Comparison of Protein Structure in the Crystal and in Solution. II. Tritium–Hydrogen Exchange of Zinc-Free and Zinc Insulin*

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ABSTRACT: The tritium–hydrogen exchange properties of zinc and zinc-free insulin were measured in the solid phase and in solution at pH between 2 and 9. Reaction in solution followed an EX_2 mechanism (Hvidt, A., and Nielsen, S. O., (1966), *Advan. Protein Chem. 21*, 287) for which the rate-limiting step is exchange of an exposed amide. Reaction in the crystal was consistent with an EX_1 mechanism, for which exchange is governed by the rate of opening of the native conformation. Differences between solution and crystal are in the motility of the protein, the average conformation being invarient to changes in pH and phase. pH-shift measurements were introduced to examine differences between solid and solution. Binding of zinc reduced the exchange rate by about 5% at pH 7, but it had no significant effect at acid or alkaline pH. The amorphous precipitate of zinc-free insulin behaved like the crystalline zinc protein. Aggregation did not affect exchange behavior in solution, suggesting that exchange should be used to probe changes in subunit structure during protein association. Similarly, the hydrogens of lattice contacts in the crystal did not contribute to the differences between crystal and solution. High ionic strength did not alter exchange in solution, but it increased long-time exchange in the crystal. There was no effect of storage of the protein. The net equilibrium isotope effect was smaller than 5%.

X-Ray analysis has been enormously successful in defining the crystal structure of proteins (Davies, 1967). The solution conformation, however, is the property of general interest, making it necessary to determine what changes might have been brought about by crystallization. Since rate and equilibrium parameters depend on molecular structure, an attractive approach to the problem is comparison of reactivity in the crystal with that in solution. Several experiments of this kind have been reported (*e.g.*, Doscher and Richards, 1963; Rupley, 1964; Chance *et al.*, 1966; Quiocho and Richards, 1966; Butler and Rupley, 1967). In these studies, the observed behavior usually has reflected a single group or region of the molecule, and other parts of the protein that might have undergone changes during crystallization were not seen. Since hydrogen exchange rates are determined by over-all conformation (Hvidt and Nielsen, 1966), it is therefore of interest that Haggis (1957), using infrared spectrophotometry, was able to measure exchange in protein films and crystals. He found that about one-third of the amide protons of crystalline hemoglobin did not exchange after 6 days at 20°; crystalline seal myoglobin exchanged fewer and crystalline ribonuclease exchanged about the same number of hydrogens as in solution. Although these data are difficult to interpret owing to incomplete control of the time of sampling and pH, they suggested that it would be possible to make a quantitative comparison of exchange in crystal and solution phases.

In order to explain differences in reactivity between crystal and solution in terms of intermolecular contacts or a change in conformation, it must be assumed that diffusion is not rate limiting and that the solvent inside the crystal is identical with that outside. Since hydrogen exchange measurements usually involve times of reaction longer than 30 sec, which is greater than the time required for equilibration of solvent throughout the solid phase (Rupley, 1964; Chance *et al.*, 1966; Quiocho and Richards, 1966), diffusion is unlikely to be a problem. The identity of interior and exterior solvent is supported by the invariance to crystallization of several equilibria (*e.g.*, Rupley, 1964; Butler and Rupley, 1967) and by the absence of a volume change during crystal formation (Krivacic and Rupley, 1968). These properties are expected in view of the open and gel-like structure of protein crystals, in which there are few intermolecular contacts and about half the crystal volume is solvent (Richards, 1963).

The approach of Hvidt and Nielsen (1966) will be applied to the interpretation of hydrogen exchange data. A protein is understood to fluctuate between a number of conformational states, the probability of any one state being governed by its free energy (Linderstrom-Lang and Schellman, 1959). The states accessible to a molecule range from compact to highly open conformations. The average conformation for most proteins is understood to be dominated by a compact structure, specifically the one most probable for a native molecule, and it is this that most physical techniques measure.

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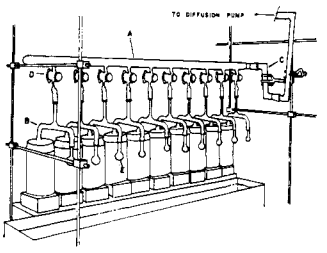

FIGURE 1: Lyophilization apparatus. See text for description.

Fluctuations in conformation can be characterized by both a *rate* of opening of the dominant (compact) structure and the *proportion* of the fluctuant conformation. "Motility" is used to describe either or both of these properties. The "conformation" of a protein, as the term is generally used, denotes average conformation. Because there can be a large change in motility without any measurable change in average conformation, the distinction between the two should be kept in mind throughout the following discussion. It must be emphasized that exchange measures both characteristics. A labile protein hydrogen must be exposed to solvent for exchange to occur, and because a large fraction of the amide protons are not (*e.g.*, Kendrew, 1962), it is evident that opening of the structure is necessary to obtain the observed extents of reaction.

Materials

Crystalline bovine zinc insulin (Eli Lilly and Co., lot no. QA 132Y) was stored in a tightly sealed container at 5°. Tritiated water (THO, 1 Ci/ml; New England Nuclear) was diluted to 4 mCi/ml and stored frozen; the same stock solution was used in all experiments. The solvent used for scintillation counting of the aqueous samples was a slight modification of that of Bray (1960): 4 g of 2,5-diphenyloxazole (scintillation grade, Packard), 0.2 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (scintillation grade, Packard), and 60 g of naphthalene (Practical, Eastman Kodak) were dissolved in *p*-dioxane (Matheson, twice refluxed over sodium, distilled, and stored in the dark); 100 ml of anhydrous methanol and 20 ml of ethylene glycol were added, and the solution was made up to 1 l. with *p*-dioxane; 25 ml of tetraethylammonium hydroxide (10% in water, Eastman Kodak) was added, and the solution was stored in the dark. Polyethylene counting vials (25 ml) were purchased from Packard. Conductivity water (resistivity 10^6 ohms) was obtained from a Heraeus Quartz Bi-distiller. All other reagents were of the highest purity commercially available. Constriction micropipets were obtained from Herr Pedersen, Copenhagen.

Methods

Crystals of insulin were grown from solutions of the protein in 0.1 M citric acid by the addition of 0.1 N NaOH until a slight permanent turbidity developed (Low and Berger, 1961). Small, well-formed crystals appeared within a few hours. After several days of crystallization at 5° the pH of the solution was adjusted over several weeks to that of minimum solubility (pH 5.5) by the addition of small aliquots of 50% NaOH. Repeated stirring during pH adjustment resulted in fragmented crystals no larger than 12 μ in length. Zinc-free insulin was obtained by dissolving 2 g of insulin in 25 ml of 0.025 N HCl and dialyzing at 3° with frequent changes of 0.025 N HCl (Laskowski *et al.*, 1960). The dialyzed protein was analyzed for zinc by the method of Kinnunen and Merikanto (1955) and was found to contain less than 0.04% zinc, to be compared with 0.43% zinc in the crystalline material. All protein preparations were stored at 5° and used over a 4–8-week period.

Insulin concentration was measured by absorbance at 280 mμ in 0.035 N HCl solution, using a Zeiss PMQII spectrophotometer. The extinction coefficient was based upon dry weight. Three to five 1-ml samples of a stock solution containing 28–40 mg/ml of protein were dried at 105° to constant weight (requiring about 6 hr). Twenty-five separate determinations, for both zinc and zinc-free insulin, led to an average molar extinction coefficient of ϵ 5220 $\pm$ 70 M^{-1}. There was no significant difference between the zinc-containing and zinc-free preparations, confirmed by the absence of a zinc-dependent difference spectrum at pH 2. Since insulin is insoluble at its isoionic point, the dry weights measured include a small contribution from counterions present (sodium or chloride, depending upon the direction in which the pH was adjusted from 5.5 in order to dissolve the crystals); correction was made for this in the calculation of ϵ, using the titration data of Tanford and Epstein (1954).

Lyophilization Apparatus. Hydrogen exchange was measured according to a modification of the method of Leach and Springell (1962), which requires exhaustive drying of the protein sample after exposure to a solution of tritiated water. The lyophilization apparatus consisted of a drying train evacuated by a two-stage, water-cooled, mercury diffusion pump (designed and built by Messrs. Nunamaker and Wise of this department), backed by a Cenco Hyvac pump, Model 91105. Except for brief maintenance shutdowns, the apparatus was operated continuously throughout the exchange studies. The train was connected to the vacuum system through a demountable safety trap, which during lyophilizations was kept at Dry-Ice–acetone temperature and after each run was cleaned of THO and mercury. The vacuum on the low-pressure side of the diffusion pump was monitored with a Delmar McLeod gauge (reading to 10^{-5} mm). The manifold (Figure 1A; 3-cm diameter) accommodated ten bifurcated arms (Figure 1B) and could be fixed in two positions by rotation of a glass elbow (Figure 1C) through 180°. After the protein was lyophilized until almost dry (train in the up position), the train was lowered 30-cm, bringing samples into the 60° bath where the last traces of water were removed. The bifurcated arms were connected to the manifold through a $^{14}/_{35}$ standard-taper joint and a 2-mm vacuum stopcock (Figure 1D). The donor or sublimation bulb containing the protein (Figure 1E) fitted into one $^{14}/_{20}$ female joint of the arm, and the acceptor bulb (kept in Dry-Ice–acetone; −60° at the position of the bulb) fitted into the other. The bulbs (2.5-cm

diameter) were constructed from $^{14}/_{20}$ joints; a 2.5-cm neck between joint and sample container was necessary in order to keep the grease seal intact during heating. High-vacuum stopcocks were used throughout the system and were lubricated with Apiezon N grease; the joint on the donor bulb that was near a 60° bath during part of the drying was lubricated with Apiezon T; Apiezon M was used for all other parts of the system.

