Polymerization Behavior of Bovine Zinc–Insulin at Neutral pH. Molecular Weight of the Subunit and the Effect of Glucose†

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ABSTRACT: Sedimentation equilibrium and sedimentation velocity experiments with bovine zinc–insulin solutions at pH 7 show that aggregates of molecular weight higher than the insulin hexamer may exist in solution under these conditions. Frontal analysis on Sephadex G-50 and sedimentation equilibrium experiments also show that insulin containing 2 g-atoms of zinc/hexamer dissociates to the monomer in solution at pH 7. Comparison of the molecular weight concentration dependence of zinc–insulin with that of zinc-free insulin at pH 7 indicates that the polymerization behavior of the two is qualitatively similar. The addition of glucose to insulin solutions at pH 7 and 2 favors dissociation, all equilibria apparently being affected. It has not been established whether the effect is due to specific binding of glucose to insulin, to changes in solvent structure, or to a combination of both. If the effect operates at physiological concentration levels it would result in an even higher ratio of insulin monomer to dimer in serum than has been indicated previously for zinc-free insulin solutions in the absence of glucose.

Bovine insulin exhibits two main types of zinc binding (Cunningham *et al.*, 1955; Summerell *et al.*, 1965; Grant *et al.*, 1972). The first, characterized by a high association constant ($K = 4.7 \times 10^6$ M^{-1} at pH 8.0, Summerell *et al.*, 1965; $K = 1.9 \times 10^6$ M^{-1} at pH 7.0, Grant *et al.*, 1972), has a capacity of about 2 g-atoms of zinc per insulin hexamer (mol wt 34,500) corresponding to the amount of zinc in crystalline zinc–insulin (Adams *et al.*, 1969). This strongly bound zinc cannot be removed by dialysis at neutral pH. In the second type of binding the zinc is less strongly bound ($K = 0.035 \times 10^6$ M^{-1} at pH 8.0, Summerell *et al.*, 1965), binds to the extent of about 6 g-atoms per insulin hexamer (Grant *et al.*, 1972), and can be removed by dialysis at pH 7 (Cunningham *et al.*, 1955).

The ability of bovine insulin to bind zinc results in a marked dependence of its polymerization behavior at neutral pH upon the amount of zinc present in solution. Pekar and Frank (1972) in a sedimentation equilibrium study have shown that at pH 7.0 zinc-free insulin solutions have weight average molecular weights below that of the insulin dimer at low concentrations and above that of the insulin octamer at the highest concentration measured. The system was described in terms of a model involving equilibria between monomer, dimer, hexamer, and higher aggregates of hexamer. Earlier sedimentation velocity (Cunningham *et al.*, 1955; Fredericq, 1956) and osmotic pressure studies (Marcker, 1960) of zinc-free insulin at near-neutral pH are in qualitative agreement with this description.

Studies of the polymerization behavior of insulin near neutral pH in solutions containing 2 g-atoms of zinc per insulin hexamer (Cunningham *et al.*, 1955; Fredericq, 1956; Creeth, 1953; Grant *et al.*, 1972) are in general agreement that under such conditions insulin is monodisperse at neutral pH and that the species in solution is the hexamer of mol wt 34,500. Creeth (1953), however, noted that dissociation may be occurring at concentrations below about 0.3 g/100 ml.

Grant *et al.* (1972) report that when the zinc concentration is higher than 2 g-atoms per hexamer the only detectable form of insulin present in the soluble phase at pH 7.0 is the 3.2S component, *i.e.* the hexamer. This is at variance with the findings of Cunningham *et al.* (1955) and Fredericq (1956) at pH values near neutrality. The former authors noted a linear increase in $s_{20,w}$ with zinc concentration for insulin at pH 7.3 and measured a sedimentation coefficient as high as 9 S with about 8 g-atoms of zinc bound per hexamer. They also reported a sedimenting boundary of higher $s_{20,w}$ comprising about 10–15% of the total area and with behavior indicating a group of zinc-protein complexes of similar form. Fredericq's (1956) observations made at pH 8.0 are in close agreement, with components of molecular weight about 72,000 and then 200,000–300,000 progressively appearing as the zinc concentration was raised. Grant *et al.* (1972) suggest that the difference in their findings and those reported above might be due to differences in pH affecting the zinc binding or the solubility of zinc–insulin polymers larger than the hexamer. This explanation loses some force when it is recalled that the results obtained by Cunningham *et al.* at pH 7.3 are essentially the same as those of Fredericq at pH 8.0.

The present work was undertaken with the intention of studying the possible dissociation of bovine insulin containing 2 g-atoms of bound zinc per hexamer at pH 7.0, especially with regard to whether the insulin monomer exists in solution under these conditions. The results of some of the sedimentation equilibrium experiments are also relevant to the question of the existence in solution of zinc–insulin polymers of higher molecular weight than the hexamer at neutral pH.

A report (Brohult, 1940) that glucose dissociates *Helix pomatia* hemocyanin to about the same extent as urea and a more recent finding that glucose promotes the dissociation of muscle phosphorylase *a* tetramers to dimers (Wang *et al.*, 1965) suggest that glucose might exert a similar dissociating effect with certain other proteins. It was of interest

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TABLE I: Zinc Content of Insulin Solutions in Phosphate Buffer at pH 7.0.

