this[21] contained 462 μg. of anti-S XIV N per ml. With lung galactan it precipitated a maximum of 34 μg. of N per ml.; the supernatant gave 392 μg. of N with S XIV. The antibody solution precipitated 13 μg. of N with jellose; the supernatant from this gave 15 μg. more N with the arabogalactan of Jeffrey pine.

The results on the cross reactions of the various polysaccharides in Type XIV anti-Pn sera do more than merely establish the types of galactose linkages which suffice to induce this reactivity. Because of their generality, as shown by the success of predictions noted above as to which gums would react, they provide a powerful aid to the carbohydrate chemist, since a simple serological test may show the presence or absence of one or more of the linkages in question in certain galactose-containing polysaccharides of unknown structure, particularly those which do not include glucose or N-acetylglucosamine. Demonstration has already been made of some of the potentialities of other anti-Pn sera in which multiple recurrences of glucuronic acid[2] or glucose[22] or cellobiuronic acid[23] in a polysaccharide involve cross reactivity, and other instances are at present under study.

(21) L. D. Felton, *J. Infect. Dis.*, **42**, 248 (1928), and earlier papers.

(22) (a) M. Heidelberger and A. C. Aisenberg, *Proc. Nat. Acad. Sci.*, **39**, 453 (1953); (b) *J. Exper. Med.*, **99**, 343 (1954).

(23) M. Heidelberger and G. L. Hobby, *Proc. Nat. Acad. Sci.*, **28**, 516 (1942).

NEW YORK 32, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Casein: Monomers and Polymers[1]

BY PETER H. VON HIPPEL[2] AND DAVID F. WAUGH

RECEIVED MARCH 7, 1955

A method is described for preparing a mixture of α- and β-caseins at constant *p*H and reduced temperature. This casein is termed soluble casein since it is in equilibrium with the casein in the casein micelles of milk and is readily soluble at neutral *p*H. The behavior of soluble casein has been examined by physico-chemical means as a function of *p*H, temperature, ionic strength and protein concentration. At *p*H 12 soluble casein is completely and reversibly dissociated into monomers having an average $S_{20}^0 = 1.18 \times 10^{-13}$ sec. and $D_{20}^0 = 7.11 \times 10^{-7}$ cm.^{2} per sec. An average monomer molecular weight of 15,000 is calculated. A detailed consideration of the mixture suggests that the molecular weight of the α-casein monomer probably lies in the range 13,000 to 15,000 and that the molecular weight of the β-casein monomer lies in the range 15,000 to 25,000. Analyzing values of S and D and of measured viscosity increments, yields an average axial ratio of 12.0 for the anhydrous particle and a hydration of 0.3 g. water per gram protein. If a casein solution at *p*H 12 and temperatures near 0° (monomers) is adjusted to progressivly lower *p*H values, a peak representing α-casein polymers appears in the ultracentrifuge at *p*H 10.8. These α-casein polymers increase in sedimentation constant from 4.0 to 5.8 S (8°) as the *p*H is decreased to *p*H 7. β-Casein remains in the monomeric form. At *p*H 7 and 0°, the ultracentrifuge and electrophoretic data are in excellent agreement. If at *p*H 7 the temperature is increased from 0°, the β-casein peak disappears and polymers containing both α- and β-casein appear, the average sedimentation constant increasing progressively from $S_{20} \sim 4.4$ S at 4° to $S_{20} \sim 9.3$ S at 32°. Beyond 32° there is a degeneration in the pattern. In all cases the polymers are characteristic of a given set of conditions and center around a preferred size. A re-examination of the properties of casein prepared from acid-precipitates suggests that such preparative procedures lead to some aggregation, particularly of α-casein, of dubious reversibility.

Since its preparation in 1838 by Mulder,[3] casein has been subjected to extensive study. Nevertheless, its physical and chemical properties have eluded precise definition. This may be due, in part, to the fact that most of these studies have been carried out on caseins prepared using modifications of the acid-precipitation method described by Hammarsten[4] in 1883. Linderstrom-Lang and Kodama,[5] in examining the solubility behavior of casein prepared in this way, first demonstrated the presence of several components. Mellander[6] and Warner[7] have since characterized these components electrophoretically, and more recently Hipp, Groves, Custer and McMeekin[8,9] have devised methods for fractionating casein by chemical means.

