Kaldor, G., and Gitlin, J. (1964), *Biochim. Biophys. Acta 81*, 181.
Kaldor, G., Saifer, A., and Westley, F. (1962), *Arch. Biochem. Biophys. 99*, 275.
Kielley, W. W., and Bradley, L. B. (1956), *J. Biol. Chem. 218*, 653.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Levy, H. M., and Ryan, E. M. (1961), *Biochim. Biophys. Acta 46*, 193.
Levy, H. M., Sharon, N., Ryan, E. M., and Koshland, D. E., Jr. (1962), *Biochim. Biophys. Acta 56*, 118.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Lowey, S., and Holtzer, A. (1959), *J. Am. Chem. Soc. 81*, 1378.
Lowry, O. H., Rosebrough, M. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Lundgren, H., and Ward, W. H. (1951), Amino Acids and Proteins, Greenberg, D. M., ed., Springfield, Ill., Thomas, p. 312.
Mandelkern, L., and Flory, P. J. (1952), *J. Chem. Phys. 20*, 212.
Mommaerts, W. F. H. M. (1958), *Methods Med. Res. 7*, 7.
Morales, M. F., Botts, J., Blum, J. J., and Hill, T. T. (1955), *Physiol. Rev. 35*, 475.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Seidel, J. C., and Gergely, J. (1963), *J. Biol. Chem. 238*, 3648.
Stracher, A., and Chan, P. C. (1961), *Arch. Biochem. Biophys. 95*, 435.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford Univ. Press, p. 39.
Weiss, M., and Sobolew, N. (1913–14), *Biochem. Z. 58*, 119.
Woods, E. F., Himmelfarb, S., and Harrington, W. F. (1963), *J. Biol. Chem. 238*, 2374.

Inter- and Intramolecular Interactions of α-Lactalbumin. I. The Apparent Heterogeneity at Acid *p*H*

MARTIN J. KRONMAN† AND RAYMOND E. ANDREOTTI

From the Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pa. (M.J.K.), and the Pioneering Research Division, U. S. Army Natick Laboratories, Natick, Mass. (M.J.K. and R.E.A.)

Received February 4, 1964

Sedimentation velocity and equilibrium measurements carried out at *p*H values acid to the isoelectric zone show that α-lactalbumin exists largely in associated or aggregated form at finite protein concentration. The association process in acid *p*H appears to be a rapidly reversed equilibrium with a negative enthalpy. It can be observed independently of the aggregation reaction by carrying out experiments at sufficiently low protein concentration (below 0.8 g/100 ml). Association persists even as low as *p*H 2, where electrostatic repulsions must be significant. This is in contrast with its behavior at *p*H 8.55, where the weight-average molecular weight shows a slight positive dependence on protein concentration but does not deviate markedly from the value of 16.2×10^3 obtained at infinite dilution. At *p*H values acid to the isoelectric point at protein concentrations in excess of 1 g/100 ml, a "heavy" component is readily seen in the ultracentrifuge. The amount of this component, an aggregate of monomeric α-lactalbumin units, increases with time and with increasing protein concentration and temperature. Association at *p*H values *alkaline* to the isoelectric zone is quite feeble with little or no temperature dependence to the process, as was the case for association at acid *p*H values. The time-dependent aggregation reaction observed at acid *p*H values is essentially absent at *p*H 5.24 and 6.00 even at higher protein concentrations than were employed at *p*H 3.00 and 2.00. It seems probable that the apparent electrophoretic heterogeneity observed previously at acid *p*H values is a consequence of association or aggregation of α-lactalbumin. The feebleness of association at *p*H values alkaline to the isoelectric zone, as well as the absence of a time-dependent aggregation reaction, suggests that the apparent electrophoretic heterogeneity observed in this region may have other origins.

Gordon and Semmett (1953) noted in their paper on the isolation of crystalline α-lactalbumin that the protein was homogeneous electrophoretically in *p*H 2 and 3 glycine buffer, *p*H 6.6 and 7.7 potassium phosphate buffer, and *p*H 8.5 Veronal, but heterogeneous in *p*H 3.3 sodium lactate buffer. At that time they raised the question as to whether the protein were really heterogeneous or if the apparent inhomogeneity might be "an artifact due to some obscure effect of lactate ion." Klostergaard and Pasternak (1957) subsequently confirmed Gordon and Semmett's findings of heterogeneity in *p*H 3.3 lactate buffer and found this also to be the case in *p*H 4.83 lactate. They were unable, however, to demonstrate any such inhomogeneity under comparable conditions in the ultracentrifuge. Both Klostergaard and Pasternak (1957) and Wetlaufer (1961) show electrophoretic evidence for heterogeneity at *p*H values above 7.

It appears from our studies that much of the apparent heterogeneity of α-lactalbumin is the result of association-aggregation processes occurring at acid *p*H. The association process will be described in this paper; the characteristics of the aggregation process will be considered in the accompanying publication (Kronman *et al.*, 1964). Subsequent papers in this series will consider the molecular conformation of α-lactalbumin

* Presented in part before the Division of Biological Chemistry at the National Meeting of the American Chemical Society, Atlantic City, N. J., September, 1962, and before the Division of Colloid Chemistry at the National Meeting of the American Chemical Society, Los Angeles, Calif., April, 1963.