Method of Preparation	Zinc Content (g-atoms/mole of Hexamer)
Information supplied by C.S.L.	4.1
Dissolved at pH 9.8, adjusted to pH 7.0, no dialysis	4.25
As above with overnight dialysis	3–4
Dissolved at pH 9.8, dialyzed to pH 7 overnight	2.3
As above with additional 48-hr dialysis	1.95
As above in buffer containing 7.76 g of glucose/100 ml	1.89

therefore to examine its effect on insulin which consists of a number of polymeric species in rapid reversible equilibrium and with which, moreover, glucose is associated in biological systems.

Materials and Methods

Bovine insulin was obtained from the Australian Commonwealth Serum Laboratories (C.S.L.) and was stated to contain 0.778 g of zinc per 100 g of anhydrous protein, 8.97 g of water per 100 g of sample, and 3–4% proinsulin as determined by polyacrylamide gel electrophoresis. The amount of the monodesamido component in this insulin has been shown to be 5% or less (Human and Leach, 1961). The ovalbumin was Sigma crystalline grade 5 salt free, the bovine plasma albumin, Armour crystallized, and the D-glucose, BDH analytical reagent grade. Analytical reagent grade chemicals and glass-distilled water were used in the preparation of all solutions. The buffer of pH 7.00, ionic strength 0.1 at 20°, was composed of 0.002 M NaH_2PO_4, 0.0028 M Na_2HPO_4 and 0.09 M NaCl while that of pH 2.00, ionic strength 0.05 at 20°, was composed of 0.0053 M glycine, 0.0353 M NaCl, and 0.0147 M HCl. Glucose-containing buffers were prepared by weighing out 7.760 g of glucose and making the volume up to 1 l with the appropriate pH 7 or pH 2 buffer, followed by adjustment of the pH to 7.00 or 2.00 if required. All buffer solutions were millipored before use. Insulin solutions of concentrations of about 0.25 g/100 ml were prepared by dissolving 0.03 g of protein (equivalent to 0.027 g of anhydrous insulin) in 10 ml of the required buffer. In the glucose-containing solutions the molar ratio of glucose to insulin (expressed as monomer) was close to 1000:1. In order to prepare zinc–insulin solutions of pH 7 it was necessary to adjust the pH of the solution to just under pH 10 before adding it to the dry insulin. About 30 min slow stirring sufficed to dissolve the insulin, which remained in solution upon subsequent readjustment of the pH to 7.00 by dialysis. Insulin dissolved more easily in buffers containing glucose and it was possible to make more concentrated solutions. It seemed that about 0.35 g/100 ml was near the upper limit of zinc–insulin which would remain in solution in the absence of glucose at pH 7 for the time required for experiments, whereas solutions containing as much as 1 g of protein/100 ml were prepared in the glucose-containing buffers. After the insulin solutions were prepared they were dialyzed in 18/32 Visking tubing at room temperature *vs.* a total of 200–300 volumes of buffer in three changes for 48 hr. Sometimes the solutions at pH 7 not containing glucose showed evidence of precipitation during dialysis. When this occurred the solution was filtered through a 5-μ, and then a 0.22-μ Millipore filter and its concentration was checked by measuring its optical density at 278 nm. If the concentration was still close to 0.25 g/100 ml the solution was given a final overnight dialysis *vs.* buffer before use in a sedimentation experiment. If a substantial amount of protein had precipitated a fresh solution was prepared and the dialysis started again. Since the solutions were originally prepared to contain 0.27 g of insulin/100 ml and all sedimentation equilibrium experiments were performed with solutions containing not less than 0.25 g of insulin/100 ml the maximum amount of protein precipitated from the solutions employed was 0.02 g/100 ml. If all of the protein lost in this way were insulin, the proinsulin content in the final solution would only increase from 4% of the total protein to about 4.5% of the total. The behavior of the zinc–insulin solutions at pH 7 was rather variable and evidently zinc–insulin aggregates have a tendency to precipitate progressively from solution. Nevertheless, as subsequently shown, by working at a protein concentration near 0.25 g/100 ml it is possible to retain in solution zinc–insulin aggregates of higher molecular weight than hexamer for at least long enough to carry out sedimentation equilibrium experiments.

Zinc determinations were carried out by atomic absorption spectroscopy and the results are given in Table I. Buffer solutions prepared as described, with or without glucose, contained no detectable zinc; thus all the zinc derived from the dissolved insulin.

The analyses show that the original insulin sample contained about 4 g-atoms of zinc per insulin hexamer and that two of these were dialyzable, in agreement with the studies previously cited (Cunningham *et al.*, 1955; Summerell *et al.*, 1965; Grant *et al.*, 1972). The zinc–insulin solutions used in the studies to be described were prepared as indicated in Table I to contain 2 g-atoms of zinc per insulin hexamer unless otherwise noted. The figures quoted in the table are values determined on the actual samples used in the reported sedimentation equilibrium experiments at pH 7.00.