Procedure. Zinc insulin crystals, grown from solutions 0.1 M in citrate, were harvested by centrifugation and resuspended in conductivity water, and the process was repeated. Suspensions of washed crystals were adjusted before reaction to the proper concentration (usually 11.5 mg/ml) and pH (Leeds and Northrup meter, Model 7664, standardized with pH 6.5 and 4.0 buffers). For studies with zinc insulin in solution at pH 6.7 or above, crystalline suspensions had to be titrated to pH above 10 before they dissolved and could be adjusted to the appropriate pH. Solutions of dialyzed zinc-free insulin were diluted to the proper concentration, and then 6 N HCl or concentrated NaOH (50%) was added to obtain the desired pH. Routinely, after the foregoing adjustments, all samples were stored at 3° for 12–18 hr; several samples that had been stored for only 20 min exhibited the same exchange as those that had been treated in the usual manner. The solution was placed in an appropriate temperature bath 10 min before the addition of THO that started exchange (0.5 ml of 4 mCi/ml of THO per 3.5 ml of protein solution). The solution was stirred about 30 sec and an aliquot (routinely 0.5 ml) transferred into a donor bulb and frozen at −60°, using vigorous swirling to obtain a thin layer of frozen solution. The bulb was capped and stored in a container of finely chopped Dry Ice. The process was repeated until three or four samples had been collected over 2–3 min. These were called zero-time samples, even though they reflect exchange over a several-minute period. In most experiments the reaction mixture was stirred continuously; crystal suspensions were stirred during sampling as well, to maintain homogeneity.

Loss of protein was prevented by a metal net placed on top of the ground-glass joint of the donor bulb (Linderstrom-Lang, 1955b). For a few of the experiments with soluble or amorphous insulin, fragmentation of the protein sheet occurred during drying. The material that flew from the bulb was trapped on the net, and after the protein was almost dry it was returned by gentle tapping of the arm. Crystalline protein behaved differently in that extensive fragmentation occurred a few minutes after the beginning of distillation, and as much as 25% of the protein was carried past the net in the vapor stream.

During the time required to establish vacuum, distillation was prevented by keeping the donor bulbs at −60°. In order to start lyophilization after operating pressure (5×10^{-4} mm) was reached, the manifold train was lifted slightly, the arms were rotated 180°, and the train was lowered to bring the acceptor bulbs into the Dry-Ice–acetone bath. After 90 min the train was lowered again to bring the donor bulbs into the 60° bath. Drying at high temperature was necessary to remove the last trace of water (Linderstrom-Lang, 1955b), and it was found that 5.5 hr at 60°, a convenient length of time, gave the same results as longer heating. Acceptor bulbs were kept at −60° throughout drying, except for the brief periods during which the train was lowered. After 7-hr lyophilization each bulb was removed from the vacuum system and the protein was dissolved immediately by adding 2 ml of 0.035 N HCl (any delay resulted in exchange with water vapor in the air; Hvidt *et al.*, 1960). Determinations were made of the pH of the reaction mixture and of the protein concentration and tritium content of the reaction mixture and samples. Tritium was analyzed as follows: 1 ml of protein solution was transferred into 20 ml of scintillation solvent, the solution was stirred, and 15 ml was transferred into a counting vial. The samples, containing approximately 15,000 cpm, were counted at −3° in a Mark I or Model 625 Nuclear-Chicago liquid scintillation spectrophotometer, using 4- or 10-min count times. A quenching correction was determined by recounting the samples after adding, as internal standard, 0.1 ml of tritiated water containing approximately 200,000 cpm.

Three aliquots were taken for each time of sampling, the measurements showing an average deviation of approximately 1% in hydrogens exchanged, except for a slightly greater error at zero time. The average deviation between experiments was larger, between 1 and 2%. The data presented are average values, usually over several experiments (the average deviation is indicated by vertical bars in figures, and the absence of a bar means the deviation was less than the diameter of the symbol).

Following each lyophilization, the bifurcated arms and metal nets were freed of grease by soaking in chloroform. The apparatus, including the elbow, was taken apart after four to six lyophilizations, freed of grease, cleaned with concentrated HNO_3, and baked along with the arms for 1 hr at 600°. The donor bulbs, micropipets, reaction vessels, and the test tubes used in determinations of the concentration of the lyophilized material were cleaned in a NaOH–ethanol–water solution for 24 hr and profusely rinsed, first with tap water and then with deionized water. The acceptor bulbs and other test tubes were soaked overnight in detergent, and then scrubbed and rinsed.

Retention of THO by Salt. Removal of THO from NaCl was incomplete under the lyophilization conditions of these experiments. The average of nine determinations on solutions of 0.58–5.8% NaCl was 345 ± 50 cpm retained/% salt. At even the highest concentrations used, the salt contributed less than 15% of the total count rate (protein plus salt), and the error introduced by variation in the salt blank is therefore small (generally less than 1%). The water adsorbed on the salt corresponds to 7 mmoles/mole of NaCl.

Absorption of Carbon Dioxide at High pH. Buffers were not used because of the correction that would be necessary for their exchangeable hydrogens. Consequently, pH drifts of 0.1–0.2 unit occurred during exchange, likely reflecting, in part, absorption of CO_2. Reaction mixtures of insulin at pH 9 suffered substantially larger changes, that were reduced to less than 0.3

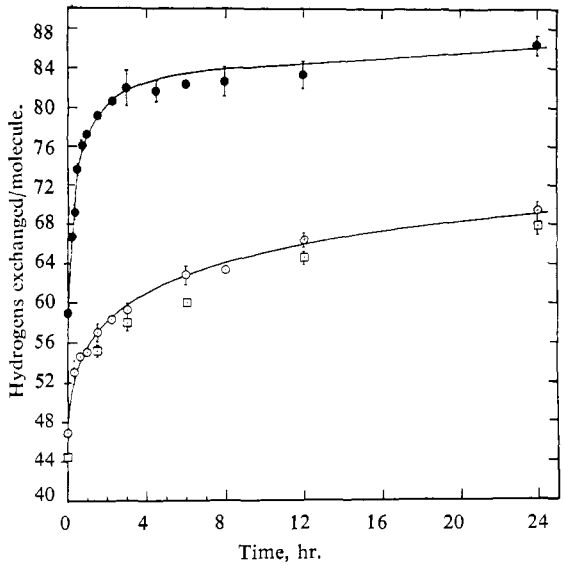

FIGURE 2: Exchange of zinc (⊙) and zinc-free (⊡) insulin at 0°, pH 3.2–3.6, and exchange of zinc insulin (●) at 38°, pH 3.1–3.3.

pH units by maintaining a nitrogen atmosphere (the solution was exposed to air only during sample withdrawal, and after this it was flushed with nitrogen for 15 min).

pH-Shift Experiments. In several experiments the pH of the reaction mixture was altered during exchange. In shifts from pH 5.5 to 3, crystals were dissolved either rapidly, by adding a small aliquot (10–20 μl) of 6 N HCl directly to the crystal suspension, or slowly, by adding over approximately 10 min 1 N HCl from a microsyringe, using a fine polyethylene delivery tube that rested on a magnetic stirring bar at the bottom of the reaction vessel. Because of the effectiveness of base catalysis of the exchange, in shifts from pH 3 to 7 it was necessary to avoid locally high concentrations of titrant, and only slow delivery of 0.2 N NaOH (over about 10 min using the microsyringe) led to satisfactory dispersion of the base.

Calculation of Hydrogens Exchanged. Calculation of the number of hydrogens exchanged is based on two assumptions (Linderstrom-Lang, 1955b): first, that the atom fraction of tritium incorporated into the protein is equal to the atom fraction of isotope in the solvent, *i.e.*, there are no equilibrium isotope effects (see Discussion); secondly, that exchange stops when the sample is frozen. The hydrogen atoms exchanged per mole of protein (N) is given by eq 1, where C_R = counts

$$N = (C_R - C_B)I_R \frac{\epsilon}{A(C_{RM} - C_B)I_{RM}} \frac{110}{} \quad (1)$$

per minute per milliliter of protein solution, C_B = counts per minute for a blank vial containing all components except protein, I = quenching correction determined by internal standard addition (range 0.97–1.04), ϵ = molar extinction coefficient of the protein, A = absorbance of the protein solution, C_{RM} = counts per minute per milliliter of reaction mixture, and 110 = gram-atoms of hydrogen per liter of water. In exchange studies with

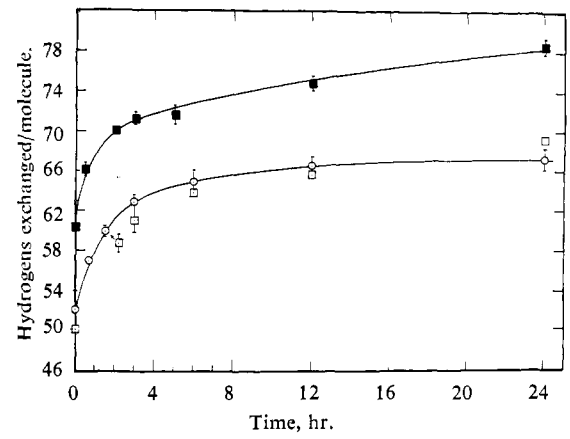

FIGURE 3: Exchange of crystalline (⊙) and amorphous (⊡) insulin at 0°, pH 5.4–5.5, and exchange of amorphous insulin (■) at 38°, pH 5.5–6.0.

salt present the count rate of a salt blank was subtracted from C_R.

