avirulent strain, as seen in Table I, and in actual total yield, shown in Table II, no difference existed between the two strains.

On the other hand, the fact that there was more, both relatively and actually, of the faster protein component (mobility 5.8–6.0) in the heated aqueous extract from the virulent than from the avirulent strain, might suggest some connection between this component and virulence. Since two proteins, B and C, are found in this peak[2,5] and since the C protein has seemed to be less specific in its potency, it is possible that the B protein may prove to be of significance as far as virulence is concerned. This will be investigated.

Acknowledgment.—This work was aided by grants from the Committee on Medical Research of the National Tuberculosis Association.

Summary

1. Electrophoretic diagrams of concentrated filtrates of unheated tuberculin serve for the evaluation of the relative amounts of the different protein, polysaccharide, nucleoprotein and nucleic acid components present, all of which have been isolated and identified.

2. The mobilities and relative proportions of these components correspond very closely in different batches made from the same strain at different times.

3. The relative percentage of the components varies greatly between different strains, even though the mobilities correspond closely.

4. In a study of differences between the undissociated H 37 and the dissociated H 37 Rv and H 37 Ra strains, the Rv strain yielded in the unheated filtrate more polysaccharide II, than did the Ra strain.

5. Extracts of the bacillary bodies of all three strains made by autoclaving them with water showed the presence of a large amount of nucleic acid not found in the original filtrates. The extract of the H 37 Rv bacilli showed the presence of more polysaccharide II and more of the faster protein with mobility 5.8–6.0, but the same amount of the slower proteins with mobilities around 3.7, and of the free nucleic acid, as did the Ra strain. The possible relationship between virulence and these findings, as well as those listed under (4), is discussed.

6. An extensive fractionation of the H 37 filtrate was carried out and further resolution of the slowest proteins was achieved.

7. Comparative skin potency tests showed that the slowest protein was the most potent one. Furthermore, unheated filtrates of the H 37, as well as of the dissociated strains, H 37 Rv and H 37 Ra, were all more potent than the heated aqueous extracts of the bacillary bodies. These latter were similar in potency to the standard PPD-S. The unheated filtrate from the H 37 Ra strain was as, or more, potent than that from the H 37 Rv strain, but it was less potent when the heated aqueous extracts of these two strains were compared.

PHILADELPHIA, PA. RECEIVED SEPTEMBER 22, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY SCHOOL OF MEDICINE]

The Interaction of Insulin with Thiocyanate and other Anions. The Minimum Molecular Weight of Insulin[1]

BY EUGÈNE FREDERICQ[2] AND HANS NEURATH

Introduction

Recent studies have indicated that the minimum molecular weight of insulin is approximately 12,000[3,4] and that in aqueous solutions, this insulin "monomer" is in equilibrium with higher, tri- or tetrameric aggregates. The equilibrium is greatly dependent on protein concentration, *p*H, and ionic strength of the medium.[4]

Preliminary to an electrophoretic investigation of insulin in the isoelectric range it was found[5] that between *p*H 5 and 8 thiocyanate ions increase appreciably the solubility of this protein. This was also reflected in a shift in the *p*H mobility curve, corresponding, at *p*H 5.5, to the binding of approximately 2 equivalents of thiocyanate ions per 12,000 g. of insulin. In contrast, in acid solutions in which insulin is highly soluble in the absence of thiocyanate, the addition of these anions resulted in almost complete precipitation of the protein. Subsequent quantitative studies on the effect of thiocyanate on the solubility of insulin,[6] carried out in relation to protein concentration, temperature and *p*H have led to the conclusion that the precipitating action of thiocyanate is directed primarily toward the tri- or tetrameric form of insulin and that any factor which shifts the molecular equilibrium toward the aggregated state likewise promotes precipitation.

In the work presented herein, an attempt was made to evaluate quantitatively the effects of

(1) Presented at the 116th meeting of the American Chemical Society, before the Division of Biological Chemistry, held in Atlantic City, N. J., September 18 to 23, 1949.

(2) Fellow of the Belgian American Educational Foundation. Present address: Institute of Physical Chemistry, University of Liege, Belgium.

(3) H. Gutfreund, *Biochem. J.*, **42**, 156, 544 (1948).

(4) E. Ellenbogen, Thesis, Harvard University, 1949. J. L. Oncley and E. Ellenbogen, paper presented at the 116th meeting of the American Chemical Society held in Atlantic City, 1949.

(5) E. Volkin, *J. Biol. Chem.*, **175**, 675 (1948).

(6) M. H. Schwert and H. Neurath, THIS JOURNAL, **72**, 2784 (1950).

thiocyanate on the physical properties of insulin, as determined by the methods of equilibrium dialysis, sedimentation in the ultracentrifuge, electrophoresis and solubility. In this analysis, the contribution of thiocyanate to the total ionic strength of the medium was considered separately from the specific interaction of this anion with the protein. The effect of thiocyanate was compared to that produced by other anions, such as iodide, chloride, acetate and dihydrogen phosphate. As a result of these measurements, the state of dispersion of insulin in the presence of dihydrogen phosphate, which is only weakly bound, if at all, was determined in detail by sedimentation and diffusion measurements, and was found to correspond to a minimum molecular weight only one-half as great as previously reported.

Experimental

Materials.—Pancreatic beef insulin five times recrystallized (lot T-2344) was obtained through the courtesy of Eli Lilly and Company, Indianapolis: Zn, 0.59; N, 16.6; biological activity, 27 units per mg.

