Effects of Zinc Ion on the Conformation of Antigenic Determinants on Insulin

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Comparison of c.d. spectra of Zn-insulin with Zn²⁺-free insulin demonstrated significant differences. It has been proposed that these differences are due to either changes in the structure of insulin monomers within aggregated insulins or the results of insulin aggregation. The effect of Zn²⁺ on the immunological activity of insulin indicated that the antigenic determinants of insulin were also altered. The apparent loss of immunological activity of monoiodotyrosylinsulin was demonstrated to be due to the loss of Zn²⁺ rather than the substitution of iodine. The immunological activity of Zn-insulin and Zn²⁺-free insulin was compared in both the radioimmune and immune haemolysis-inhibition assays by using an identical population of antibodies and concentrations of inhibitor. Relative to Zn-insulin, Zn²⁺-free insulin had a markedly attenuated immunological activity in the immune haemolysis-inhibition assay, whereas in the radioimmune assay slightly greater immunological activity was observed with the Zn²⁺-free insulin. These observations are submitted as evidence that the removal of Zn²⁺ perturbs the conformation of determinants that react with antibodies operative in the immune haemolysis-inhibition assay (immune haemolysis determinants) and has a minimal effect on the conformation of determinants that react with antibodies operative in the radioimmune assay (radioimmune determinants).

Previous studies from this laboratory demonstrated that the structure of insulin in solution (Arquilla *et al.*, 1969; Arquilla & Stanford, 1972) was similar to the structure of insulin in 2-Zn rhombohedral crystals (Adams *et al.*, 1969; Blundell *et al.*, 1972). Alterations in the structure of insulin from that delineated by X-ray crystallography resulted in decreased biological activity (Arquilla *et al.*, 1969; Blundell *et al.*, 1972; Gliemann & Gammeltoft, 1974). There have been several c.d. studies (Hamlin & Arquilla, 1974; Goldman & Carpenter, 1974) that demonstrated significant differences between Zn²⁺-free insulin and Zn-insulin. These observations were of insulin in solution at concentrations where it is aggregated. However, insulin in circulation is at concentrations where it probably exists as a monomer (Goldman, 1971). Influences by Zn²⁺ on insulin at very low concentrations would therefore be of direct interest with regard to its action and metabolism *in vivo*.

Experiments on preparations of monoiodotyrosylinsulin initially suggested that iodine substitution caused a decrease in the immunological activity of insulin as measured by the immune haemolysis-inhibition and radioimmune assays (Arquilla *et al.*, 1972). It was subsequently demonstrated that the

addition of Zn²⁺ restored the immunological activity of purified preparations of monoiodotyrosylinsulin (Hamlin & Arquilla, 1974).

The results of these preliminary experiments indicated that Zn²⁺ induced significant alterations in the tertiary structure of insulin and also affected its binding to antibodies. Experiments were therefore designed to study the effects of Zn²⁺ on the immunological activity of insulin at concentrations where it is thought to exist predominantly as a monomer. Two immunological systems, the immune haemolysis-inhibition assay and radioimmune assay, were used to measure the ability of antibodies to bind to different regions on the insulin molecule (Arquilla *et al.*, 1976).

Experimental

Antisera

The insulin antiserum (A) used in the immune haemolysis experiments and the first group of immune haemolysis-inhibition experiments was a pool from ten mongrel guinea pigs. The insulin antiserum (LW4175) used to compare the reactivity of the various insulins in the immune haemolysis-inhibition assay and the radioimmune assay was a

pool from 15 mongrel guinea pigs. In both cases the guinea pigs were immunized with $10 \times$ recrystallized bovine Zn-insulin (Novo Industri A/S, Copenhagen, Denmark; lot 012269) and antisera prepared as previously described (Brugman & Arquilla, 1973). The Zn^{2+} content of this preparation was 0.76% (equivalent to 0.6 atom of Zn^{2+} per molecule of insulin). It is unknown whether the Zn^{2+} crystals were in the 2-Zn or 4-Zn form or mixtures of both.