† Present address: Pioneering Research Division, U. S. Army Natick Laboratories, Natick, Mass.

and its relationship to properties such as susceptibility to aggregation and resistance to denaturation.

Experimental

Materials.—α-Lactalbumin was prepared from pooled commercial unpasteurized milk by either of two methods: preparations 2–69 and 3088 by the procedure of Aschaffenburg and Drewry (1957); preparations 42G, 44G, and 45G by a method developed in our laboratory (Robbins and Kronman, 1964). (Like that of Gordon and Semmett, the new procedure avoids the heating and low *p*H precipitation required for the Aschaffenburg method. Unlike the procedure of Gordon and Semmett it does not require a long-term dialysis [10–14 days] with consequential danger of bacterial attack of the protein.) Some experiments were also done with α-lactalbumin prepared by the method of Gordon and Semmett (1953). In all cases the final steps were crystallization of the protein from ammonium sulfate at *p*H 6.6. Except where noted the protein was stored in the refrigerator (2–5°) as crystals wet with *p*H 6.6, three-fourths-saturated ammonium sulfate solution from which they had been precipitated. An atmosphere of toluene vapor was kept over the protein to prevent bacterial growth. Homogeneity of these preparations was routinely verified by ultracentrifugation at *p*H 8.55 (see Results, Molecular State of α-Lactalbumin at *p*H 8.55) and by starch-gel electrophoresis (Robbins and Kronman, 1964). The latter procedure, which was carried out in *p*H 3.0, 0.05 sodium formate buffer, 5 M in urea, showed a "trace" component in every preparation which moved slightly slower than the main band. In nonurea gels at alkaline *p*H a similar trace band moved slightly faster than the main band (M. J. Kronman and R. Vitols, unpublished data). This is presumably the minor component reported by Aschaffenburg and Drewry (1957) as occurring in paper electrophoresis at *p*H 8.6 and the fast component observed by Wetlaufer (1961) in phosphate buffer at *p*H 7.5.

All solutions were prepared with glass-distilled water. Reagent grade chemicals were used throughout, except in the large-scale protein preparations where "purified" ammonium sulfate was employed.

Protein Concentration.—Protein concentration was routinely determined by measuring the ultraviolet absorption at 280 mμ in the Beckman DU spectrophotometer. An extinction coefficient, $E^{1\%}_{1\text{ cm}}$, of 20.1 was obtained in a buffer having the following composition: *p*H 6.9, $NaH_2PO_4 \cdot H_2O$, 5.20 g/liter, Na_2HPO_4, 7.70 g/liter. The extinction coefficient was obtained for solutions prepared by weighing out salt-free lyophilized α-lactalbumin, the actual concentrations being corrected for the residual moisture of the dried protein. Moistures were determined by drying separate samples to constant weight in an oven set at 105°. The value of 20.1 is in reasonable agreement with that of 20.9 previously reported (Wetlaufer, 1961), although strictly speaking no comparison can be made since the buffer employed by Wetlaufer is not stated.

Sedimentation-Velocity Measurements.—Measurements were carried out in a Spinco Model E ultracentrifuge equipped with temperature-measurement and -control systems and schlieren-phase-plate optics. The former were routinely calibrated against a Bureau of Standards certified thermometer. Unless otherwise stated, velocity measurements were carried out at a speed of 59,780 rpm. Kel-F centerpiece cells (12 mm) were generally used except for very high or very low protein concentrations. In the former case at *p*H values between 5 and 7, 3-mm Dural cells were employed. For low protein concentrations 30-mm Kel-F cells were used at a speed of 50,740 rpm.

Sedimentation constants were obtained from measurement of the position of the "peak" using a Gaertner two-dimensional traveling microscope. Values were corrected to 20° using experimentally determined values of solvent density and viscosity. The partial specific volume employed, 0.735, was that reported by Gordon and Semmett (1953).

Relative amounts of sedimenting components were estimated from the area distribution in the schlieren diagram. These were obtained by planimetric measurement of enlarged tracings of the centrifuge photographs. No corrections were made for Johnson-Ogston effects.

Sedimentation-Equilibrium Measurements in 0.15 M KCl.—Molecular weights were determined by the short-column procedure (Van Holde and Baldwin, 1958) using standard double-sector cells and schlieren optics. Column lengths of 0.8–1.0 mm were employed. Molecular weights were calculated from the equation (Van Holde and Baldwin, 1958):

$$M = \frac{1}{\bar{r}c_o}\left(\frac{dc}{dr}\right)_{r=\bar{r}} \frac{RT}{\omega^2(1 - \bar{V}e)} \qquad (1)$$

The use of equation (1) presupposes ideality, an assumption which may be far from valid at high concentrations and low *p*H. The direction of the deviation from ideality, however, can generally be inferred from the conditions of the experiment.