Binding Studies.—The equilibrium dialysis method was essentially the same as that described by Klotz, Walker and Pivan.[7] Equilibrium was reached after twenty-four hours at 25°, with occasional stirring by hand. The protein concentration was 0.5% and the ionic strength 0.1. Thiocyanate determinations were made by two procedures: (1) photometrically as described by Schreiber,[8] and (2) titrimetric using the oxidation of thiocyanate by excess of iodate and back titration with thiosulfate.[9] The first procedure was found more accurate for thiocyanate concentrations below 0.005 *N*.

Solubility.—Samples of 2 cc. of suspensions containing 50 mg. of insulin were shaken for twenty-four hours in a water-bath at 25°. After centrifugation, analysis of the supernatant solution was performed photometrically, by measuring the absorption at 276 mμ in a Beckman, D. U. photoelectric quartz spectrophotometer, as recently described.[6]

Electrophoresis.—A Tiselius apparatus, equipped with the Philpot-Svensson optical system was used, as described by Sharp, Cooper and Neurath.[10] Unless otherwise stated, the buffer solutions contained 0.02 *N* glycine hydrochloride and 0.08 *N* sodium chloride, *p*H 2 to 3.1. Below *p*H 2, sodium chloride (0.1 ionic strength) was used. The potential gradient was about 3 volts per cm.

Sedimentation Analysis.—The electrically-driven ultracentrifuge, manufactured by the Specialized Instrument Corporation, Model E, was used.[11] The centrifugal field was 262,000 g., the temperature 23 to 27°. The sedimentation constant was computed from measurements of the displacement of the maximum ordinate of the boundaries.[12] The boundaries were nearly symmetrical. In order to prevent precipitation of the insulin, the cell was coated with a thin layer of paraffin. The sedimentation constants were corrected for solvent viscosity and density and calculated for a standard temperature of 20°. Similar corrections were applied to the diffusion constant.

Diffusion.—These measurements were performed at 1.3°, using the Tiselius electrophoresis apparatus manufactured by Frank Pearson Associates, equipped with the Longsworth scanning system.[13] A Tiselius cell with hemispherical ball joints (Pyrocell Manufacturing Company) was used. The diffusion constants were calculated by conventional methods,[14] after proper correction for zero time,[15] from enlarged tracings of the refractive gradients.

Results and Discussion

The Binding Curve.—The amount of thiocyanate ions bound by insulin, as determined by equilibrium dialysis measurements[7] at 25°, is shown in Fig. 1. These experiments were performed in 0.5% insulin solutions, within the range of *p*H 2.7 to *p*H 3.6, at a constant ionic strength of 0.1. Within this *p*H range, the extent of binding was practically independent of *p*H. The experimental points are somewhat scattered because binding occurs at relatively high thiocyanate concentrations of which only a small fraction is bound by the protein. For this reason it was impossible to fit the data unequivocally to the equations which have been recently proposed for various types of binding, *i. e.*, those due to statistical distribution,[7] electrostatic interactions[16] or heterogeneity of binding constants.[17] As a first approximation, the data were resolved by one of the equations of Klotz, *et al.*,[7] for the purely statistical distribution of the ions bound by the protein, by plotting the reciprocal of the moles of bound anions per mole of protein, *versus* the reciprocal of the concentration of free anions. The best straight line was calculated by the method of least squares and converted to the coördinates of Fig. 1. Comparison of the resulting solid line with the experimental points in Fig. 1 shows that, in the range of thiocyanate concentration which was investigated, deviations from statistical binding are apparently slight. Calculations give a value of 6 to 7 for the maximum number of sites available per 12,000 molecular weight unit of insulin. Although such calculations are subject to considerable error,[17] it is interesting to note that the total number of (α

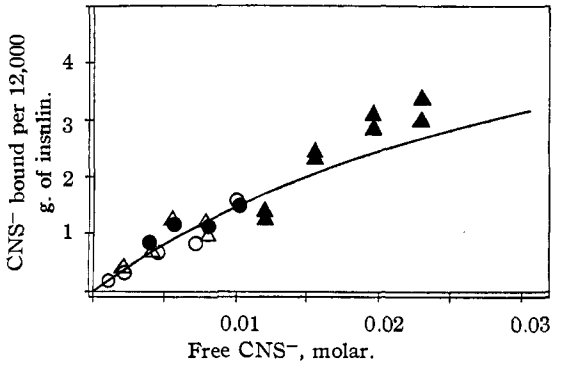

Fig. 1.—Amount of thiocyanate ions bound by insulin as a function of the concentration of free anions, at *p*H 2.7–3.6, ionic strength 0.1.

