solutions having previously been brought to the temperature desired in a carefully regulated water-bath).

At appropriate times an aliquot of 4 ml. was removed from each reaction vessel and added to a tube containing 3 ml. of 2×10^{-4} *M* mercuric chloride solution, which stopped the enzymatic action. The unhydrolyzed galactoside was estimated from the solution density at its absorption maximum[2] (232 or 236 mμ) in the Beckman DU spectrophotometer following dilution when necessary. Correction was made for absorption due to the enzyme and reagents. The velocity *v* is expressed in millimoles per liter per 30 minutes; and the substrate concentration, *S*, in millimoles per liter. The results were plotted and the slope and intercept calculated by the method of least squares.

Acid Hydrolysis.—The experiments were run in a water-bath regulated to ±0.2°. To 25.0 ml. of 0.02 *N* hydrochloric acid preheated five minutes at bath temperature was added 25.0 ml. of a solution of approximately 140 mg. of glycoside per liter, preheated for the same period. At the end of one minute a 5.0-ml. sample was withdrawn and placed in 20.0 ml. of 0.1 *M* *p*H 7.0 Clark and Lubs buffer. Samples were removed at 15-minute intervals, so that five samples in all were obtained. Using a Beckman DU spectrophotometer, the ultraviolet absorption at the absorption maximum of each buffered sample was determined in a 1-cm. quartz cell against a blank composed of 10.0 ml. of 0.02 *N* hydrochloric acid, 10.0 ml. of water, and 80.0 ml. of 0.1 *M* *p*H 7.0 Clark and Lubs buffer. Duplicate hydrolyses of each isomer were performed. Optical density readings were converted to logarithms of concentration, plotted against time, and the linear relationship characteristic of first-order reactions was established. Slopes were derived statistically by the method of least squares and the average of two determinations for each compound was taken. Rates are expressed in Briggsian logarithms and minutes, according to custom in carbohydrate studies. Heats of activation were calculated from these average rates of hydrolysis using the integrated form of the Arrhenius equation.

Acid Hydrolysis of the Saponified Glucoside (V).—To a solution of 38.0 mg. of I in about 20 ml. of water was added 0.153 meq. of sodium hydroxide, and the solution was kept at room temperature (25°) for 45 minutes. An equivalent amount of hydrochloric acid was added and the solution was diluted to 100 ml. This treatment effects complete saponification of the methyl ester, as indicated by the change of the absorption coefficient to a constant value. (The optical rotation was followed on a more concentrated sample and came to a constant value in about the same length of time).

The determination and the calculation of the hydrolysis rates were conducted as described above. The extinction coefficient for the saponified glucoside was calculated from the average of the optical densities at zero time for the two runs, $\epsilon = 7.05 \times 10^3$ at 236 mμ.

Acknowledgment.—We are indebted to Dr. Joshua Lederberg for the dried cells from which the enzyme extract was prepared.

MADISON, WISCONSIN RECEIVED NOVEMBER 8, 1951

[CONTRIBUTION FROM THE GIBBS LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

The Association of Insulin. I. Preliminary Investigations

BY PAUL DOTY, MARTIN GELLERT AND BERNARD RABINOVITCH

A light scattering investigation of insulin solutions show that below *p*H 2.2 the monomer-dimer (molecular weight 12,000 and 24,000) equilibrium can be isolated. Data covering a fifty fold range in concentration can be fitted with a single equilibrium constant. At higher *p*H values tetramer and probably trimer exist. The tetramer species is dominant at *p*H 3.17 above a concentration of 0.3%. The effect of varying the ionic strength is illustrated. It is shown that earlier data on less highly purified insulin preparations may not be in conflict with the behavior just summarized. This behavior is in fair agreement with published osmotic pressure measurements but it is in disagreement with the current interpretation of sedimentation constants.

For more than a decade the molecular state of insulin in solution has been in dispute. Meanwhile great advances have been made in our knowledge of the internal structure of insulin and it has become increasingly evident that association and dissociation reactions of proteins in general deserve critical investigation by a variety of physical methods. Consequently we have undertaken an investigation of insulin solutions by light scattering methods.

