Biochem. J. (1972) **126**, 433–440
Printed in Great Britain

Differences in the Nature of the Interaction of Insulin and Proinsulin with Zinc

By P. T. GRANT, T. L. COOMBS and B. H. FRANK*
*Natural Environment Research Council, Institute of Marine Biochemistry, St Fittick's Road, Aberdeen AB1 3RA, U.K., and *The Lilly Research Laboratories, Indianapolis, Ind. 46206, U.S.A.*

(*Received* 10 *August* 1971)

1. The reversible interaction of zinc with pig insulin and proinsulin has been studied at pH7 by equilibrium dialysis (ultrafiltration) and by sedimentation equilibrium and velocity measurements in the ultracentrifuge. Binding values calculated from equilibria, where the ratio of free to bound zinc was varied in the range 0.01:1–10:1, indicated that proinsulin and insulin each contained two main orders of zinc binding with very different affinities for the metal. 2. In equilibria containing low concentrations of free zinc (free: bound ratios of 0.01–0.1:1) both insulin and proinsulin aggregated to form soluble hexamers containing firmly bound zinc (up to 0.284g-atom/monomer) with an apparent intrinsic association constant of 1.9×10^6 M^{-1}. 3. Higher concentrations of zinc (free: bound ratios of 0.1–10.0:1) resulted in a progressive difference in the zinc binding, aggregation and solubility properties of the metal complexes of insulin and proinsulin. At the highest concentration of free zinc, proinsulin bound a total of more than 5.0g-atom/monomer and aggregated to form a mixture of soluble polymers (mainly 5.1S). In contrast, insulin bound a total of only 1.0g-atom/monomer and was almost completely precipitated from solution. 4. These results would indicate that the presence of the peptide segment connecting the insulin moiety in proinsulin does not prevent the firm binding of zinc to the insulin moiety and the formation of hexamers of zinc–proinsulin. At the same time although the connecting peptide contains additional sites of lower affinity for zinc, which should facilitate inter- and intra-molecular cross-linking, the general conformation of the zinc–proinsulin hexamer must preclude the formation of very large and close-packed aggregates that are insoluble in solutions at equilibrium.

It is well established that the β-cells of the islets of Langerhans from several species derive insulin from its biosynthetic precursor (proinsulin) by an intracellular process involving the hydrolysis of specific peptide bonds. The formation of insulin appears to take place within the β-cell after proinsulin is packaged into membrane-limited vesicles or granules (Grant & Coombs, 1970; Kemmler & Steiner, 1970; Grant *et al.*, 1971). Some functional role for Zn^{2+} in this conversion process may be inferred from the observations that the granules are rich in Zn^{2+} (Logothetopoulos *et al.*, 1964) and that the high degree of order observed in the electron-dense granule (Greider *et al.*, 1969) may be explained in terms of close-packed arrays of zinc–insulin crystals.

Zinc is normally essential for the crystallization of insulin *in vitro* and it has been shown by equilibrium dialysis (Cunningham *et al.*, 1955; Summerell *et al.*, 1965) that commercial preparations of insulin exhibit two main orders of Zn^{2+} binding. In the presence of low concentrations of Zn^{2+}, the metal was firmly bound and resulted in the formation of a hexameric polymer containing about 2g-atoms per hexamer; additional Zn^{2+} added to the hexamer was less firmly bound and resulted in the formation of higher polymers with subsequent precipitation of the zinc–insulin complex (Cunningham *et al.*, 1955). In contrast, although proinsulin can also form hexamers in the presence of low concentrations of Zn^{2+} (Frank & Veros, 1970) and may be crystallized under special conditions (Fullerton *et al.*, 1970), it was found that the proinsulins of ox, pig and cod did not form detectable precipitates in the presence of an excess of Zn^{2+} under conditions that resulted in the ready precipitation or crystallization of the corresponding insulin (P. T. Grant & T. L. Coombs, unpublished work).

For these reasons, the present report is concerned with the quantitative differences in the degree of metal-binding and sedimentation behaviour of the complexes formed by the interaction of highly purified (single-component) preparations of pig insulin and proinsulin at pH7 with a wide range in the concentration of Zn^{2+}. The results for insulin are in general agreement with previous binding studies that used commercial preparations. These probably contained small but significant amounts of proinsulin. A brief account of some of this work has been given (Coombs *et al.*, 1971).

