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Osmotic Pressure Measurements on Insulin: Anomalous Results Indicate that the Monomer is Preferentially Adsorbed

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Abstract

The concentration dependence of the number average molecular weight of insulin at pH 2, ionic strength 0·05, and 20°C as determined by osmotic pressure measurements indicates that the hormone is a homogeneous protein of molecular weight close to that of the dimer. Since sedimentation equilibrium experiments confirm what is well known, namely that insulin is a self-associating protein dissociating to monomer under these conditions, an explanation for the anomaly was sought in the possible loss of protein from solution by adsorption. Analysis of the results strongly supports this conclusion and consideration of the adsorption properties of insulin in terms of hydrophobic interactions shows them to be consistent with the behaviour of insulin as a self-associating protein. The monomer appears to be the primary molecular species responsible for insulin adsorption.

Introduction

Adsorption of insulin from aqueous solutions to materials such as paper (Berson *et al.* 1950), glass and talc (Cuatrecasas and Hollenberg 1975), polyethylene and a variety of plastics (Cecil and Robinson 1975) is well documented. The phenomenon has important clinical implications because adsorption to materials commonly used in infusion apparatus (Hirsch *et al.* 1977) may result in delivery of less than the nominal dose of the hormone. More seriously, adsorption may represent the initial event in the aggregation of insulin solutions leading to actual blockages in infusion tubing and pumps, a problem which has been investigated by Lougheed *et al.* (1980) and by Sefton and co-workers (Sefton and Nishimura 1980; Sefton 1982). More recently, Sefton and Antonacci (1984) have measured isotherms for insulin adsorption onto hydrophobic and hydrophilic materials at different values of pH and temperature in an attempt to quantify the effect and to understand the underlying mechanism, especially as it relates to nucleating aggregate formation.

Loss of protein from solution is also a potentially serious source of errors in laboratory measurements, especially in physicochemical studies of systems showing concentration dependence. Mammalian insulin dissolved in aqueous buffers is well-known as a system of this type, exhibiting association behaviour over a wide range of conditions of pH, ionic strength and temperature (Jeffrey and Coates 1966; Jeffrey *et al.* 1976; Goldman and Carpenter 1974). As a result of studies of the self-association of insulin it has been clear for many years that at low concentrations insulin dissociates to the true monomer (M_r = 5733 for bovine insulin) at pH's ranging from acid to alkaline (Jeffrey and Coates 1966; Jeffrey 1974; Goldman and Carpenter 1974; Jeffrey *et al.* 1976). It is therefore of interest in the present context that some of

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the earlier studies of the molecular weight of insulin in aqueous solution, notably those utilizing the technique of osmometry (Gutfreund 1948, 1952; Marcker 1960), indicated that the smallest subunit in aqueous solution was what is now known to be the dimer with a molecular mass of about 12 kDa. The present work is a report of an osmotic pressure study of the concentration dependence of the molecular weight distribution of bovine insulin at acid pH leading to just this result. Its interpretation in terms of loss of protein from solution by adsorption provides a rationalization of the anomaly and leads to some further insights into the adsorption phenomenon itself.

Materials and Methods

Materials

Bovine insulin was obtained from the Australian Commonwealth Serum Laboratories. It contained less than 5% each of proinsulin and monodesamido insulin and the equivalent of about 4 g-atoms of zinc per insulin hexamer. The zinc was removed by exhaustive dialysis against 0·01 M HCl at 4°C as shown by atomic absorption spectroscopy. The buffer used in all experiments was NaCl–HCl–glycine of pH 2·00, ionic strength 0·05, at 20°C and was prepared from analytical reagent grade chemicals and glass-distilled water. Insulin solutions for osmometry were prepared by dissolving a weighed amount of freeze-dried, zinc-free insulin in the appropriate volume of buffer to make the concentration close to 10 g/l, filtering through a 0·22 μm Millipore filter and determining the concentration by measuring the optical density at 278 nm [$E^{1\%}_{1\,cm}$ = 10·5 (Frank and Veros 1968)]. A series of more dilute solutions was then prepared by accurate weight dilutions from the stock solutions.