Goldberg (1953) has shown that the sedimentation equilibrium equation can be put in the form

$$\frac{1}{(\bar{M}_w)_{app}} = \frac{1}{\bar{M}_w} + Bc \qquad (2)$$

where $(\bar{M}_w)_{app}$ is the apparent molecular weight, as calculated from equation (1), for example, and B is a virial coefficient describing the interactions identified with the nonideal behavior. Since the virial coefficient, B, is essentially identical with that of the usual light-scattering equation, we can make use of such measurements in considering sedimentation equilibrium in nonideal systems. This is particularly useful since neither activity coefficient data nor detailed information about sedimentation equilibrium in nonideal protein systems are usually available. In general, in systems in which strong repulsive interactions occur, B will be large and positive. For example, the light-scattering curve for α-lactalbumin in salt-free solution on the alkaline side of its isoelectric point has a large positive B value (Timasheff and Kronman, 1959). Thus it is to be anticipated that for proteins at *p*H values removed from their isoelectric point $(\bar{M}_w)_{app} < \bar{M}_w$. Conversely, in systems with strong attractive interactions B will be negative and $(\bar{M}_w)_{app} \leq \bar{M}_w$.

*p*H *Measurements.*—For experiments carried out at 25°, *p*H was measured at ambient temperature (22–27°) using a Beckman Model G meter equipped with internal electrodes. In the case of experiments carried out at other temperatures the Radiometer TTT-1c unit equipped with scale expander was employed using Radiometer external electrodes. For these latter measurements calibration and *p*H measurements were carried out in a jacketed vessel through which water could be circulated from a refrigerated constant-temperature bath. Calibration of both *p*H meters was made with Fisher or with Beckman standard buffers.

Preparation of Solutions for Ultracentrifuge Measurements.—Solutions were prepared for sedimentation-velocity measurements by dissolving wet solid α-lactalbumin in an appropriate amount of the desired

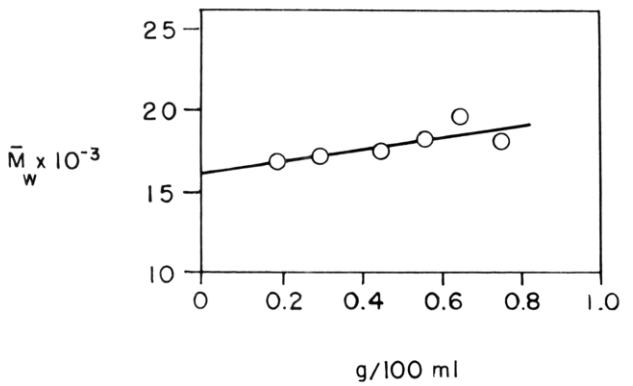

FIG. 1.—Molecular weight of α-lactalbumin at *p*H 8.55, 0.15 M KCl, preparation 45G.

salt (generally 0.15 M KCl). The protein solution was then dialyzed in the cold room (2–4°) for 24–48 hours against two to five changes of 4000-fold volume excesses of the same salt solution. The protein solution was then adjusted to the desired *p*H by the addition of 1 M HCl or KOH in very small increments with efficient stirring. In the cases of experiments carried out at 10° the solutions were never allowed to rise above this temperature; the *p*H was adjusted at this temperature and all manipulations were made with chilled glassware. Protein concentrations were determined on aliquots of the solution after adjustment of *p*H. Where experiments were carried out with anions other than chloride, the *p*H was adjusted with the corresponding acid with the exception of thiocyanate ion, where HCl was employed.

Results and Discussion

Molecular State of α-Lactalbumin at pH 8.55.—To provide a basis for evaluating the extent of association of α-lactalbumin at acid *p*H its molecular state was determined at *p*H 8.55, where preliminary work indicated that the molecule probably existed in monomeric form. Examination of α-lactalbumin preparations at *p*H 8.55 in 0.15 M KCl revealed a single symmetrical boundary in the ultracentrifuge. The concentration dependence of the sedimentation constant as obtained from fourteen points can be described by:

$$s_{20,w} = 1.92_1 \pm 0.01_9 - (6.5 \pm 1.0) \times 10^{-2}c$$

The equation was obtained from a linear least-squares fit of the data. The concentration, *c*, is in g/100 ml; $s_{20,w}$ at infinite dilution is in good agreement with Wetlaufer's (1961) value of 1.87. A very small amount of heavy material was noted as a slight thickening of the pattern on the fast side of the boundary. This appears to amount to less than 2% of the protein.

Sedimentation-equilibrium measurements were likewise made as a function of concentration at *p*H 8.55 in 0.15 M KCl. These data are shown in Figure 1. As can be seen, the apparent molecular weight shows a small increase with increase in concentration of protein. Such an increase might be due to weak association of monomeric units. As we have indicated in the experimental section, however, apparent molecular weights calculated using equation (1) assume ideality for the protein. The positive slope of the curve in Figure 1 may be thus due to nonideality arising from attractive interactions. The value of 16.2×10^3 obtained for the weight-average molecular weight at infinite dilution (Figure 1) is in good agreement with the number-average values of 16.3×10^3 obtained by osmotic pressure (Wetlaufer, 1961) and 15.5×10^3 from amino acid analysis (Gordon and Ziegler, 1955). It would thus appear that at *p*H 8.55 α-lactalbumin exists largely in monomeric form.