The apparent specific volume of insulin was determined in the pH 7 phosphate buffer in the presence and absence of glucose, using dialyzed solutions. Measurements of the densities of the insulin solutions and dialysates were made at 20° to six decimal places using a DMA 02 C precision density meter (Anton Paar, Graz). The concentrations of the insulin solutions were determined from measurements of their refractive index increments using dialysate as reference and a specific refractive index increment of 0.001849 dl/g. The concentration of the insulin solution containing 7.76 g of glucose/100 ml was 0.3389 g/100 ml while that of the solution without glucose was 0.3445 g/100 ml, and the values of the apparent specific volumes were 0.720 and 0.725 ml/g, respectively. As a check on the instrument the apparent specific volume of a solution of bovine plasma albumin (0.9352 g/100 ml) in 0.1 M KCl was determined at 20°. The value of 0.734 ml/g obtained is in good agreement with that of 0.733 ml/g measured in a careful study by Hunter (1966).

Frontal analysis experiments were done with columns of Sephadex G-50 of about 60-ml volume, thermostated at 20°, and with a flow rate of 30 ml/hr. In order to achieve a plateau of concentration equal to that of the original protein solution, a volume of 30 ml of solution was loaded for each

concentration investigated. The elution was monitored refractometrically by means of an LDC refractomonitor calibrated with sucrose solutions. Weight average elution volumes were determined from the median bisector of the ascending boundary (Nichol and Winzor, 1972). The void volumes of the columns were determined using blue dextran.

Sedimentation velocity experiments were carried out at 20°, 56,000 rpm, in a double-sector synthetic boundary cell with dialysate in the solvent sector, using the schlieren optical system of the Spinco Model E analytical ultracentrifuge. The densities of solvent with and without glucose were measured in the DMA 02 C precision density meter at 20° and their viscosities in an Ostwald viscometer at 20°. The values were, for the phosphate buffer, ρ_{20} = 1.0027 g/ml and η^{20} = 1.0254 cP, and for the same buffer plus 7.76 g of glucose/100 ml, ρ_{20} = 1.0370 g/ml and η^{20} = 1.2632 cP. Weight average sedimentation coefficients were evaluated from the rate of movement of the second moment of the schlieren curves taking measurements of the height of the gradient above the base line at 20–25 equally spaced radial increments for each exposure. These sedimentation coefficients were corrected to water at 20° by making use of the expression

$$s_{20,w} = \frac{s\eta(1 - \bar{v}\rho)_{20,w}}{\eta_{20,w}(1 - \bar{v}\rho)}$$

where s is the measured sedimentation coefficient, η is the viscosity of the solvent at the temperature of the experiment, $\eta_{20,w}$ is the viscosity of water at 20°, $\rho_{20,w}$ is the density of water at 20°, and ρ is the density of the solution at the temperature of the experiment.

Sedimentation equilibrium experiments were done in Al-filled Epon double-sector cells using the Rayleigh interference optical system of the ultracentrifuge. One sector contained dialyzed protein solution and the other its equilibrium dialysate. Initial concentrations of the protein solutions were determined in a capillary-type synthetic boundary cell immediately before the sedimentation equilibrium experiment, the synthetic boundary cell and the equilibrium cell being filled at the same time directly from the dialysis sac. A photograph of the fringe pattern was taken as soon as the columns equilibrated in the synthetic boundary experiment so that the fringe fraction could be determined before any significant dilution had occurred. The overspeeding technique (Howlett and Nichol, 1972) and 3-mm solution columns were used to reach equilibrium in less than 24 hr. Interference fringe patterns were measured at equal fringe increments for the low-speed sedimentation equilibrium experiments and concentrations determined in this way were corrected by the hinge point method suggested by Richards *et al.* (1968) after employment of the conservation of mass condition. Apparent weight average molecular weights were evaluated by use of the expression $(M_{w,app})r = [\mathrm{d} \ln c/\mathrm{d}(r^2)_r][2RT/(1 - \bar{v}\rho)\omega^2]$, where c is the concentration (in Rayleigh interference fringes in the 12-mm ultracentrifuge cell), r is the radial distance from the axis of rotation, T is the absolute temperature, R is the gas constant, ω is the angular velocity in radians/second, $\bar{v}$ is the partial specific volume of the protein and ρ is the solution density. The ln c *vs.* r^2 data were fitted to a quadratic expression by the technique of least squares. The measurement and calculation of molecular weights from the meniscus depletion experiment were done exactly as described by Yphantis (1964). Rayleigh interference fringe patterns were recorded on Kodak II G plates and schlieren patterns on Ilford R-40 plates. Measurements were made on a Gaertner microcomparator.