The lysozyme antiserum pool was prepared with $3 \times$ recrystallized hen's egg-white lysozyme (Pentex, Kankakee, IL, U.S.A.; lot 24, code EZ1962) as the antigen. The antisera were raised in ten guinea pigs in the same manner as the insulin antisera.

Complement

The complement used in all the immune haemolysis experiments was a single pool of fresh guinea-pig sera from 15 animals that was adsorbed on sheep erythrocyte stroma as described in Arquilla & Finn (1963).

Preparation of insulin and insulin derivatives

Zn^{2+} -free insulins were prepared by chelation with EDTA (Sluyterman, 1955), precipitation with cold acetone (Carpenter, 1958) or column chromatography (Hamlin & Arquilla, 1974). All of these preparations had similar c.d. spectra and were indistinguishable immunologically. The Zn^{2+} content of several of these preparations, as measured by atomic absorption, was never greater than 1 atom of Zn^{2+} per 15 molecules of insulin (Elek Micro-analytical Laboratories, Torrance, CA, U.S.A.).

$[^{125}\text{I}]$ monoiodotyrosylinsulin and carrier-free $[^{125}\text{I}]$ monoiodotyrosylinsulin were prepared by a modification (Sodoyez *et al.*, 1975) of the method of Hamlin & Arquilla (1974). These preparations contained 1 atom of iodine per molecule of insulin. The unchanged insulin isolated by this procedure formed a single band on polyacrylamide gels (monocomponent insulin) (Hamlin & Arquilla, 1974; Sodoyez *et al.*, 1975). The $[^{125}\text{I}]$ monoiodotyrosylinsulin and monocomponent insulin obtained in this manner had the same immunological activity and c.d. spectra identical with those of the Zn^{2+} -free insulin prepared by the methods of Carpenter (1958) and Sluyterman (1955). The carrier-free $[^{125}\text{I}]$ monoiodotyrosylinsulin used as an immunological indicator in the radioimmune assay contained approx. 250 mCi/mg (Sodoyez *et al.*, 1975).

The Zn-insulins used in the c.d. studies were prepared by dissolving Zn^{2+} -free insulin in half the final volume of 0.01 M-HCl, which contained the appropriate amount of ZnSO_4 (reagent grade, Mallinckrodt Co., St. Louis, MO, U.S.A.) and then adjusted to the final volume with 0.22 M-sodium

phosphate buffer, pH 7.4. The final concentration of insulin was 0.17 mM in all cases. All insulin concentrations refer to insulin in its monomeric form.

The Zn-insulin used to prepare the immunological indicator (Zn-insulin-sheep-erythrocyte conjugates) in the immune haemolysis experiments was prepared by equilibrating Zn^{2+} -free insulin at a concentration of 1.0 mg/ml (A_{276} 1.04) in 1.5 ml of 0.11 M-sodium phosphate buffer, pH 7.4, with 0.01 ml of 15.7 mM- ZnSO_4 in deionized water. The mixture was left to incubate for 1 h in a gyratory water bath at 4°C . This insulin had the same molar ratio of Zn^{2+} /insulin (0.6) as the insulin used to raise antibodies and was therefore considered to be homologous.

The Zn-insulin used in the first group of immune haemolysis-inhibition experiments was prepared by adding 0.01 ml of 0.523 mM- Zn^{2+} to 0.05 ml of 1.0 mg of Zn^{2+} -free insulin/ml and incubated for 1 h before being diluted to the appropriate concentrations with Veronal-buffered saline (Kabat & Mayer, 1961) containing 1.5 g of bovine plasma albumin/litre (VBS/albumin medium).

C.d. studies

U.v. c.d. spectroscopy of insulin was performed in both the near- and far-u.v. regions with a Beckman c.d. spectrophotometer. All spectra used for analysis were averaged by a Hewlett-Packard Signal Analyzer from at least 250 scans. Measurements in the near-u.v. region (310–250 nm) were performed by using a 1 cm optical-path-length, whereas a 0.02 cm path-length was used in the far-u.v. region (250–200 nm).