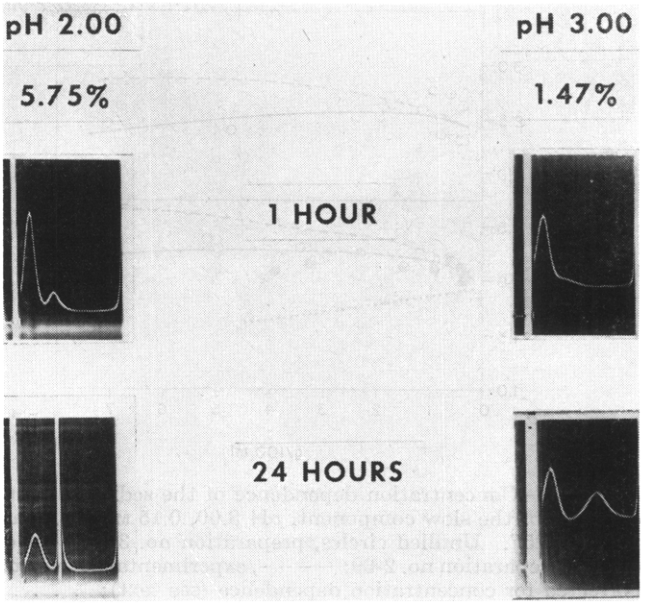

FIG. 2.—Effect of time of exposure on aggregation of α-lactalbumin at 25.0° in 0.15 M KCl. Bar angle, 60°; time, 48 minutes; *p*H 2.00 solution measured in a 4-mm Dural cell; *p*H 3.00 solution measured in a 12-mm Kel-F cell; preparation 3088.

State of Aggregation at Acid pH.—Careful acidification of α-lactalbumin to *p*H values less than 4 yields solutions which are essentially free of insoluble protein. Examination of such solutions in the ultracentrifuge reveals patterns such as those shown in Figure 2. These were obtained for solutions equilibrated at the appropriate temperature for 1 and for 24 hours prior to ultracentrifugation. At sufficiently low protein concentration only small amounts of heavy material are present, with the amount observed increasing markedly with time. (The reference to "heavy" component is made only for sake of convenience and should not be taken to imply that this necessarily corresponds to a single molecular species. Sedimentation constants calculated from the boundary position therefore represent average values for a distribution of components. Likewise, the use of the terms "aggregation" and "association" are arbitrary. By "aggregation" we mean formation of heavy component. We reserve the use of the term "association" for formation of low-molecular-weight polymers such as dimers, trimers, etc.) Since α-lactalbumin was shown to be homogeneous at *p*H 8.55, the presence of this heavy component must be the consequence of polymerization of monomeric units. The marked increase with time in the amount of heavy material indicates that the aggregation is a rate process rather than an equilibrium. As a consequence of this, while aggregation at *p*H values as low as 2 is relatively slow, at sufficiently high protein concentration virtually all of the protein will move as heavy component. The characteristics of this aggregation process, including the effects of *p*H, ion species, and temperature, will be considered in the companion paper (Kronman *et al.*, 1964). At sufficiently low concentration the majority of protein moves as a single boundary. This boundary, however, does not correspond to the monomeric molecule. This can be seen from the concentration dependence of s_{20} for the slow component as illustrated in Figure 3. These were obtained for solutions 1 hour after *p*H adjustment. Shown also for compari-

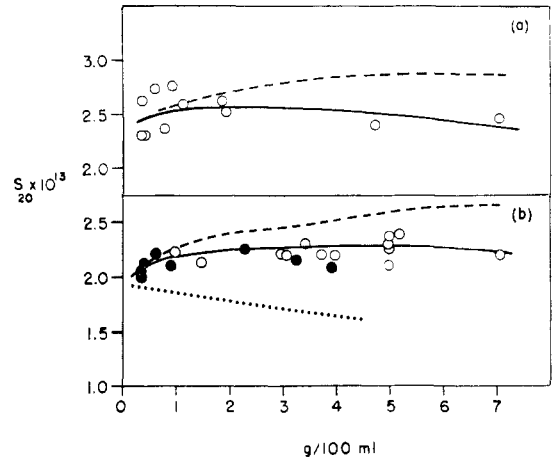

FIG. 3.—Concentration dependence of the sedimentation constant of the slow component, *p*H 3.00, 0.15 M KCl. (a) 10°, (b) 25°. Unfilled circles, preparation no. 3088; filled circles, preparation no. 2-69; – – – –, experimental constants corrected for concentration dependence (see text); . . . . , *p*H 8.55 data.

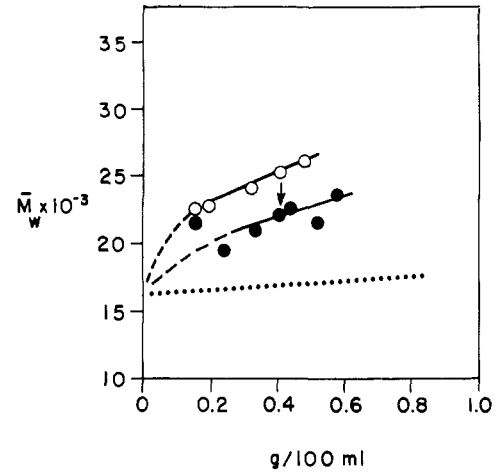

FIG. 4.—Molecular weights of α-lactalbumin at *p*H 3.00, 0.15 M KCl. O, 10°; ●, 25°; preparation 45G.

son is the curve obtained at *p*H 8.55. The s_{20} at both 10 and 25° shows little concentration dependence and lies significantly higher than those observed at *p*H 8.55 (dotted curve). These characteristics suggest that the slow "component," although present as a single boundary, is not a single molecular species and certainly not the monomeric molecule.