Osmometry

Osmotic pressure measurements were made in a Melabs model CSM2 osmometer maintained at 20°C; the procedure followed was exactly as suggested by the manufacturer and determinations were repeated at each protein concentration until a constant osmotic pressure was obtained to allow for dilution of the sample with that previously run. Normally only two or at most three repeat loadings were required. Membranes were cut from washed Visking dialysis tubing that had previously been shown not to pass insulin in dialysis experiments at pH 2·00. Number average molecular weights, $\bar{M}_n$, were evaluated as a function of the total protein concentration, C, from the expression derived for thermodynamically ideal heterogeneous systems (Jeffrey 1981):

$$\Pi/C = \mathbf{R}T/\bar{M}_n \qquad (1)$$

where Π, the osmotic pressure, is measured in centimetres of solvent, C is measured in grams per millilitre, T is the absolute temperature and R, the gas constant, is expressed as $8{\cdot}478 \times 10^4$ g cm mole^{-1} degree^{-1} for these units. The operation of the instrument was checked by a determination of the molecular weight of a sample of ovalbumin (Pentex, five times crystallized) at pH 7·0 in phosphate buffer of ionic strength 0·1. The graph of Π/C versus C was horizontal over the concentration range 0·015–0·8 g/100 ml and extrapolated to infinite dilution to give a molecular weight of 46 000. The instrument was therefore judged to be operating satisfactorily.

Sedimentation Experiments

Equilibrium sedimentation experiments of the Chervenka long-column, meniscus-depletion type (Chervenka 1970) were carried out at 20°C in a Spinco model E analytical ultracentrifuge equipped with a Rayleigh interference optical system. Solutions of insulin in the buffer were dialysed to equilibrium overnight and dialysed solution and its equilibrium dialysate were employed as solution and solvent, respectively, in a capillary-type, double-sector synthetic boundary cell. The photographic records of concentration distributions at sedimentation equilibrium were measured with a Nikon microcomparator and, after subtraction of baseline, readings were expressed in terms of vertical displacement, y, in micrometres of the interference fringes versus radial distance, r, from the axis of rotation.

Point weight average molecular weights, $\bar{M}_w$, were evaluated by the procedure suggested by Yphantis (1964) or by drawing tangents to a plot of ln y versus r^2 from the equation applicable to a thermodynamically ideal heterogeneous system (Jeffrey 1981)

$$\bar{M}_w = [2\mathbf{R}T/(1 - \bar{v}\varrho)\omega^2][\mathrm{d}\ln y/\mathrm{d}(r^2)]\,, \qquad (2)$$

where $\bar{v}$ is the partial specific volume of the protein, ω is the speed of angular rotation and ϱ is the solution density. A value for $\bar{v}$ of insulin of 0·72 ml/g measured at 20°C in this laboratory with an Anton-Paar DMA-02C precision density meter was used.

Results

Weight average molecular weights as a function of insulin concentration measured in two sedimentation equilibrium experiments at pH 2·00, 20°C, ionic strength = 0·05, are plotted in Fig. 1. Two widely differing sets of experimental conditions of speed of rotation and initial concentration were used and the good continuity between the two sets of results together with the general form of the curve show that under these conditions insulin is a self-associating protein, as expected at pH 2. The values of the molecular weight at low concentration show that dissociation proceeds to the insulin monomer. These results can be fitted very nicely to a reaction scheme consisting of monomer, dimer, and tetramer in reversible equilibrium, with a dimerization constant, k_2, equal to 4×10^4 M^{-1}, and a tetramerization constant, k_4, equal to $1{\cdot}5 \times 10^2$ M^{-1}, as shown by the solid line in Fig. 1. The