Immune haemolysis experiments

In these experiments either Zn^{2+} -free insulin or Zn-insulin was conjugated to sheep erythrocytes with bisdiazobenzidine (Arquilla & Finn, 1965). Serially diluted antisera, conjugated erythrocytes (8.7×10^6 sheep erythrocytes) and VBS/albumin medium were mixed and incubated for 1 h at 4°C . Excess of complement was then added and the incubation resumed for another 30 min at 37°C . The reaction was stopped with cold phosphate-buffered saline (0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4). The intact erythrocytes were pelleted by centrifugation at 1000 g for 10 min. The haemoglobin in the supernatant was measured spectrophotometrically at 414 nm. A fuller account of this method can be found in Brugman & Arquilla (1973). The immunological activity of Zn^{2+} -free insulin was calculated from the ratio of the amount of antiserum required to haemolyse 50% of the Zn-insulin-erythrocyte conjugates to the amount of antiserum required to haemolyse 50% of the Zn^{2+} -free-insulin-erythrocyte conjugates in the presence of excess of complement.

Immune haemolysis-inhibition experiments

These were basically the same as the immune haemolysis experiments, except that only Zn-insulin-erythrocyte conjugates were used as the immunological indicator. Various amounts of inhibitor (antigen) were added to the antiserum immediately before addition of the Zn-insulin-erythrocyte conjugates (Arquilla *et al.*, 1976).

In this first group of experiments antiserum A was used at a dilution sufficient to lyse 80% of the 8.7×10^6 Zn-insulin-erythrocyte conjugates in the presence of an excess of complement (Brugman & Arquilla, 1973; Arquilla *et al.*, 1976).

The percentage of immunological activity was calculated from the ratio of the amount of homologous inhibitor (Zn-insulin) to the amount of heterologous inhibitor (Zn^{2+} -free insulin) required to inhibit 50% of the immune haemolytic activity induced by the pre-selected amount of antiserum.

To compare results of the immune haemolysis-inhibition assay with those from the radioimmune

assay (see below), a second series of immune haemolysis-inhibition experiments was performed, which differed from those just described in three ways. The antiserum used was lot LW4175 and its initial dilution was sufficient to lyse 95% of 8.7×10^6 Zn-insulin-erythrocyte conjugates. The Zn-insulin inhibitor was diluted in VBS/albumin medium containing $10 \mu\text{M}$ - ZnSO_4 (approximate concentration of Zn^{2+} in the circulation; Underwood, 1962). These changes were necessary for effects on the two immunological assay systems to be tested with the same dilution of the same antiserum, and at a constant concentration of Zn^{2+} .

Radioimmune assay

The radioimmune assay was a modification of the charcoal/dextran method of Herbert *et al.* (1965) with carrier-free [^{125}I]monoiodotyrosylinsulin as the indicator (Arquilla *et al.*, 1976). The antiserum pool used was lot LW4175 at a dilution necessary to bind 32% of the [^{125}I]monoiodotyrosylinsulin indicator.

Fig. 1. Near- and far-u.v. c.d. spectra of 0.17 mM- Zn^{2+} -free insulin to which various concentrations of ZnSO_4 were added. The near- and far-u.v. path-lengths were 1 cm and 0.02 cm respectively. The ZnSO_4 concentrations were: $16 \mu\text{M}$ (—), $50 \mu\text{M}$ (---), 0.1 mM (.....) and zero (-.-.-).