Since the available data on the problem have been reviewed recently by several authors[1-3] our references to earlier work need not be comprehensive. Crowfoot's[4] X-ray studies in 1938 showed the unit cell molecular weight to be 37,600. Later revision of the moisture content changed this value to 36,000. The existence of trigonal symmetry led to the suggestion that three molecules of 12,000 molecular weight are arranged with this symmetry in the unit cell and may constitute the kinetic unit which exists in solution.

The determination of the mean size of the kinetic unit in solution has been the subject of numerous investigations using sedimentation and diffusion and osmotic pressure measurements. In neutral solutions molecular weights in the range of 36,000 to 46,000 are obtained,[3,5] the differences arising principally from the use of different values of the partial specific volume. Gutfreund[6] reports osmotic pressure molecular weights of 48,000 in this same range at concentrations of 0.5 to 0.8%. However, at lower concentrations dissociation was evident. Other experiments, mostly by these same workers, have shown that the degree of association is greatly decreased in acid solutions, in particular by decreasing the *p*H, the ionic strength and the dielectric constant. There is, however, no quantitative agreement on the dominant species present. Thus at about *p*H 2.5 and ionic strength 0.1 Oncley and Ellenbogen[3] consider insulin to be in the form of monomer (molecular weight 12,000), Pedersen[5] in the form of dimer while Fredericq and Neurath[2] propose the existence of a half-monomer unit when sodium dihydrogen phosphate is the added electrolyte. Gutfreund's osmotic pressure data indicate an average molecular weight of a dimer under these conditions. As conditions are changed to favor association there is agreement, except for

(1) F. Sanger, *Ann. Reports Chem. Soc., Long*, 283 (1948).

(2) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(3) J. L. Oncley and E. Ellenbogen, *J. Phys. Colloid Chem.*, **56**, 85 (1952).

(4) D. Crowfoot, *Proc. Roy. Soc.* (*London*), **A164**, 580 (1938).

(5) K. D. Pedersen, *Cold Spring Harbor Symposium on Quantitative Biology*, **14**, 148 (1949).

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

Pedersen, that the trimer is dominant. Inasmuch as a labile association should be quite concentration dependent, it is surprising that there are only marginal indications of this in the concentration dependence of sedimentation constants. Such a dependence is clearly shown by the osmotic pressure work, however.

The light scattering method having been applied successfully to the determination of the molecular weight and interaction of a number of proteins,[7] it was of interest to see if its application to the insulin problem would lead to any clarification. In particular we hoped that conditions could be found in which only two species in equilibrium would exist so that it could be characterized thermodynamically by measuring its concentration and temperature dependence.

Reference is made to a recent review[7] for theoretical and experimental details of the light scattering method. In the work reported here, we have simply applied the basic light scattering relation

$$Kc/R_{90} = (1/M) + 2Bc \qquad (1)$$

to insulin solutions in which the ionic strength was maintained at 0.1 in order to minimize the contribution of the charge effects to the value of B. Indeed experiments with bovine serum albumin and other proteins[7] show that under these conditions B is essentially zero. Using this approximation we have then determined the weight average molecular weight at numerous concentrations under several sets of conditions.

Experimental

Insulin.—The experiments discussed in the next section, the only ones which are quantitatively interpreted, were performed with pancreatic beef insulin five times recrystallized (lot T-2344) obtained through the courtesy of the Eli Lilly Co., Indianapolis. Its zinc content was 0.59% and was not removed. The other samples referred to in a later section were another pancreatic beef insulin from the Lilly Co. (lot 280177) and a mixed beef-pork insulin also from the Lilly Co. (lot 491516). Both contained normal amounts of zinc.

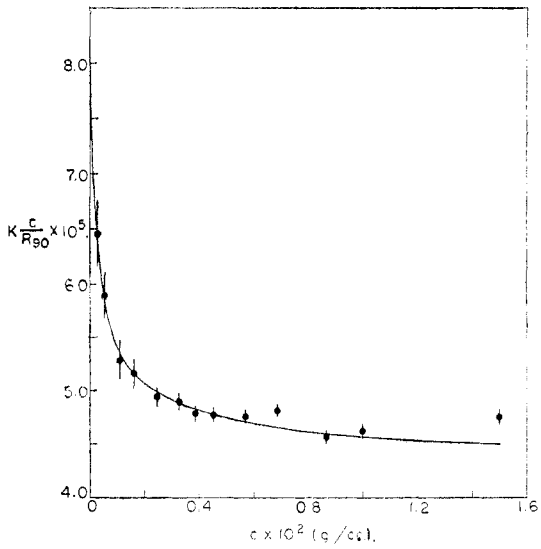

Fig. 1.—Light scattering data for insulin (T-2344) in 0.1 M KCl at pH 1.96. Full line is drawn for $K_{21} = 1.67 \times 10^{-5}$.