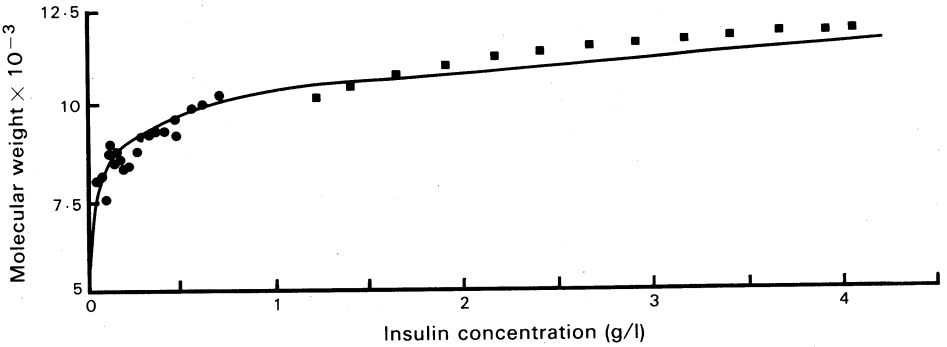

Fig. 1. Weight average molecular weight of bovine insulin as a function of insulin concentration at pH 2·00, ionic strength = 0·05, temperature 20°C. Points show results from two sedimentation equilibrium experiments: ● Initial concentration = 2·37 g/l, speed 22 000 rpm. ■ Initial concentration 0·50 g/l, speed 56 000 rpm. The line is calculated for a monomer–dimer–tetramer equilibrium with $k_2 = 4 \times 10^4$ M^{-1}, $k_4 = 1{\cdot}5 \times 10^2$ M^{-1}.

availability of these values allows calculation of the concentrations of the individual insulin species and hence the concentration dependence of $\bar{M}_n$, the number average molecular weights evaluated experimentally by osmotic pressure measurements and application of equation (1). The marked discrepancy between predicted result and the experimentally determined molecular weights (Fig. 2) reflects the results of osmotic pressure measurements on insulin solutions previously reported (Gutfreund 1948, 1952; Marcker 1960). The experimental result would be interpreted, incorrectly, as defining a homogeneous system with the molecular weight of the insulin dimer.

The experimental error in reading osmotic pressures is indicated by the error bars in Fig. 2 and is a maximum of about ±10% at the lowest concentration. The pressures read were corrected for dilution by solvent flow in the instrument (3×10^{-6} litres per centimetre of pressure) but this is a trivial correction, amounting only to less than 0·2% up to a concentration of 1 g/l. A correction was also applied to the number average molecular weights calculated from the osmotic pressure measurements, taking into account the fact that insulin molecules have a net positive charge at pH 2. The charge on the insulin monomer was taken to be +5 under these

conditions (Tanford and Epstein 1954) and it was assumed that charge is conserved on polymerization of insulin. This correction is less than 5% in $\bar{M}_n$ from the measured apparent value.

The aggregate effect of the errors involved in deriving the number average molecular weights at low insulin concentrations is quite insufficient to explain the discrepancy between measured and expected values. These results *can* be understood in terms of anomalous measurements arising from loss of protein from solution by adsorption. This is a plausible mechanism since in the osmometer the solution is in contact with glass, stainless steel and a cellulose membrane, materials to which insulin is known to be adsorbed. The design of the osmometer is such that the concentration of the solution is measured before it is injected and it cannot be checked subsequently because it is not possible to recover it from the instrument unmixed with previous solutions or solvent. It can be seen from equation (1) that, if an inappropriately high value of C is employed in the calculation, too high a value of $\bar{M}_n$ for the measured Π will result. Thus, in qualitative terms, a fall in concentration of a solution after it is put into the osmometer would be expected to lead to the kind of effect illustrated in Fig. 2.