(7) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).
(8) H. Schreiber, *Biochem. Z.*, **168**, 241 (1925).
(9) R. Lang, *Z. anorg. allgem. Chem.*, **142**, 289 (1925).
(10) D. G. Sharp, G. R. Cooper and H. Neurath, *J. Biol. Chem.*, **142**, 203 (1942).
(11) G. W. Schwert, *ibid.*, **179**, 655 (1949).
(12) J. L. Oncley, *Ann. N. Y. Acad. Sci.*, **41**, 121 (1941).
(13) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).
(14) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford (1940); H. Neurath, *Chem. Revs.*, **30**, 357 (1942).
(15) L. G. Longsworth, *Ann. N. Y. Acad. Sci.*, **41**, 267 (1941).
(16) G. Scatchard, *ibid.*, **51**, 660 (1949).
(17) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

+ ϵ) amino groups per 12,000 molecular weight unit of insulin is likewise 6.[18] The free energy change for the binding of the first anion is approximately −3000 cal. per mole. This value may be compared to Luck and Welsh's value of −1,800 cal. per mole for the binding of chloride ions by serum albumin.[19] Considerably lower values were reported for the binding of organic anions by serum albumin.[7,17,19,20]

At *p*H 7 and under otherwise identical conditions, no binding of thiocyanate by insulin could be detected. This finding is not due to some irreversible changes of insulin at that *p*H, for the protein regained its full binding ability after the *p*H was readjusted to *p*H 3.0. Klotz and Urquhart[21] likewise reported that insulin does not bind methyl orange at *p*H 8.2. Although the limited solubility of insulin between *p*H 4 and 7 precluded equilibrium dialysis measurements in this intermediate *p*H range, it is of interest to note that Volkin's electrophoretic measurements indicated that at *p*H 5.5, in the presence of 0.15 *M* thiocyanate, 2 moles of anions were bound per 12,000 g. of insulin.[5] This effect of *p*H on ion binding is similar to that observed by Klotz and Urquhart[21] for serum albumin.

Solubility.—Previous experiments by M. H. Schwert and Neurath[6] have already shown that potassium thiocyanate decreases markedly the solubility of crystalline zinc-insulin within the *p*H range of 1.7 to 3.9, and that any factor which promotes polymerization of the monomer (increasing *p*H and protein concentration, decreasing temperature) likewise promotes precipitation. Additional experiments carried out as part of the present investigation showed that the precipitating action of thiocyanate was not related to the presence of zinc in this protein. At *p*H 1.7 and 2.7, the solubility of amorphous insulin (containing less than 0.1% zinc) in the presence of thiocyanate was the same as that of the crystalline zinc-insulin. The data are shown in Fig. 2 for measurements at *p*H 2.7 (right-hand curve), together with additional experiments on the crystalline protein in solutions of varying ionic strength.

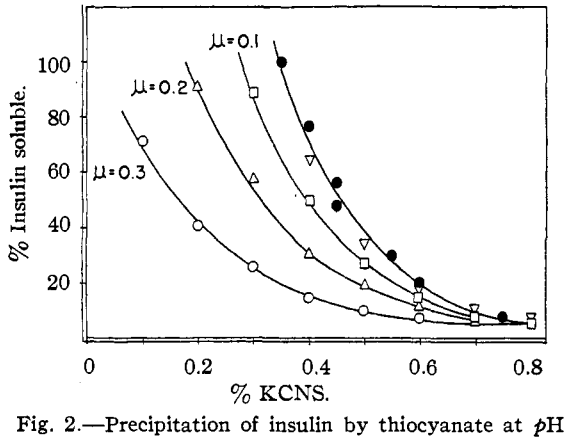

Fig. 2.—Precipitation of insulin by thiocyanate at *p*H 2.7 and various ionic strengths: full circles, amorphous insulin; other points, crystalline zinc-insulin.

In order to evaluate the specific precipitating effect of thiocyanate independent of those effects caused by the contribution of this ion to the ionic strength of the medium, experiments such as those shown in Fig. 2 were carried out also at *p*H 1.7 and *p*H 3.8. At each *p*H, the concentration of thiocyanate was varied and the ionic strength, calculated as a first approximation from the concentration of the salts, controlled by the addition of sodium chloride. At constant *p*H and constant ionic strength, the experimental points followed the linear equation

$$\log S = \beta' - K'(\mathrm{SCN}^-) \qquad (1)$$

where S is the solubility of insulin in g. per liter and (SCN^-) is the molarity of thiocyanate. β' and K' are constants. Typical results are shown in Fig. 3 for measurements at *p*H 1.7 and 3.8, at 25°.

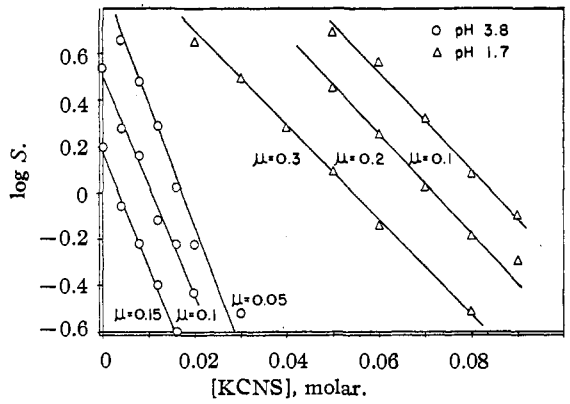

Fig. 3.—A plot of the logarithm of the solubility of insulin in g. per liter *versus* thiocyanate concentration at various ionic strengths and at *p*H 1.7 and 3.8, respectively.

Equation (1) is a generalization of the well-known salting-out equation.[22] β represents the value of log S in the absence of thiocyanate, and as in typical salting-out phenomena, K' should be independent of *p*H and ionic strength. In the present case, however, K' tends to increase above *p*H 2.7, as shown in Table I in which the calculated values of β' and K' are given for the entire series of experiments. This apparent disagreement between theory and experimental results has to be ascribed to the change in the state of aggregation of insulin as the salt concentration (thiocyanate and/or sodium chloride) is varied.