(This is the same dilution used in immune haemolysis-inhibition experiments.) The buffer used was 7mM-sodium barbital/sodium acetate, pH7.4, with 0.15M-NaCl and 0.15% human serum albumin (Hyland, Costa Mesa, CA, U.S.A.). The albumin was dialysed against 10mM-EDTA in sodium barbital/acetate buffer with 0.15M-NaCl to remove bivalent ions and subsequently dialysed against the same buffer to remove EDTA. Both Zn^{2+} -free insulin and Zn-insulin inhibitors were diluted immediately before use in buffers containing $10\mu\text{M}$ - ZnSO_4 (approximate concentration of Zn^{2+} in the circulation). Thus constant Zn^{2+} concentrations were maintained throughout. The charcoal/dextran pellet was washed once with 0.5ml of the buffer, and the two supernatants were combined. The radioactivity of the antibody-bound insulin in the supernatants and of the free insulin adsorbed on the pellet was measured in a Beckman Biogamma Counter. The ratio (C_0/C_i) of the number of counts remaining in the supernatant (bound to antibody) in the absence of any inhibitor (C_0) to the number of counts in the presence of inhibitor (C_i) was plotted against the concentration of inhibitor. The percentage immunological activities in the radioimmune assay of Zn^{2+} -free insulin and Zn-insulin were calculated at a ratio of C_0/C_i equal to 2, at which point half the indicator was displaced from the antibody by the inhibitor.

Concentrations of Zn^{2+} , and other conditions such as preparation and concentration of inhibitor, temperature and duration of the initial reaction, were similar to those in the immune haemolysis-inhibition experiments.

Results

Effects of Zn^{2+} on the c.d. spectra of insulin

The near- and far-u.v. c.d. spectra of insulin equilibrated with different concentrations of Zn^{2+} are presented in Fig. 1. The c.d. minimum of the near-u.v. spectrum at 276nm was progressively increased with increasing Zn^{2+} concentrations. A maximum effect was noted at a Zn^{2+} concentration of 0.1mM, at which the molar ratio of Zn^{2+} /insulin is 0.6. The presence of additional Zn^{2+} did not further alter the c.d. spectrum significantly. The far-u.v. spectrum of Zn^{2+} -free insulin was significantly different from that of each of the Zn-insulins (Fig. 1).

The effect of Zn^{2+} on the c.d. spectra in the near-u.v. was reversed with EDTA (Fig. 2). The addition of equimolar EDTA to 0.1mM- Zn^{2+} resulted in a c.d. spectrum identical with that recorded for Zn^{2+} -free insulin. EDTA added to half the molarity of the added Zn^{2+} yielded a spectrum comparable with that recorded for the solution containing $50\mu\text{M}$ -Zn-insulin (Fig. 2).

Fig. 2. Near-u.v. c.d. spectra of 0.17mM- Zn^{2+} -free insulin to which 0.1mM- ZnSO_4 and various concentrations of EDTA were added

The EDTA concentrations were: zero (—), $50\mu\text{M}$ (— · — · —) and 0.1mM (----).

Effect of Zn^{2+} on the conformation of antigenic determinants on insulin studies by immune haemolysis and immune haemolysis inhibition

In immune haemolysis experiments approx. 7 times more antiserum was needed to haemolyse 50% of the Zn^{2+} -free-insulin-erythrocyte conjugates than was required to haemolyse 50% of the Zn-insulin-erythrocyte conjugates (Fig. 3).

This decreased ability of antibodies to combine with Zn^{2+} -free-insulin-erythrocyte conjugates compared with Zn-insulin-erythrocyte conjugates is submitted as evidence that Zn^{2+} induces structural changes that involve the conformation of exposed antigenic determinants on insulin. As it is likely that aggregated forms of insulin cross-linked by bisdiazobenzidine were coupled to the surface of these erythrocyte preparations, the reaction of antibodies

Fig. 3. Immune haemolysis of Zn-insulin-erythrocyte conjugates (●) and Zn²⁺-free-insulin-erythrocyte conjugates (○) by insulin antiserum

The percentage cross-reactivity was calculated from the ratio of the amount of antiserum required to haemolyse 50% of the Zn²⁺-free-insulin-erythrocyte conjugates to the amount of the antiserum required to haemolyse 50% of the homologous Zn-insulin-erythrocyte conjugates. The immunological activity of Zn-insulin was considered to be 100%. The immunological activity of Zn²⁺-free insulin was $14.5 \pm 0.9\%$ (mean $\pm$ S.E.M.; $n = 14$).