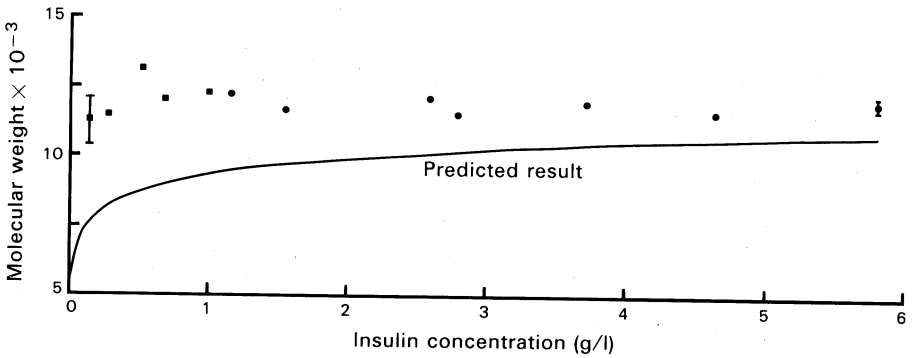

Fig. 2. Number average molecular weight of bovine insulin as a function of insulin concentration at pH 2·00, ionic strength = 0·05, temperature 20°C. Points (●, ■) show the results of two separate osmotic pressure experiments with bars indicating the maximum estimated error at the low and high end of the concentration range. The solid line shows the dependence predicted from the sedimentation equilibrium results in Fig. 1.

The magnitude of the loss in concentration at each total concentration required to produce the effect on the number average molecular weight at that concentration can be calculated as follows. In the osmotic pressure experiments the primary quantity measured, Π, gives values of $(C/\bar{M}_n)_{op}$ versus C. The relationship between these quantities can also be obtained independently by utilizing values of $\bar{M}_n$ as a function of concentration calculated from the sedimentation equilibrium results to give $(C/\bar{M}_n)_{true}$ versus C not affected by the suspected adsorption. From these values plotted as a curve, the true value of C corresponding to a given $(C/\bar{M}_n)_{op}$ can be read off and compared with the value of C that actually generated it in the osmotic pressure experiment. In this way it is possible to construct the plot of ΔC (the difference in concentration between the solution put into the osmometer and that calculated as described) versus C, shown in Fig. 3. It can be seen that in all of the solutions used ΔC was positive, that is, the system behaved as if the

concentrations of the solutions giving rise to the observed osmotic pressures were actually lower than those measured prior to injection into the instrument. Moreover, the effect is evidently linear. This result provides strong support for the idea that adsorption of insulin is giving rise to the anomalous results presented in Fig. 2.