The dependence of log S on *p*H and thiocyanate concentration at constant total ionic strength is

(18) A. C. Chibnall, *J. Intern. Soc. Leather Trades' Chemists*, **30**, 1 (1946).

(19) Quoted in J. M. Luck, *Trans. Faraday Soc.*, in press.

(20) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **174**, 653 (1948); **177**, 383 (1949).

(21) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

(22) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," New York, N. Y., 1943.

TABLE I

VALUES OF CONSTANTS IN EQUATION (1)

Ionic strength	pH 1.7 β'	pH 1.7 K'	pH 2.7 β'	pH 2.7 K'	pH 3.8 β'	pH 3.8 K'
0.05	..	..	...	..	0.87	51
.1	1.77	21	1.4	25	.53	50
.15	..	..	...	..	.19	50
.2	1.50	21	1.1	22	..	..
.3	1.12	20	0.7	20	..	..

shown in Fig. 4 in which log S is plotted against pH. The experimental points follow reasonably

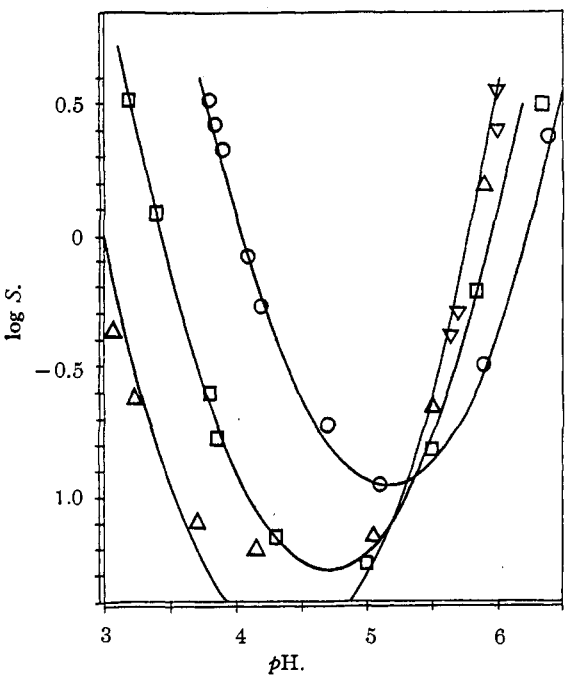

Fig. 4.—Effect of pH on the logarithm of the solubility of insulin at various concentrations of thiocyanate and sodium chloride, total ionic strength 0.1. The solid lines are calculated from text equation (2): O, 0.1 N sodium chloride; □, 0.08 N sodium chloride–0.02 N potassium thiocyanate; △, 0.05 N sodium chloride–0.05 N potassium thiocyanate; ▽, 0.1 N potassium thiocyanate.

well the solid curves which were calculated from the equation

$$\log S = \log S_0 - K(p\mathrm{H} - p\mathrm{H}_0)^2 \qquad (2)$$

where S_0 is the solubility minimum at $p\mathrm{H}_0$ and K is a constant. The values of S_0 and $p\mathrm{H}_0$ were estimated by extrapolation of the data in Fig. 4, and K was chosen so as to make the resulting curves coincide as closely as possible with the experimental points. The best value of K was 0.81 and was independent of thiocyanate concentration. According to this finding, the presence of thiocyanate does not change the shape of the solubility curves but merely shifts these curves toward lower values of pH and solubility. The value of $p\mathrm{H}_0$ probably corresponds to the pH at which the net charge of the protein is zero. In the presence of 0.1 N sodium chloride and 0.02 and 0.05 N potassium thiocyanate, respectively, $p\mathrm{H}_0$ is 5.15, 4.70 and 4.40, suggesting that, in comparison with solutions containing only sodium chloride, thiocyanate decreases the net charge by combination with positively charged groups on the protein.[5]

According to an equation derived by Linderstrøm-Lang,[23] the value of K in equation (2) is related to the molecular weight of the protein by

$$K = 1/2\ Mw \times \mathrm{d}h/\mathrm{d}p\mathrm{H} \qquad (3)$$

where Mw is the molecular weight of the protein, and h is the equivalents of hydrogen ions bound per g. of protein. From the present value of K and from the titration curve of insulin,[24] a molecular weight of 8,000 is calculated from equation (3). Although the conditions required by equation (3) (absence of interaction between protein and salt ions) are not fulfilled by the present system, it is remarkable that the calculated molecular weight is of the same order of magnitude as that of the insulin monomer which presumably prevails in solution in this range of concentration (*vide infra*).

In the pH range below pH 2.5, the solubility of insulin in the presence of 0.05 and 0.06 N thiocyanate reaches, approximately, a constant value (Fig. 5), presumably since, under these conditions, the protein has attained its maximum charge.

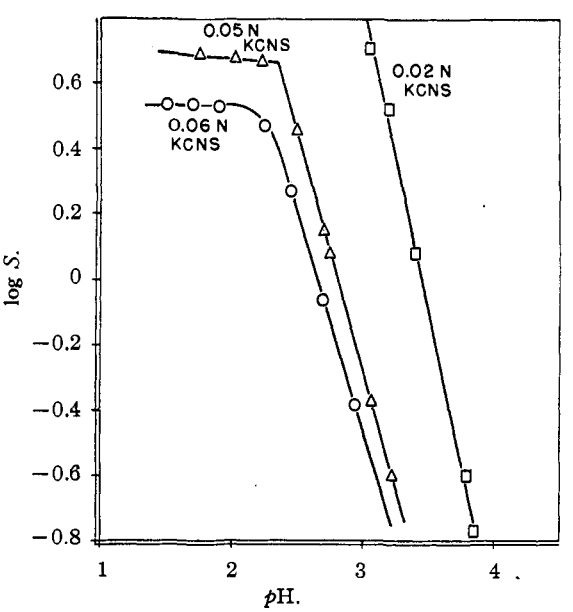

Fig. 5.—The logarithm of the solubility of insulin in the region of low pH, measured in solutions of varying thiocyanate concentrations, total ionic strength 0.1.

