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Insulin association in neutral solutions studied by light scattering

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Molecular weights and weight distributions of sulfated, Zn-free, and 2Zn insulins have been measured at pH 7.3 as a function of concentration from 0.1 to 2 mg/ml by use of a combination of light scattering, refractometry, and size-exclusion chromatography. Results show that sulfated insulin is monomeric over the studied concentration range. Weight average molecular weights between those of a monomer and a hexamer were found for both zinc-free and 2Zn insulins. Zinc stabilizes the hexamer, and the dimer-hexamer equilibrium constant is approx. 400-times higher in the presence of zinc than in its absence. An average hydrodynamic radius of 5.6 nm, close to the crystallographic size of the insulin hexamer, was determined from dynamic light scattering of 2Zn insulin solutions.

1. Introduction

Insulin has been the subject of extensive investigations with respect to its structure and function, as discussed in comprehensive reviews [1,2]. The biologically active form of the hormone appears to be the insulin monomer, which has a molecular weight of 5778 g/mol for porcine insulin [1,2]. Insulin associates in solution and can crystalize as a 2Zn form, which consists of a hexamer with two bound Zn^{2+}. Its crystal structure has been determined [1]. Information about the association behavior of insulin in solution at near neutral pH stems primarily from sedimentation equilibrium studies [3–8] and stopped-flow measurements [9]. These studies show that insulin without zinc forms dimers with a typical equilibrium constant 4×10^4 M^{-1}, and that insulin associates further. Some data have been analyzed with a model involving monomer-dimer-hexamer and higher order aggregates [3,6], whereas other results favor a model with a monomer-dimer equilibrium and a further indefinite association of either dimers [7] or monomers [8]. With zinc present the hexamer is stabilized, and aggregates of even higher molecular weights are inferred from sedimentation equilibrium studies [4,5].

Light scattering (LS) offers an alternative way to investigate the association of macromolecules in solution. Early LS studies of insulin focused on its properties in acidic solutions, where insulin is found as monomers and dimers [10]. A few studies have been performed in solutions near neutral pH [11-12]. These results indicate a very complex time dependent association pattern and the formation of higher molecular weight aggregates.

Insulin has a well-known tendency to precipitate on surfaces and interfaces [2,13], and traditional LS is very sensitive to traces of dust or particles. These considerations prompted us to perform low-angle laser light scattering on insulin solutions using a flow system with a size-exclusion chromatography (SEC) column to ensure that any large particles were effectively removed from the insulin. An advantage of low-angle laser LS, besides the elimination of an extrapolation to

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vanishing angles, is that the scattering volume is very small compared with traditional static LS in glass cells, where scattering from dust or particles in a large volume is averaged and added to the scattering from the macromolecule under study. We report here the results of an investigation of three forms of insulin. Both zinc-free and 2Zn porcine insulin, and sulfated bovine insulin were investigated. Sulfated insulin is used to suppress insulin association [14]. A recent study [15] shows that phenols affect the conformation of zinc insulins in the crystalline state. Phenols are often included as anti-bacterial agents in insulin solutions for pharmaceutical use [2]. The studies reported here were performed in a solvent which contained *m*-cresol, in order to compare properties with published results in solutions without phenols. Information about the association properties is obtained from the intensity of scattered light and its dependence on concentration.

2. Materials and methods

2.1. Samples and solvents

All insulin samples were a generous gift from Dr B. Welinder, Hagedorn Research Laboratory, Denmark. Zinc-free porcine insulin was supplied as a highly purified (> 98%) sodium insulinate powder. Porcine insulin with a zinc content of approx. two Zn^{2+} per six insulin monomers was received as a crystalline powder with a water content of 5.4% or as an approx. 3.7 mg/ml solution in the buffer described below. The sulfated bovine insulin was a commercial grade from Connaught NOVO, Canada. This solution was found by LS to contain some very high molecular weight components and was purified by SEC on a Sephadex G-75 column (described below) before use.

The solvent used contained 1/75 M sodium phosphate (pH 7.3) buffer with 1.63% glycerol and 0.3% *m*-cresol by weight. Solid samples were dissolved overnight at 5 °C in buffer, typically to 3.5 mg/ml. The refractive index of the solvent was measured to be 1.3345, by use of an Abbe refractometer with a red light source. The density of the solvent was determined by use of a pycnometer to be 1.0055 g/ml at 21° C.