Extinction Coefficient.—The value of the extinction coefficient at the maximum of the 280 mμ absorption band was taken as 11.3 from the work of Ellenbogen.[8] Our concentration measurements are based on this value.

Refractive Index Increment.—The value of the refractive index increment, required for the evaluation of K in equation (1), was found to be 0.202 at λ 436 mμ using a differential refractometer.

Preparation of Solutions.—Solvents were prepared by dissolving appropriate amounts of salts in doubly glass-distilled water and adjusting the pH to the desired value. Insulin was then added to a part of this solvent to make a solution of the highest concentration to be employed in the light scattering measurements. The pH of the insulin solution was then adjusted to that of the solvent and both solution and solvent were centrifuged at 20,000 g. for at least eight hours.

Light Scattering Photometer.—Scattering measurements were made on a slightly modified Brice–Spicer light scattering photometer[9] using the blue mercury line (λ 436 mμ). The calibration of the photometer has been reported previously.[9,10] Having established that the photometer cell was optically clean, centrifuged solvent was introduced into it and the reduced intensity at 90°, R_{90}, was determined. Solution was then added stepwise, the concentration being determined by weighing and use of the extinction coefficient determined on the centrifuged solution. In some cases, in order to make measurements at higher concentrations, the centrifuged solution was placed in the cell and diluted stepwise with solvent.

The Monomer–Dimer and Other Equilibria

Our more recent work has been with the best characterized of the samples, T-2344, and it is only in these experiments that the optical conditions and the required precision have been sufficiently fulfilled to justify quantitative interpretation of the data. Consequently, these results are presented first.

In Fig. 1 are plotted the data obtained at pH 1.96 in 0.1 M KCl. We note that the concentration dependence of Kc/R_{90} is marked, particularly at low concentration and although the measurements extend down to 0.03% an extrapolation to zero concentration would be quite inaccurate. At higher concentrations, however, the values appear to approach a value of about 4.5×10^{-5} which, subject to our assumption that $B = 0$, corresponds to a weight average molecular weight of 22,000. This suggests that the dimer may be the highest molecular species prevalent under these conditions. Its dissociation to the monomer upon dilution could apparently account qualitatively for the observed data.

To see if this could provide a quantitative explanation for the data, we proceed to define α as the degree of dissociation of the dimer in the equilibria represented by $I_2 = 2I$. The thermodynamic equilibrium constant is then given by

$$K_{21} = (4\alpha^2 m/1 - \alpha) \qquad (2)$$

where m is the total concentration expressed in moles of dimer per liter. For a given value of K the weight concentrations of the monomer, c_1, and the dimer, c_2, can be calculated and the reciprocal weight average molecular weight, equivalent to Kc/R_{90} evaluated from the equation

(7) P. Doty and J. T. Edsall, *Advances in Protein Chem.*, **6**, 35 (1951).

(8) E. Ellenbogen, Thesis, Harvard University, 1949.

(9) B. A. Brice, M. Halwer and R. Speiser, *J. Optical Soc.*, **40**, 768 (1950).

(10) P. Doty and R. F. Steiner, *J. Chem. Phys.*, **18**, 1211 (1950).

$$\frac{1}{M} = \frac{c}{12{,}000\,(c_1 + 2c_2)} \qquad (3)$$

Choosing a value of $K_{21} = 1.67 \times 10^{-5}$, the line drawn in Fig. 1 is obtained. The fit is seen to be within the probable experimental error which varies from $\pm 5\%$ at the lowest concentration to $\pm 1\%$ at the highest. (Our systematic error due to uncertainties in the calibration may be as high as 5%.[10]) The conclusion follows that under these conditions of *p*H 1.96 and 0.1 *M* KCl the state of insulin, for this particular sample at least, is that of an equilibrium between monomer and dimer, showing a normal concentration dependence. The two species exist in equal weight concentration at about 0.08 g./100 ml. of insulin.