In view of the findings of Lens,[25] the solubility test for purity was applied to the present preparation of crystalline zinc-insulin. At pH 4.0, in the presence of 0.1 N sodium chloride, the curves obtained when the solubility was plotted against the

(23) K. Linderstrøm-Lang, *Arch. Biochem.*, **11**, 191 (1946).
(24) W. F. H. M. Mommaerts and H. Neurath, in preparation.
(25) J. Lens, *Biochim. et Biophys. Acta*, **2**, 76 (1948).

total concentration of insulin in the system were those characteristic of a single component.

In contradistinction to Lens' presumption,[25] no evidence for irreversible changes due to the addition of hydrochloric acid was found. In 0.1 *N* sodium acetate, *p*H 4.0, however, the solubility was considerably higher and varied with the total concentration of insulin, in confirmation of Lens' results. In the presence of thiocyanate, at *p*H 2.2, the solubility of insulin was likewise dependent on insulin concentration.

Electrophoresis.—Electrophoretic patterns of insulin, between *p*H 1.6 and 3.1, in sodium chloride and glycine buffer, were typical of a single component. However, after prolonged electrophoresis (three hours at 3 volts per cm.) a slight asymmetry of the patterns was observed. The appearance of the patterns was unaffected by 0.02 *N* thiocyanate. The *p*H–mobility curve shows a marked decrease in the net charge in the presence of thiocyanate (Fig. 6). This finding is in qualitative agreement with the results of Volkin[5] obtained between *p*H 5 and 8. Quantitative interpretations in terms of the change in valence of the protein will not be attempted in view of the effects of thiocyanate and *p*H on the state of aggregation of the insulin molecule.

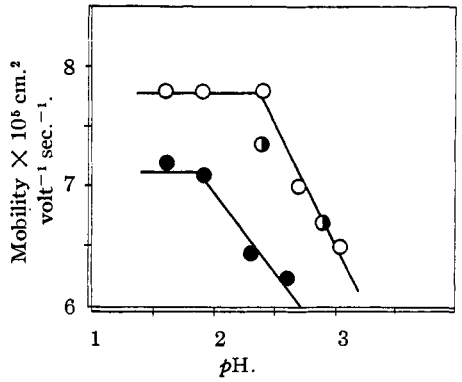

Fig. 6.—Effect of *p*H on the electrophoretic mobility of insulin in different salt solutions at ionic strength 0.1: ○, 0.08 *N* sodium chloride–0.02 *N* glycine hydrochloride; ◑, 0.1 *N* sodium dihydrogen phosphate; ●, 0.02 *N* potassium thiocyanate–0.06 *N* sodium chloride–0.02 *N* glycine hydrochloride.

Sedimentation Analysis.—The effect of thiocyanate on the sedimentation rate of insulin was determined at four different *p*H values (Fig. 7). Because of the dependence of the state of aggregation on ionic strength,[4] all sedimentation analyses were carried out at a constant total ionic strength of 0.1 which was maintained by the addition of sodium chloride. The data plotted in Fig. 7 show that in the acid *p*H region the addition of thiocyanate promotes association of the insulin monomer until an experimentally limiting value of $S = 3$ is approached beyond which precipitation occurs. The molecular size alone, however, is not

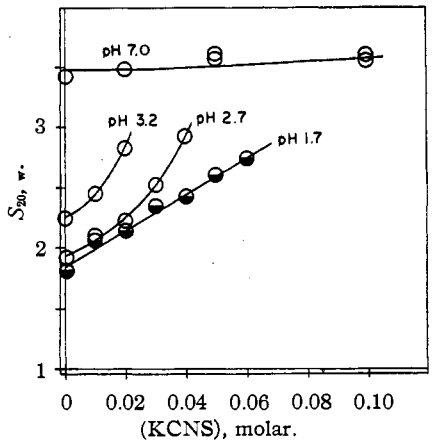

Fig. 7.—Effect of thiocyanate concentration on the sedimentation constant of 0.25% insulin at ionic strength 0.1 and various *p*H values.

the sole factor which determines the stability of the solution, for higher aggregates can exist at higher ionic strength[4] or on the alkaline side of the isoelectric point. At *p*H 7, there is almost no effect of thiocyanate on the sedimentation rate which agrees with the fact that there is no measurable binding of this anion at that *p*H.