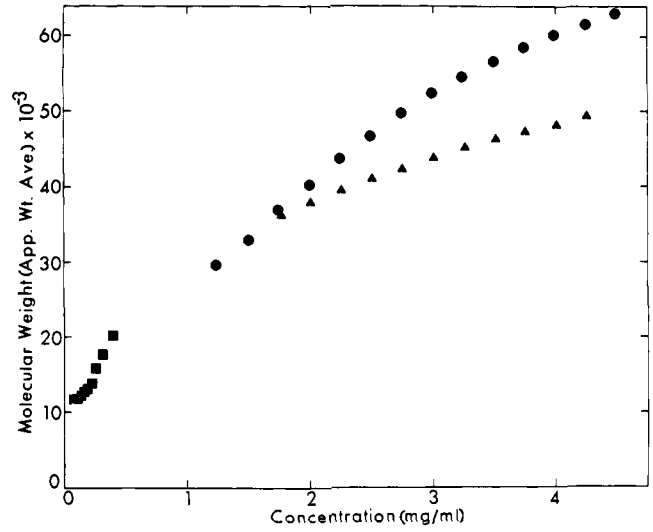

FIGURE 1: Molecular weight *vs.* protein concentration for zinc-insulin at pH 7. Sedimentation equilibrium experiments in NaH_2PO_4–Na_2HPO_4–NaCl buffer of ionic strength 0.1 at 20°: (●) 3–4 g-atoms of zinc/hexamer, 14,000 rpm; (■) 3–4 g-atoms of zinc/hexamer, 56,000 rpm; the initial concentration of the insulin solution was 0.8 mg/ml; (▲) 2 g-atoms of zinc/hexamer, 14,000 rpm.

Results and Discussion

The results of sedimentation equilibrium experiments at pH 7.0 with solutions containing insulin with 3–4 g-atoms of bound zinc per hexamer are presented in Figure 1. It is evident that species of higher molecular weight than the insulin hexamer and of lower molecular weight than the tetramer are present in solution. The lowest concentration reached in the meniscus depletion experiment does not allow a decision as to whether the dimer or the monomer is the smallest unit present. The form of the dependence of the apparent weight average moleular weight on concentration and comparison of the high-speed and low-speed results suggest that under these conditions insulin forms a series of polymers in equilibrium with each other plus some high molecular weight aggregates. The experiment with insulin containing 2 g-atoms of zinc per hexamer also shown in Figure 1 gave a similar result, the apparent weight average molecular weights being somewhat lower as expected with a lower zinc content.

It is relevant in relation to the results presented in Figure 1 to inquire whether weight average molecular weights greater than 35,000 are due to the presence of polymeric forms of zinc–proinsulin which are known to be soluble at pH 7. Solutions containing 2 g-atoms of zinc per hexamer have been found to contain zinc–proinsulin species of molecular weight 55,000, while zinc–proinsulin species of molecular weight up to about 100,000 may exist in solutions of higher zinc content (Grant *et al.*, 1972). It was noted earlier that the solutions used in the present study may contain just under 5% of proinsulin. However, in order to account for the measured weight average molecular weight at a concentration of 3 mg/ml, for example, even if it were assumed that all of the zinc–insulin were in the form of the hexamer (molecular weight 35,000), 50% of the total protein would need to be the zinc–proinsulin hexamer (molecular weight 55,000) in the solution containing 2 g-atoms of zinc per

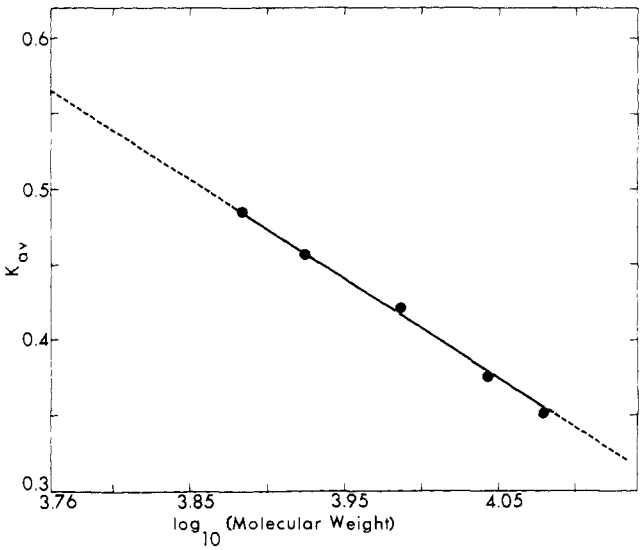

FIGURE 2: Calibration graph for insulin on Sephadex G-50 determined at pH 2 as described in text.