All of the carboxyl groups of insulin are titrated at about *p*H 2.20.[8] In the view of Oncley and Ellenbogen,[3] the repulsion of like-charged insulin monomers is the principal energy factor causing dissociation of the associated form. On this basis one would expect therefore that changing the *p*H from 1.96 to 2.20 would not significantly alter the equilibrium. The results under these conditions are shown in Fig. 2. These data fall from 0 to 5% below the data at *p*H 1.96. (The scatter of the data is somewhat greater than that usually obtained and is indicative of a slight contamination with dust.) There is no discernible trend toward molecular weights greater than the dimer at higher concentrations. It appears therefore that the monomer–dimer equilibrium persists at this *p*H as expected. The line drawn in Fig. 2 corresponds to $K_{21} = 0.625 \times 10^{-5}$, a compromise between fitting the low concentration data where contamination was less likely and the higher concentration data which are more numerous. The dashed line in Fig. 2 reproduces the line in Fig. 1.

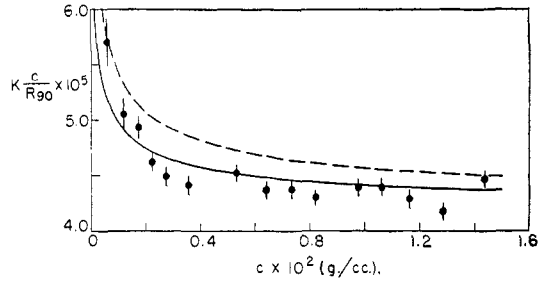

Fig. 2.—Light scattering data for insulin (T-2344) in 0.1 *M* KCl at *p*H 2.20. Full line is drawn for $K_{21} = 0.625 \times 10^{-5}$, dashed line for $K = 1.67 \times 10^{-5}$.

Upon passing to higher values of *p*H, the net charge is diminished and association should be more pronounced. The data shown in Fig. 3 are the result of measurements at *p*H 3.17 also in 0.1 *M* KCl where the charge is reduced by about 30%.[8] We note at once that higher molecular weight species must be present. The higher concentration data fall considerably below the value of Kc/R_{90} corresponding to a trimer (2.78×10^{-5}) and approach close to the value corresponding to a tetramer (2.08×10^{-5}). Thus it appears that the tetramer is predominant at higher concentrations; the possibility of fitting the data with equilibria involving the tetramer and lower molecular weight species should be explored.

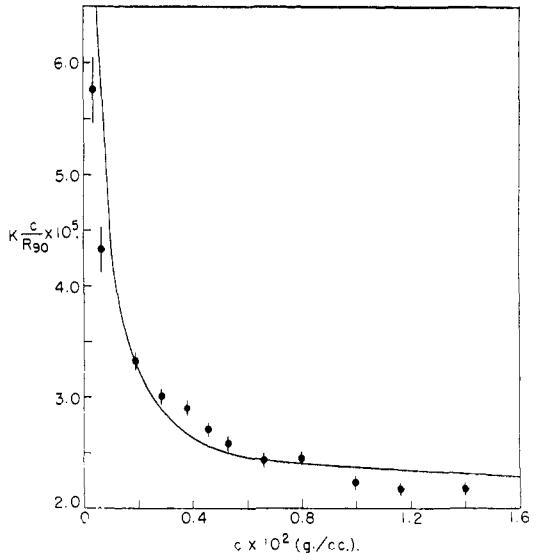

Fig. 3.—Light scattering data for insulin (T-2344) in 0.1 *M* KCl at *p*H 3.17. Full line is drawn for $K_{41} = 1.85 \times 10^{12}$.

Unfortunately instead of having only one possible equilibrium to investigate as at lower *p*H values, several possibilities are now open and it is unlikely that the data shown here are sufficiently precise and extensive to discriminate between the various possibilities. Among these possibilities we might consider first the most obvious one, a tetramer–monomer equilibrium in which the bonds are of equal energy. This implies that the monomer units lie in a linear sequence. This is indeed unlikely since it does not account for the absence of higher forms under conditions which lead to a high percentage of dimer nor does it provide a reason for tetramers being the most highly associated species. However, it is of interest to see how well this simplest equilibrium involving only one constant can fit the present data; the best fit with this equilibrium is shown in Fig. 3. Except for the two lowest concentration points, the fit is seen to be comparable to that obtained previously.