2.2. Flow analysis system

A schematic diagram of the flow and data acquisition system is given in fig. 1. It consists of the following parts: a 5 l buffer reservoir flushed with helium in order to remove air; an HPLC Technology pump (type RR/065) typically operated at 0.2–1 ml/min; a 12 cm × 4 mm PW2000 (Toyo Soda) column to increase the pump pressure to insure a stable flow rate; a Rheodyne 7120 sample injection unit with loops ranging from 0.1 to 3 ml; an SEC column (2.8 cm diameter) packed with either Sephadex (Pharmacia) G-15 or G-75 materials, a 0.22 μm Millipore filter mounted directly on the scattering cell of a KMX-6 (Chro-

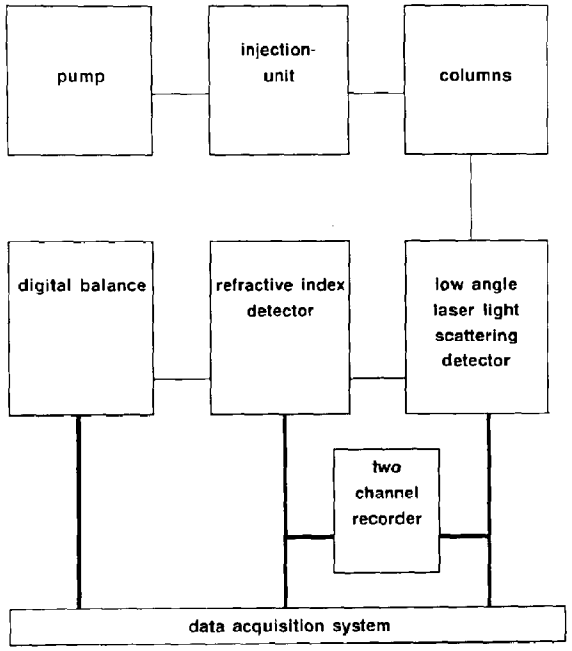

Fig. 1. Schematic diagram of the flow system used in this study. Flow of solvent indicated with fine line. Insulin solutions are fractionated on Sephadex columns and the eluents analyzed by light scattering and refractive index dectors. The integrated flow is monitored on a balance. Signals, indicated with heavy lines, are sampled on a computer and also shown on a chart recorder.

matix) light scattering instrument; an Optilab 5902 (Tecator, Sweden) refractometer with a 0.2 or 10 mm measuring cell and a wavelength of 633 nm; and a Mettler type AE200 balance, which enables a continuous monitoring of the elution volume. The individual parts were connected with polyethylene tubing.

The KMX-6 low-angle laser LS instrument with a wavelength of 633 nm was used with a 4.93 mm flow-through scattering cell. The intensity of the scattered light between 6 and 7° of scattering angle were recorded. Due to differences in the refractive index between solvent and the quartz scattering cell this range of scattering angles results in an average scattering angle in solution of 4.8°. The signals from the KMX, the refractometer, and the balance were sampled by a Hewlett Packard HP 3421A data acquisition unit and analyzed and stored on an HP 9121 microcomputer. Data analysis was performed in a batch mode.

2.2.1. Size-exclusion chromatography

Two types of SEC columns were used, depending on whether it was desired to separate the insulin according to size or just to use the column as a buffer exchange column, needed for accurate RI concentration determinations. A 33 cm long column with Sephadex G-75 with a diameter of 2.8 cm was used for fractionation purposes. A short 5 cm long and 2.8 cm diameter column packed with Sephadex G-15 was used for buffer exchange. In this case insulin elutes in the void volume, since the exclusion limit for Sephadex G-15 is approx. 1500 g/mol for globular proteins. The void and total volumes of columns were determined by injecting buffer containing Blue Dextran (Phamacia) and 1% D_2O. Insulin samples were diluted with buffer from stock solutions and allowed to equilibrate at 5°C for 18 h before measurements.

2.2.2. Dynamic light scattering

Translational diffusion constants for 2Zn insulins were measured by dynamic light scattering in the homodyne mode, using a BI-2030 correlator with associated optics and photomultiplier (Brookhaven Instruments, Holtsville NY). The intensity autocorrelation function of 633 nm laser light scattered at 90° was calculated with a delay time of 2 μs per channel and analyzed by fitting to a single-exponential function, as described elsewhere [16]. The diffusion constant, D, is obtained from the decay rate of the intensity autocorrelation, Γ, and the scattering vector, k, using the relation $\Gamma = Dk^2$. An experimental baseline, determined from a 1024 channel delay, was used for fitting of data. All measurements were carried out at room temperature (21°C). Insulin solutions (1 ml) were filtered through a 0.22 μm Millipore filter directly into the scattering tubes.