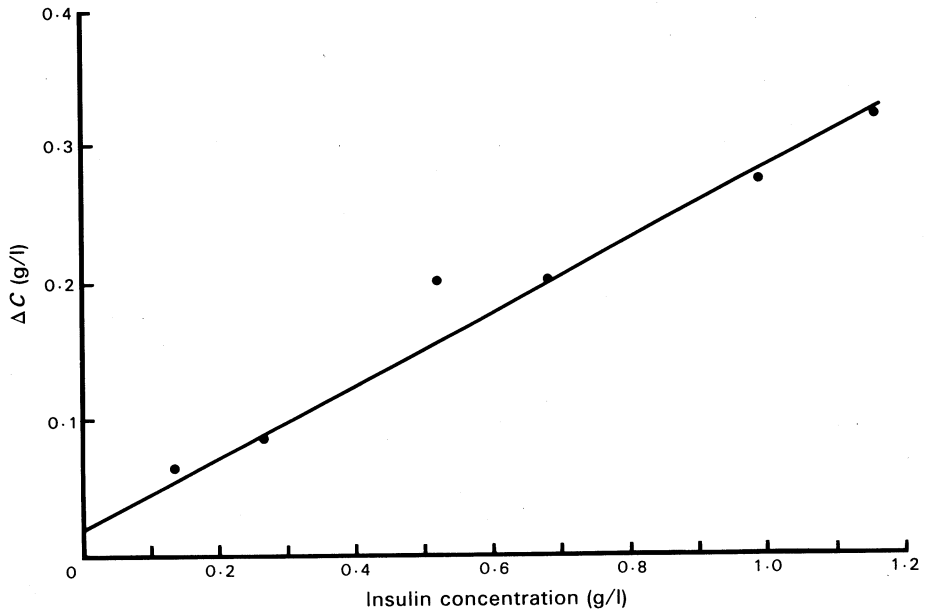

Fig. 3. Concentration change (loss) in insulin solutions at pH 2·0 as a result of assumed adsorption, calculated as described in the text. The line was obtained by linear least-squares regression and is plotted from the equation $\Delta C = 0{\cdot}019 + 0{\cdot}27C$, where C is the insulin concentration. The s.e. of the intercept is $\pm 0{\cdot}014$ and of the slope $\pm 0{\cdot}020$.

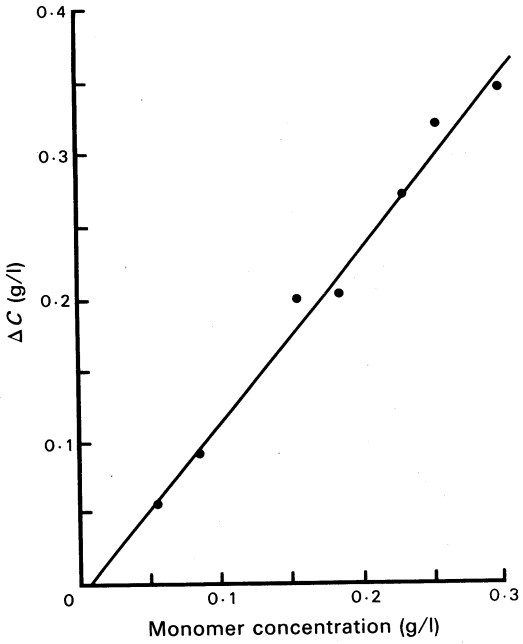

Fig. 4. Concentration change (loss) in insulin solutions at pH 2·0 as a result of assumed adsorption plotted as a function of the insulin monomer concentration calculated as described in the text. The line was obtained by linear least-squares regression and is plotted from the equation $\Delta C = -0{\cdot}010 + 1{\cdot}23C_m$, where C_m is the monomer concentration. The s.e. of the intercept is $\pm 0{\cdot}01$ and of the slope $\pm 0{\cdot}06$.

Since insulin dissociates as the concentration is lowered it is logical to test the idea that the form favoured by low concentration, the monomer, is preferentially adsorbed. The availability of a model for the self-association of insulin, together with the required equilibrium constants, allows construction of a plot of the monomer concentration, C_m, versus the total insulin concentration. This information allows the construction of the plot of ΔC versus C_m shown in Fig. 4. Not only is this relationship linear, like Fig. 3, but the regression line has a slope of 1·23 compared with that of only 0·27 for the plot of ΔC versus the *total* concentration. Thus, the change in concentration of the solution, ascribed to adsorption onto components of the instrument, can be accounted for almost entirely by adsorption of the insulin monomer.