Materials and Methods

Proteins

Pig insulin (lot no. 615-1082B-37C), pig proinsulin (lot no. 759-1035B-2001) and ox proinsulin (lot no. 615-1070B-212-2) were each isolated by gel filtration and chromatography on DEAE-cellulose (Bromer & Chance, 1967; Chance *et al.*, 1968). Ox insulin (Lot no. 12779N) was a crystalline preparation obtained from Boots Pure Drug Co., Nottingham, U.K., and was purified and separated from traces of proinsulin by gel filtration on Sephadex G-50 with 1.0M-acetic acid as solvent (Grant & Reid, 1968). The preparations were repeatedly freeze-dried from aqueous suspension to remove traces of acetic acid. Each protein migrated as a single component on electrophoresis in polyacrylamide gel at pH8.3. In each case they contained $8\pm2\%$ moisture and <0.04 and <0.002g-atom of Zn^{2+}/mol of proinsulin and insulin respectively as determined by atomic absorption spectroscopy.

Buffer solutions

Buffer was prepared with water treated with a mixed-bed ion-exchange resin and subsequently distilled in an all-glass apparatus. Stock buffer solutions were prepared from analytical grades of tris, NaCl and glass-distilled 6M-HCl and the solution was extracted with dithizone dissolved in CCl_4 to remove traces of free Zn^{2+}. The buffer used in all experiments contained 0.1M-tris and 0.1M-NaCl, adjusted to pH7 with 6M-HCl. All solutions were stored in polyethylene containers that had been previously soaked in 1M-HCl and well washed with water.

Buffers containing defined concentrations of $^{65}Zn^{2+}$ were prepared by the addition of small volumes of $^{65}ZnCl_2$ (100–250mCi/g; The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.1M-HCl and unlabelled analytical grade $ZnCl_2$ to stock buffer solutions, followed by dilution. Before any experiment, the concentration and specific radioactivity of Zn^{2+} as well as the pH of the final buffer were redetermined.

Ultrafiltration procedures

Weighed amounts of the protein were dissolved directly in the buffer solution, and the concentration checked by measurement of E_{276}. The extinction coefficients used were 1.05 and 0.66 for a 1% (w/v) solution of insulin and proinsulin respectively (Frank & Veros, 1968).

'*Wash-in*' *procedure.* In all experiments, 5ml of a 0.1mM solution of insulin or proinsulin was placed in the cell of an ultrafiltration apparatus (Amicon Corp., Lexington, Mass., U.S.A.) that was constructed essentially as described by Blatt *et al.* (1968). The membrane of the cell, UM-2, completely retained both insulin and proinsulin but was freely permeable to Zn^{2+} and buffer ions. There was no detectable absorption of either protein by the material of the membrane.

The cell contents were continuously ultrafiltered at 20 ± 2°C and at constant volume by supplying buffer containing 0.01–1.0mM-$^{65}Zn^{2+}$ to the cell under a pressure of N_2 at 350kN·m^{-2} (50lb/in^2), and collecting the ultrafiltrate in a fraction collector. Volume changes in the cell were minimized by a device that equalized the pressure in the buffer reservoir with that in the cell. The cell contents were continuously mixed by a magnetic stirrer. Equilibrium of the protein with Zn^{2+} was attained when the specific radioactivity (c.p.m./ml) in the ultrafiltrate was equal to that of the buffer entering the cell from the reservoir. At equilibrium the amount of protein-bound Zn^{2+} was calculated after determining the specific radioactivity of a measured volume of the cell contents.

'*Wash-out*' *procedure.* In all experiments, 5ml of a solution containing 0.1mM-insulin or -proinsulin in equilibrium with buffer containing 0.01–1.0mM-$^{65}Zn^{2+}$ was placed in the cell, and ultrafiltered at 20 ± 2°C under pressure with a zinc-free buffer solution as described above. When the specific radioactivity in the ultrafiltrate had reached a minimum value, it was assumed that a new equilibrium condition had been established. The amount of protein-bound Zn^{2+} was calculated after determining the specific radioactivity of a measured volume of the cell contents, by making the assumption that the concentration of free Zn^{2+} in the cell is the same as that of the last fraction of the ultrafiltrate collected before the experiment was terminated.

In both 'wash-in' and 'wash-out' procedures the progress of the protein solution towards equilibrium with $^{65}Zn^{2+}$ was measured by expressing graphically the value of $\log(C_f/C_f-C)$ for 'wash-in' or $\log(C_0/C)$ for 'wash-out', as a function of the volume of the ultrafiltrate (Figs. 1 and 2). C_f is the concentration of $^{65}Zn^{2+}$ in the buffer reservoir, C_0 is the concentration of $^{65}Zn^{2+}$ in the cell at the start of the experiment and C is the concentration of $^{65}Zn^{2+}$ in the ultrafiltrate. In each procedure, the graph for an unretarded species such as $^{65}Zn^{2+}$ is a straight line with an intercept on the axis of the ultrafiltrate volume equal to the apparent retention volume of the system (Blatt *et al.* 1968).