3. Method of data analysis

The molecular weight of the samples was calculated by an analysis of the refractometer and LS signals. The difference between the refractive index of a solution and solvent, Δn, depends on the differential refractive index increment and concentration at low concentrations as

$$\Delta n = (\mathrm{d}n/\mathrm{d}c) \cdot c \tag{1}$$

The weight average molecular weight, M_w, is obtained from the Rayleigh ratio, R, at low concentrations as

$$Kc/R = 1/M_w + 2A_2 \cdot c \tag{2}$$

where A_2 is the second virial coefficient. The constant K in eq. 2 is given by

$$K = (2\pi^2 n^2/\lambda^4 N)(\mathrm{d}n/\mathrm{d}c)^2(1 + \cos^2\theta) \tag{3}$$

where n is the solvent refractive index, λ the wavelength of light in vacuum, N Avogadro's number, and θ the scattering angle. Eq. 2 is only exact at vanishing scattering angles, but it is an excellent approximation, since insulins are much smaller than the wavelength of light and since θ is only 4.8°.

The differential refractive index increment was determined by use of the refractometer. Six solutions of 2Zn insulins were prepared gravimetrically in the buffer system at concentrations ranging from 0.3 to 1.5 mg/ml. 3 ml were injected in the flow system where the filter and the KMX

instrument had been removed. The refractive index increments were determined and analyzed by a linear least-squares fit to eq. 1. After a correction for the water content in the lyophilized insulin sample, the differential refractive index increment was determined to be 0.183 ± 0.003 ml/g.

A computer program calculates the weight average molecular weight from the LS and refractive index signals at each elution volume by use of eqs 1–3. A correction for the small dead-volume (230 μl) between the KMX and the refractometer is made. The result of the calculation is c and M_w at 1704 elution volume fractions in the chromatogram.

4. Results

The scattering profile in fig. 2 for a 2Zn insulin solution fractionated on a Sephadex G-75 column shows spikes due to particles passing through the scattering volume. These spikes are removed by use of boolean smoothing and the resulting curve is shown in the lower part of fig. 2. The refractive index as a function of elution volume is also shown. The excess scattering and refractive index signals due to insulin are found by subtraction of the respective solvent baselines. It is seen that the LS peak is somewhat asymmetric and has its maximum at a slightly earlier elution volume than the refractive index. A mono-disperse sample would result in similarly shaped LS and RI elution profiles. The LS signal is proportional to cM_w as seen from eq. 2, if effects due to the second virial coefficient can be neglected. The refractive index increment, on the other hand, is proportional only to c, as shown in eq. 1. The ratio of the two signals is thus proportional to M_w. It is therefore immediately seen that the insulin sample in fig. 2 has a non-uniform molecular weight distribution