Concentration Correction for Lyophilized Protein. The absorbance of insulin was increased slightly by lyophilization, determined by comparing the absorbance of the solution of each lyophilized sample with that of the reaction mixture. Since the absorbance enters the calculation for the number of exchanged hydrogens, results were corrected by determining for the set of samples in a given experiment the average difference between the absorbance of the lyophilized and unlyophilized protein, and then applying this average correction to the absorbance of each sample. Because loss of protein occurred during lyophilization of zinc insulin crystals, an average correction was used for these experiments, 3.5 ± 0.8% (found in studies with soluble zinc-free and zinc insulin, and with amorphous zinc-free insulin). Leach and Hill (1963) have found that the extinction coefficient of ribonuclease decreased in lyophilization by approximately 15%, which they attributed to a volatile impurity. Hnojewyj and Reyerson (1961) have observed that exhaustive drying under high vacuum can alter the conformation of a protein.

Kinetic Analysis. Exchange data were analyzed in accord with the following rate equation (Hvidt and Nielsen, 1966) (eq 2). n_0 is the total number of exchange-

$$n_0 - n(t) = \sum_i n_i e^{-k_i t} \quad (2)$$

able hydrogens; $n(t)$ is the number exchanged at time t; n_i is the number which exchange at a rate defined by the first-order constant, k_i. Experimental exchange values were plotted as a function of time, and a smooth curve was drawn through the data. At pH 6.7 or higher, complete exchange was reached in 24 hr; at lower pH, the exchange at infinite time was determined by constructing plots of the rate of exchange as a function of the number of exchanged hydrogens and then extrapolating to zero rate. Values for the hydrogens exchanged taken from the experimental progress curve were subtracted from the infinite-time value, and the logarithm of the hydrogens unexchanged was plotted

against time. Linear extrapolation of the long-time portion of the semilog plot to zero time gave the number of protons in the slowest exchanging class, and the rate constant for their exchange was determined from the slope of the line. The hydrogens contributed by this class to the exchange were then substracted from the progress curve. The resulting new set of exchange values, representing all the faster exchanging classes, were replotted, and the above process was repeated. In this fashion two or three classes of slowly exchanging hydrogens were defined. It must be noted that analyses of this kind are approximate and limited by the accuracy of the data; in particular, it is unlikely that the exchangeable hydrogens of a protein are cleanly divided into such a small number of classes.

Results

The hydrogen exchange of zinc and zinc-free insulin was measured at 0° between pH 2 and 9.5. At least two runs were made with zinc insulin at each pH, and the reaction mixture was sampled at various times up to 24 hr. In most studies of zinc-free insulin, samples were taken only at zero time, 3.0 hr, and 24 hr. The insulin monomer (mol wt 5733) was the basis in calculation of the data presented.

pH 2.1, 0°. Reproducible exchange measurements could not be obtained at pH 2 in that, except for one run (Table I), internal agreement between replicate analyses within a run was poor. A. Hvidt (personal communication) has suggested that at this low pH a substantial amount of HCl might vaporize during lyophilization, leading to modification of the protein. In support of this idea, it was noted that protein flaked during drying at pH 2.1, in contrast to the smooth sheet formed from soluble insulin at other pH values.

TABLE I: Hydrogen Exchange of Zinc Insulin in Solution, pH 2.15, 0°.

Time (hr)	Hydrogens Exchanged/ Molecule
Zero time	59.4 ± 0.6
0.33	62.1[a]
1.5	62.8[a]
3.0	67.7 ± 0.7
6.0	71.8 ± 0.1
12	75.2 ± 0.9
24	79.5 ± 0.8

[a] Single value.

pH 3.1–3.6, 0 and 38°. Figure 2 gives exchange data for zinc and zinc-free insulin at 0° and pH 3.2–3.6. Only a slight difference between these proteins was observed. Data for zinc insulin at 38° and pH 3.1–3.3 are also shown in Figure 2.

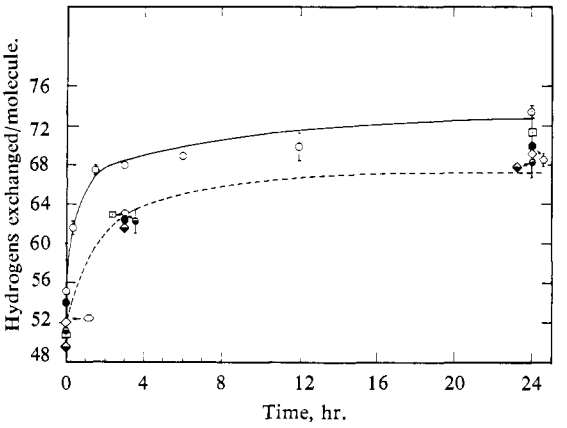

FIGURE 4: Exchange of crystalline and amorphous insulin at 0°, pH 4.6–6.6. All values are corrected for the contribution of soluble insulin to the exchange. Crystalline zinc insulin: (⊙) pH 6.6, (◈) pH 6.0, (○) pH 6.25, (●) pH 6.4–6.5, and (◒) pH 4.6–4.7. Amorphous zinc-free insulin: (⊡) pH 6.4 and (⧫) pH 4.9. The dashed curve is from Figure 3 and represents data obtained at pH 5.5 for crystalline zinc and amorphous zinc-free insulin.

pH 4.6–6.6, 0 and 38°. Between pH 4 and 6.6 zinc insulin crystallizes and zinc-free insulin forms an amorphous precipitate. The solubilities of the crystalline and amorphous protein at 0° were determined in each exchange experiment shortly after the zero-time and 24-hr samples had been taken; an aliquot of the protein suspension was centrifuged at 5° and protein concentration was determined in the supernatant. The solubilities of crystalline and amorphous insulin at pH 4.5 and 5.5 agreed with those of zinc insulin in 0.1 N KCl at 25°, determined by Fredericq and Neurath (1950). At pH 6–6.5 the solubility was about half of that found in the earlier work. The lack of agreement between the two sets of data at pH above 5.5 may reflect the different ionic strengths and temperatures used. The solubility at pH 6.6 was 1.2 mg/ml, about 12% of the total protein, and at this pH, soluble protein might therefore make a substantial contribution to exchange.

Figure 3 gives exchange data for crystalline zinc and amorphous zinc-free insulin at 0° and pH 5.4–5.5. The low solubility of insulin near pH 5.5 (0.1 mg/ml or less)

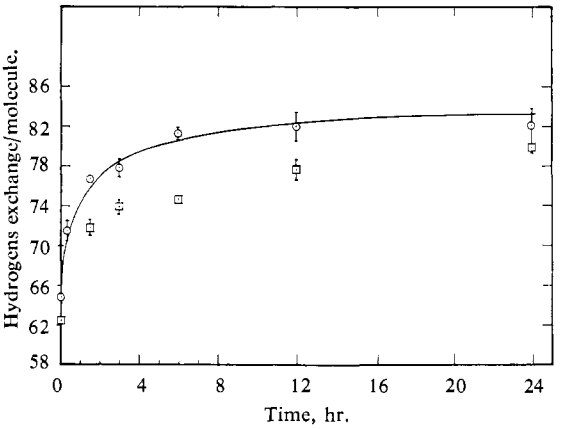

FIGURE 5: Exchange of zinc (⊙) and zinc-free (⊡) insulin at 0°, pH 6.75–7.0.