The observation of a single boundary is no criteria for absence of association reactions leading to higher-molecular-weight components. As has been amply demonstrated, from both theoretical and experimental points of view, association occurring in systems in which the rate of re-equilibration of components is rapid leads to patterns which do not quantitatively reflect component distribution (Fujita, 1962). This point will be considered further in the following paper (Kronman *et al.*, 1964) in considering the relationship between slow and fast components.

The flatness of curves such as those illustrated in Figure 3 can be attributed to the opposition of two effects: (a) the normal decrease in sedimentation constant with increasing concentration arising from changes in solvent density and viscosity and backflow (see, for example, Fujita, 1962, Schachman, 1959); and (b) an increase in sedimentation constant occurring upon association. Quantitatively, the shape of such an *s* versus *c* curve will depend upon the relative magnitudes of the equilibrium constants for the association reactions and the concentration dependences of the sedimentation constants. While in favorable situations association may be detectable through the observation of a positive slope to the *s* versus *c* curve or through the appearance of a maximum, it may be evident only through the deviation of the slope from an anticipated value.

If we assume that the inherent concentration dependence of the sedimentation constant is the same at *p*H 8.55 and 3.00, one can obtain an estimate of the dependence of the sedimentation constant on concentration and indirectly the extent of association. (The assumption of comparable slopes of the *s* versus *c* curve at *p*H 8.55 and 3.00 appears to be a reasonable one to a first approximation. Estimation of the net charge at *p*H 3 and 8.55 from the amino acid data of Gordon and Ziegler [1955] indicates that the absolute value of the protonic charge is about 20% lower at *p*H 8.55. Anion binding would tend to minimize this difference and hence make the sedimentation-potential effects more nearly alike.) The dashed curves were obtained from the smoothed experimental data by subtracting the concentration-dependent portion of the sedimentation constant, using the value obtained at *p*H 8.55. A comparison of the values of s_{20} shown in the dotted curves with the value of 1.93 S obtained at infinite dilution at *p*H 8.55 indicates that association occurs to a significant extent at *p*H 3.00, even at relatively low concentration. The value of the sedimentation constants at 10° are significantly higher than those at 25°, suggesting that association is stronger at the lower temperature.

Molecular Weight of α-Lactalbumin at Acid pH.—In order to determine if the slow component did indeed represent associated protein, equilibrium-sedimentation measurements were made by the short-column procedure. This was selected as the most reasonable method for our purpose since measurements could be made relatively rapidly at concentrations below 1 g/100 ml. In this way it is possible to minimize the effect of the time-dependent aggregation. Since the molecular weight of the heavy component is in excess of 300,000 (Kronman *et al.*, 1964), very small amounts of the latter may make significant contribution to $\bar{M}_w$.

Shown in Figure 4 are values of $\bar{M}_w$ obtained at *p*H 3.00 in 0.15 M KCl. The dotted curve represents the smoothed data obtained at *p*H 8.55 (Fig. 1). As Figure 4 indicates, the molecular weights at both 10 and 25° are significantly higher than at *p*H 8.55. A comparison of the concentration dependence of the sedimentation constants at these two temperatures (Fig. 3) led us to predict that association was stronger at 10° than at 25°. This is borne out by the molecular-weight data of Figure 4.

While there may be some contribution of the high-molecular-weight aggregate to the apparent molecular weight, it cannot be solely responsible for the enhancement of the molecular weight at *p*H 3.00. This follows from the observation that the molecular weight at 10° is significantly higher than at 25°, in spite of the fact that the rate of aggregation at 10° is considerably lower than at 25° (Kronman *et al.*, 1964).

The association observed at low concentration is readily reversed in acid solution. An α-lactalbumin solution of concentration 0.4 g/100 ml, after equilibration at 10° for 6 hours, was warmed to 25° for 1 hour before ultracentrifuge measurements were made. The points connected by the arrow in Figure 4 were the molecular weights obtained at 10 and 25°. As can be

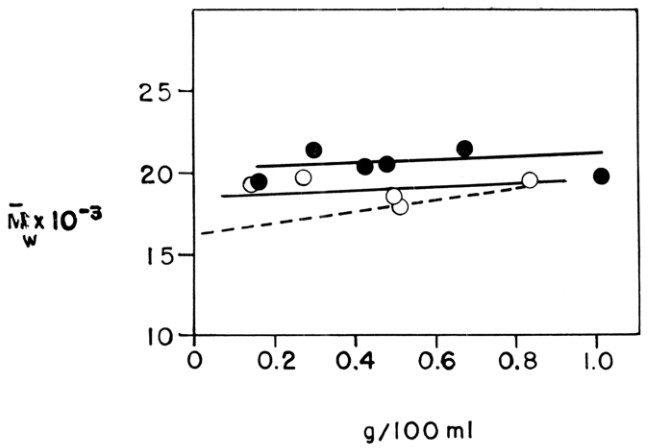

FIG. 5.—Molecular weight of α-lactalbumin at *p*H 2.00, 0.15 M KCl. ●, 10°; ○, 25°; preparation 45G.

mented to the bottom of the cell, thereby reducing the concentration in the liquid column to values lower than the initial ones. Conditions favorable to the formation of aggregate were therefore avoided.)