There is a strong dependence of the sedimentation constant of insulin on the protein concentration, as has been pointed out previously by Ellenbogen and Oncley.[4] The data given in Fig. 8 show the marked decrease in sedimentation constant with decreasing insulin concentration at *p*H 1.7 and 2.7, in the presence of thiocyanate and phosphate, respectively (total ionic strength, 0.1). At relatively high protein concentrations a nearly constant degree of molecular aggregation is attained but the well known concentration effect on sedimentation rates[14] tends to decrease somewhat the apparent sedimentation constant. Below about 1% protein, the sedimentation constant de-

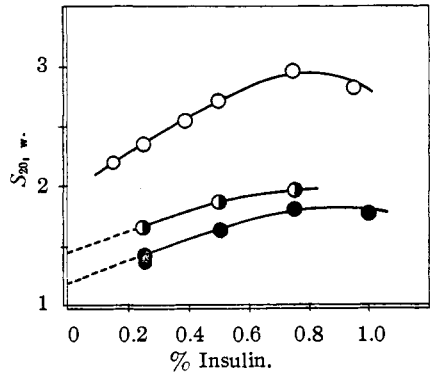

Fig. 8.—Effect of protein concentration on the sedimentation constant of insulin in different salt solutions, total ionic strength 0.1: ●, 0.1 *N* sodium dihydrogen phosphate—phosphoric acid, *p*H 2.6; ◑, 0.1 *N* sodium chloride, *p*H 2.4; ○, 0.03 *N* potassium thiocyanate–0.07 *N* sodium chloride *p*H 1.7.

creases with decreasing protein concentration because of molecular disaggregation, approaching a limiting minimum value. As the apparent sedimentation constant in 0.25% insulin is not greatly different from the extrapolated value at infinite dilution this concentration was used for the remaining sedimentation studies of this investigation.

The sedimentation rate of insulin is a function not only of the *p*H but also of the nature of the anions. This is evidenced by the results shown in Fig. 9 for the *p*H range of *p*H 1.5 to 4.1. In the presence of 0.1 *N* sodium chloride as well as in the presence of 0.02 *N* thiocyanate–0.08 *N* sodium chloride, the degree of disaggregation reaches a constant minimum level below *p*H 2.5, the presence of 0.02 *N* thiocyanate causing a shift of the disaggregation curves toward lower *p*H values. At even higher thiocyanate concentrations (*i. e.*, 0.06 *N*), the ascending part of the curve cannot be reached because complete precipitation occurs at and above *p*H 2.5.

The effects of thiocyanate on insulin are fully reversible. After removal of thiocyanate by dialysis, insulin regains its original sedimentation constant, solubility, electrophoretic mobility and biological activity.

The effect of binding on the state of aggregation of insulin is not limited to the thiocyanate ion. Iodide, which is strongly bound by serum albumin,[26] has likewise a marked effect which, however, is weaker than that of thiocyanate. Thus in the presence of 0.1 *N* sodium iodide, the sedimentation constants of 0.25% insulin at *p*H 2 and 2.5 were, respectively, $S = 2.55$ and 2.65, as compared to $S = 2.7$ in the presence of 0.06 *N* thiocyanate at *p*H 2 and $S = 1.6$ and 1.7 in the presence of 0.1 *N* sodium chloride at *p*H 2 and 2.5, respectively.

Ellenbogen and Oncley[4] explain the molecular aggregation of insulin by assuming that constant attractive forces (mainly of the nature of interfacial forces) tend to associate the monomer units while electrostatic repulsive forces act in the opposite way, the effect of the latter being related to the amount of protons bound. In accordance with this hypothesis, the effect of thiocyanate on insulin can be accounted for by assuming that the binding of thiocyanate, and of other anions, decreases the net positive charge and, consequently, shifts the disaggregation toward lower *p*H values.

We may now examine whether the effect of potassium thiocyanate on the measured physical properties can be wholly accounted for by the change in net charge of the protein which results from the binding of this anion. According to this postulate, insulin in the presence and absence of thiocyanate, respectively, will have the same net charge at two different *p*H values which are conjugate to the same physical constant. Thus, for instance, according to the data plotted in Fig. 9, the sedimentation constant of insulin in 0.02 *N* thiocyanate–0.08 *N* sodium chloride at *p*H 2.95 is the same as that of insulin in 0.1 *N* sodium chloride at *p*H 3.4 ($S = 2.6$). Assuming that the state of aggregation is solely a function of the net charge, it is apparent that the intrinsic increase in the net positive charge resulting from the binding of additional protons at *p*H 2.9 must have been compensated by a decrease in the net positive charge due to the binding of an equivalent amount of thiocyanate anions. In other words, the shift along the *p*H axis observed for any of the present physical constants under the influence of thiocyanate ions corresponds, as a first approximation, to the number of protons bound.

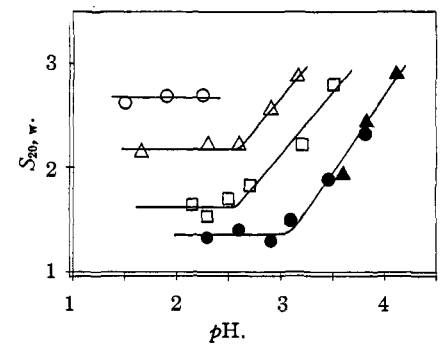

Fig. 9.—Effect of *p*H on the sedimentation constant of 0.25% insulin in different salt solutions at ionic strength 0.1: ○, 0.06 *N* potassium thiocyanate–0.04 *N* sodium chloride; △, 0.02 *N* potassium thiocyanate–0.08 *N* sodium chloride; □, 0.1 *N* sodium chloride; ●, 0.1 *N* sodium dihydrogen phosphate; ▲, 0.1 *N* sodium acetate.