with either the Zn-insulin- or Zn²⁺-free-insulin-erythrocyte conjugates involved determinants on the surface of aggregated insulin complexes. Studies in which albumin was conjugated to erythrocytes by bisdiazobenzidine have demonstrated that multiple-functional groups on the albumin molecule were involved in the conjugation (Gelewitz *et al.*, 1954; Tabachnick & Sobotka, 1960). It is therefore reasonable to assume that the insulin aggregates conjugated to the erythrocytes were oriented in many different ways. Determinants buried because of insulin aggregation, such as the residues involved in dimer and hexamer formation, were probably not accessible to antibodies, whereas exposed determinants on the surface of the hexamer or dimer would be accessible. The relatively larger number of antibodies necessary to lyse Zn²⁺-free-insulin-erythrocyte conjugates is evidence that the removal of Zn²⁺ perturbs the determinants accessible to antibodies in the immune haemolysis reaction.

The effects induced by Zn²⁺ measured by c.d. spectroscopy and immune haemolysis were observed at insulin concentrations of $167 \mu\text{M}$. At this concentration, Zn-insulin is aggregated as hexamers (Arquilla *et al.*, 1969). Of particular interest with respect to insulin action *in vivo* is the possibility that Zn²⁺ can induce structural changes at very low concentrations of insulin. Immune haemolysis-inhibition experiments were therefore performed with Zn-insulin and Zn²⁺-free insulin as inhibitors at concentrations ranging from 1.74 nM to $1.74 \mu\text{M}$.

In these experiments less Zn-insulin was required

to inhibit haemolysis by antibodies than Zn²⁺-free insulin (Fig. 4). These results further substantiate the theory that removal of Zn²⁺ perturbs those determinants on insulin that interact with antibodies operative in the immune haemolysis-inhibition assay. In addition, this effect persists at concentrations where insulin is predominantly a monomer. In the absence of Zn²⁺, insulin dimers begin to form at concentrations of $8.33 \mu\text{M}$ (Goldman, 1971). Preliminary data from Goldman (1971) suggest that Zn-insulin at concentrations of $16.7 \mu\text{M}$ exists mainly in the dimer form. It is not known whether Zn-insulin exists as a dimer at the significantly lower concentration of Zn-insulin (43.5 nM) used to inhibit 50% of the antibodies in the immune haemolysis-inhibition assay.

Possible non-specific effects that Zn²⁺ might have on the immune haemolysis assays were tested in a system that was independent of insulin and insulin antibodies. This system consisted of lysozyme as the antigen and pooled guinea-pig lysozyme antisera as the source of antibodies. The Zn-lysozyme-erythrocyte conjugates and Zn²⁺-free-lysozyme-erythrocyte conjugates were prepared in the same manner as the Zn-insulin- and Zn²⁺-free-insulin-erythrocyte conjugates. No significant difference in the ability of the lysozyme antibodies to haemolyse either Zn²⁺-free-lysozyme-erythrocyte or Zn-lysozyme-erythrocyte conjugates was observed. We therefore feel that the results noted with the immune haemolysis and the immune haemolysis-inhibition experiments were due to alterations in the conformation of the antigenic determinants on insulin instead of possible non-specific effects.

Fig. 4. Inhibition of immune haemolysis by Zn-insulin (●) and Zn²⁺-free insulin (○)

The percentage cross-reactivity was calculated from the ratio of the amount of homologous inhibitor (Zn-insulin) to the amount of heterologous inhibitor (Zn²⁺-free insulin) required to inhibit 50% of the immune haemolytic activity induced by a given dilution of antiserum. The immunological activity of Zn-insulin was 100% by definition. The immunological activity of Zn²⁺-free insulin was measured as $3.7 \pm 0.86\%$ (mean $\pm$ S.E.M.; $n = 6$).

Fig. 5. Effect of Zn^{2+} on the inhibition of immune haemolysis by insulin and $[^{127}\text{I}]$ monoiodotyrosylinsulin. Zn-insulin-erythrocyte conjugates were the indicator. The percentage cross-reaction of Zn- $[^{127}\text{I}]$ monoiodotyrosylinsulin ($\blacksquare$, $105.56 \pm 0.25\%$), Zn-insulin ($\bullet$, taken as 100% activity), Zn^{2+} -free $[^{127}\text{I}]$ monoiodotyrosylinsulin ($\square$, $5.94 \pm 0.03\%$) and Zn^{2+} -free insulin ($\circ$, $4.87 \pm 0.01\%$) was calculated from the amount of each insulin inhibitor required to inhibit 50% of maximal immune lysis ($n > 5$ in all cases, except for Zn^{2+} -free $[^{127}\text{I}]$ monoiodotyrosylinsulin where $n = 3$).