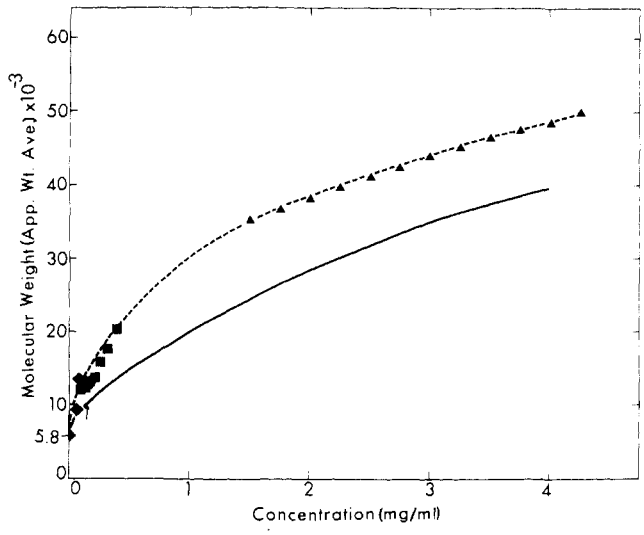

FIGURE 3: Molecular weight *vs.* protein concentration for insulin at pH 7. Sedimentation equilibrium experiments in NaH_2PO_4–Na_2HPO_4–NaCl buffer of ionic strength 0.1 at 20°: (▲) 2 g-atoms of zinc/hexamer, 14,000 rpm; (■) 3–4 g-atoms of zinc/hexamer, 56,000 rpm; the initial concentration of the insulin solution was 0.8 mg/ml. Frontal analysis on Sephadex G-50 in the same buffer at 20°: (♦) 2 g-atoms of zinc/hexamer. The dashed line is drawn to facilitate comparison with the sedimentation equilibrium results obtained for zinc-free insulin (shown as the solid line) by Pekar and Frank (1972). The experiments of Pekar and Frank were done at pH 7 in Tris-NaCl buffer at 25° and the lowest concentration achieved in their experiments is shown by the arrow.

hexamer. Similarly the weight average molecular weight at the concentration 3 mg/ml in the solution containing 3–4 g-atoms of zinc per hexamer would be accounted for in terms of proinsulin only by requiring 30% of the total protein to be in the form of a zinc-proinsulin polymer of molecular weight 100,000. It seems, therefore, that although there is presumably some contribution to the high measured weight average molecular weight from polymers of zinc-proinsulin the major contribution is from polymers of zinc-insulin of greater size than the hexamer.

Molecular Weight of the Insulin Subunit in the Presence of Zinc. It was not possible to measure weight average molecular weights of zinc-insulin at pH 7 at low enough concentrations by sedimentation equilibrium experiments with the Rayleigh interference optical system to decide whether the monomer or dimer was the smallest insulin unit in solution. The technique of frontal analysis gel filtration offered an alternative and was used as follows. A column of Sephadex G-50 was equilibrated with a buffer solution of pH 2 and the elution volumes of insulin solutions of concentrations between 0.03 g/100 ml and 0.2 g/100 ml equilibrated with the same buffer were determined by frontal analysis. These elution volumes were expressed as $K_{av} = (V_e - V_0)/(V_t - V_0)$ with the use of the void volume, V_0, and the total volume, V_t, of the Sephadex bed. The weight average molecular weights of insulin at pH 2 in the same buffer, at the concentrations used for the frontal analysis experiments, were determined from sedimentation equilibrium experiments (Figure 6). This allowed a calibration graph of K_{av} *vs.* molecular weight to be constructed for Sephadex G-50 over the molecular weight range 7,000–11,000 (Figure 2). Frontal analysis experiments with insulin solutions containing 2 g-atoms of zinc per hexamer at pH 7 were then performed on a Sephadex G-50 column equilibrated with the pH 7 buffer over the insulin concentration range 0.002–0.008 g/100 ml using refractometric monitoring to follow the elution. The determination of elution volumes and hence K_{av} values for zinc-insulin solutions at pH 7 in this way allowed the corresponding molecular weights to be estimated at extremely low insulin concentrations from the previously established calibration graph. The molecular weights found in such experiments are plotted in Figure 3 where it is seen that the dissociation of zinc-insulin solutions at pH 7 proceeds to the monomer. The dependence of elution volume on insulin concentration also indicates that there is a series of insulin polymers in equilibrium under the conditions employed. That is, the dependence of weight average molecular weight on concentration observed in the sedimentation equilibrium experiments is not just the result of the centrifugal field acting on a nonreacting mixture of insulin polymers.

The gel filtration experiments at pH 7 were done with insulin solutions prepared as described under Materials and Methods. Initially, when a large volume of such a solution was loaded on a Sephadex G-50 column the elution profile showed a peak at the void volume followed by the profile normally expected from a frontal analysis experiment. The calibration carried out at pH 2 showed that the void volume of such a column corresponded to a molecular weight of about 37,000, *i.e.* species of molecular weights greater than the insulin hexamer would be totally excluded. It therefore seemed that the peak referred to above represented aggregated forms of zinc-insulin larger than the hexamer. Subsequent frontal analysis experiments at pH 7 were performed with solutions from which the material eluting at the void volume had been removed by gel filtration on Sephadex G-50. These frontal analysis experiments were done immediately after the removal of the high molecular weight material and showed no detectable influence of such material on the elution profiles subsequently obtained in experiments lasting about 2 hr. The gel filtration experiments with insulin solutions containing 2 g-atoms of bound zinc per insulin hexamer therefore indicate that such solutions contain aggregates larger than the insulin hexamer which are apparently not in rapid equilibrium with the rest of the system and that the dissociation proceeds to the monomer of molecular weight 5800 (Figure 3). The results of the frontal analysis experiments are not expected to be exactly in agreement with those of the sedimentation equilibrium ex-

periments because polymeric forms of molecular weight greater than hexamer were removed in the former. The good agreement observed between the molecular weights derived from gel chromatography and those at the lowest concentrations from sedimentation equilibrium may be ascribed to the fact that the latter were evaluated under meniscus depletion conditions where the higher molecular weight species not involved in rapid equilibria are concentrated near the cell base.