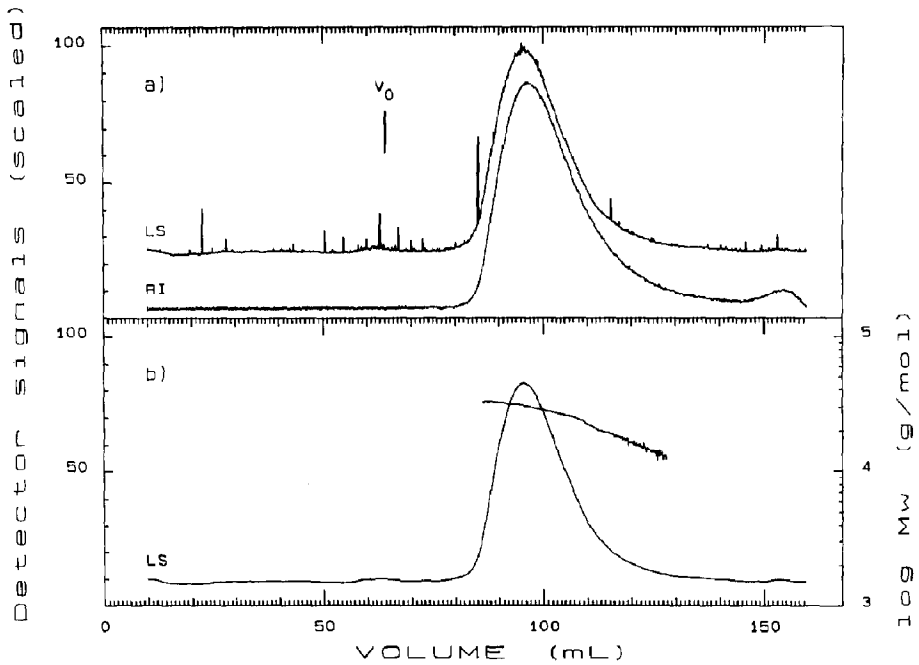

Fig. 2. Chromatogram of 2 ml of 3.7 mg/ml 2Zn insulin injected and fractionated on a Sephadex G-75 column at a flow rate of 0.6 ml/min. The void volume of the column is indicated by V_0. Top part (a) shows light scattering (LS) signal before noise reduction and the refractive index (RI) in arbitrary scales. LS signal after noise reduction is shown in the bottom part (b) together with log of the calculated weight average molecular weight (right scale) for each fraction in the elution peak.

and that the parts with higher molecular weights are eluted at lower volumes, as expected for a size-exclusion column. With the assumption of a negligible contribution from the second virial coefficients at the small concentrations used (maximum concentration in fig. 2 is 0.135 mg/ml) the molecular weight is computed as a function of elution volume, as shown on a log scale in the lower part of fig. 2.

The zinc-free insulin also shows asymmetric elution peaks and evidence for a molecular weight distribution. The peak elution was observed at slightly larger volumes (109 ml) when compared with 2Zn insulin solutions (97 ml). Sulfated insulin, however, shows only one symmetrical peak at 127 ml in both detection systems and no signs of a molecular weight distribution. Both 2Zn and zinc-free insulin have a non-vanishing RI signal at the elution volume of sulfated insulin, suggesting that these solutions also contain insulin with a molecular weight similar to that of sulfated insulin.

The molecular weight distribution of the insulin samples was calculated from the determined values of M_w and c in the chromatogram. Molecular weights were rounded to nearest thousand for each fraction, and for each rounded value corresponding concentrations were summed over the chromatogram. Fig. 3 illustrates results of such calculations for the three types of insulin in the form of histograms. Both zinc-free and 2Zn insulin contain broad molecular weight distributions in the range from 6000 to 35 000, whereas sulfated insulin has a narrow molecular weight distribution around 5000–6000. It is seen that neither 2Zn nor Zn-free insulin contains significant concentrations of material with molecular weights in excess of the hexamer (35 000).

The results shown in figs. 2 and 3 suggest that the insulin solutions consist of insulin oligomers with between one and six insulin monomers. It is not possible, however, to use the chromatograms for a more detailed analysis, since the solution of oligomers is a complex system where kinetic factors are important. This was illustrated by the observation that when the peak fraction of 2Zn insulin was collected and rechromatographed, the insulin re-elution peak came later in the chromatogram and had a peak molecular weight of only 12 700. This indicates that the system, given sufficient time and additional dilution during the column run, would dissociate to mainly monomers or dimers. In order to minimize these complicating factors the short Sephadex G-15 columns were used. Fig. 4 shows the LS chromatogram and molecular weight distribution for a zinc-free solution. It is seen that the molecular weight distribu-