Effects of Zn^{2+} on the conformation of antigenic determinants of monoiodotyrosylinsulin as measured by immune haemolysis-inhibition assay

The marked effect of Zn^{2+} on the immunological activity of insulin in the immune haemolysis-inhibition assay was an unexpected finding in a series of studies designed to prepare homogeneous $[^{127}\text{I}]$ monoiodotyrosylinsulin (Hamlin & Arquilla, 1974). Preliminary studies at that time indicated that the immunological activity of Zn^{2+} -free insulin was significantly decreased in comparison with Zn-insulin in the immune haemolysis-inhibition assay. Experiments were therefore designed to study the effects of Zn^{2+} on the immunological activity of both insulin and monoiodotyrosylinsulin by immune haemolysis inhibition.

The results of these experiments are shown in Fig. 5. Approx. 20 times as much Zn^{2+} -free insulin (4.9% cross-reaction) or Zn^{2+} -free $[^{127}\text{I}]$ monoiodotyrosylinsulin (5.9% cross-reaction) was required to inhibit the same amount of insulin antibodies as Zn-insulin (100%) or Zn- $[^{127}\text{I}]$ monoiodotyrosylinsulin (105.6%). No significant differences were noted between Zn-insulin and Zn- $[^{127}\text{I}]$ monoiodotyrosylinsulin or between Zn^{2+} -free insulin and Zn^{2+} -free $[^{127}\text{I}]$ monoiodotyrosylinsulin with pool A antiserum. The very similar immunological activity of Zn-insulin compared with Zn- $[^{127}\text{I}]$ monoiodotyrosylinsulin and Zn^{2+} -free insulin compared with Zn^{2+} -free $[^{127}\text{I}]$ monoiodotyrosylinsulin is presented as evidence that iodine substitution on insulin does not significantly alter the conformation of antigenic

Fig. 6. Immunological activities of Zn-insulin ($\bullet$) and Zn^{2+} -free insulin ($\circ$) by immune haemolysis-inhibition assay. The percentage cross-reaction is calculated from the ratio of μg of Zn-insulin to μg of monocomponent insulin needed for 50% inhibition. Zn-insulin was assumed to be 100% active.

determinants on insulin that are operative in the immune haemolysis-inhibition assay. Removal of Zn^{2+} , however, does significantly alter these determinants. Previous reports from our laboratory (Arquilla *et al.*, 1972) proposed that iodine substitution altered the conformation of antigenic determinants involving the A-chain tyrosine residues at A-14 and A-19. Our present observations demonstrate that the previous results were due to the effects of Zn^{2+} and not as a result of iodine substitution.

Effect of Zn^{2+} on antigenic determinants of insulin as measured by immune haemolysis-inhibition and radioimmune assays

Antibody populations directed toward determinants involved in the radioimmune assay can be distinguished from determinants involved in the immune haemolysis-inhibition assay (Arquilla *et al.*, 1976). To determine whether Zn^{2+} alters the radioimmune determinants to a greater degree than the immune haemolysis determinants or vice versa, Zn^{2+} -free insulin was compared with Zn-insulin, in each of the assays. The same dilution of a single antiserum and the same concentrations of inhibitor were used in each of these assay systems. In all cases Zn-insulin (homologous antigen) was considered to have 100% immunological activity.