Even at *p*H 2.00, where the α-lactalbumin molecule carries a near maximum charge, association persists. This is illustrated by the molecular-weight data shown in Figure 5. Shown also for comparison is the curve obtained at 25° at *p*H 8.55 (dotted curve). As was the case at *p*H 3.00, association is stronger at low temperature (Figs. 4 and 5). The molecular weights at *p*H 2 represent *minimum* values since they were obtained at finite protein concentration where we might anticipate negative deviations from ideality due to repulsive interactions.

State of Aggregation on the Alkaline Side of the Isoelectric Point.—The marked tendency of α-lactalbumin to associate and aggregate on the acid side of the isoelectric point (4.2–4.5) led us to examine its molecular

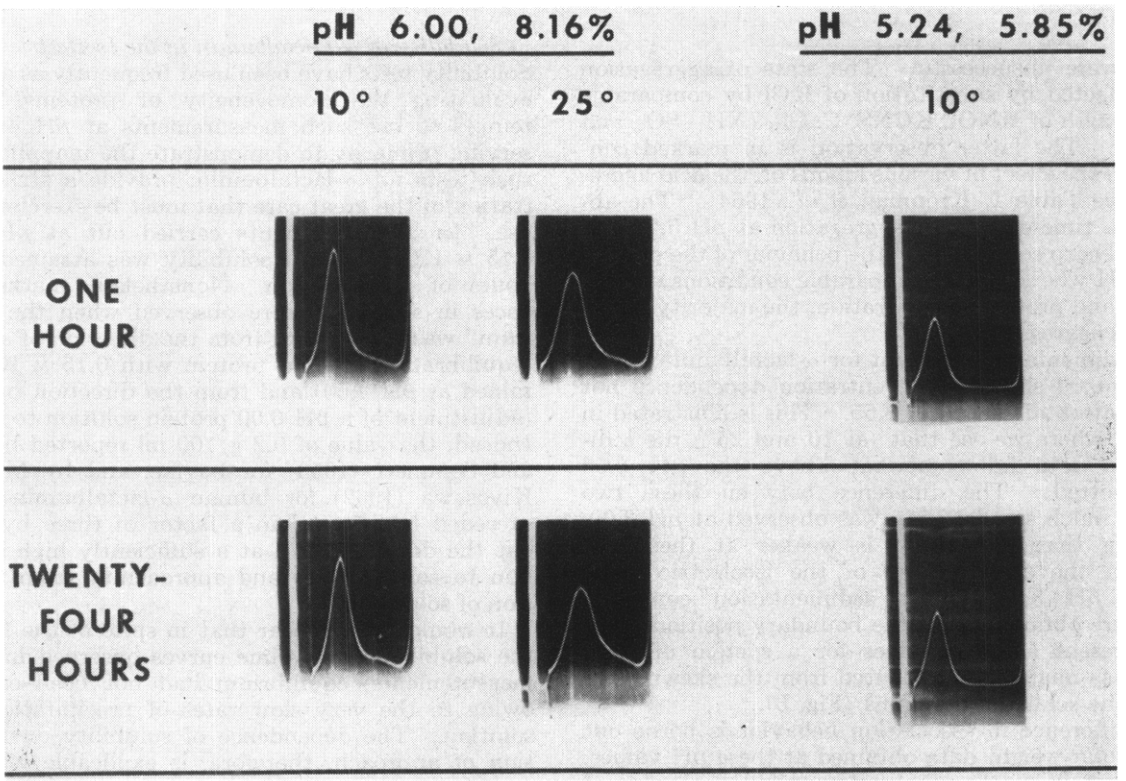

FIG. 6.—Sedimentation pattern of α-lactalbumin at *p*H 5.24 and 6.00. Time of photography: 10°, 112 min; 25°, 48 min; preparation 3088.

seen, raising the temperature from 10° to 25° resulted in dissociation of the complex to yield a lower molecular weight. The rate of dissociation must be relatively rapid since the molecular weight was obtained after 1 hour of sedimentation (2 hours after warming to 25°) and remained unchanged for the remainder of the experiment (approximately 5 more hours). By similar reasoning one can conclude that the rate of association is relatively rapid. (In all sedimentation equilibrium studies reported in this paper equilibrium was established 64 minutes after the centrifuge was brought to speed, yielding a constant gradient, dc/dr [see equation 1] at the mid-point of the liquid column for 5 succeeding hours. In a few experiments at *p*H 3.00 and 25° at concentrations exceeding 0.6 g/100 ml prolongation of the runs to times in excess of 8 hours resulted in small decreases in dc/dr. This presumably was due to slow formation of small amounts of aggregate which sedistate just alkaline to this zone, where the net charge might be expected to be lower than *p*H 2, for example. The time-dependent aggregation observed at acid *p*H values is essentially absent at *p*H 5.24 and 6.00. This is illustrated by the ultracentrifuge patterns shown in Figure 6. At 10° and *p*H 5.24 the patterns are virtually symmetrical and show no change during the course of 24 hours. Essentially, the same observations were made at *p*H 6.00 except at the highest concentration studied, 8.16 g/100 ml, where some heavy material was present, the amount of which increased slightly over a 24-hour period. Presumably a similar observation would have been made at *p*H 5.24 if the measurements had been carried out to the same high concentration. Likewise, at *p*H 6.00 and 25° (Fig. 6b) a small amount of heavy material is present, the amount of which increases slightly during 24 hours. Attempts to promote drastic aggregation at *p*H 6.00 through changes in ion