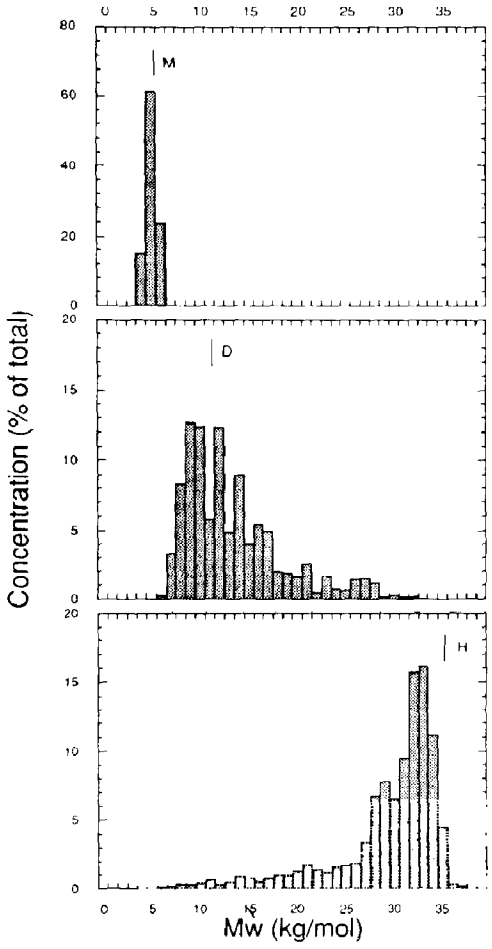

Fig. 3. Weight average molecular weight distributions for sulfated insulin (top); zinc-free insulin (middle); 2Zn insulin (bottom). The histograms give concentration found in Sephadex G-75 chromatograms in 40 molecular weight ranges from 0 to 40 000 g/mol. The molecular weights of insulin monomer (M), dimer (D), and hexamer (H) are shown as bars.

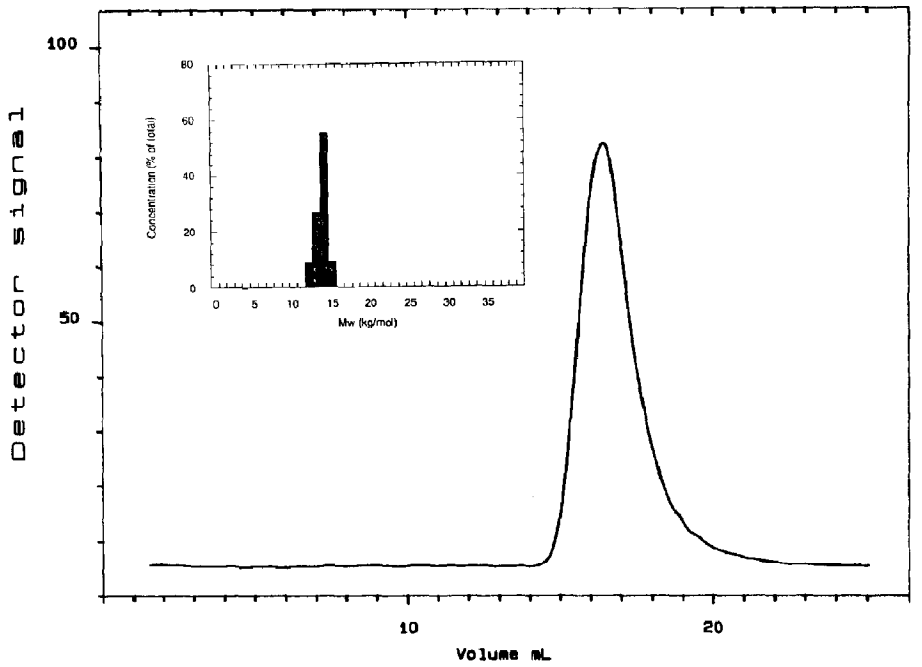

Fig. 4. Zinc-free insulin chromatogram on a Sephadex G-15 buffer exchange column. The light scattering signal is plotted vs elution volume. The corresponding weight average molecular weight histogram is shown in the inset. The molecular weight and concentration at the elution peak are 14540 g/mol and 0.332 mg/ml, respectively.

tion is narrow and that this column does not result in a fractionation of the sample. The samples are diluted somewhat during the passage of the column, and molecular weights and concentrations were calculated at the elution peaks. For 1 ml sample, the volume used in figs. 4 and 5, the peak concentration was close to one half of the injection concentration.

Fig. 5 shows a plot of R/Kc vs concentration, calculated from the RI signal, for the three systems investigated on the short G-15 column. If the second virial coefficient is neglected, R/Kc equals M_w. It is seen that sulfated insulin has the lowest molecular weight and only slight concentration dependence. Zn-free and 2Zn insulins have higher molecular weights, and at high concentrations 2Zn approaches a value characteristic of an insulin hexamer. It is also seen that the two forms of insulins have a very strong concentration dependence of the molecular weight, and that Zn-free insulin at the lowest concentrations has a weight average molecular weight close to that of a dimer.

Dynamic light scattering was performed on 2Zn insulin solutions at concentrations from 1.5 to 3.7 mg/ml. The intensity correlation function as a function of delay time is shown in fig. 6. The decay is seen to follow a single exponential closely, which suggests a low polydispersity, even at the highest concentration studied. The decay rates were independent of concentrations in the studied range, and an average translational diffusion constant of $(7.9 \pm 0.2) \times 10^{-7}$ cm^2/s was obtained.