Ultracentrifugation

The centrifuge experiments were done at 20 ± 1°C with a Beckman model E analytical ultracentrifuge equipped with both absorption and schlieren optics. The sedimentation-equilibrium experiments were performed by using u.v. optics. The partial specific volumes used in the calculation of molecular weight were calculated from the known amino acid composi-

tions of each protein (Frank & Veros, 1968). The sedimentation boundaries were monitored at 276nm and with schlieren optics. To obtain the desired zinc/protein ratios, microlitre volumes of the appropriate stock $ZnCl_2$ solutions were added to the buffered protein solution so that there was less than 3% dilution of the protein solutions and no significant change in pH. The final Zn^{2+} content of these solutions was checked periodically by atomic-absorption analysis. In the sedimentation-velocity experiments, when Zn^{2+} was added, duplicate runs were made either 30min or 3h after the addition of Zn^{2+}. The centre-pieces of the centrifuge cells were composed of either Kel-F or charcoal-filled Epon. These were used to obviate surface absorption of the protein and metal-ion contamination of the zinc-free protein solutions.

Determination of radioactivity

Fractions of the ultrafiltrate (either 1 or 2ml) were directly collected in standardized glass vials and the radioactivity was determined by a scintillation counter with a NaI crystal well (Panax Equipment Ltd., Redhill, Surrey, U.K.). At least 10000 counts were recorded and the specific radioactivity was expressed as c.p.m./ml above background.

Determination of zinc

The concentrations of Zn^{2+} were measured directly with a Techtron AA-5 atomic-absorption spectrophotometer (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.).

Results

Relative rate of interaction with zinc

In all experiments, the progress of the system towards an equilibrium state was monitored by measurement of the concentration of $^{65}Zn^{2+}$ (c.p.m./ml) in successive fractions of the ultrafiltrate passing through the membrane of the ultrafiltration cell. With the 'wash-in' procedure there was initially a marked retention, followed by a slower interaction of both proteins with $^{65}Zn^{2+}$ entering the cell from the buffer reservoir, when compared with that obtained in control experiments when no protein was present in the ultrafiltration cell. This effect is more easily seen when the buffer in the reservoir contained low concentrations of Zn^{2+}. Similarly in the 'wash-out' procedure there was initially a rapid rate of appearance of $^{65}Zn^{2+}$ in the ultrafiltrate followed by a slower rate when zinc-free buffer was present in the reservoir

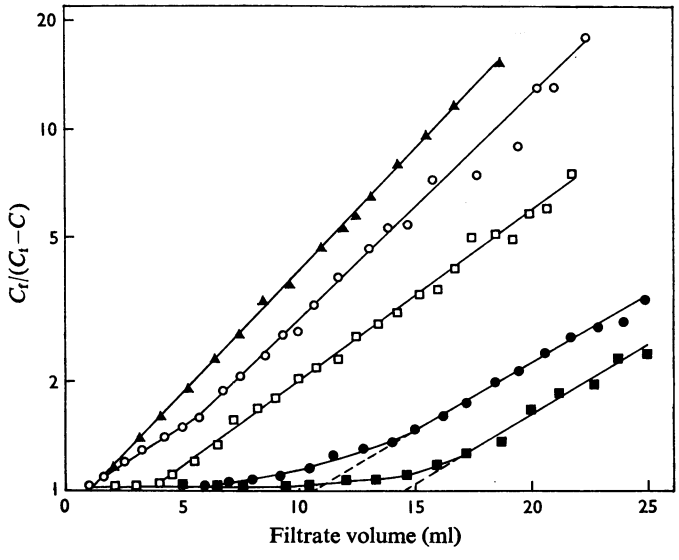


Fig. 1. *Equilibration of zinc with insulin and proinsulin*

The relative rate of equilibration of $^{65}Zn^{2+}$ with a 0.1 mM solution of either insulin or proinsulin was measured with a cell whose membrane retained the polypeptides. This 'wash-in' procedure is described in the Materials and Methods section. Insulin in cell and either 0.1 mM (□) or 0.01 mM (■) $^{65}Zn^{2+}$ in buffer in feed reservoir; proinsulin in cell and either 0.1 mM (○) or 0.01 mM (●) $^{65}Zn^{2+}$ in buffer in feed reservoir; buffer in cell and 0.01 mM (▲) $^{65}Zn^{2+}$ in buffer in feed reservoir.