The results of Pekar and Frank (1972) with zinc-free insulin solutions at pH 7.0 by sedimentation equilibrium are shown in Figure 3 for comparison. As mentioned previously these results were interpreted in terms of a set of rapid, reversible equilibria between insulin monomers, dimers, hexamers, and higher aggregates. It seems that the results with the zinc-containing insulin solutions of the present study could be explained in similar terms with the addition of some nonreacting or slowly reacting aggregates. No attempt has been made to carry out such a detailed analysis because of the ill-defined nature of the aggregates and the possible variation in their amounts in solutions of different ages.

Dissociation of Insulin by Glucose at pH 7. Sedimentation velocity experiments were done at pH 7.0 with zinc-insulin solutions of concentration 0.3 g/100 ml. In one of the experiments the solution contained 7.76 g of glucose/100 ml. Single symmetrical peaks were obtained in the presence and absence of glucose and the weight average sedimentation coefficients corrected for density and viscosity were $\bar{s}_{20,w}$ = 4.14 S without glucose and $\bar{s}_{20,w}$ = 3.69 S with glucose. The magnitude of the sedimentation coefficient obtained in the absence of glucose is consistent with the presence of insulin species of molecular weight greater than the insulin hexamer (sedimentation coefficient about 3.2 S) in solution. However, the absence of any shoulders or resolved peaks in the schlieren profile suggests that such species are smaller than the gross aggregates observed by Cunningham *et al* (1955) and Fredericq (1956). As with the sedimentation equilibrium experiments under these conditions, the amount of proinsulin present in the solution is far too low for zinc proinsulin polymers to make any significant contribution to the observed sedimentation coefficients. The significant drop in the weight average sedimentation coefficient upon the addition of glucose indicates that some dissociation has occurred, but species larger than hexamer are apparently still making a contribution to the observed sedimentation coefficient.

A comparison of the results of sedimentation equilibrium experiments with zinc insulin at pH 7 in the presence and absence of glucose is made in Figure 4. There is a significant drop in the apparent weight average molecular weights measured in the glucose-containing solution, in qualitative agreement with the sedimentation velocity experiments. The concentration of glucose is quite high in these experiments and there are several conceivable ways in which the apparent weight average molecular weight might be affected without invoking dissociation of the macromolecule. Those considered are a difference in glucose concentration in solution and solvent sufficient to give rise to spurious refractive index gradients, the presence of a density gradient due to redistribution of glucose, a difference of partial specific volumes of insulin in the presence and absence of glucose due to preferential solvation, and a large second virial coefficient in concentrated glucose solutions.

The sedimentation equilibrium experiments were analyzed using the Rayleigh interference optical system and were conducted with dialyzed solution and its equilibrium dialysate in the solution and solvent sectors, respectively. While calculations and experiments showed that an excess of glucose in the insulin solution compared with the solvent would result in a decrease in the measured apparent weight average molecular weight, measurements on glucose-containing insulin solutions and buffers dialyzed for the same lengths of time as those used in the sedimentation equilibrium experiments showed no detectable difference in glucose concentration in solution and solvent. The refractometric method used was capable of detecting reliably differences in glucose concentration five times smaller than those required (about 0.075 g/100 ml) to produce the depression of the apparent molecular weight.

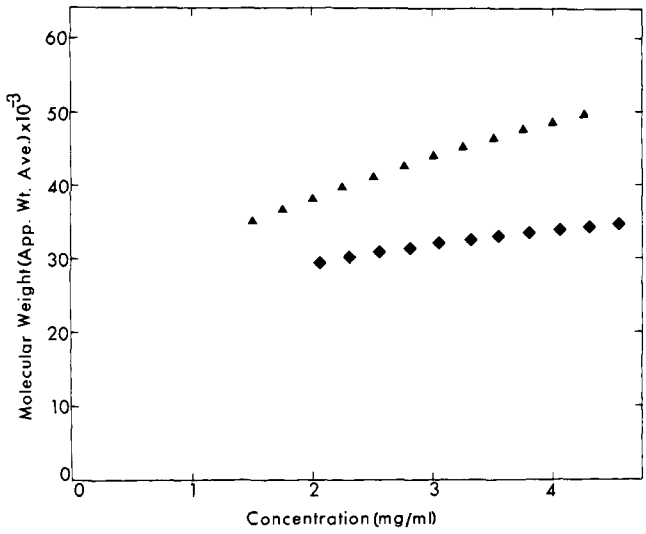

FIGURE 4: Molecular weight *vs.* protein concentration for zinc-insulin at pH 7 showing effect of glucose. Sedimentation equilibrium experiments in NaH_2PO_4–Na_2HPO_4–NaCl buffer of ionic strength 0.1 at 20°: (▲) 2 g-atoms of zinc/hexamer, 14,000 rpm; (◆) 2 g-atoms of zinc/hexamer, 14,000 rpm; 7.76 g of glucose/100 ml.

The concentration distribution of glucose was calculated from its physical parameters for the conditions used in the sedimentation equilibrium experiments and found to give rise to a density difference of only 0.0006 g/ml between the ends of the solution column, which is much too small to affect significantly the evaluation of the molecular weight of the macromolecular component. As noted in Materials and Methods the apparent specific volumes of zinc insulin at pH 7.0 in the presence and absence of glucose were 0.720 and 0.725 ml/g, respectively. These figures are in good agreement with each other and with the value 0.72 ml/g given by Oncley *et al.* (1952).