Two other possibilities may be mentioned. In one the tetramer would be formed from the union of two dimers. In the other, the trimer may form from a dimer and monomer and the tetramer would in turn form from the trimer by addition of another monomer as suggested by Oncley and Ellenbogen.[3] The former would require two and the latter three equilibrium constants to fit the dissociation curve. The present data do not permit any choice between these two more plausible views, but future work may lead to a decision.

Decreasing the ionic strength offers one way of increasing the dissociation. However, to be effective the decrease must be so great that the second virial coefficient, *B*, becomes quite large and complicates the interpretation of the data. As an illustration we show in Fig. 4 data obtained at a total gegenion concentration (Cl^-) of 0.016 *M* at *p*H 2.30. It is seen that the data at higher concentration fall on a straight line which extrapolates to 4.0×10^{-5} ($\bar{M} = 25{,}000$) indicating the existence of essentially pure dimer at concentrations

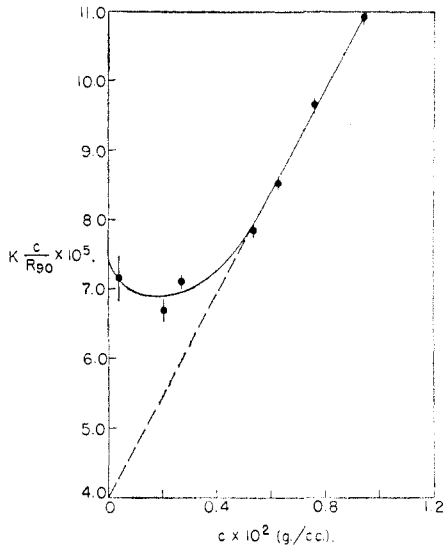

Fig. 4.—Light scattering data for insulin (T-2344) in 0.016 M chloride ion at pH 2.30.

above 0.6%. At lower concentrations dissociation is clearly evident although the data are not sufficient to establish the curve with high precision. Assuming that B has the same value for the dimer as for the monomer, as would be the case for purely electrostatic interactions, the dissociation appears to be greater than exhibited in Fig. 2. The extrapolated value of Kc/R_{90} corresponds to $\bar{M}$ = 13,500 but at these low concentrations the probable error is so great that this is not in conflict with a real value of 12,000.

In current experiments in this Laboratory Mr. George Myers has shown that the dissociation is increased by replacing chloride ions by dihydrogen phosphate[2] and by raising the temperature. By changing the conditions of measurement in this manner, the dissociation may be sufficiently shifted to allow a direct measurement of the monomer molecular weight.

Earlier Experiments

In our initial investigation of this problem, which was begun in 1949, two insulin samples were found to behave differently from each other and from sample T-2344. It now appears that reasons can be advanced in each case to show that the behavior is not necessarily different from that shown above. In particular, one set of this early data which has already been published[7] is almost certainly in error by a constant factor and a retraction and explanation are in order. For these reasons and in order to illustrate some of the difficulties arising in this work, we summarize here one set of data on each of these insulin samples. It is important to emphasize that in this section we only wish to show that the earlier data may not be in conflict with the behavior outlined above. However, it should be kept clearly in mind that it is quite possible that different insulin samples may exhibit different equilibrium constants either due to intrinsic differences or to slight denaturation that might occur during the long period of preparation. Indeed we are at present examining several different purified insulin samples to see how much variation exists.

In Fig. 5 are shown data for Lilly insulin No. 491516 at pH values of 2.20 and 3.17. In comparison with Sample T-2344 this sample shows a higher mean molecular weight which at pH 2.20 exceeds that of a dimer and at pH 3.17 exceeds that of a tetramer. Although both curves clearly show dissociation at lower concentrations, it does not appear to be sufficiently pronounced to permit an extrapolation to the monomer (Kc/R_{90} = 8.33 × 10^{-5}). Such a behavior