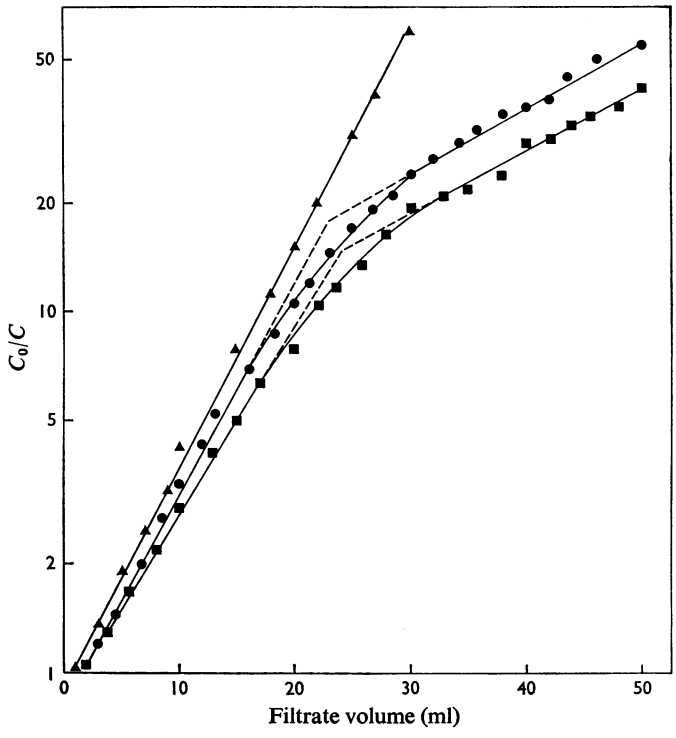


Fig. 2. *Re-equilibration of zinc–insulin and zinc–proinsulin*

The relative rate of re-equilibration was measured by using an equilibrium mixture containing 0.1 mM free $^{65}Zn^{2+}$ and 0.1 mM of the zinc complexes of either insulin or proinsulin, with a zinc-free buffer in the feed reservoir. This 'wash-out' procedure is described in the Materials and Methods section. Cell contents: ■, $^{65}Zn^{2+}$–insulin and free $^{65}Zn^{2+}$; ●, $^{65}Zn^{2+}$–proinsulin and free $^{65}Zn^{2+}$; ▲, free $^{65}Zn^{2+}$.

and the cell initially contained an equilibrium mixture of free and protein-bound Zn^{2+} (Fig. 2). These results would imply that both insulin and proinsulin each contain at least two types of metal-binding site with distinctly different affinities for zinc. Zinc bound to the site or sites of higher affinity in both proteins was firmly complexed as only traces of $^{65}Zn^{2+}$ were released during extensive ultrafiltration with zinc-free buffer.

It is not possible to make any conclusions about the similarity or otherwise of the types of binding sites present in insulin and proinsulin from the results given in Figs. 1 and 2. The small observed differences in behaviour may be partially attributed to the fact that the initial rate of ultrafiltration varied slightly between experiments because of pressure adjustments to the constant value.

Zinc binding at equilibrium

The amount of Zn^{2+} bound at equilibrium to either insulin or proinsulin as a function of the concentration of free Zn^{2+} is shown in Fig. 3(*a*). At low concentrations the degree of Zn^{2+} binding was similar for both pig proteins but at 0.1 mM free Zn^{2+} there was a small but significant difference and at 1.0 mM free Zn^{2+} the equilibrium binding capacity of pig proinsulin was more than five times that of the corresponding insulin. This progressive difference between pig insulin and proinsulin in the range of concentration from 0.1–1.0 mM free Zn^{2+} was also reflected in differences in the phase-states of the equilibrium mixtures. At any concentration of Zn^{2+}, the equilibrium mixtures with pig proinsulin remained optically clear for at least 24h, at either 20° or 4°C. In contrast, the solubility of the pig zinc–insulin complex was critically dependent on the concentration of free Zn^{2+} (Fig. 3*b*). Under comparable conditions similar differences were also observed in the solubilities of the zinc complexes of insulin and proinsulin derived from the ox and cod.

It has also proved possible to obtain further information about the nature and number of zinc-binding sites in both pig proteins by using the equilibrium

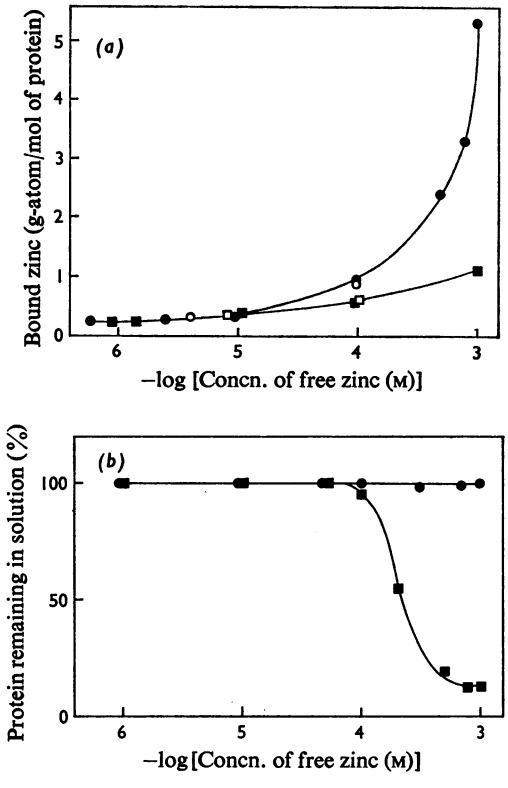


Fig. 3. *Effect of increasing the concentration of free zinc on the amount of zinc bound to either insulin or proinsulin at equilibrium*