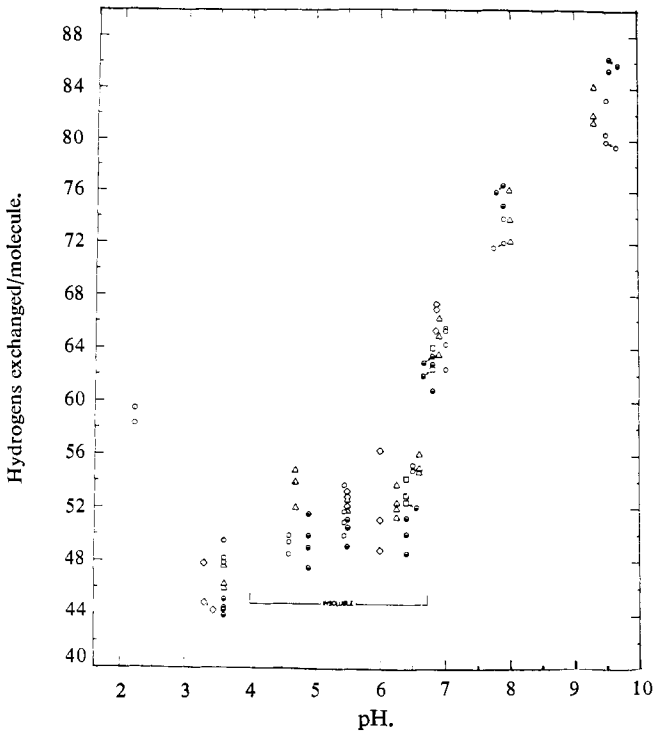

FIGURE 6: Zero-time exchange at 0° for zinc and zinc-free insulin as a function of pH. Open symbols refer to zinc-containing protein and half-darkened ones to zinc free. The data are not averaged, but are given for each sample analyzed. A distinguishable symbol is used for each run. The same symbols are used for Figure 7.

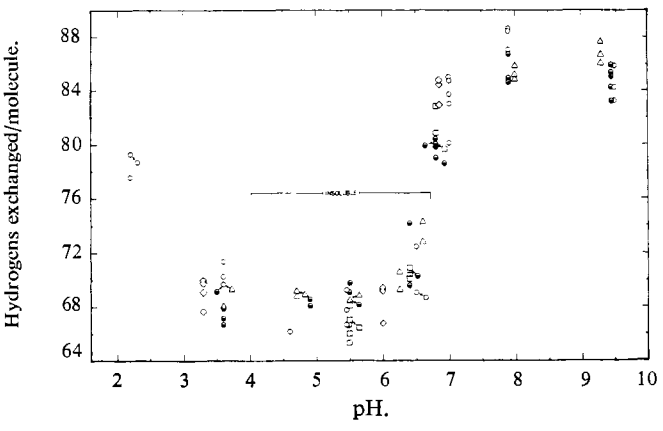

FIGURE 7: Exchange (24 hr) at 0° for zinc and zinc-free insulin as a function of pH. Symbols as in Figure 6.

ensures that exchange in this pH region is of the crystalline protein. Crystalline insulin at 38° displayed unusual behavior in that the pH of the suspension increased 0.5–1 unit in 24 hr, and during lyophilization as much as 70% of the protein was carried into the acceptor bulb by the vapor stream. Exchange results were not only scattered for any one time, but after 6 hr the number of exchangeable hydrogens decreased. Better data at this temperature were obtained for amorphous zinc-free insulin (Figure 3), but even in this case the pH of the suspension increased by 0.5 unit in 24 hr. The increase occurred with samples maintained under nitrogen and equilibrated at 38° overnight before the addition of tritiated water. The effect is unexplained. The instability of crystalline insulin at room temperature has been noted by Low *et al.* (1966), who reported that insulin crystals containing UO_2 or Hg rapidly deteriorate according to crystallographic criteria; metal-free crystals did not behave in this way, and the damage could be reduced by cooling the metal-containing crystals to 0°.

The exchange of crystalline and amorphous insulin at pH 4.6–4.9 (Figure 4) is within experimental error the same as that at pH 5.5. Figure 4 also contains data for exchange between pH 6 and 6.6. Since in this region the solubility of insulin becomes appreciable, correction was made for the soluble protein by assuming it exchanged as at pH 6.8. Between pH 6.0 and 6.5, exchange of the crystalline protein was the same as that found under more acid conditions. At pH 6.6, however, three to five more hydrogens were exchanged. At this pH the crystalline protein was 12% soluble and lyophilized as a smooth sheet with no fragmentation and loss of material, behavior characteristic of the soluble protein and unlike that of the crystalline protein at pH 6.5 (7% soluble) or lower pH. It is important to note that the almost identical exchange behavior of crystalline zinc and amorphous zinc-free insulin stands in contrast to their different lyophilization properties, which suggests that exchange was not affected by or dependent upon the drying process. Consequently, the greater exchange at pH 6.6 compared to pH 6.5 cannot be simply explained. It may be that the rates of dissolution and reformation of the crystals are strongly pH dependent; the protein in solution at pH 6.6 might "turn over" during the time of exchange, which might not happen at lower pH. Alternatively, the conformation of the crystalline protein may change with pH as the region of crystal instability is approached.

pH 6.75–7.0, 0°. The exchange of zinc and zinc-free insulin in solution at 0° and pH 6.75–7 is shown in Figure 5. It is only in this pH range that a substantial effect of metal binding can be seen. The higher values found for zinc insulin might reflect the retention of THO by the metal, which suggested the following experiment. Zinc-free insulin was allowed to react with THO at pH 6.8, samples were taken after 24 hr, a small aliquot of $ZnCl_2$ solution was added to give 0.5 g-atom of zinc/mole of insulin (approximately that in the zinc insulin), and samples were taken after reaction for 0.5 hr. Because of the acidity of the $ZnCl_2$ solution (10^{-2} M HCl), the final pH was 6.2, and the insulin was largely precipitated. The zinc-free protein exchanged 82.7 ± 2.2 hydrogens, while the zinc-containing insulin exchanged 82.1 ± 0.7 hydrogens.

An effect of crystallization on exchange behavior is evident on comparing the data of Figures 4 and 5. This is also shown in Figures 6 and 7, in which zero-time and 24-hr values, respectively, are presented as a function of pH. The transition from solid-to-solution phase is accompanied by a substantial increase in exchange. Ignoring the pH 6.6 data, upon solubilization near neutral pH there is an increase of 10–12 in the number of protons reacting. The minimum in exchange rate near pH 3 (Figures 6 and 7) has been observed generally for proteins and polypeptides (Hvidt and Nielsen, 1966).

TABLE II: Hydrogen Exchange of Zinc and Zinc-Free Insulin in Solution, pH 9.3–9.5, 0°.

Time (hr)	Hydrogens Exchanged/Molecule: Zinc Insulin	Hydrogens Exchanged/Molecule: Zinc-Free Insulin
Zero time	82.3 ± 1.3	85.9 ± 0.4
0.33	86.1 ± 0.2	
1.5	83.1 ± 0.2	
3.0	84.6 ± 1.7	86.3 ± 0.2
6.0	84.3 ± 0.8	
12	83.1 ± 0.5	
24	85.8 ± 1.0	84.9 ± 1.1

pH 7.9–8.0, 0°. Exchange is rapid at pH 8 and essentially complete after 3 hr (Figure 8).[1] At this point it is appropriate to discuss the number of exchangeable hydrogens contained by insulin. There are 92 in the fully protonated molecule at pH 2 (Ryle *et al.*, 1955). The titration curve of bovine insulin (Tanford and Epstein, 1954) can be used to determine the number of hydrogens that can exchange at higher pH, which are 90, 84, 82, and 81 at pH 3, 7, 8, and 9. The theoretical limit is apparently exceeded by four hydrogens at pH 9 (see below) and by five hydrogens at pH 8 (determined by extrapolation to $dn/dt = 0$). The excess exchange might reflect fractionation of the tracer isotope between insulin and solvent (an equilibrium isotope effect). As an alternative explanation, the titration of insulin was at 25° and ionic strength 0.075, but exchange was followed at 0° in solutions of low ionic strength (≤ 0.01). Above the isoionic point (about pH 5.3 for insulin) increased ionic strength would lead to greater dissociation of hydrogen ions (Tanford, 1961). An increase in temperature would have the same effect in the alkaline region. At pH 3 and 38° the value for complete exchange was 89 (by extrapolation to $dn/dt = 0$), in accord with the estimate from titration data and the expected effect of ionic strength and temperature. Hvidt and Nielsen (1966) have reviewed the evidence that full exchange is obtained under these conditions. The total number of exchangeable hydrogens therefore was specified for pH at which it could not be measured by interpolating between 3 and 8–9 (*e.g.*, 88 for pH 5.5 and 87 for pH 7).

pH 9.0–9.5, 0°. At pH 9–9.5 there was no significant time dependence of the exchange (Table II), which apparently was complete at zero time, a conclusion supported by the comparison between 0 and 25° of the total number of exchanged hydrogens of zinc-free insulin. At the lower temperature, 85.1 ± 0.7, and at 25°, 85.2 ± 0.3 hydrogens had reacted (the difference in temperature should lead to no more than one or two fewer exchangeable hydrogens at 25° than at 0°; Cohn and Edsall, 1943).

[1] In one experiment in which a nitrogen atmosphere was not maintained, the pH of the reaction mixture decreased to 7.4 after 24 hr, but there was no effect on exchange, perhaps because this was complete within the early stages of reaction.

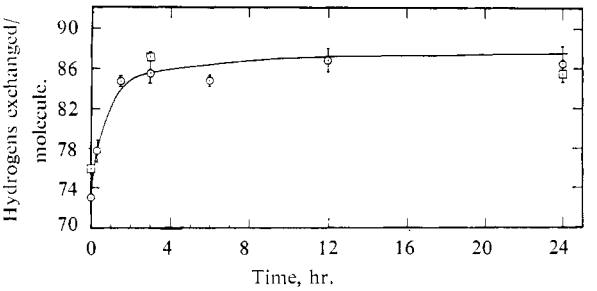

FIGURE 8: Exchange of zinc (⊙) and zinc-free (⊟) insulin at 0°, pH 7.9–8.0.