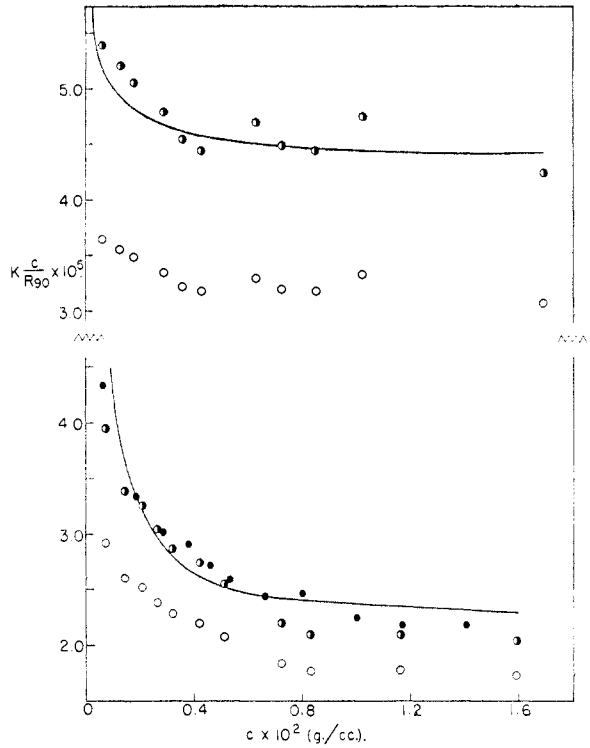

Fig. 5.—Light scattering data for insulin (491516) in which contamination with a non-dissociating impurity was suspected in 0.1 M KCl at pH 2.20 and 3.17: ○, observed data; ◑, corrected data; ●, observed data for insulin (T-2344) from Fig. 3. Full lines taken from Figs. 2 and 3.

would be expected if a non-dissociating impurity were making a significant contribution to the scattering. This possibility could be tested by determining whether the observed data could be brought into coincidence with the data of sample T-2344 by subtracting a particular constant value of R_{90} from the observed value of R_{90} for sample 491516. Whereas in a non-dissociating system such a procedure would be completely arbitrary, the introduction of a constant factor in this case must not only raise the mean value of Kc/R_{90} to that observed for sample T-2344 but to be tentatively acceptable must also produce a very special concentration dependence in the Kc/R_{90} data. Upon examination of the data it is found that an assignment of 9000 to the contribution of the non-dissociating impurity to R_{90}/Kc transforms the data as shown in Fig. 5. It is seen that the agreement with the full lines taken from Figs. 2 and 3 is quite good. Since in Fig. 3 there was some discrepancy between the line and the data, the points from this graph have also been included in Fig. 5. Indeed the agreement between the two sets of points is even closer than with the line. This strongly suggests that this insulin sample is behaving as sample T-2344 would if it were contaminated with an impurity whose R_{90}/Kc value was 9000. Of course no estimate of the mean molecular weight of the impurity is possible since its concentration is unknown.

As a second example we refer to Lilly sample 280177 for which our earliest results at pH values of 2.29, 2.85 and 3.42 in 0.1 M KCl have been published. These results, then stated to be only qualitative, were tentatively interpreted in terms of a monomer–trimer equilibrium between molecules which at pH 2.29 was completely dissociated. We had, however, strong reservations about these data because the sensitivity of our photometer was only about one tenth its present value, our calibration was not reliable and the photometer was not properly adjusted to compensate for the color of the solutions. The need to check this work under present conditions was evident but efforts to do so were restricted because only 50 mg. of the sample was available. However measurements using this amount were carried out at pH 2.29 and are shown in Fig. 6 as open circles together with a line taken from Fig. 3 corresponding to

sample T-2344 at *p*H 2.20. The data show a dissociation whose concentration dependence is quite like that of sample T-2344 and whose absolute values of Kc/R_{90} lie only a few per cent. below. Part of this difference would be expected from the difference in *p*H.