(*a*) Equilibrium was attained by either the 'wash-in' or 'wash-out' procedure (see Figs. 1 and 2). Subsequently, the concentrations of total, free and bound zinc in the residual solution contained in the cell were determined as described in the Materials and Methods section. ●, Pig proinsulin; ○, ox proinsulin; ■, pig insulin; □, ox insulin. (*b*) After equilibrium had been attained by the 'wash-in' procedure, a sample of the residual solution contained in the cell was centrifuged and the E_{276} of the clear supernatant was measured. ●, Pig proinsulin; ■, pig insulin.

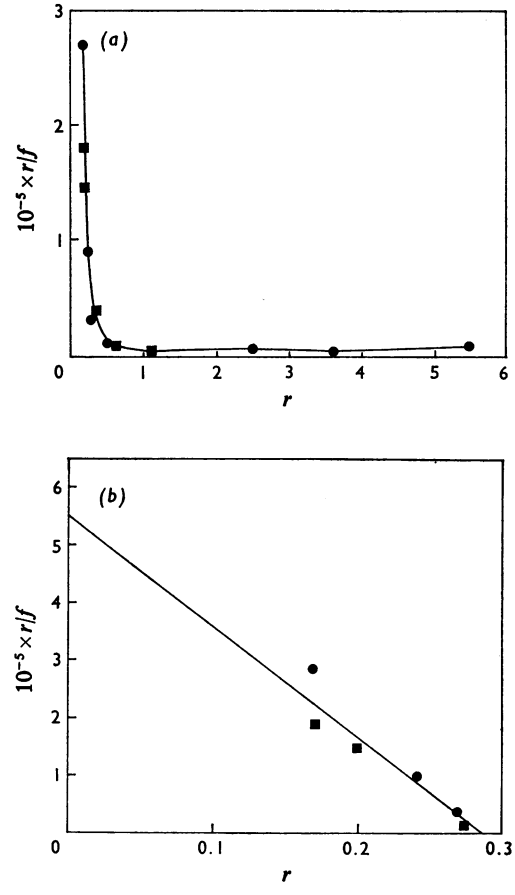


Fig. 4. *Binding parameters of zinc–insulin and zinc–proinsulin complexes*

(*a*) Over a wide range and (*b*) over a narrow range of zinc concentration. Details and symbols are given in Fig. 3(*a*) and in the text.

results (Fig. 3*a*) in the Scatchard equation (Hughes & Klotz, 1956): $r/f = nk - k$, where r is the Zn^{2+} bound (g-atom/mol of protein), f is the molar concentration of free Zn^{2+}, k is the intrinsic association constant and n is the amount of Zn^{2+} bound at saturation (g-atom of Zn^{2+}/mol of protein). For a single set of non-interacting binding sites, the graph of r/f against r is a straight line where the intercept on the r axis gives the value of n and the intercept on the r/f axis is the value of nk. When the equilibrium results from Fig. 3(*a*) are expressed in this form, it can be seen (Fig. 4*a*) that the graph contains two almost linear segments joined by an inflexion point. This result lends further support to the concept derived from direct binding results (Figs. 1 and 2) that pig insulin and proinsulin each contain at least two sets of binding sites with very different affinities for Zn^{2+}.

The equilibrium results for both pig proteins at low Zn^{2+} concentration (<0.01 mM free Zn^{2+}) have been replotted on an expanded scale (Fig. 4*b*). At first sight it seemed possible that there could be small differences between the association constants for insulin and proinsulin. However, when a regression line was fitted to all six experimental points by the method of least mean squares, all the points were

within twice the standard deviation so that any differences between the results for insulin and proinsulin were not significant. Within the limitation of the available results we have concluded that the site or sites of higher affinity in both insulin and proinsulin are very similar; the capacity for Zn^{2+} at saturation (n) is 0.284g-atom/mol of protein and the apparent association constant ($k_{app.}$) is $1.9\times10^{6}M^{-1}$. The latter value is in general agreement with that obtained at pH8 ($4.7\times10^{6}M^{-1}$) by Summerell *et al.* (1965). These calculations make no allowance for any competitive binding of Zn^{2+} by tris and Cl^{-} anions present in the buffer solution nor for any contribution by Zn^{2+} binding to the site of lower affinity in the proteins. There is, however, a 100-fold difference between the affinities of the two sites in insulin for Zn^{2+} (Summerell *et al.*, 1965) so that although these corrections will not be large, the values given here for n and k must be accepted with these reservations.