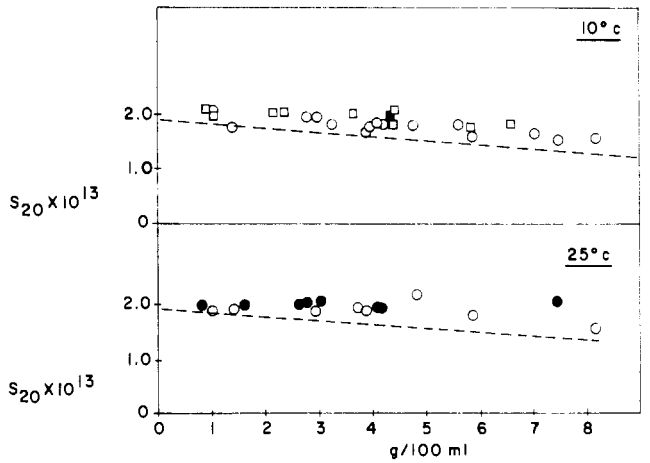

FIG. 7.—Concentration dependence of s_{20} for α-lactalbumin in 0.15 M KCl. □, pH 5.24; ○, pH 6.00. Filled symbols, preparation 2-69; unfilled symbols, preparation 3088.

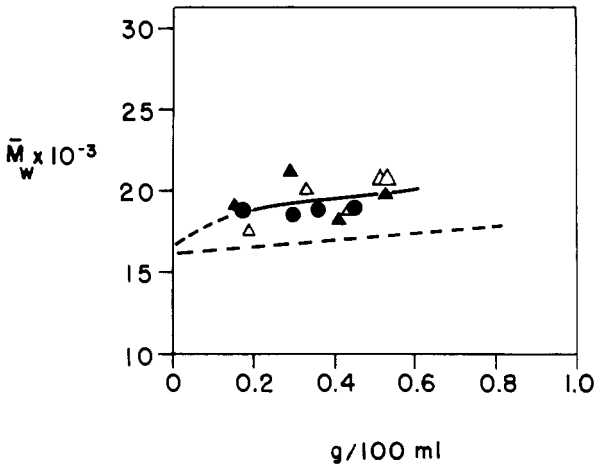

FIG. 8.—Molecular weight of α-lactalbumin in 0.15 M KCl. ▲, pH 6.00, 10°; ●, pH 5.24, 10°; △, pH 6.00, 25°. Preparation 45G. – – – –, pH 8.55.

binding were unsuccessful. The state of aggregation was uneffected by substitution of KCl by comparable ionic strength of KNO_3, KCNS, $CaCl_2$, $(NH_4)_2SO_4$, and Tris-HCl. The latter observation is in marked contrast with the effect of various anions on the acid aggregation (see Table I, Kronman *et al.*, 1964). The absence of a time-dependent aggregation at pH 5.24 and 6.00 is in sharp contrast with the behavior of the protein at acid pH where, under comparable conditions of temperature and protein concentration, the majority of the protein is aggregated.

The sedimentation constant for α-lactalbumin at pH 5.24 and 6.00 shows a concentration dependence not unlike that obtained at pH 8.55. This is illustrated in Figure 7, where we see that (at 10 and 25°) the sedimentation data fall somewhat above the pH 8.55 curve (dotted). The difference between these two curves is much smaller than was observed at pH 3.00, suggesting that association is weaker at these pH values on the alkaline side of the isoelectric point than at pH 3.00. These sedimentation constants which were obtained from the boundary positions may well represent average values for a system of components, as might be anticipated from the skewness of some of the schlieren diagrams (Fig. 6).

This difference in association behavior is borne out by molecular-weight data obtained at these pH values. As Figure 8 illustrates, molecular weights observed at pH 5.25 and 6.00 at low protein concentration are somewhat higher than those at pH 8.55 (dotted curve), indicating weak association. Comparison with the molecular-weight data at pH 3.00, however (Figure 4), reveals that at the acid pH, association occurs to a significantly greater degree.

Furthermore, there is no temperature dependence of the degree of association discernible at pH 6.00, the values of $\bar{M}_w$ at 10 and 25° being essentially the same. This is to be contrasted with the association at pH 3.00 and 2.00 (Figures 4 and 5), where it was quite evident that association occurred to a greater extent at low temperature.

The difference in the association and aggregation behavior of α-lactalbumin on the acid and alkaline sides of the isoelectric point is quite striking and is paralleled by differences in molecular properties such as spectra, fluorescence, and rotatory dispersion. Consideration of the mutual relationships of these properties will be discussed in the accompanying paper (Kronman *et al.*, 1964).