With the exception of the electrophoretic studies, upon which are based the designations of the various casein fractions as α-, β- and γ-casein, the results of physical examinations have not been in satisfactory agreement. Svedberg, Carpenter, and Carpenter[10,11] and Pedersen[12] examined casein by ultracentrifugation and obtained only polydisperse sedimentation patterns, highly dependent in form on small modifications in preparative procedure. These patterns generally exhibited from six to nine peaks, representing components ranging upward in molecular weight from about 75,000. On the other hand, osmotic pressure measurements by Burk and Greenberg[13] on acid-precipitated casein

(1) Appreciation is expressed to the Massachusetts Institute of Technology and to the National Dairy Research Laboratories, Oakdale, Long Island, New York, for supporting this research. The authors are pleased to acknowledge the technical assistance of Miss Vilma Grube.

(2) Predoctoral Fellow of the National Science Foundation, 1952–1955. The work reported here will constitute part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A portion was reported in: P. H. von Hippel, Master's Thesis, Department of Biology, Massachusetts Institute of Technology, June, 1953.

(3) G. J. Mulder, *Ann. Pharm.*, **28**, 73 (1838).

(4) O. Hammarsten, *Z. physiol. Chem.*, **7**, 227 (1883).

(5) K. Linderstrom-Lang and S. Kodama, *Compt. rend. trav. lab. Carlsberg*, **16**, No. 1 (1925).

(6) O. Mellander, *Biochem. Z.*, **300**, 240 (1939).

(7) R. Warner, THIS JOURNAL, **66**, 1725 (1944).

(8) N. Hipp, M. Groves, J. Custer and T. McMeekin, *J. Dairy Sci.*, **35**, 272 (1952).

(9) N. Hipp, M. Groves, J. Custer and T. McMeekin, THIS JOURNAL, **72**, 4928 (1950).

(10) T. Svedberg, L. Carpenter and D. Carpenter, *ibid.*, **52**, 241 (1930).

(11) T. Svedberg, L. Carpenter and D. Carpenter, *ibid.*, **52**, 701 (1930).

(12) K. Pedersen, *Biochem. J.*, **30**, 948 (1936).

(13) N. Burk and D. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

dissolved in 6.66 *M* urea yielded a molecular weight of 33,600, a fact which suggested that the higher molecular weights observed in other systems might be due to complex formation or aggregation. Warner[7] and Nitschmann and Zurcher,[14] through analyses of electrophoretic patterns, have demonstrated complex formation in unfractionated casein and Perlmann,[15] using enzymatic techniques, has substantiated their findings. In addition Halwer[16] has recently demonstrated electrolyte dependent aggregation in solutions of both α- and β-caseins. Sullivan, *et al.*,[17] have reported on α- and β-caseins fractionated from the acid-precipitated starting material. They found the α-casein fraction to have a minimum molecular weight of 121,800 and the β-casein fraction a minimum molecular weight of 24,100.

During the course of an examination of the colloidal chemistry of skim milk,[18] it became apparent that the casein in the casein micelle is in equilibrium with soluble casein (*i.e.*, casein which does not centrifuge out with the micelles) and is also in equilibrium with ionic calcium and phosphate. It became clear that dilution of skim milk causes a dissociation of the micelle with the release of soluble casein, that the addition of ionic calcium forces soluble casein into the micellar form and that, contrary to suggestions published by several investigators, inorganic phosphate is not involved in the stabilization of the micelle. In fact, the addition of phosphate increases the concentration of soluble casein. Thus inorganic phosphate must be considered as in competition with casein for ionic calcium. The fact that one can manipulate the distribution of casein between the soluble and the micellar forms suggested that this property be taken advantage of in preparing casein free of whey protein and other contaminants at constant *p*H, thus avoiding any possible aggregation which might result from isoelectric precipitation.