Aggregation of zinc complexes

Further insight into the interaction of Zn^{2+} with insulin and proinsulin is provided by an examination of the sedimentation behaviour of equilibrium mixtures in the ultracentrifuge. In the absence of added Zn^{2+}, the schlieren patterns of both pig insulin and proinsulin (Fig. 5*a*) are asymmetric and show marked trailing edges. The sedimentation coefficients ($s_{20,w}$) are 2.3 and 3.2S respectively, corresponding to weight-average molecular weights of 23000 and 31000. Under these conditions, both proteins exhibit strong and quite similar self-association behaviour and exist in solution as an equilibrium mixture of various polymeric aggregates (B. H. Frank & A. H. Pekar, unpublished work). Earlier studies (Zühlke & Behlke, 1968; Frank & Veros, 1968) have shown that the degree of self-association of these proteins is a function of pH, ionic strength, temperature and protein concentration.

Equilibrium mixtures containing low concentrations of Zn^{2+} (0.1mM total zinc or 0.05mM free zinc) exhibit a single and sharp symmetrical boundary for both insulin ($s_{20,w}=3.2S$) and proinsulin ($s_{20,w}=3.4S$; Fig. 5*b*) and a similar behaviour has been observed in a different buffer system (Frank & Veros, 1970). Sedimentation equilibrium studies would indicate that each of the zinc–protein complexes is a homogeneous entity with a molecular weight of 35000 for insulin and 55000 for proinsulin. Thus both

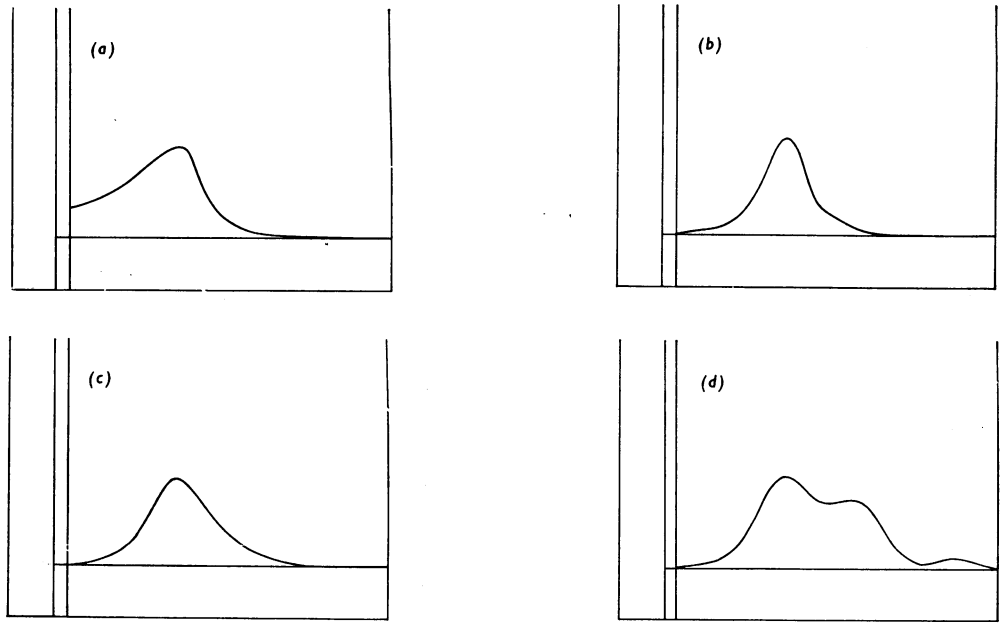


Fig. 5. *Ultracentrifugation of pig proinsulin in zinc solutions*

The figure shows typical schlieren patterns for 0.1mM solutions of pig proinsulin in buffer and in equilibrium with buffer, pH7, containing different concentrations of free Zn^{2+} ions. All sedimentation is from left to right. (*a*) No added Zn^{2+}; (*b*) 0.1mM total Zn^{2+}, or 0.05mM free Zn^{2+} at equilibrium; (*c*) 0.6mM total Zn^{2+}, or 0.4mM free Zn^{2+} at equilibrium; (*d*) 1.8mM total Zn^{2+} or 1.2mM free Zn^{2+} at equilibrium.

proteins associate with zinc to form a soluble hexameric polymer containing 2.0g-atoms of Zn^{2+}/hexamer.