The previously published values of Kc/R_{90}[7] at *p*H 2.29 were about 80% higher than those in Fig. 6 and scattered so much in the low concentration range that the shape of the curve in the region could not be determined. Since the available data on this sample obtained under current conditions are essentially the same as for sample T-2344, two possible explanations of the earlier data are possible. One is that the equilibrium constant was altered in preparing the solutions for the earlier work; the other that the calibration constant and effect of the color of this particular insulin solution led to an over-all error of a factor of about 0.55 in the evaluation of the data. While the former cannot be excluded the latter can be shown to be quite possible in the following way. If the data on sample 280177 at representative concentrations of 0.3 and 0.8% are multiplied by 0.55 and plotted as a function of *p*H and the corresponding values from Figs. 2, 3 and 4 are also plotted, it is found that the six points at each of the two concentrations fall on a continuous line of slight curvature. Thus, if these early measurements are considered to be in error by a factor of 0.55, the two sets of data on different insulin samples each at three values of *p*H form a consistent picture. The earlier data at *p*H 2.29 corrected in this manner are shown as filled circles in Fig. 6. It is interesting to note that the early data include the *p*H value of 2.85. On the basis of the T-2344 data this is the condition under which trimer would be expected and indeed it appears to be the dominant form above 0.3% but more precise work will be necessary to substantiate this conclusion.

Discussion

Accepting the equilibrium association of insulin to be that described for sample T-2344, a comparison with studies by other methods becomes of interest.

Insofar as comparisons with the osmotic pressure measurements of Gutfreund[6] can be made there is fair agreement. In particular at *p*H 2.2 in the concentration range of 0.5–1.0%, he states that a molecular weight of 22,000 is found. This is precisely what we would conclude from Fig. 2. In addition we conclude that this result is due to the nearly complete association of monomer to dimer whereas from Gutfreund's work one can only conclude that this is an average value due to a particular distribution in the possible species from monomer to tetramer. Indeed if both his result and ours are valid, the occurrence of essentially pure dimer follows in order that a number average and weight average measurement may lead to the same value.

At *p*H 3.0, however, Gutfreund reports that the mean molecular weight is still 22,000, whereas at a little higher *p*H (3.17) we find the mean to lie between trimer and tetramer. Part of this difference is due to the measurement of different averages but an essential discrepancy remains. At *p*H 2.5 Gutfreund shows that the mean molecular weight varies from monomer to at least trimer with increasing concentration. When our measurements in this region are completed, they may well agree with these, but at present it would appear unlikely that we will find as great dissociation at lower concentrations as he does.

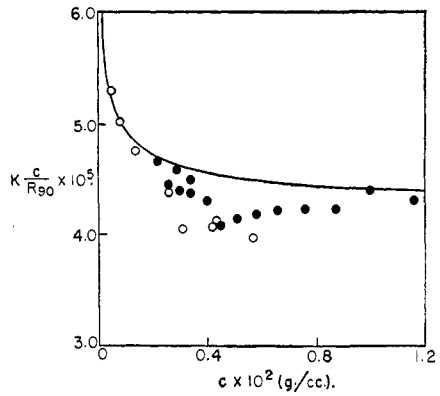

Fig. 6.—Light scattering data for insulin (280177) in 0.1 *M* KCl at *p*H 2.20: ○, recent data with more sensitive photometer and improved technique; ●, corrected data from reference 7. Full line is taken from Fig. 2.

In view of the divergent results obtained by sedimentation and diffusion studies[2,3,5,8] and our plans to join with Professor Oncley in the study of identical insulin solutions by both sedimentation and diffusion and light scattering methods, it seems premature to attempt a detailed comparison at present. In summary it may be stated that at *p*H 2 present sedimentation and diffusion measurements have not been carried to sufficiently low concentration to cover the range in which most of the dissociation of the dimer occurs according to the foregoing result. At concentrations greater than 0.4 g./100 cc. we are in agreement with Pedersen's[5] conclusion, which presumably applies to the same range, that dimers dominate at least down to a *p*H 1 to 2 when the acidification has been made with hydrochloric or phosphoric acid. However, the data to support his conclusion have not yet appeared.

Finally two further points should be considered in the comparison of data on insulin association. First the attractive forces involved in the association may differ in magnitude in insulin derived from different sources or because different extents of damage may have occurred in different preparations. Secondly, the additional pressure ($\sim$ 100 atm.) exerted on the solution in the ultracentrifuge may be sufficient to shift the equilibrium appreciably if a specific volume change accompanies the association.

Acknowledgments.—The authors wish to thank Mr. George Myers for helpful discussions and for performing the last set of measurements on Sample 280177, and the Eli Lilly Co. for financial support.

CAMBRIDGE, MASS. RECEIVED NOVEMBER 5, 1951