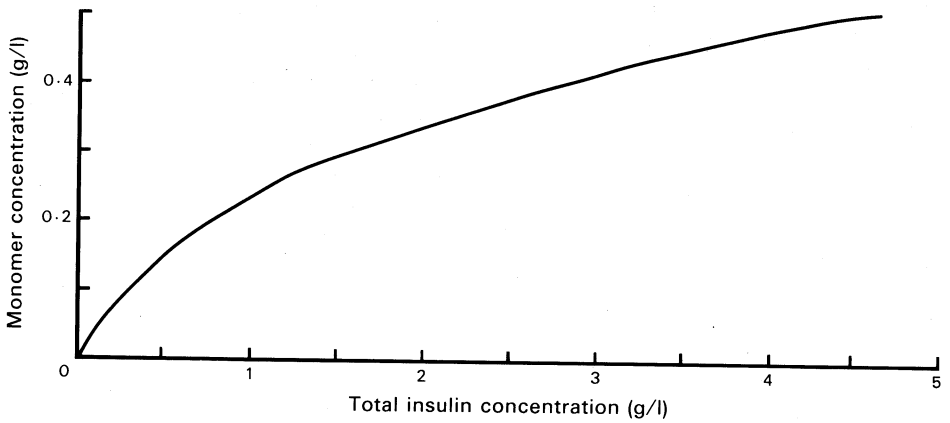

Fig. 5. Variation of the insulin monomer concentration with total concentration at pH 2·00, ionic strength = 0·05, temperature 20°C. The concentration 4 g/l is equivalent to about 100 international units, the maximum concentration used by Sefton and Antonacci (1984) in their adsorption experiments.

Discussion

The preferential adsorption of insulin monomers at low total protein concentration implied in Fig. 4 does not arise simply because dissociation has resulted in a solution containing little else other than monomers. In Fig. 5 the concentration of insulin monomer is plotted as a function of total concentration over the concentration range covered in Fig. 4. It is seen that monomers account for only from about 40 to 20% of the total protein in solution over this range. Fig. 5 also serves to demonstrate that, although the fraction of insulin present as monomer naturally decreases as the total insulin concentration increases, the absolute monomer concentration, of course, continues to increase. Thus, at the highest insulin concentration employed by Sefton and Antonacci (1984) in their adsorption experiments (100 i.u. ≡ 4 g/l) there is almost 0·5 g/l of monomer in solution at pH 2.

The slope of the line in Fig. 3 indicates that even at a total insulin concentration as high as 1 g/l (0·17 mM) almost one-third of the protein has been lost from solution. It is reasonable to ask whether the surface area available in the osmometer cell can account for adsorption of this quantity of insulin. The volume of protein solution held by the cell (0·5 ml) represents 5×10^{16} monomer molecules at a total concentration of 0·17 mM. The surface area of the cell and membrane is estimated from measurements to be 220×10^{16} $(Å)^2$, though this does not, of course,

take into account surface roughness or membrane porosity. Using these figures the surface area available is 44 $(Å)^2$ per insulin molecule for a close-packed monomolecular protein layer. The surface area of an insulin monomer occupied by hydrophobic residues can be estimated to be 200–300 $(Å)^2$ (Blundell *et al.* 1972; Cecil and Robinson 1975) from which it would be deduced that perhaps one-fifth of the total insulin (in the form of monomers) can be removed from solution by adsorption at the concentrations used in the osmotic pressure experiments before signs of saturation become evident. However, the present results are consistent with those obtained by Sefton and Antonacci (1984) where the amount of insulin adsorbed on a hydrophobic surface exceeded by a factor of six an estimated plateau surface concentration, suggesting multilayer adsorption of insulin molecules. If the adsorption represents the first stage in a process of aggregation, as suggested by those authors, a progressive removal of insulin from solution by the formation of insoluble particles or fibrils could account for the apparent non-saturability of the adsorption phenomenon.

In their study, Sefton and Antonacci (1984) found that there was more adsorption of insulin to hydrophobic than to hydrophilic surfaces; that adsorption was increased when the pH was lowered from 7·4 (phosphate) to 3·5 (acetate) and that an increase in temperature resulted in a decrease in adsorption. The effect was accordingly identified as originating in a primarily hydrophobic interaction between insulin molecules and the adsorbing surface. The authors noted that a full understanding of the effect and its relationship to the formation of aggregates would require, among other things, a determination of the association state of insulin solutions (Sefton and Antonacci 1984).