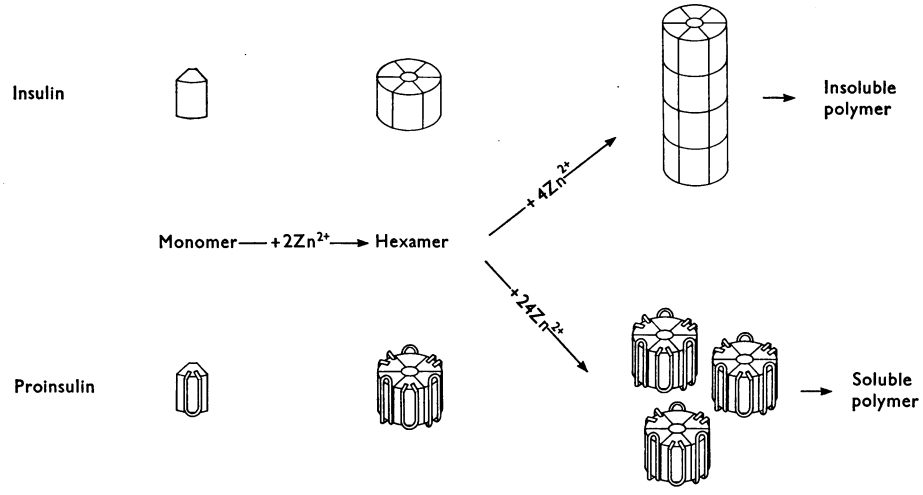
Higher concentrations of Zn^{2+} in the equilibrium mixture with insulin (up to 1.8mM total zinc or 1.7mM free zinc) cause precipitation of the complex (see Fig. 3*b*). The only detectable form present in the soluble phase is the 3.2S component. Aggregates of higher molecular weight, up to 8S, have been observed for soluble complexes of zinc–insulin at pH7.3 (Cunningham *et al.*, 1955) and at pH8.0 (Fredericq, 1956). The absence of these more rapidly sedimenting components in the present experiments at pH7 may be due to the difference in pH affecting either the amount of Zn^{2+} bound to insulin or the solubility of the zinc–insulin polymers larger than the hexamer. In this connexion, Goldman (1971) has shown that the degree of binding of Zn^{2+} by insulin is very dependent on pH, the amount of Zn^{2+} bound increasing with increasing pH over the range 6.0–8.0.

In contrast with the results with insulin at pH7, higher concentrations of Zn^{2+} result in the formation of a number of large soluble polymers of proinsulin. Thus, with 0.6mM total zinc or 0.4mM free zinc at equilibrium the schlieren pattern shows a marked leading edge with an observed sedimentation coefficient of 4.6 (Fig. 5*c*). With 1.8mM total zinc or 1.2mM free zinc at equilibrium there are two distinct peaks, about 3.6 and 5.1S, together with a trace of material sedimenting more rapidly than the 5.1S component (Fig. 5*d*). Sedimentation-equilibrium studies would also indicate the heterogeneous nature of the zinc–proinsulin polymers made up of components with weight-average molecular weights ranging from 60000 to 98000.

Discussion

The present results show that in the presence of excess of Zn^{2+}, pig proinsulin binds more than 5g-atom/mol of protein and polymerizes to form soluble aggregates of high molecular weight under conditions where the corresponding insulin binds only 1g-atom/mol and is almost completely precipitated from the equilibrium solution. This clear-cut difference only becomes apparent when the concentration of free Zn^{2+} in the equilibrium is such that more than 0.3g-atom of Zn^{2+} is bound per mol of protein (Figs. 3*a* and 3*b*). These differences should be viewed in the context of the conclusion that proinsulin, like insulin (see also Cunningham *et al.*, 1955; Summerell *et al.*, 1965), exhibits at least two main orders of zinc binding.

With low concentrations of free Zn^{2+}, less than 0.3g-atom is bound per mol of protein. The site or sites of higher affinity for Zn^{2+} in insulin and pro-



Scheme 1. *Diagrammatic representation of polymers formed as pig insulin and proinsulin bind increasing amounts of zinc*

It is assumed that the conformation of both insulin and proinsulin in solution are the same as that found for crystals of zinc–insulin by X-ray diffraction (Adams *et al.*, 1969). For this reason, the peptide segment connecting the *N*-terminus of the A chain to the *C*-terminus of the B chain in the constitutive insulin moiety of proinsulin is depicted as a peripheral loop on the exterior surface of each monomer. Additionally, the hexamers are depicted as consisting of monomers arranged in an alternate anti-parallel manner. For details, see the Discussion section.

insulin would appear to be very similar if not identical because the apparent intrinsic association constant and other properties of the zinc–protein complexes can be equated one with the other. This conclusion is consistent with the proposal that the conformation of the insulin moiety in the molecule of proinsulin is the same as that in the molecule of free insulin (Frank & Veros, 1968). Comparisons of the physical properties of insulin and proinsulin (Frank & Veros, 1970) would also indicate that both proteins polymerize to hexameric aggregates in the presence of low concentrations of Zn^{2+}. On this basis it seems probable that the peptide segment in proinsulin, that links the *N*-terminus (glycine) of the A chain and the *C*-terminus (alanine) of the B chain in the constitutive insulin moiety, is located on the outer surface of the proinsulin monomer in such a way that it does not interfere with the binding of Zn^{2+} to the sites of higher affinity and the consequent formation of the zinc–proinsulin hexamer. This is illustrated diagrammatically in Scheme 1 and indirect support for this idea may be derived from X-ray diffraction studies on zinc–insulin crystals where it was shown that the *N*-terminus of the A chain and the *C*-terminus of the B chain are on the exterior surface of the monomer (Adams *et al.*, 1969) and only about 1.1 nm (11Å) distant from one another (G. G. Dodson, personal communication).