5. Discussion

Light scattering is traditionally used to determine molecular weights and sizes, together with the second virial coefficient. It becomes exceed-

ingly complex to separate effects due to the association and the virial coefficients in a multi-component associating system. Only sulfated insulin behaves like a simple system giving a symmetric peak in the elution chromatogram with a constant molecular weight, and a nearly linear dependence of R/Kc vs c. From a linear least-squares fit of Kc/R vs c, a weight average molecular weight of 5670 ± 90 g/mol and a second virial coefficient of -6×10^{-6} mol l g^{-2} were obtained. The molecular weight is in good agreement with the native

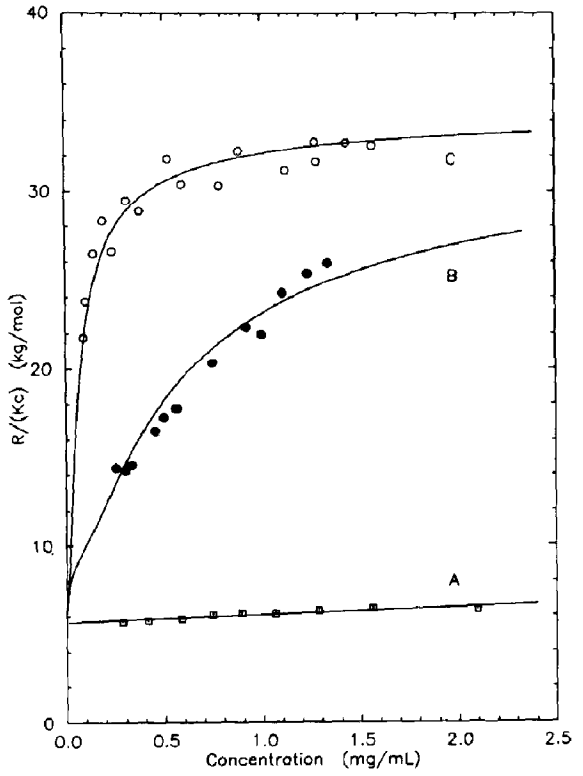

Fig. 5. Plots of R/Kc vs concentration for sulfated (A; squares), Zn-free (B; filled circles), and 2Zn (C; open circles) insulin solutions. A linear fit is shown for sulfated insulin. The curve for zinc-free insulin is computed by use of eqs 6 and 7 with the values of $K_{1,2}$ and $K_{2,6}$ determined by Pekar and Frank [3] and given in table 1. The curve shown for 2Zn insulin is the least-squares fit to the data with respect to $K_{2,6}$ using the same $K_{1,2}$ as for zinc-free insulin. Values of the two constants are given in table 1.

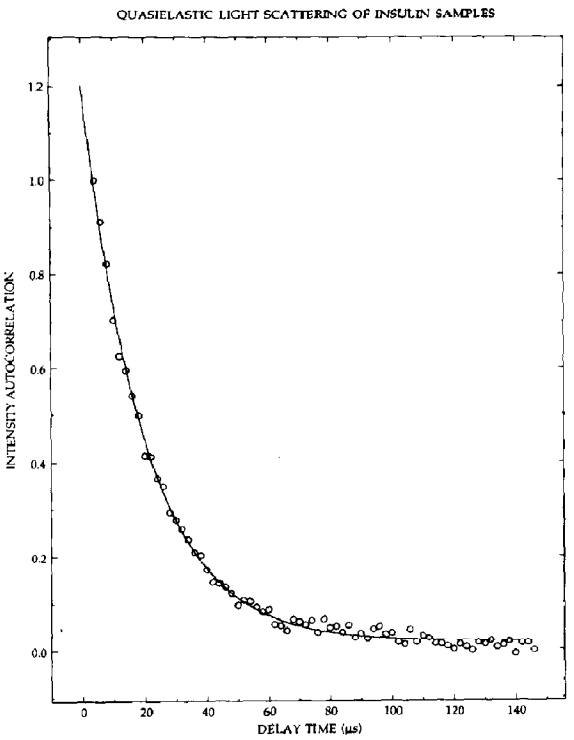

Fig. 6. Dynamic light scattering intensity autocorrelation function plotted vs delay time for a 3.7 mg/ml 2Zn insulin solution. Curve is least-squares single-exponential fit to the data. A translational diffusion constant of 7.7×10^{-7} cm^2/s is obtained from the fitted decay rate.

insulin monomer molecular weight of 5778. The results are taken as evidence for the dominance of sulfated insulin monomers up to a concentration of at least 2 mg/ml.