The shift in the *p*H of minimum *solubility* in 0.02 *N* thiocyanate is 0.45 *p*H unit which, from titration curves,[24] corresponds to the binding of 1.4 equivalents of protons per 12,000 g. of insulin. Similarly, the shift in the ascending portion of the *sedimentation curve* in 0.02 *N* thiocyanate (Fig. 9) corresponds to the same value of 0.45 *p*H unit. The *electrophoretic mobility* (Fig. 6) of insulin in 0.02 *N* thiocyanate at *p*H 2.60 is equal to that of insulin in 0.1 *N* sodium chloride (glycine buffer) at *p*H 3.20, corresponding to a *p*H difference of 0.6 *p*H unit, or to a difference in proton binding of 1.8 equivalents per 12,000 g. of insulin. Comparison of electrophoretic mobilities appears to be justified in this case since at the two experimental conditions under consideration, the sedimentation constants are the same. Finally, *binding studies* indicate that in the presence of 0.02 *N* potassium thiocyanate, 2.8 moles of anions are bound per mole of protein (Fig. 1). The marked discrepancy between the latter value and the preceding ones requires consideration. It is to be noted that no account was taken in these calculations of the possible binding of chloride ions by insulin. If it is assumed that in the presence of 0.1 *N* sodium chloride, 1 mole of chloride ions is bound per mole of insulin, a value which is lower

(26) G. Scatchard and E. S. Black, *J. Phys. and Colloid Chem.*, **53**, 88 (1949).

than that found by Scheinberg and Armstrong[27] for the chloride binding of serum albumin, one additional unit would have to be added to the charge changes calculated from solubility, sedimentation and electrophoresis measurements. This results in values of 2.4, 2.4 and 2.8 equivalents of hydrogen ions bound as compared to the value of 2.8 derived from binding studies. These considerations are based on the implicit assumption that the binding of anions has no effect on the isoionic point of the protein, an assumption which is not necessarily valid.[26]

The postulated binding of chloride ions by insulin was subjected to experimental tests by determining the sedimentation rate of insulin in the presence of dihydrogen phosphate and of acetate. These anions were chosen since the results of Klotz and Urquhart[28] showed that phosphate and acetate ions had low binding affinities for serum albumin, as compared to the other ions tested by these authors. The right-hand curve in Fig. 9 shows, indeed, a considerably lower degree of association of insulin in 0.1 N sodium dihydrogen phosphate and in 0.1 N sodium acetate, as compared to sedimentation rates in 0.1 N sodium chloride at the same pH.

The Minimum Molecular Weight of Insulin.— Osmotic pressure measurements of Gutfreund[3] and ultracentrifugal and diffusion measurements of Oncley and Ellenbogen[4] indicate that in dilute solutions, the molecular weight of insulin approaches a minimum value of about 12,000. Oncley and Ellenbogen's[4] value was obtained by extrapolation of sedimentation rate measurements, in 0.1 N NaCl, to zero protein concentration, yielding a value of $S = 1.85$. This constant, together with the revised value for the partial specific volume ($V = 0.707$) and of the diffusion constant of $D_{20} = 13.5 \times 10^{-7}$ cm.^{2} per sec., gave a molecular weight of 11,500.

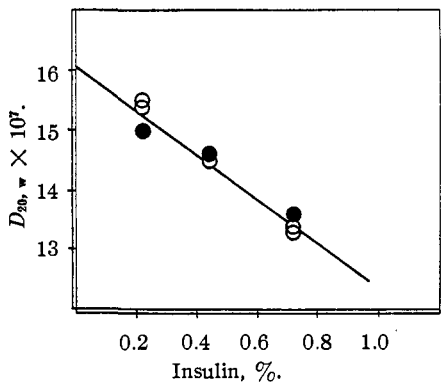

Fig. 10.—Effect of protein concentration on the diffusion constant of insulin in 0.1 N sodium dihydrogen phosphate at pH 2.6: O, calculated by the maximum ordinate–area method; ●, calculated by the method of moments.

(27) Quoted in (26).

(28) I. M. Klotz and J. M. Urquhart, *J. Phys. and Colloid Chem.*, **53**, 100 (1949).

Since the present measurements (Fig. 9) showed that in the presence of 0.1 N dihydrogen phosphate, the sedimentation constant of 0.25% insulin was significantly lower than the minimum extrapolated value reported by Oncley and Ellenbogen in the presence of 0.1 N NaCl, the concentration dependence of the sedimentation constant of insulin in dihydrogen phosphate at pH 2.6 was determined (Fig. 8). The extrapolated value at zero protein concentration was $S = 1.2$. Diffusion constants were similarly determined in the same phosphoric acid—dihydrogen phosphate buffer, pH 2.6, and are shown in Fig. 10. The relatively large concentration dependence of the diffusion constant in regions of relatively low protein concentration may also be considered as being indicative of molecular disaggregation. The extrapolated value of $D_{20} = 16 \times 10^{-7}$ cm.^{2} per sec., together with $S = 1.2$, and $V = 0.707$[4] yields a molecular weight of insulin in 0.1 N dihydrogen phosphate pH 2.6, of 6,300, and a frictional ratio of $f/f_0 = 1.1$. In view of the relatively large error which attends sedimentation rate measurements of small protein molecules in low protein concentrations, it is reasonable to assume that the actual minimum molecular weight is approximately 6,000.