Aggregation and Ionic Strength. The aggregation of insulin is a function of pH and ionic strength. Under most conditions the average particle consists of two to six monomeric units (Reithel, 1963). On the other hand, insulin crystals may be considered aggregates of thousands of these subunits. If the ionizable and polar side chains of proteins are exposed to solvent, they exchange their hydrogens in times less than 1 min (Hvidt and Nielsen, 1966). In the transition from solution to crystal, a fraction of these surface groups must be buried in the intermolecular contacts that generate the crystal lattice. The effect of this upon hydrogen exchange was considered through the following experiments. First, crystalline protein was dissolved at low pH after 24 hr of exchange at pH 5.5 (Table III). Immediately after dissolution there was no increase in the hydrogens exchanged, and at long times the exchange did not go above the value found for solutions maintained entirely at pH 3. Secondly, the average molecular weight of insulin in solution at pH 2.65 and 25° is 2.5 times greater in 0.20 M KCl than in 0.05 M KCl (Steiner, 1952). Exchange in the presence of comparable concentrations of salt was measured at 0 and 25°, at pH near 3 (Tables IV and V). The only significant effect of ionic strength was seen for the zero-time exchange at 0°, which was four hydrogens greater in the presence of 0.24 M NaCl. It should be noted that owing to the high initial rate of exchange the zero-time values are the least accurate. At 0° soluble insulin was partially precipitated in 0.24 M NaCl (30% soluble after 16 hr); the lyophilization properties were characteristic of soluble protein, *i.e.*, fragmentation of the protein sheet did not occur during distillation. Finally, exchange was measured for crystal and solution in 0.87 M NaCl at pH 6–7 and 0°. Comparison of the results (Table VI) with data for low ionic strength show that there was no appreciable effect of 0.87 M NaCl on insulin in solution at pH 7 (Table VI), except for a small increase in the zero-time value. Exchange in the crystal at this high salt concentration is difficult to interpret, owing to the effect of ionic strength on the solubility. Although the pH range of insolubility was broader in the presence of salt (*i.e.*, some precipitation at 0° was observed at pH 2.9 in 0.24 M NaCl and at pH near 7 in 0.87 M NaCl), the solubility of the protein was increased by salt (in 0.87 M NaCl at pH 6.4, the solubility was 3.3 mg/ml; in the absence of the salt,

TABLE III: Effect of Change in Phase and pH on the Exchange.[a]

	Hydrogens Exchanged/Molecule					
	Expt 1	Expt 2	Expt 3	Expt 4	Av of All Values	Determined in Expt without Shifts[b]
pH in crystal	5.4	5.5	5.5	5.3		
pH in solution	2.7	2.7	2.6	2.9		
24-hr crystal	67.6 ± 0.7	65.3 ± 0.3	66.8 ± 0.0	66.5 ± 0.4	66.5 ± 0.6	67.2 ± 1.1
Zero-time solution	68.0 ± 1.3	68.5 ± 0.9	67.1 ± 0.9	65.6 ± 0.7	67.3 ± 0.9	
3-hr solution	70.3 ± 0.6	70.5 ± 0.4			70.4 ± 0.5	
24-hr solution		71.5 ± 0.8		68.9 ± 0.5	70.2 ± 1.3	69.3 ± 0.9

[a] Crystalline zinc insulin was exchanged for 24 hr at pH near 5.5, then dissolved at pH near 3 and the exchange followed for 24 hr. [b] Values taken from Figures 2 and 3.

the solubility was 0.7 mg/ml). After correction of the exchange for soluble protein, values for crystalline insulin in 0.87 M NaCl (Table VI) differed significantly from those found for low salt, in particular at zero-time and 24 hr. Results for short times of exchange contain the largest correction for soluble protein, and interpretation is difficult. However, salt did substantially increase the extent of exchange in the crystal at 24 hr, conditions under which the correction for soluble protein was small. It might be relevant that in the absence of salt an increase in the exchange rate of the crystalline protein was observed as the pH approached the region of solubility (at pH 6.6; discussed above).

Shift during Exchange from pH 3 to 7. Insulin in solution at pH 3.3 was treated with THO for 24 hr, and samples were taken. Then 0.2 N NaOH was added slowly from a microsyringe until the precipitate disappeared (pH 6.6). Samples were taken immediately, and again at 3 and 24 hr (expt 1, Table VII). In expt 2, THO was added to an insulin solution at pH 3.2 and the solution was immediately adjusted as above, *i.e.*, less than 1-min exchange at the most acid pH. Samples were taken as in expt 1.

Hydrogen Exchange during Lyophilization. The possibility of exchange during the drying of insulin was explored. Tritiated water was added to a zinc insulin solution at 0° and pH 3.2; six zero-time samples were withdrawn, immediately frozen, and separated into two sets. The temperature of three samples was kept at −20° for the first 40 min of lyophilization, then at −17° for 20 min, and finally at −8° for 40 min. After 40 min at −20° the protein appeared dry. The other set of three samples was lyophilized with no external control of temperature (normal drying conditions). All six samples were then dried as usual for another 5.5 hr at 60°. The samples kept at −20° for 40 min exchanged 47.1 ± 1.5 hydrogens; the others that had been treated normally exchanged 48.4 ± 1.0 hydrogens.

Discussion

Aggregation and Exchange. It was expected that the pH-dependent aggregation of insulin might affect hydrogen-exchange rates in two ways: by changing the average conformation and motility of the interacting subunits, or by retarding the exchange of labile hydrogen atoms that are freely exposed in the subunit but are involved in intermolecular contacts in the complex. Changes in aggregation were found to be unimportant. First, increased ionic strength favors association (Oncley *et al.*, 1952) but had little or no effect on exchange in solution (Tables IV–VI). Secondly, when the crystal

TABLE IV: Effect of Salt on the Exchange of Insulin in Solution at 25°.

	Hydrogens Exchanged/Molecule	
Time (hr)	No Salt Added pH 3.0	0.20 M NaCl pH 3.0
Zero time	49.8 ± 1.3	51.0 ± 0.6
3	72.9 ± 0.4	70.9 ± 1.0
24	83.9 ± 1.3	82.0 ± 1.0

TABLE V: Effect of Salt on the Exchange of Insulin in Solution at 0°.

	Hydrogens Exchanged/Molecule	
Time (hr)	No Salt Added pH 3.3–3.6	0.24 M NaCl pH 2.9
Zero time	46.9 ± 1.3	51.0 ± 1.3
3	59.2 ± 0.6	59.8 ± 1.4
24	69.3 ± 0.9	71.0 ± 1.2

TABLE VI: Effect of Salt on the Exchange of Insulin in Solution and in the Crystal at 0°.

	Hydrogens Exchanged/Molecule					
	Solution		Crystal			
			0.87 M NaCl, pH 6.4[b]		No Added Salt	
Time (hr)	0.87 M NaCl pH 7.0	No Added Salt pH 6.7–7.0[a]	Uncor	Cor[c]	pH 6.6[d]	pH 6.0–6.5[d]
Zero time	69.2 ± 0.6	64.8 ± 1.2	54.3 ± 1.7	48.9	55.1 ± 0.5	52.9 ± 1.4
3	78.1 ± 1.9	77.8 ± 0.9	68.6 ± 0.9	64.5	68.1 ± 0.2	62.4 ± 0.3
24	82.4 ± 0.3	82.0 ± 1.8	78.7 ± 1.6	78.0	73.5 ± 0.7	69.8 ± 1.0

[a] Values taken from Figure 5. [b] 33% soluble after 6-hr reaction. [c] Corrected for soluble protein (see text). [d] Values taken from Figure 4.

TABLE VII: Effect of Change in pH from 3 to 7 on the Exchange.

	Hydrogens Exchanged/Molecule		
	Expt 1[b]	Expt 2[c]	Exchange Determined in[a] Expt without Shifts
pH at start	3.3	3.2	
pH at end	6.6	6.8	
24 hr, pH 3	67.3 ± 1.3		69.3 ± 0.9 at pH 3.3
First sample, pH 7 (≤ 10 min)	69.3 ± 0.7	69.4 ± 0.7	64.8 ± 1.2 at pH 6.8
3.0 hr, pH 7	75.4 ± 0.2	78.3 ± 0	77.8 ± 0.9 at pH 6.8
24 hr, pH 7	81.2 ± 0.3	81.4 ± 0.3	82.0 ± 1.8 at pH 6.8

[a] Values taken from Figures 2 and 5. [b] Expt 1: A solution of zinc insulin was exchanged for 24 hr at pH 3.3 and 0° and then the solution adjusted over 10 min to pH 6.6. [c] Expt 2: THO was added to a solution of zinc insulin at pH 3.2 and 0° and the solution adjusted immediately over 10 min to pH 6.8.

after 24-hr exchange at pH 5.5 was dissolved at pH 3[2] (Table III), there was no burst of exchange as would be expected if labile hydrogens had been immobilized in intermolecular contacts. Thirdly, the hydrogens exchanging instantaneously (by the time of the first measurement) at pH near 3 are likely to include all side-chain and some amide hydrogens (see below). These are most likely atoms on the surface of the protein (Hvidt and Nielsen, 1966) and should include hydrogens participating in intermolecular contacts in the crystal. Consequently, the large zero-time value for the crystal, five hydrogens greater than in solution at pH 3 (Figures 2 and 3), is evidence that lattice contacts do not necessarily alter exchange properties of the groups involved.