Solubility of α-Lactalbumin in the Isoelectric Region.—Solubility tests have been used frequently as a means of evaluating the homogeneity of proteins. Our attempts to use such measurements at pH 4.50, while serving primarily to demonstrate the inapplicability of such tests for α-lactalbumin, provide a striking illustration of the great care that must be exercised in their use. In *all* experiments carried out at pH 4.50 in 0.15 M KCl constant solubility was attained after 20 hours of equilibration. Nonetheless, marked differences in solubility were observed when the "equilibrium" was approached from the direction of saturation (equilibration of solid protein with 0.15 M KCl maintained at pH 4.50) and from the direction of solution (adjustment of a pH 6.00 protein solution to pH 4.50). Indeed, the value of 0.2 g/100 ml reported by Gordon and Semmett (1953) for bovine, and by Maeno and Kiyosawa (1962) for human α-lactalbumin could be exceeded by more than a factor of three by carrying out the determination at a sufficiently high solid protein–to–solvent ratio and approaching from the direction of solution.

It would thus appear that in spite of the flatness of the solubility-versus-time curves observed in *all* these measurements, equilibrium had not been established owing to the very slow rates of precipitation and of solution. The dependence of solubility on the direction of approach, therefore, is explicable without recourse to the hypothesis of heterogeneity. This, of course, does not eliminate such a possibility. It does mean, however, that the solubility of α-lactalbumin in this pH region must be regarded as an empirical quantity having no thermodynamic significance.

Heterogeneity of α-Lactalbumin.—As we have shown, α-lactalbumin in the pH region below its isoelectric point exists largely in an associated or aggregated form. While the association may be minimized at low protein concentration and low pH, aggregation will not necessarily be absent even under these conditions since the process is time dependent. These characteristics of α-lactalbumin in acid solution render any evaluation of the homogeneity by transport methods such as electrophoresis, ultracentrifugation, and chromatography quite uncertain. Since the rates of aggregation and disaggregation (Kronman *et al.*, 1964) are sufficiently slow to permit resolution of components in the ultracentrifuge, one would anticipate that α-lactalbumin polymers could even be separated from unaggregated protein by chromatography on cellulosic exchangers or on Sephadex.

It seems probable that the apparent electrophoretic heterogeneity observed in lactate buffer in acid solution (Gordon and Semmett, 1953; Klostergaard and Pasternak, 1957) is a consequence of association or aggregation of the protein. In light of the sensitivity of the intermolecular interactions of α-lactalbumin to anion binding (Kronman *et al.*, 1954), the fact that the protein was found to be homogeneous in glycine buffers at *p*H 2 and 3 (Gordon and Semmett, 1953) may not be surprising. The use of isoelectric solubility measurements as an index of homogeneity of alpha lactalbumin likewise involves a high degree of ambiguity since solubilities in this region are not true thermodynamic quantities.

The feebleness of the association reaction of α-lactalbumin at *p*H 5.24 and 6.00, particularly at low temperature, as compared to acid *p*H values, suggest that the electrophoretic heterogeneity on the alkaline side of the isoelectric zone (Klostergaard and Pasternak, 1957; Wetlaufer, 1961) may have other origins, e.g., strong reversible interactions with buffer as suggested by Wetlaufer.

While protein-buffer interactions may play some role, it seems doubtful if they can entirely account for the apparent heterogeneity at alkaline *p*H. Wetlaufer (1961) himself concluded this from his observation that different preparations of α-lactalbumin gave different amounts of the fast "trace" component. More recently, starch-gel electrophoresis carried out in our laboratory on about eighty different samples of α-lactalbumin prepared from the milk of individual dairy cows of widely different genetic history likewise showed different amounts of the "trace" component without any systematic variation being apparent (M. J. Kronman and R. Vitols, unpublished data). It is of interest to note that this "trace" component was observed in both *p*H 3.0, 0.5 sodium formate, 5 M urea and in *p*H 8.55, 0.1 M Veronal, indicating that this minor component most likely does not represent an association species of α-lactalbumin.

It is apparent that the majority of observations of heterogeneity of α-lactalbumin at present can be accounted for on the basis of protein-protein or ion-protein interactions. However, in order to definitely exclude the possibility of molecular "microheterogeneity" such as exists for β-lactoglobulin, for example (Aschaffenburg and Drewry, 1955), further work of a rather critical nature will be required. In such an evaluation, recognition of the marked propensity of α-lactalbumin toward association and aggregation will be of the utmost importance.

References

Aschaffenburg, T., and Drewry, J. (1957), *Biochem. J. 65*, 273.

Aschaffenburg, R., and Drewry, J. (1955), *Nature 176*, 218.

Fujita, H. (1962), *in* Mathematical Theory of Sedimentation Analysis, New York, Academic.

Goldberg, R. J. (1953), *J. Phys. Chem. 57*, 194.

Gordon, W. G., and Semmett, W. F. (1953), *J. Am. Chem. Soc. 75*, 328.

Gordon, W. G., and Ziegler, J. (1955), *Arch. Biochem. Biophys. 57*, 80.

Klostergaard, H., and Pasternack, R. A. (1957), *J. Am. Chem. Soc. 79*, 5674.

Kronman, M. J., Andreotti, R. E., and Vitols, R. (1964), *Biochemistry 3*, 1152 (this issue, following paper).

Maeno, M., and Kiyosawa, I. (1962), *Biochem. J. 83*, 271.

Robbins, F., and Kronman, M. J. (1964), *Biochim. Biophys. Acta 82*, 186.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, Academic.

Timasheff, S. N., and Kronman, M. J. (1959), *Arch. Biochem. Biophys. 83*, 60.

VanHolde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.

Wetlaufer, D. B. (1961), *Comp. Rend. Trav. Lab. Carlsberg 32*, 125.