In the immune haemolysis-inhibition assay, Zn-insulin (100%) was a much more effective inhibitor than Zn^{2+} -free insulin (0.86% s.e.m. $\pm$ 0.08%; Fig. 6). On the other hand, in the radioimmune assay, Zn^{2+} -free insulin was a slightly more effective inhibitor (125.21% s.e.m. $\pm$ 10.38%) than Zn-insulin (100%) (Fig. 7). Immunological activities of insulin and Zn-insulin complexes were determined at a point where 50% of the antibodies were inhibited

Fig. 7. Representative plot of the immunological activities of Zn-insulin (●) and Zn²⁺-free insulin (○) by radio-immune assay

C₀ represents the number of counts in the supernatant in the absence of insulin or any inhibiting material. C₁ represents the radioactivity in the supernatant in the presence of Zn²⁺-free insulin or Zn-insulin. The immunological activity was calculated from the ratio of μg of Zn-insulin to μg of monocomponent insulin needed for C₀/C₁ = 2. Zn-insulin was assumed to be 100% active (correlation coefficient = 0.9952 for Zn-insulin, and 0.9853 for Zn²⁺-free insulin).

and paired-data analysis of four experiments demonstrated that the immunological activity of Zn²⁺-free insulin is greater than that of Zn-insulin in the radioimmune assay (0.05 < P < 0.1 by Student's *t* test). The radioimmune assay and immune haemolysis-inhibition assay experiments were performed with identical concentrations of the same insulin antiserum and inhibitor. We are therefore confident that comparison of the immunological activities in the respective assays is valid.

Zn²⁺-free insulin reacted with antibodies operative in the immune haemolysis-inhibition assay to a lesser degree than did Zn-insulin, indicating that the absence of Zn²⁺ significantly altered the immune haemolysis determinants. In contrast, Zn²⁺-free insulin reacted with antibodies operative in the radioimmune assay to a slightly greater degree than did Zn-insulin, indicating that in the absence of Zn²⁺ the radioimmune determinants become more reactive. The divergence of the regression curves for Zn-insulin and Zn²⁺-free insulin in the radioimmune assay (Fig. 7) demonstrates that the increased reactivity of Zn²⁺-free insulin was greater at high inhibitor concentrations.

Discussion

Our c.d. studies confirmed and extended earlier findings that Zn²⁺ induced changes in the c.d. spectra of insulin (Hamlin & Arquilla, 1974;

Goldman & Carpenter, 1974; Strickland & Mercola, 1976). The c.d. changes observed in the near-u.v. region induced by Zn²⁺ involved the environment of the tyrosine residues (Strickland & Mercola, 1976). The changes seen in the far-u.v. region at 222nm involved the β-structure (Quadrifoglio & Urry, 1968), a feature of the dimer. The changes at 208nm involved the α-helix (B10-B19), a feature of the monomer (Ettinger & Timasheff, 1971; Goldman & Carpenter, 1974). The c.d. differences could therefore be explained by Zn²⁺-induced changes in the conformation of the monomer and/or dimer, changes in the state of aggregation, or a combination of changes that involve conformation and aggregation.

Antisera used in these experiments were raised in guinea pigs against 10 × recrystallized Zn-insulin. This insulin preparation contained 0.76% Zn²⁺, equivalent to 0.6 atom of Zn²⁺ per molecule of insulin. It is not known whether the Zn²⁺ crystals were in the 2-Zn or 4-Zn form or a mixture of both. It is reasonable to assume that most of the antibodies directed against insulin were primarily against the Zn²⁺ form, as it was present in the material used as the antigen (Freunds adjuvant). Consequently, the Zn²⁺-free insulin used in the experiments was considered to be the heterologous antigen and the Zn-insulin the homologous antigen.

The slight but significantly increased immunological reactivity of Zn²⁺-free insulin in the radioimmune assay, observed at the higher concentrations of inhibitors, could be explained by the presence of antibodies directed against determinants involving the dimer region (Brugman & Arquilla, 1973; Arquilla *et al.*, 1976). The relative immunological reactivity between Zn²⁺-free insulin and Zn-insulin was negligible at the lower concentrations of inhibitor. This suggested that, with increasing concentrations of inhibitor, Zn-insulin dimers were formed, decreasing the accessibility of radioimmune determinants involving the dimer site. Because the differences between Zn²⁺-free and Zn-insulin in the radioimmune assay were slight, it is likely that there were relatively few antibodies involved in this proposed interaction. Probably there were other antibodies directed against determinants operative in the radioimmune assay, which were also operative in the immune haemolysis assay, such as the A-chain α-loop (A8-A9-A10). Berson & Yalow (1959) and Grodsky *et al.* (1959) observed radioimmune-assay differences in the immunological reactivity of various mammalian insulins that differed only in this region. There were, in addition, determinants on the A-chain solvent surface that were operative in the immune haemolysis assay, but which were not expressed to a significant degree in the radioimmune assay. These determinants were markedly perturbed in the absence of Zn²⁺.