The lack of an effect of aggregation suggests that contacts can be disrupted sufficiently frequently for immeasurably fast exchange of the labile hydrogens contained in them. This is not unexpected, considering the high exchange rates of side-chain groups (Hvidt and Nielsen, 1966); these rates might be reduced by several orders of magnitude and still be too fast to measure by the techniques usually applied to proteins. In turn, this suggests a particular application of exchange measurements. They appear able to measure the structural properties of subunits in isolation from the association process, *i.e.*, exchange rates show the effect of association upon the internal structure as distinguished from an effect upon exposed (surface) groups.

Other measurements have indicated, in accord with the exchange properties of insulin, that significant changes in average conformation do not occur in association of the dimer to higher polymers (these are the only equilibria that can be important at the relatively high protein concentrations used in these exchange studies; Oncley *et al.*, 1952; Steiner, 1952). First, the optical rotation of zinc insulin between pH 7.5 and 10, conditions under which it is highly associated, was as low as that found for the dimer at pH 2 (Schellman, 1958). Secondly, no changes in optical density were observed in association of the insulin dimer into higher aggregates (Rupley *et al.*, 1967). Thirdly, the nuclear

[2] Throughout the discussion, the pH at which exchange was carried out will be indicated, where context permits it, by rounded values; *e.g.*, pH 3 for the range pH 2.6–2.9 in Table III.

magnetic resonance spectrum of insulin indicates that aggregation does not interfere with internal motions of the molecule (Kowalsky, 1962).

Exchange in Solution. Exchange of insulin in solution was followed below pH 3.6 and above pH 6.7, approximately the solubility limits in solutions of low ionic strength. Insulin exhibits under these conditions the general exchange behavior found for most proteins, *i.e.*, for a given time of reaction the number of protons unexchanged decreases with increasing pH (Figure 6) and temperature (Figures 2 and 3). This behavior may reflect either a pH-dependent change in average conformation or the influence of protein motility (Hvidt and Nielsen, 1966).

The similarity of the optical rotation at pH 2 and 7–10 (Schellman, 1958) suggests that over this range there is no substantial change in conformation of the insulin subunit. The conclusion is supported by the following experiment. If conformational differences exist between insulin molecules at pH 3 and 7, they should be brought out by changing from one pH to the other during exchange. The results of such a pH shift were presented in Table VII and are summarized in the following observations. First, the hydrogens that do not exchange in 24 hr at pH 3 are nearly equal in number (20 = 89 − 69) to those that are slowly or nonexchanging at pH 7 (22 = 87 − 65).[3] Secondly, comparison of the first samples taken after the shift with zero-time samples at pH 7 suggests that four to five of the hydrogens that exchange slowly at pH 3 might exchange at a measurable rate at pH 7. Some of these can be accounted for by the 2-hydrogen difference between the numbers given under the preceding point. The first sample after adjustment of the pH is, however, not a true zero-time sample (pH adjustment took 10 min, and there may have been locally high concentrations of base); this can plausibly explain the remaining discrepancy. Finally, the set of exchanging hydrogens at pH 3 does not include hydrogens that react after 3 hr at pH 7. To summarize, nearly all of the approximately 20 hydrogens that are slowly exchanging and presumably buried at pH 7 are also buried at pH 3. It is these hydrogens, representing the set of groups that are most deeply buried, that because of the cooperative character of the protein structure should be most strongly affected by any substantial changes in conformation.

As a consequence of the foregoing considerations, a mechanism of hydrogen exchange that does not involve changes in average conformation would be the most satisfactory explanation for insulin. Discussion of the data is simplified by having in mind a specific model for this kind of exchange process, and the most appropriate seems to be that of Hvidt and Nielsen (1966), which is based upon conformational fluctuations (motility) of the protein. These authors suggest that

$$\mathrm{N} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{I} \xrightarrow{k_3} \text{exchange} \qquad (3)$$

where N is a folded conformation in which a given hydrogen is shielded from exchange, and I is an unfolded conformation in which this hydrogen is fully exposed and exchanges at a rate characteristic of a free group. Two limiting cases for this reaction scheme were specified as EX_1 and EX_2. In the EX_1 mechanism the exchange rate of the mth hydrogen is as shown in eq 4,

$$k_m = k_1 \qquad k_3 \gg k_2, k_1 \qquad (4)$$

i.e., the rate of exchange is governed by the rate of opening of the compact structure that shields a hydrogen from solvent. In the EX_2 mechanism

$$k_m = \frac{k_1}{k_2} k_3 \qquad k_2 \gg k_3, k_1 \qquad (5)$$

i.e., the rate of exchange is governed by the equilibrium between N and I forms and by the rate of exchange of the exposed group. Only amide groups were thought likely to contribute to the set of slowly exchanging hydrogens, and therefore a single constant (k_3) should describe all hydrogens that react at measurable rates. k_3 is pH dependent, with a minimum near pH 3. As a consequence of eq 4 and 5, large changes in exchange behavior are possible without change in average conformation, and exchange rates therefore can be understood as reflecting either protein motility or average conformation.

Table VIII lists the simplest set of class sizes and rate constants that fit the data for the hydrogen exchange of insulin at 0° and pH 3–8. These numbers are approximate in that they reflect the accuracy of the data; the average deviation of between 1 and 2% allows the number of hydrogens in a class to be changed by about 2, or the rate constant to be adjusted equivalently. Although detailed interpretation of the parameters of Table VIII may therefore be impossible, some useful general conclusions can be reached. For example, the following points suggest that exchange of insulin in solution is unlikely to occur through an EX_1 mechanism. (1) Using eq 4 and the data of Table VIII, a lower limit can be set on the ratio k_1/k_2, *i.e.*, greater than 1 for all exchanging hydrogens at pH 3. Since these include two-thirds of the amide protons, at least half of the time an insulin molecule would have to be in a conformation that has this many amides accessible to solvent, a possibility that seems unlikely, by analogy with the proteins of known crystal structure. (2) Exchange in the crystal probably proceeds through an EX_1 mechanism (see below), and if the same kinetic behavior obtains at pH 3 in solution, then there is no appreciable change in the exchange properties between pH 3 (in solution) and pH 6.5 (in the crystal). It then would be difficult to explain the substantial change in behavior over only 0.3 pH unit above pH 6.5 (Figures 6 and 7). In contrast, an EX_2 mechanism easily accounts for the dependence of exchange upon pH in that the minimum rate at pH 3 is in accord with the minimum in k_3. Also, reasonable values of k_1/k_2 are obtained, *i.e.*, at pH 3 the ratio is 0.1 or less for classes D and E, so that two-thirds of the amide hy-

[3] The estimates for the total number of exchangeable hydrogens were made according to the procedure described under Results.

TABLE VIII: Numbers of Hydrogens and Rate Constants for Each Exchange Class.

pH	Temp (°C)	Class A[a]	Class B	$k_B \times 10$ min^{-1}	Class C	$k_C \times 10^2$ min^{-1}	Class D	$k_D \times 10^3$ min^{-1}	Class E[a]	Total No. of Exchanging H's[b]	k_3 (min^{-1})[c]
3.3–3.6	0	47	5	1.0	7	1.3	14	1.1	16	89	0.013
5.4–5.5	0	52	0		9	1.4	7	2.2	20	88	1.6
6.75–7.0	0	65	5	1.6	8	1.3	6	1.4	3	87	32
7.9–8.0	0	73	3	1.0	8	2.1	3	3.6	0	87	400
3.1–3.3	38	59	12	0.6	10	1.9	8	1.7	0	89	1.3
5.4–5.5[d]	38	60	3	1.1	7	1.8	11	1.3	7	88	160

[a] Class A represents the hydrogens that exchange instantaneously (*i.e.*, before the zero-time sample was taken), and class E those that do not exchange. [b] Estimated as described in Results. [c] Calculated from eq 5 of Hvidt and Nielsen (1966). [d] Zinc-free insulin; all other class sizes and rate constants refer to the exchange of zinc insulin.

drogens are shielded by a folded conformation. An EX_2 mechanism for insulin is in accord with the exchange behavior of most proteins, as suggested by Hvidt and Nielsen (1966), and it agrees with the interpretation by these authors of previous data for insulin exchange (Hvidt and Linderstrom-Lang, 1955; Linderstrom-Lang, 1955a).

Comparison of Exchange in Crystal and Solution. Several explanations may be offered for a change in hydrogen exchange behavior upon transfer of a protein from solution to the solid phase: (1) intermolecular interactions in the crystal might prevent the exchange of labile hydrogen atoms that are involved in a contact; (2) the average conformation of the molecule might differ between crystal and solution; and (3) the conformational freedom might be reduced upon crystallization, *i.e.*, there might be differences in the motility of the protein.