The effect of dihydrogen phosphate on the molecular weight of insulin is reversible. After removal of dihydrogen phosphate by dialysis and readjustment of the ionic strength to 0.1 with sodium chloride, the sedimentation constants are the same as those obtained by direct dissolution of insulin in sodium chloride. The lower sedimentation constants observed in the presence of dihydrogen phosphate cannot be ascribed to electrostatic effects[14] since in these measurements the protein concentration was of low order, and since the rate measurements were extrapolated to zero protein concentration. It is recognized that under such conditions, charge effects become nullified.[14]

While there is no doubt that in the presence of 0.1 N NaCl the insulin exists in a higher degree of association than in 0.1 N dihydrogen phosphate, one may question whether in the presence of sodium chloride, a definite molecular weight (*i. e.*, 12,000) may be assigned to the protein. While in and above 0.5% protein solutions, the present sedimentation measurements agree with those obtained by Oncley and Ellenbogen,[4] in lower concentrations, *i. e.*, 0.25% protein, lower sedimentation constants were consistently obtained in the present work ($S = 1.6$), yielding an extrapolated value of $S = 1.5$, and perhaps even somewhat lower. A sedimentation constant of $S = 1.6$ was likewise obtained by M. H. Schwert[29] for complexes of insulin with cetyltrimethylammonium bromide and a value of 1.5 for insulin methyl ester.[24] It appears, therefore, that even in 0.1 N sodium chloride, in sufficiently dilute so-

(29) M. H. Schwert, Ph.D. Thesis, University of Minnesota, 1949

lutions, the average molecular weight of insulin is lower than 11,500.

The proposed value of 6,000 for the minimum molecular weight of insulin is in apparent contradiction to the results of the amino acid analysis of this protein, which likewise yielded a minimum molecular weight of about 12,000.[18] However, since each 12,000 molecular weight units of insulin purportedly contains four polypeptide chains,[18] one would have to assume merely that one pair of polypeptide chains differs slightly in amino acid composition from the other pair. Closer inspection of the data of Brand,[30] Chibnall[18] and Fromageot[31] reveals, however, certain divergences in the analyses of the different authors.

In most instances, the number of moles of amino acid residues per 12,000 g. of insulin is an even one, thus permitting of a minimum chemical molecular weight only one-half as great as reported. In the few instances in which an odd number of moles of amino acid residues has been reported, there is disagreement between the three authors,[18,30,31] with the single exception of glycine for which a value of 7 has been consistently obtained. Since the glycine analyses have been considered by one of the authors[18] to give the least reliable results, it appears that even the analytical data are not in significant disagreement with a minimum molecular weight for insulin of 6,000. It is evident that additional analytical results have to be awaited before the minimum chemical molecular weight of this protein can be definitely established.

It is unlikely that the molecular weight of 6,000, observed in solutions of dihydrogen phosphate, is a result of a specific disaggregating effect of this anion. It is more likely that the molecular weight of the insulin monomer is actually 6,000 and that the presence of ions which are bound by the protein, such as chloride, thiocyanate or iodide, suppresses disaggregation into the monomer by a lowering of the net charge of the protein molecule.

Acknowledgment.—We are indebted to The Lilly Research Laboratories, Eli Lilly and Company, for support of this work and for the supply of crystalline zinc-unsulin.

Summary

The reversible binding of thiocyanate ions by crystalline zinc-insulin has been investigated by use of the methods of equilibrium dialysis, sedimentation in the ultracentrifuge, electrophoresis and solubility.

From the binding curve at *p*H 2.7–3.6, interpreted on the assumption of purely statistical binding, a value of 6 has been calculated for the maximum number of thiocyanate ions bound per 12,000 molecular weight unit of insulin. No binding occurred at *p*H 7.

The effect of thiocyanate on the solubility of insulin has been determined as a function of the total ionic strength of the solution, and interpreted by an empirical relation which bears formal resemblance to the salting-out equation. At constant ionic strength, the logarithm of the solubility is related parabolically to the *p*H of the solution, the resulting curves being shifted toward lower *p*H values with increasing thiocyanate concentrations.

The electrophoretic patterns of insulin between *p*H 1.5 and 3.0, in the presence of thiocyanate, were as symmetrical as those obtained in the presence of sodium chloride alone. However, the cationic electrophoretic mobilities were markedly reduced in the presence of thiocyanate.

On the acid side of the isoelectric point, the sedimentation constants of insulin, determined under specified conditions of protein concentration, *p*H and ionic strength, were increased in proportion to the concentration of thiocyanate. At *p*H 7, the addition of thiocyanate was virtually without effect on the sedimentation constant. The effect of iodide is qualitatively the same as that of thiocyanate. Theoretical considerations suggest that the effects of thiocyanate on the physical properties of insulin may be wholly accounted for in terms of the effect of binding of thiocyanate (and of chloride) on the net charge of the protein.

In the presence of dihydrogen phosphate, below *p*H 3, the molecular disaggregation of insulin proceeds farther than in the presence of chloride ions. Sedimentation and diffusion measurements at *p*H 2.6 in dihydrogen phosphate, after extrapolation to zero protein concentration, yield a minimum molecular weight of insulin of approximately 6,000. This value is discussed in relation to other pertaining experimental data and is proposed to represent the monomer unit of this protein. The association of this unit to dimers and higher polymeric forms is related to its binding affinity for anions.

DURHAM, NORTH CAROLINA RECEIVED OCTOBER 27, 1949

(30) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(31) C. Fromageot, "Cold Spring Harbor Symposia on Quantitative Biology," 1949, in press.