The most pronounced effect of Zn^{2+} was observed when Zn^{2+} -free insulin was treated with antibodies in the direct immune haemolysis and immune haemolysis-inhibition assays. These assays measured antibody-insulin interactions on an insoluble matrix (erythrocytes) and in solution respectively. The attenuated immunological activity of Zn^{2+} -free insulin in both of these systems reinforced the interpretation that removal of Zn^{2+} significantly perturbed the antigenic determinants on insulin.

Previous work from our laboratory (Brugman & Arquilla, 1973; Arquilla *et al.*, 1976) indicated that determinants operative in the immune haemolysis assay involved the solvent-exposed portions of insulin as it exists in 2-Zn crystals. This portion of insulin is comprised predominantly of the surface A-chain residues. Therefore it follows that the attenuated behaviour of Zn^{2+} -free insulin in the immune haemolysis and immune haemolysis-inhibition experiments (Figs. 3, 4 and 6) were due to alterations of this portion of the A-chain. However, the interaction that caused these structural alterations of the A-chain determinants are not readily explained by our knowledge of the structure of insulin, as it is known in either 2-Zn crystals or 4-Zn crystals (G. Dodson, personal communications).

The observed immunological changes may possibly be explained by Zn^{2+} -induced aggregation. The reports by Goldman & Carpenter (1974) and Blundell *et al.* (1972), however, indicated that the most favourable concentration for dimer formation in the absence of metals occurs at insulin concentrations of $50 \mu\text{g/ml}$ ($8.33 \mu\text{M}$). In the immune haemolysis-inhibition assay and radioimmune assay experiments, insulin inhibitors were used at concentrations ranging from 1.74 nM to $1.74 \mu\text{M}$. Less than 1% dimer formation would be expected for Zn^{2+} -free insulin at the higher concentration used. It can be argued that Zn-insulin crystals remain aggregated when diluted. It is known that Zn^{2+} induces aggregation at concentrations lower than those observed with Zn^{2+} -free insulin (Goldman, 1971; Fredericq, 1956). Zn-insulin exists in the dimer form at concentrations of $16 \mu\text{M}$ -insulin, whereas Zn^{2+} -free insulin exists mainly as a monomer at this concentration (Goldman, 1971). In the studies described in this report it is not known whether Zn-insulin could exist as a dimer at the substantially lower concentrations of inhibitor (43.5 nM) used to inhibit 50% of the insulin antibodies.

It is likely that the Zn-insulin complex is not essential for hormonal activity (Goldman & Carpenter, 1974). However, the possibility that Zn^{2+} may modulate the action and turnover of insulin cannot be excluded. In most mammalian species, insulin is stored in the β -cells as Zn crystals (Howell *et al.*, 1969; Lazaris *et al.*, 1974; Berglund & Hellman, 1976). It is therefore likely to be secreted

into the portal circulation and reach the liver within seconds in a Zn-insulin form. The degree to which such a Zn-insulin might influence degradation and binding by the liver is of significance with respect to the molecular action and turnover of insulin.

In preliminary experiments, it was possible to demonstrate that more ^{125}I -labelled insulin was bound faster and its degradation inhibited in the livers of the Zn^{2+} -treated mice than the Na^+ -treated mice (Arquilla *et al.*, 1977; Tarmas *et al.*, 1977). Thus there is a possibility that Zn-insulin will be metabolized and have molecular interactions different from Zn^{2+} -free insulin *in vivo*.

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