Experiments already discussed in connection with the possible effects of aggregation show that intermolecular contacts are not likely to contribute to exchange differences between crystalline and soluble insulin. In particular, if labile hydrogens are immobilized in contacts, then they should react upon crystal dissolution. There was no immediate increase in the number of exchanged hydrogens when crystalline insulin was dissolved at pH 3 after 24-hr exchange at pH 5.5 (Table III). This experiment also stands against a change in average conformation upon crystallization, owing to arguments like those advanced in discussing the similar shift from pH 3 to 7. After 24 hr, crystalline insulin at pH 5.5 and soluble insulin at pH 3 have exchanged nearly the same number of amide protons (Figures 2 and 3, and Table III). The additional few hydrogens that did exchange at pH 3 after 24-hr preexchange in the crystal were expected (*i.e.*, the nonexchanging class, E of Table VIII, differs by 4 between pH 3 and 5.5, within experimental error of the 3 found in the shift experiment). The lack of significantly greater exchange after the pH shift suggests that peptide groups that do not exchange at pH 3 also do not at pH 5.5. The correspondence between the most deeply buried hydrogens supports the identity of conformation in crystal and solution. Furthermore, because no instantaneous exchange occurs upon dissolution, hydrogens that exchange rapidly at pH 3 must be in exchanging classes at pH 5.5. Insulin crystals dissolve only slowly at pH 7, and exchange is too fast to be of interest at higher pH where solubilization is rapid. However, the information that could be obtained from a pH 5.5 to 7 shift is available indirectly through the shift from pH 3 to 7, which demonstrated that the nonexchanging hydrogens at pH 3 were slowly exchanging and presumably also buried at pH 7 (discussed above). To summarize, the *average* conformation of insulin between pH 3 and 7 appears to be constant and the same in crystal and solution.

In order to explain exchange in the crystal we are thus compelled to consider mechanisms based on protein motility. This follows from the discussion of the preceding paragraph and from reflection on the restraints of the crystal lattice, because of which changes in average conformation with pH and other environmental factors must be negligible. The following argument supports an EX_1 mechanism. First an EX_2 mechanism, because of the constant exchange behavior between pH 4.5 and 6.5 (Figure 4), would require that

$$\left[\frac{\mathrm{d}\log(k_1/k_2)}{\mathrm{d\ pH}}\right]_T = \left[-\frac{\mathrm{d}\log k_3}{\mathrm{d\ pH}}\right]_T = -1$$

This is possible, but such exact compensation that holds for all hydrogens seems unlikely. An EX_1 mechanism does not exhibit this difficulty, since k_1 can be independent of pH, and indeed this behavior would agree with the pH invariance of the average conformation. An EX_1 mechanism gives limits of k_1/k_2 that are reasonable for the several classes of hydrogens. Specifically, for an EX_1 mechanism k_2 is limited by k_3 ($k_2 \ll k_3$); at pH 4.5, $k_3 = 0.15$ (Hvidt and Nielsen, 1966), and if $k_2 \leqslant 0.015$, then k_1/k_2 is of order 1 for class C, 0.1 for class D, and smaller for class E.

Rate constants obtained for exchange through an EX_1 mechanism are the rates of opening the exchanging units (eq 4), and they indicate that the half-lives for this reaction are 45, 300, and $\geq$ 7000 min for classes C–E, respectively (Table VIII).

It is of interest to compare values of k_2 for solution and crystal. If EX_2 and EX_1 mechanisms, respectively, are followed

$$k_2(\text{solution}) \gg k_3(\text{pH } 3.3) = 10^{-2}\ \text{min}^{-1}$$

$$k_2(\text{crystal}) \ll k_3(\text{pH } 4.5) = 15 \times 10^{-2}\ \text{min}^{-1}$$

Assuming at least a tenfold separation of the constants k_2 and k_3, $k_2(\text{solution}) \geq 7k_2(\text{crystal})$. Apparently, then, it is possible to associate a change in motility with the difference in exchange properties between crystal and solution. This is in accord with the preceding discussion, which suggested that during crystallization no change in average conformation occurs.

Other experiments have indicated that upon crystallization the motility of a protein can decrease without a change in conformation.[4] Azide binding to crystalline ferrihemoglobin is for one phase of the reaction 21 times slower than in solution (Chance and Ravilly, 1966). Rupley and Gates (1968) measured the equilibrium constant for the binding in the crystal and found it to differ by less than 20% from that for the soluble protein. The essentially identical equilibrium behavior suggests that a change in average conformation does not occur upon crystallization and that the reduction in rate is a consequence of an effect upon protein motility. A number of other reports of reactions in the crystal suggest a general pattern of behavior: (1) an invariance to crystallization of the equilibrium properties of a protein;[5] (2) a change upon crystallization in kinetic properties, in particular those that in the course of the reaction might require disturbance of the internal structure.[6] The data support the conclusion that the average conformation in solution is the same as in the crystal, and that a principal effect of crystallization is to increase the barriers to fluctuations of structure. It is of interest to note that comparison of the rate of a reaction in the crystal and in solution is likely to be useful for specifying whether reaction depends upon motility. Chance (1966), in a discussion of azide binding, first brought this matter forward. Specifically, if for a protein one or more equilibrium parameters can be shown invariant to crystallization, a lower rate in the solid would indicate that opening up of the structure is necessary. The converse, however, does not follow, demonstrated by the lack of a difference between the hydrogen exchange properties of soluble and crystalline lysozyme (Praissman and Rupley, 1968). Several enzymic reactions are among those that exhibit kinetic differences between solution and crystal (ribonuclease S (Doscher and Richards, 1963; Winstead and Wold, 1965), carboxypeptidase A (Quiocho and Richards, 1966), and alcohol dehydrogenase (Theorell *et al.*, 1966)).

Comparison of Zinc and Zinc-Free Insulin. At pH 8 and 9 exchange was complete after 24 hr. The absence of an effect of zinc on the number of protons exchanged under these conditions (Figure 8 and Table II) indicates that in the dried protein either (1) there is no water bound to the metal and the metal is coordinated by groups that are at this pH ionized in the free protein, or (2) the protons of water that is bound are balanced by protons displaced from side-chain groups. Since histidine (Tanford and Epstein, 1954; Summerell *et al.*, 1965) and α-amino groups (Marcker, 1960) are those that have been implicated in zinc binding, the absence of exchange differences between zinc and zinc-free insulin at alkaline pH supports the first alternative. At more acid pH it is possible that groups coordinating the zinc are protonated in the uncomplexed molecule. The ratio of zinc to insulin monomer is approximately 0.5 g-atom/mole, and any difference would be small, probably no more than two protons. Zinc-free insulin at pH 6.8 exchanged about four fewer hydrogens than the zinc protein (Figure 5). At acid pH in both crystal and solution the zinc-free protein consistently exchanged one to two fewer hydrogens than zinc insulin, which is at the limit of experimental error. However, if at acid pH the metal displaces protons, the difference might be as large as the 4 found at pH 6.8.

There are several explanations that are possible for differences appearing at pH near 7 but not at more alkaline pH. First, as the pH is increased, less motile regions of the molecule undergo exchange (EX_2 mechanism). If the differences between zinc and zinc-free insulin are restricted to regions of a particular motility, then at pH 8 the classes which show the differences might exchange too fast to measure. Alternatively, there may be a change in average conformation between pH 7 and 8 that abolishes the different behavior, but following the preceding discussion, this is to be considered unlikely. Arguments of the same type hold if exchange in the acid region is indeed identical for zinc and zinc-free protein. Although the effect of zinc on exchange properties could come through a change in average conformation (Weil *et al.*, 1965), an argument against this explanation is the observation that zinc can be removed from crystals of zinc insulin without disruption of the lattice (Netter, 1939; Schlichtkrull, 1956). In this connection it is of interest that the amorphous

[4] This possibility has been put forward by several authors: Praissman and Rupley (1964), Chance *et al.* (1966), and Quiocho and Richards (1966).

[5] The binding of protons to the tyrosyl groups of methemoglobin (Rupley, 1964), the binding of saccharides to lysozyme (Butler and Rupley, 1967), the protein volume (Krivacic and Rupley, 1968), the two histidine ionizations of insulin (R. Pecoraro and J. A. Rupley, unpublished data), and most of those of hemoglobin (Rupley, 1968).

[6] In addition to hydrogen exchange, the reaction of azide with myoglobin (Chance *et al.*, 1966) as well as hemoglobin (Chance and Ravilly, 1966), and the enzymic activity of carboxypeptidase A (Quiocho and Richards, 1966). Not all kinetic properties are altered by crystallization, however: the reaction of myoglobin with iodoacetate (Banaszak *et al.*, 1963), the enzymic activity of triosephosphate dehydrogenase (Murdock, 1967), and the hydrogen exchange of lysozyme (Praissman and Rupley, 1968); in such cases it may be that the motility of kinetically important regions is not affected, or that reaction involves only surface groups.

zinc-free and crystalline zinc proteins exhibit nearly identical exchange characteristics.