Insulin monomers have two largely non-polar surfaces along the approximate two-fold axes designated OP and OQ in the crystal (Blundell *et al.* 1972). Thus, the interactions between monomers involve mainly non-polar surfaces and, indeed, in the process of self-association there is a progressive burial of non-polar groups in the interior until the surface of the hexamer is almost entirely polar (Blundell *et al.* 1972). There are currently two basic models for the self-association of insulin. One involves the interaction of monomers to form dimers and the subsequent addition of dimers to form tetramers, hexamers and higher even-numbered species (Jeffrey and Coates 1966; Goldman and Carpenter 1974; Jeffrey *et al.* 1976); the second model envisages two pathways for dimer formation (interaction about OP and OQ, respectively) and subsequent addition of monomers to form a series of polymers involving odd-numbered as well as even-numbered insulin species (Nichol *et al.* 1984). Both schemes provide excellent descriptions of the experimental results over the range of conditions for which the insulin self-association has been studied and, in both, the composition of aqueous solutions responds qualitatively in the same way to changes in variables such as pH, temperature and ionic strength. For the conditions used in the adsorption studies and over the concentration ranges for which adsorption and osmotic pressure measurements are being considered, the insulin solutions contain mostly monomers and dimers with some contribution from tetramers in the first scheme and trimers in the second, at the higher end of the concentration scale. Calculations carried out on the basis of either model give the result shown in Fig. 4.

All insulin species present in solution, whatever scheme is considered, have non-polar groups exposed but the significant point is that dissociation, with its concomitant

increase in exposed hydrophobic surfaces, is favoured by a decrease in pH from 7 to 2 (Jeffrey and Coates 1966; Jeffrey *et al.* 1976) and of temperature (Jeffrey and Coates 1966). Thus, the self-association behaviour of insulin is entirely consistent with the effects observed in the adsorption studies and, as has now been demonstrated by analysis of the osmotic pressure results, the monomeric molecule seems to be capable of accounting for almost all of the adsorption. This is understandable because it is the molecular species with relatively most exposed non-polar surface and its presence in solution is favoured by the same conditions that lead to increased adsorption. This conclusion is supported by the work of Helmerhorst and Stokes (1986) who reported specific adsorption of porcine insulin monomer to Sephadex G25 at pH 8. These investigations showed that the adsorption persisted in the presence of other proteins and concluded that an 'aromatic interaction' is responsible, specifically one involving the tyrosines B16 and B26. Other effects may operate also, in particular the electrostatic charge on insulin molecules may be a modifying influence on the adsorption phenomenon. The net charge changes from negative to positive on passing from neutral to acidic conditions and it may be modified by specific binding of ionic species present in solution. Sefton and Antonacci (1984) reported that the presence of glutamic or aspartic acid in insulin solutions at pH 3·5 has the effect of reducing the adsorption and that an effect of these amino acids in reducing aggregation in insulin solutions has been observed in another study (Bringer *et al.* 1981). A possible reason advanced by Sefton and Antonacci (1984) for the effect on adsorption was that the amino acids could bind to insulin and that the resulting complex, being more hydrophilic, would be adsorbed less readily. The binding of these acids to insulin monomers is plausible and would provide an explanation for their effect on both the adsorption and the aggregation by modifying the hydrophobic surfaces involved in self-association. This possibility deserves investigation by binding experiments and by detailed studies of the effect on the self-association of the presence of these amino acids.

The findings described in this work demonstrate that in experiments with proteins the possibility of adsorption should be taken into account where it may affect results. This includes sedimentation equilibrium experiments of the type requiring a measurement of the initial concentration independent of the actual equilibrium experiment. The problem may be particularly suspected in experiments with self-associating proteins where low concentrations favour species that are very likely to have large non-polar areas on their surfaces. For insulin, in applications where it is desirable to minimize or eliminate adsorption, especially where it may be a factor in nucleating aggregation, it seems clear that attention should be directed towards the insulin monomer as the primary adsorbed species.

References

Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K. (1950). Insulin-I^{131} metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J. Clin. Invest.* **35**, 170–90.

Blundell, T., Dodson, G., Hodgkin, D., and Mercola D. (1972). Insulin: the structure in the crystal and its reflection in chemistry and biology. *Adv. Protein Chem.* **26**, 279–402.

Bringer, J., Heldt, A., and Grodsky, G. H. (1981). Prevention of insulin aggregation by dicarboxylic amino acids during prolonged infusion. *Diabetes* **30**, 83–5.