Sedimentation equilibrium experiments with ovalbumin at pH 7.0 with and without added glucose were undertaken to investigate the effect of glucose on the apparent weight average molecular weight of a nonpolymerizing protein. Ovalbumin was chosen because its molecular weight (45,000) is close to the weight average molecular weight of insulin in the concentration range of interest and because gel electrophoresis experiments showed that it contained no aggregated material. The molecular weights determined for the ovalbumin solutions (Figure 5) showed no concentration dependence either in the absence or presence of glucose and gave mean molecular weights of 43,200 and 44,200, respectively. The mean of these values, 43,600, is in excellent agreement with that of 43,500 obtained for ovalbumin by

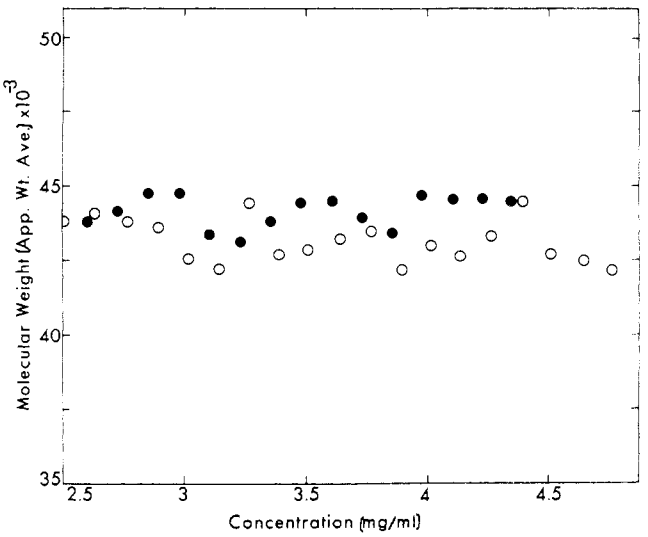

FIGURE 5: Molecular weight *vs.* protein concentration for ovalbumin at pH 7. Sedimentation equilibrium experiments in NaH_2PO_4-Na_2HPO_4-NaCl buffer of ionic strength 0.1 at 20°, 10,000 rpm: (○) without glucose, (●) 7.76 g of glucose/100 ml. Values of d ln j/d(r^2) were obtained by least-squares quadratic fit to ln j *vs.* r^2 points taken in groups of 5.

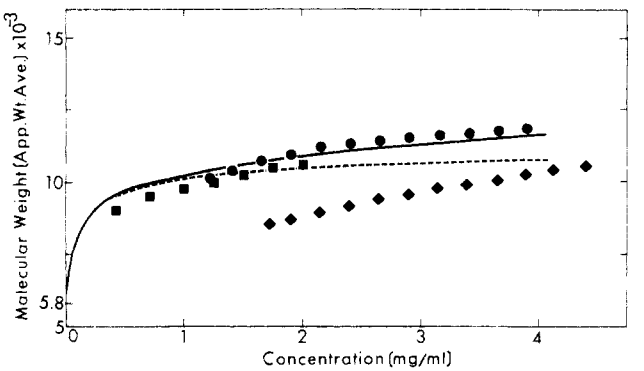

FIGURE 6: Molecular weight *vs.* protein concentration for insulin at pH 2 showing effect of glucose. Sedimentation equilibrium experiments in glycine-NaCl buffer of ionic strength 0.05 at 20°: (●) 22,000 rpm; (■) 30,000 rpm; (♦) 22,000 rpm; 7.76 g of glucose/100 ml. The solid line is calculated for a monomer-dimer-tetramer equilibrium with association constants of K_2 = 14.0 (mg/ml)$^{-1}$ and K_4 = 2.62 × 10^{-2} (mg/ml)$^{-1}$. The dashed line shows the effect of putting K_4 = 0.

sedimentation equilibrium by Svedberg and Pedersen (1940). It is evident that the presence of 7.76 g of glucose/100 ml of solution produces no change in the molecular weight determined for ovalbumin by sedimentation equilibrium and it is therefore extremely unlikely that the depression of apparent weight average molecular weight observed in glucose-containing insulin solutions is due to a high second virial coefficient associated with the presence of glucose. It was concluded that the addition of glucose does bring about some dissociation of polymeric forms of insulin at pH 7 and the question arises as to whether it is only the nonreacting aggregates of molecular weight higher than hexamer which are affected.

Dissociation of Insulin by Glucose at pH 2. Figure 6 shows the effect of glucose on the apparent weight average molecular weight of insulin as measured by sedimentation equilibrium at pH 2.0. It is well established that at pH 2 insulin solutions consist of a series of even-numbered polymeric forms in equilibrium with the insulin monomer (Jeffrey and Coates, 1966a,b). The species of lower molecular weight are highly favored under these acidic conditions and zinc does not play a part, not being bound by insulin at pH 2. It is seen from Figure 6 that the addition of glucose to insulin solutions has the same effect at pH 2 as at pH 7, namely to favor dissociation. The solid line in Figure 6 shows that it is possible to describe the concentration dependence of the weight average molecular weight of insulin at pH 2, ionic strength 0.05, on the basis of rapid reversible equilibria between the insulin monomer, dimer, and tetramer. The dashed line shows the dependence expected if the association constant for tetramer formation is put equal to zero. Since the apparent weight average molecular weights measured in the presence of 7.76% glucose lie below the latter curve it can be concluded that the glucose affects the dimerization reaction. Arguing on the basis of the results at pH 2 it seems probable that at pH 7 the effect of glucose in lowering the apparent weight average molecular weight of insulin at all concentrations results from decreased formation constants for all the reactions, not just on aggregated forms larger than the hexamer.