At high concentrations of Zn^{2+} (1.0mM), the four- to five-fold difference in the amount of zinc bound to the site or sites of lower affinity in insulin and proinsulin, together with the difference in solubility of the zinc–protein complexes, must be related in some way to a difference in the amino acid composition or conformation of the two proteins. The connecting peptide in proinsulin contains one lysine and four glutamic acid residues (R. E. Chance, personal communication). One possible explanation is that the γ-carboxyl groups will be fully ionized at pH7 and would be available, in addition to other sites present in the insulin moiety of proinsulin, to form weak inter- and intra-molecular complexes with Zn^{2+}. Polymers larger than the hexamer of zinc–proinsulin occur in equilibrium solutions (Fig. 5*d*), but it is suggested (Scheme 1) that both the configuration of the peptide segment on the exterior surface of the hexamer and the binding of a large number of Zn^{2+} ions, where some may retain a positive charge, must preclude the formation of very large and close-packed insoluble aggregates as observed with insulin.

Irrespective of the reason for the solubility of zinc–proinsulin polymers, this phenomenon may be of some relevance in the sequence of events in the β-cell leading up to the probable formation of zinc–insulin crystalline deposits in membrane-limited granules. There is some evidence that limited or restricted proteolysis of proinsulin takes place in membrane-limited vesicles (Grant *et al.*, 1971; Kemmler & Steiner, 1970) and a role for Zn^{2+} in this process has been suggested and discussed by Grant & Coombs (1970). Although there is no direct evidence in support of this suggestion, it has been shown by Yip (1971) that the presence of Zn^{2+} minimizes the further degradation of insulin formed from bovine proinsulin by a purified protease. This enzyme had been isolated from an extract of whole bovine pancreas.

We are greatly indebted to Dr. R. E. Chance, Lilly Research Laboratories, Indianapolis, for the generous supply of insulin and proinsulin. T.L.C. thanks the British Diabetic Association for their financial support.

References

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B. & Sheat, S. (1969) *Nature (London)* **224**, 491

Blatt, W. F., Robinson, S. M. & Bixler, H. J. (1968) *Anal. Biochem.* **26**, 151

Bromer, W. W. & Chance, R. E. (1967) *Biochim. Biophys. Acta* **133**, 219

Chance, R. E., Ellis, R. M. & Bromer, W. W. (1968) *Science* **161**, 165

Coombs, T. L., Grant, P. T. & Frank, B. H. (1971) *Biochem. J.* **125**, 62P

Cunningham, L. W., Fischer, R. L. & Vestling, C. S. (1955) *J. Amer. Chem. Soc.* **77**, 5703

Frank, B. H. & Veros, A. J. (1968) *Biochem. Biophys. Res. Commun.* **32**, 155

Frank, B. H. & Veros, A. J. (1970) *Biochem. Biophys. Res. Commun.* **38**, 284

Fredericq, E. (1956) *Arch. Biochem. Biophys.* **65**, 218

Fullerton, W. W., Potter, R. & Low, B. W. (1970) *Proc. Nat. Acad. Sci. U.S.* **66**, 1213

Goldman, J. (1971) Ph.D. Thesis, University of California, Berkeley

Grant, P. T. & Coombs, T. L. (1970) *Essays Biochem.* **6**, 69

Grant, P. T. & Reid, K. B. M. (1968) *Biochem. J.* **110**, 281

Grant, P. T., Coombs, T. L., Thomas, N. W. & Sargent, J. R. (1971) in *Subcellular Organization and Function in Endocrine Tissues* (Heller, H. & Lederis, K., eds.), p. 481, Cambridge University Press

Greider, M. H., Howell, S. L. & Lacy, P. E. (1969) *J. Cell Biol.* **41**, 162

Hughes, T. R. & Klotz, I. M. (1956) *Methods Biochem. Anal.* **3**, 265

Kemmler, W. & Steiner, D. F. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1223

Logothetopoulos, J., Kaneko, M., Wrenshall, G. A. & Best, C. H. (1964) in *Structure and Metabolism of the Pancreatic Islets* (Brolin, S. E., Hellman, B. & Knutson, H., eds.), p. 333, Pergamon Press, Oxford

Summerell, J. M., Osmand, A. & Smith, G. H. (1965) *Biochem. J.* **95**, 31P

Yip, C. C. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 1312

Zühlke, H. & Behlke, J. (1968) *FEBS Lett.* **2**, 130