Cecil, R., and Robinson, G. B. (1975). The 'specific' binding of insulin to polythene and other materials. *Biochim. Biophys. Acta* **404**, 164–8.

Chervenka, C. H. (1970). Long column meniscus depletion sedimentation equilibrium technique for the analytical utracentrifuge. *Analyt. Biochem.* **34**, 24–9.

Cuatrecasas, P., and Hollenberg, M. D. (1975). Binding of insulin and other hormones to non-receptor materials: saturability, specificity and apparent negative cooperativity. *Biochem. Biophys. Res. Commun.* **62**, 31–41.

Frank, B. H., and Veros, A. J. (1968). Physical studies on proinsulin-association behaviour and conformation in solution. *Biochem. Biophys. Res. Commun.* **32**, 155–60.

Goldman, J., and Carpenter, F. H. (1974). Zinc binding, circular dichroism, and equilibrium sedimentation studies on insulin (bovine) and several of its derivatives. *Biochemistry* **13**, 4566–74.

Gutfreund, H. (1948). The molecular weight of insulin and its dependence upon pH, concentration and temperature: *Biochem. J.* **42**, 544–8.

Gutfreund, H. (1952). The reversible dissociation of insulin and its minimum molecular weight. *Biochem. J.* **50**, 564–9.

Helmerhorst, E., and Stokes, G. B. (1986). The self-association of insulin: determinations based on a different gel adsorption procedure. *Arch. Biochem. Biophys.* **245**, 238–47.

Hirsch, J., Fratkin, M., Wood, J., and Thomas, R. (1977). Clinical significance of insulin adsorption by polyvinyl chloride infusion systems. *Am. J. Hosp. Pharm.* **34**, 583–8.

Jeffrey, P. D., and Coates, J. H. (1966). An equilibrium untracentrifuge study of the self-association of bovine insulin. *Biochemistry* **5**, 489–98.

Jeffrey, P. D. (1974). Polymerization behaviour of bovine zinc-insulin at neutral pH molecular weight of the subunit and the effect of glucose. *Biochemistry* **13**, 4441–7.

Jeffrey, P. D., Milthrope, B. K., and Nichol, L. W. (1976). Polymerization pattern of insulin at pH 7·0. *Biochemistry* **15**, 4660–5.

Jeffrey, P. D. (1981). Equilibrium methods. In 'Protein–Protein Interactions'. (Eds C. Frieden and L. W. Nichol.) Ch. V. pp. 213–56. (John Wiley and Sons: New York.)

Lougheed, W. D., Woufe-Flanagan, H., Clement, J. R., and Albisser, A. M. (1980). Insulin aggregation in artificial delivery systems. *Diabetologia* **19**, 1–9.

Marcker, K. (1960). Association of Zn-free insulin. *Acta Chem. Scand.* **14**, 194–6.

Nichol, L. W., Sculley, M. J., Jeffrey, P. D., and Winzor, D. J. (1984). Indefinite self-association of a solute in linear and branched arrays. *J. Theor. Biol.* **109**, 285–98.

Sefton, M. V. (1982). Implantable micropump for insulin delivery: effect of a rate-controlling membrane. *Adv. Chem.* **199**, 511–22.

Sefton, M. V., and Antonacci, G. M. (1984). Adsorption isotherms of insulin onto various materials. *Diabetes* **33**, 674–80.

Sefton, M. V., and Nishimura, E. (1980). Insulin permeability of hydrophilic polyacrylate membranes. *J. Pharm. Sci.* **69**, 208–9.

Tanford, C., and Epstein, J. (1954). The physical chemistry of insulin. 1. Hydrogen ion titration curve of zinc-free insulin. *J. Am. Chem. Soc.* **76**, 2163–76.

Yphantis, D. A. (1964). Equilibrium ultracentrifugations of dilute solutions. *Biochemistry* **3**, 297–317.

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