Conclusions

The weight average molecular weights and the weight average sedimentation coefficient measured for zinc-insulin at pH 7 show that zinc-insulin species of molecular weight greater than the insulin hexamer can remain in solution at least for the time required to conduct a sedimentation equilibrium experiment. This finding together with the observation from frontal analysis that the high molecular weight aggregates are not in rapid equilibrium with the rest of the system and the variable precipitation behavior noted on dialysis suggest that the differing results obtained for zinc-insulin solutions at pH values near neutrality by Cunningham *et al.* (1955), Fredericq (1956), and Grant *et al.* (1972) may be explained by the retention in solution of different amounts of the high molecular weight zinc-insulin aggregates. Some of the disagreement may also arise from the presence of unknown amounts of proinsulin in the samples used in the earlier studies. As stated earlier the amount of proinsulin (3-4%) in the insulin sample used in the present work is too low to account for the high molecular weights observed.

The use of frontal analysis in conjunction with sedimentation equilibrium experiments shows that insulin containing 2 g-atoms of zinc/hexamer dissociates to the monomer at pH 7. Comparison of the molecular weight dependence of zinc-insulin with that of zinc-free insulin at pH 7 indicates that the polymerization behavior of the two is qualitatively similar.

The addition of glucose to insulin solutions at pH 7 and 2 favors dissociation, as found previously for *Helix pomatia* hemocyanin (Brohult, 1940) and muscle phosphorylase *a* (Wang *et al.*, 1965). As with the latter two proteins, it is not possible to decide from the kinds of experiments reported whether the effect on the insulin polymerization is due to changes in the solvent structure or direct interaction of the glucose with the protein, or a combination of both. It might be enquired whether the ability of glucose to bring about increased dissociation of insulin has any physiological significance. This is unlikely if a change of solvent structure is involved because the concentration of glucose in serum would be too low to exert a significant effect. On the other hand, the molar ratio of glucose to insulin in serum is several orders of magnitude higher than that (1000:1) used in the experiments reported in this study and an effect could be expected if specific binding of glucose to insulin occurs.

It is perhaps worth noting in this connection that hexoses appear to be near the critical size for penetration into the three-dimensional structure of proteins (Giles and McKay, 1962) and thus a specific interaction cannot be ruled out.

Pekar and Frank (1972) have pointed out that on the basis of their calculated association constants for zinc-free insulin, the ratio of insulin monomer to dimer at the insulin concentration levels in serum would range from 26,000:1 (under glucose challenge) to 80,000:1 (fasting) providing strong evidence that the monomer is the active form of insulin. If the glucose effect demonstrated in the present work operates at physiological concentrations these ratios (particularly the lower one) would be even higher.

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Sequence Homology between Mitochondrial DNA and Nuclear DNA in the Yeast, *Saccharomyces cerevisiae*†

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ABSTRACT: Sequence homology between mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in a haploid, respiratory sufficient strain of the yeast, *Saccharomyces cerevisiae*, has been investigated by means of DNA-DNA and DNA-cRNA filter hybridization. [^{3}H]nDNA and mtDNA, both highly purified by CsCl density gradient centrifugation, were hybridized at low temperature with formamide for 48 hr. The resulting mtDNA-nDNA hybrids were examined for the fidelity of the hybrid formed (absence of nonhomologous AT or mismatched sequences) by thermal dissociation. The method was checked by comparison with *Clostridium* DNA-nDNA hybrids. Hybridization results with either high or low molecular weight nDNA, after correction for nonhomologous hybridized sequences, indicate that approximately two mtDNA genome equivalents of homology are present in the nuclear genome. This homology was shown not to result from cross contaminating DNA sequences. *In vitro* complementary RNA (cRNA), templated from highly purified mtDNA, was also hybridized with CsCl fractionated, high molecular weight nDNA in an independent assay. Identical amounts of homology were detected. Hybridization to gradients containing sheared nDNA resulted in a shift in buoyant density of homologous sequences. These results show that significant amounts of mtDNA is homologous with nDNA and that this mtDNA is inserted within nDNA.

Mitochondrial inheritance and transmission is generally assumed to be regulated by an extranuclear cytoplasmic system. The possibility of a nuclear control acting either independently or in concordance with a cytoplasmic system has not been ruled out, however. Wilkie has proposed (Wilkie, 1963, 1970) that in yeast a "master copy" of mtDNA,[1] associated with the nucleus, might have some role in mitochondrial biogenesis. Genetic evidence has yet to substan-

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[1] Abbreviations used are: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; cRNA, *in vitro* synthesized complementary mitochondrial RNA; SDS, sodium dodecyl sulfate; PVP, polyvinylpyrrolidone; SSC, 0.15 M NaCl-0.015 M sodium citrate.