Because a group coordinated to the zinc is less free to move about, binding of zinc should affect the motility of the protein. If it affects the unfolding of all regions of the molecule, then there should be correlated changes in the rates of exchange of all buried protons. The binding constant of zinc ion to insulin at neutral pH is approximately 10^6 (Summerell *et al.*, 1965). Since there is no comparable effect on the exchange (only about 10% of the buried hydrogens could have had their rate of reaction reduced by 10^6), it must be that the motility of the regions containing buried hydrogens is largely independent of the region that binds zinc. The contacts developed in the aggregation of insulin (considered earlier in this discussion) also do not affect the opening of the exchanging regions. A similar conclusion (Praissman and Rupley, 1968) is reached from data for the exchange of lysozyme in the presence and absence of tri(*N*-acetylglucosamine), a saccharide that forms a stable complex with the enzyme. Insulin has as great a proportion of buried hydrogens (estimated as the slow and non-exchanging) and the same general exchange behavior as larger proteins,[7] for example, lysozyme (Hvidt and Kanarek, 1963; Praissman and Rupley, 1968), myoglobin (Benson, 1959), serum albumin (Benson *et al.*, 1964), and yeast alcohol dehydrogenase (Hvidt and Kagi, 1963). Apparently, a large surface to volume ratio does not greatly increase the rate of amide exchange. Behavior of this kind is also consistent with independent fluctuations of small structural units. However, it is apparent that development of a set of contacts at a sufficient number of points distributed over the molecular surface can indeed lead to general inhibition of fluctuations. This is seen in the change from an EX_2 mechanism in solution to an EX_1 mechanism in the crystal.

The similar exchange of amorphous zinc-free and crystalline zinc insulin stands in contrast to the different reactivities of crystalline and amorphous carboxypeptidase A (Quiocho and Richards, 1966). Perhaps the solid phases of the two insulin species differ only in long-range order, and the immediate surroundings of a molecule may be the same.

Effect of Ionic Strength. There was no significant effect of ionic strength on the exchange of insulin in solution (experiments at 0, 0.20, 0.24, and 0.87 M NaCl, Tables IV–VI); since a difference of 1.5 hydrogens is within the average error for zero-time data, the one to four additional hydrogens that exchanged instantaneously in the presence of salt should not be weighted heavily. In contrast, exchange in the crystal shows an effect of salt (Table VI). After 24 hr in 0.87 M NaCl solution, eight more hydrogens had exchanged than in the absence of added salt. The difference cannot be explained by an effect of salt on k_3 or on the ionization of side-chain groups, which in both cases would be to reduce the number of exchanging hydrogens. Increased ionic strength should lower the apparent pK of groups titrating at pH above the isoionic point of insulin (about 5.3), and it should reduce k_3 for the base-catalyzed reaction. Moreover, salt had little or no effect in solution, and exchange by an EX_1 mechanism in the crystal is not dependent on k_3. Consequently, it is more likely that salt affects the conformation of the crystalline protein. Owing to restrictions imposed by the crystal lattice, it is unlikely that the *average* conformation can change. The increased exchange can be explained, however, by assuming that some regions of the crystalline protein are more motile under conditions of high ionic strength. If the low exchange at zero time in high salt is in fact meaningful,[8] then other regions may be less motile. A different effect of salt on crystalline and soluble protein is in accord with different motility and exchange mechanisms in the two phases.

Effect of Storage. Stracher (1960) and Hvidt and Kanarek (1963) have found an effect of storage and source of protein on the exchange properties of ribonuclease and lysozyme, respectively. The insulin experiments described in this paper stretched over 15 months, and often 3–6 months elapsed between exchange experiments at the same pH (the same sample of insulin was used, stored at 5° in a tightly sealed container). In contrast to the substantial variations found for the two other proteins just mentioned, with insulin the results of runs at different dates at the same pH agree within 1–2%. In all zinc insulin experiments protein solutions and crystal suspensions were prepared from citrate crystals that had been grown in sufficient quantity to last for several months. In a few experiments the data for crystalline insulin were not only highly scattered but the number of hydrogens exchanged appeared to decrease after 3 hr. The difficulty was traced to a particular batch of crystals, but the cause could not be determined. Soluble insulin obtained from these crystals exchanged normally. It is of interest that a similar problem was encountered for all preparations of crystalline insulin in exchange at 38°. In this case the origin of the difficulty might be disruption of the structure at higher temperature (Low *et al.*, 1966).

Hydrogen Exchange during Lyophilization. Several investigators have suggested that hydrogen exchange may occur during lyophilization of proteins (Bryan and Nielsen, 1960; Leach and Springell, 1962; Hallaway and Benson, 1965). Hallaway and Benson (1965) found exchange of bovine serum albumin continued at −5°. Leach and Springell (1962) reported 15 fewer instantaneously exchangeable hydrogen atoms in ribonuclease kept at −20° during lyophilization. Additional exchange of this sort has been attributed to labile hydrogen atoms that react at an appreciable rate in frozen solutions at temperatures just below 0°, *e.g.*, poly-DL-alanine had exchanged approximately seven-tenths hydrogen atom per peptide group after 2 hr at −5° (W. P. Bryan and S. O. Nielsen, unpublished data, cited in Hvidt and Nielsen, 1966). An alternative explanation of

[7] The molecular unit important in the exchange of insulin is not larger than the dimer (mol wt 11,400), as considered earlier in the discussion.

[8] Four fewer hydrogens exchange in 0.87 M NaCl at zero time; because of a large correction for soluble protein and the lower accuracy of zero-time data, this difference may not be significant.

exchange during freeze drying is that the removal of water *vapor* might be inefficient. Transfer of vapor through a drying protein shell depends upon the concentration gradient, that in turn is a function of the pressure at the sample surface. If the gradient is low, a substantial concentration of vapor might develop throughout the shell of dried solid, and exchange of solid protein could become significant (exchange of dry protein has been reported by Hvidt *et al.* (1960) and Hnojewyj and Reyerson (1961)). Hallaway and Benson (1965) found no exchange of dry protein with water vapor in equilibrium with ice at Dry-Ice–acetone temperature. However, it is the vapor *traversing* a dried protein shell that would be of importance, *i.e.*, there might be a high steady-state concentration of vapor during lyophilization but a low equilibrium concentration after drying is complete. Furthermore, the temperature of the sample depends upon the rate of water transfer. In order to increase efficiency, the lyophilization system for this work was constructed with a minimum of stopcocks, with wide bore tubing, and with a two-stage mercury diffusion pump. The possibility of additional exchange during the drying of insulin was examined, using the approach of Leach and Springell (1962), with the following results. At pH 3.3 and at zero time, insulin exchanged 47.1 hydrogens when the protein was kept at $-20°$ for the first 40 min of drying, and 48.4 hydrogens when the temperature was not controlled. The lack of significant additional exchange at the higher temperature indicates that the vacuum system was adequate. Irreproducible results were obtained in preliminary experiments with a single-stage diffusion pump and with more obstacles to vapor flow.

Equilibrium Isotope Effects. When tracer amounts of tritium are used to measure hydrogen exchange, equilibrium isotope effects might be large (Hvidt and Nielsen (1966) have reviewed the literature). Estimates for proteins of the excess above random distribution of isotope have ranged between 0 (Englander, 1963) and 28% (Leach *et al.*, 1964),[9] and comparable or higher values have been found for small compounds that are analogs of the exchanging groups of proteins. An approximate calculation of the equilibrium constant for the amide exchange reaction (which accounts for more than half of the exchange for a protein) can be made using the zero-point energies and symmetry numbers of reactants and products (Melander, 1960). The vibrational frequencies of the three normal modes of H_2O, DHO, and THO have been reported by Urey (1947); the frequencies for NH and ND by Schneider *et al.* (1965); the frequencies for NT can be estimated from the mass-ratio approximation. The constant for H–D exchange was calculated as 1.2, and for N–T as 1.8.

The equilibrium isotope effect for insulin is shown by experiment to be small, at most four hydrogens (5%) at pH 8 and 9 (as was indicated in Results, this is likely to be an overestimate, because ionic strength and temperature were not considered in the calculation of the theoretical number of exchangeable hydrogens). Hvidt and Nielsen (1966) argue that both positive and negative deviations from the distribution of isotope in small compounds can come through participation of a proton in internal bonding. If indeed there is a significant equilibrium isotope effect for a free amide, then in the case of insulin the excesses and deficits of isotope in bonded and unbonded groups must sum to near zero. No isotope effect for lysozyme was observed at pH 4.7 (Praissman and Rupley, 1968), using the same exchange and lyophilization procedure as in these experiments.

Previous Work on Insulin. The hydrogen exchange of pig insulin has been investigated by Linderstrom-Lang (1955a) and Hvidt and Linderstrom-Lang (1955). Comparison of this and the present work is possible at 0° and pH 3, 3.5, and 7.1, and at 20 and 30° at pH 3. Agreement is excellent at long times of exchange (the average difference at 20 hr is two hydrogens). Agreement is, however, poor at shorter times (at zero time, excluding 30° data, the average difference is 11). Since the amino acid sequences of pig and beef insulin differ by only two residues, the discrepancy is most easily understood as originating in exchange during the lyophilizations of the earlier procedure (Bryan and Nielsen, 1960).

[9] Incomplete drying will lead to an apparent excess of isotope in the protein, perhaps accounting for the range of values.

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