthalpies and entropies, suggests that the adsorption process cannot be solely accounted for by electrostatic interactions or hydrophobic interactions. The exothermic nature of the adsorption process is consistent with the formation of van der Waals interactions [25]. Recent modeling studies on the adsorption of lysozyme to a planar, charged surface support the contention that van der Waals interactions can play an important role in electrostatic-mediated adsorption processes [24].

The thermodynamic parameters, $\Delta_{ads}H$ and $\Delta_{ads}S°$, become increasingly negative under conditions that mask or reduce the electrostatic interactions between soluble insulin and NPH, *e.g.*, high pH, high ionic strength, or low protamine concentration. This is most likely due to increasing importance of van der Waals interactions in the adsorption process under weak electrostatic conditions.

The effect of temperature on the relative contributions of $\Delta_{ads}H$ and $\Delta_{ads}S°$ to $\Delta_{ads}G°$ is consistent with an adsorption process that has a large negative heat capacity change. A plot of $\Delta_{ads}H$ (kcal/mol) as a function of T (K) at pH 7.0 was used to determine $\Delta_{ads}C_p$. The linear least-squares fit ($R^2 = 0.98$) of the data yielded a $\Delta_{ads}C_p = -2475$ cal/mol·K. This negative heat capacity is most likely due to the contribution from the hydrophobic and van der Waals interactions as well as the partial immobilization of insulin hexamer that results in a damping of the low frequency vibrational modes that subsequently "tighten" the protein structure [23, 24]. At lower temperature (T = 20°C) the adsorption is most likely facilitated by hydrophobic association due to a more ordered hydration structure around both the insulin hexamer and NPH surface. Under these conditions, $\Delta_{ads}H$ and $\Delta_{ads}S°$ are less negative due to the disruption of an ordered hydration layer around hydrophobic residues that consequently compensates for the negative $\Delta_{ads}S°$ and $\Delta_{ads}H$ due to van der Waals interactions and vibrational contributions. However, increasing the temperature (T = 40°C) diminishes the hydrophobic effect leaving only the dampened soft vibrational contributions to the entropic response, consequently making $\Delta_{ads}S°$ increasingly more negative [24, 25].

In addition to the temperature effects, an enthalpy-entropy compensation plot (Figure 5) of the adsorption data yields a linearity coefficient value, T_c (compensation temperature), of 266 K. This value is consistent with data from other binding processes and supports the contention that rearrangement of the hydration layer on the surface of the insulin and NPH plays a significant role in the adsorption process [26]. The enthalpy/entropy values observed in these adsorption studies fall within a region that is consistent with an enthalpy-driven process [27].

In conclusion, the thermodynamic fingerprint of soluble insulin adsorption suggests that hydrophobic, electrostatic, and van der Waals interactions all contribute to an enthalpy-driven adsorption process.

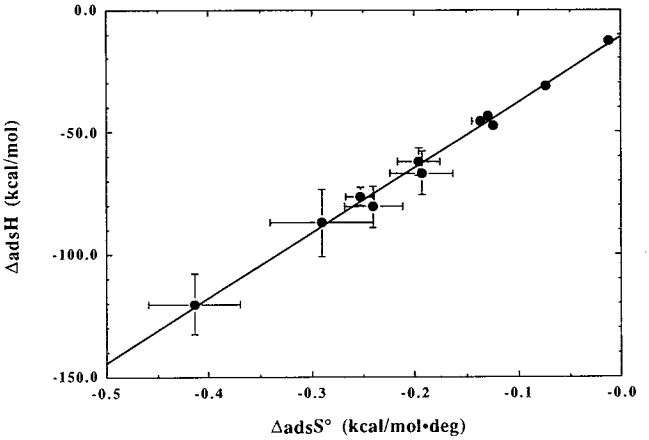

Figure 5: Enthalpy-entropy compensation plot of the adsorption data. The line represents a linear least-squares fit of the data to the equation $\Delta_{ads}H = T_c \cdot \Delta_{ads}S° + \alpha'$ ($R^2 = 0.99$), where $T_c = 266.2$ K and $\alpha' = -11.3$ kcal/mol. T_c refers to the compensation temperature.

Implications on Physiological Release of Soluble Insulin from Crystalline/Soluble Insulin Mixtures

Based on the results presented in this paper, the formulation conditions used in NPH and NRI mixtures, *i.e.*, low ionic strength and low temperature, promote the adsorption process ($K_{R6,ads} \geq 10^5$). Under these strongly adsorbing formulation conditions, the desorption of soluble insulin from the NPH surface would be expected to be quite slow because of a multiplicity of interactions, with interaction energies in the range of 1kT per segment [1, 28]. These conditions would consequently be expected to affect the pharmacokinetics of the formulation after a subcutaneous injection. However, the pharmacokinetics of each component in premixed formulations, NRI and NPH, have been shown to be unaffected by the adsorption phenomenon [29, 30]. This can be reconciled by taking into consideration the temperature and ionic strength dependence of the equilibrium constant, $K_{R6,ads}$. Studies performed under physiological conditions, ionic strength = 150 mM, pH 7.4, and 37°C, yielded the weakest association measured in these studies, $K_{R6,ads} = 3 \times 10^4$ M^{-1}. This weaker association is due in part to an increased rate of desorption that is facilitated by the reduction in the number of segmental interactions occurring between the soluble insulin and NPH surface as well as a reduction in the strength of the segmental interaction (<1kT). This reduction in the equilibrium constant coupled with the dilution effect that occurs upon injection supports the contention that under physiological conditions the adsorption process becomes reversible on a time frame that does not impact the pharmacokinetic responses of the individual insulin formulations relative to separate simultaneous injections of each.

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