The two other insulin samples in fig. 5 show a decreasing molecular weight with decreasing concentrations. It has been proposed that the dominating insulin species in neutral solutions are monomers (M), dimers (D), and hexamers (H) [3,6], which are in a dynamic equilibrium, characterized by the dimer, $K_{1,2}$ and hexamer, $K_{2,6}$, equilibrium constants

$$K_{1,2} = [D]/[M]^2 \quad (4)$$

$$K_{2,6} = [H]/[D]^3 \quad (5)$$

The weight concentration is given by

$$c = M_0([M] + 2[D] + 6[H]) \quad (6)$$

where M_0 is the insulin monomer weight and the brackets denote molar concentrations. The weight average molecular weight, determined in light scattering, depends on the concentration of the three insulin forms, and is given by

$$M_w = M_0([M] + 4[D] + 36[H]) / ([M] + 2[D] + 6[H]) \quad (7)$$

Introducing eqs 4 and 5 into eqs 6 and 7, c and M_w can be expressed as a function of $K_{1,2}$ and $K_{2,6}$. The expressions in eqs 6 and 7 and the data shown in fig. 5 can be used to test the association constants for Zn-free insulin, determined from sedimentation data by Pekar and Frank [3]. Their values are summarized in table 1 and were introduced in the above equations in order to obtain M_w as a function of concentration. The resulting curve in fig. 5, based solely on sedimentation data, is seen to be in nearly quantitative agreement with the measured data in light scattering. The data were also compared with curves computed with association constants determined for bovine insulin [6]. The data are in poor agreement (not shown) with predictions based on this set of association constants. This suggests either a difference in association constants between porcine and bovine insulins, or it may reflect different association behavior in the solvents used in their study and ours.

The data for 2Zn insulin were used to estimate the hexamer association constant, on the assumption that the dimer association constant is unaffected by zinc. $K_{2,6}$ was varied until the best least-squares fit was obtained with the data. The estimated constant is 15×10^{10} M^{-2} and the resulting curve is shown in fig. 5. The hexamer association constant is seen to be approx. 400-times higher with zinc present than in the zinc-free case. This illustrates a significant hexamer stabilizing effect of zinc. M_w for 2Zn insulin in fig. 5 is seen nearly to level off at a value close to the insulin hexamer molecular weight at concentrations above 0.5 mg/ml, suggesting that such solutions primarily consist of hexamers. The translational diffusion constant of 7.9×10^{-7} cm^2/s corresponds [16] to an average hydrodynamic diameter, d, of 5.6 nm, since $D = kT/(3\pi\eta d)$, where k and T have their usual meanings and η is the solvent viscosity, taken to be that of water. The crystal structure of 2Zn insulin hexamers [1] corresponds to an oblate spheroid with diameter and height of about 5 and 3.6 nm, respectively. The measured hydrodynamic diameters are seen to be in reasonable agreement with such sizes, with part of the difference most likely due to bound water.

The insulins studied here do not show any indications of higher aggregates than hexamers, as reported by others [3,8,12]. Results to be published elsewhere, however, show that prolonged storage or mechanical shaking of neutral insulin solutions can result in very high molecular weight aggregates. Our results are in qualitative agreement with published sedimentation data, especially for the zinc-free case [3]. This suggests that the cresol included in our buffer system does not have a large effect on the association behavior of zinc-free insulin, even though it influences the conformation of insulin both in crystals and in solution [15]. Lack of information about the virial coefficients of the various insulin oligomers, and the limited concentration range possible in light scattering make an independent determination of the association constants difficult. Light scattering demonstrates, however, in a very direct way, the qualitative differences in association between the three insulin forms studied. Furthermore, the molecular weight and hydrodynamic diameter of 2Zn insulin determined from static and dynamic light scattering are, taken together, strong evidence for the existence of stable insulin hexamers also in solution.

Table 1

Dimer and hexamer association constants for insulin

Insulin form	$K_{1,2}$ (M^{-1})	$K_{2,6}$ (M^{-2})
Porcine, Zn-free [3]	14×10^4	4×10^8
Bovine, Zn-free [6]	1×10^4	1×10^8
Porcine, 2Zn, this study	14×10^4 [a]	15×10^{10}

[a] Assumed value (see text).

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