A method for preparing casein at constant *p*H, at reduced temperatures, in an aqueous environment, and utilizing only cations normally present in the casein micelle, is described below. It should be noted at once that this procedure yields a mixture of α-, β- and possibly γ-caseins. It will become apparent that a study of this mixture of α- and β-caseins has yielded valuable information, for the monomers of each are physically similar and an understanding of their interactions and polymerizations has been found useful in clarifying many previously unresolved conflicts. The fractionation of the mixture described here, and the characterization of the isolated fractions, are currently in progress.

Materials and Methods

Preparation of Casein.—For convenience the preparative method is divided into sections, each of which is subdivided into steps. All centrifugal fields are calculated for the radial distance to the bottom of the centrifuge tube. The quantities given in the following procedure are based on one liter of skim milk.

I. **Separation of the Casein Micelles.**—1. Raw pooled milk (*p*H ~ 6.6) is centrifuged for 30 min. at 5° and 895 × *g* (2000 r.p.m.) in an International Refrigerated Centrifuge.

2. To each liter of skim milk at 5° is added 60 ml. of 2.0 *M* $CaCl_2$. The *p*H change here is less than 0.1 *p*H unit since most of the casein is already combined with calcium in the form of micelles. The shift of soluble casein into the micellar form is relatively complete at an added $CaCl_2$ concentration of 0.07 *M*.

3. Micelles are removed by centrifuging for 90 minutes at 5° and 45,000 × *g* in a Spinco Preparative Centrifuge (Model L). Rotor #20 is used at 18,000 r.p.m. Supernatant whey protein is decanted, residual cream is carefully wiped away and the gel removed with a glass spatula.

II. **Washing of the Casein Micelles.**—1. The gel resulting from I-3 is resuspended in 250 ml. of a solution 0.076 *M* in NaCl and 0.133 *M* in $CaCl_2$. Resuspension is accomplished by blending for 2 minutes in a Waring Blendor modified to prevent foaming.

2. The dispersed micelles are recovered by centrifuging for 60 minutes at 5° and 90,000 × *g* (Spinco Preparative Centrifuge, rotor #30, 28,000 r.p.m.). The precipitate is carefully recovered as described under I-3.

3. The gel resulting from II-2 is resuspended in 250 ml. of 0.085 *M* NaCl by blending. See II-1.

4. Micelles are recovered by centrifugation. See II-2.

III. **Solubilization of Casein.**—1. The micelles are resuspended by blending (see II-1) into 250 ml. of 0.085 *M* NaCl.

2. Calcium is removed from the suspended micelles by adding 100 ml. of 1.5 *M* potassium oxalate and simultaneously sufficient 0.1 *M* oxalic acid to maintain the *p*H between 6.6 and 7.0. The titration is carried out with constant stirring at ~0°, *p*H being determined continuously.

3. Calcium oxalate, present in a finely divided state, is removed in two stages: an initial centrifugation for 30 minutes at 5° and 895 × *g* (International Centrifuge, bucket type rotor, 2,000 r.p.m.) followed by a centrifugation for 90 minutes at 5° and 45,000 × *g* (Spinco Preparative Centrifuge, rotor #20, 18,000 r.p.m.).

4. The supernatant from III-3, containing the soluble casein, is dialyzed against two 6-liter lots of 0.085 *M* NaCl, each dialysis continuing for 8 hours with constant agitation. Residual oxalate ions are completely removed by this step.

IV. **Reprecipitation of Calcium Caseinate.**—1. To the solution resulting from III-4 is added 60 ml. of 2.0 *M* $CaCl_2$ and sufficient 0.1 *M* NaOH to maintain the *p*H between 6.6 and 7.0 at all times. (Titration procedure as in III-2.)

2. Calcium caseinate is removed by centrifuging for 40 minutes at 5° and 895 × *g* (International Centrifuge, bucket rotor, 2,000 r.p.m.).

3. Steps III-1, III-2, III-3 and III-4 are repeated (resuspension, solubilization, removal of calcium oxalate, removal of potassium oxalate by dialysis). The final dialysis medium is 0.15 *M* potassium chloride.

V. **Storage of Product.**—1. A completely stable product may be obtained by shell-freezing the final casein solution in liquid nitrogen. The frozen material is stored at −20°. In solution at 5° stability is limited to a period of from 2 to 4 weeks.

Measurement of Casein Concentration.—Direct measurement of concentration by means of ultraviolet absorption was found to be impractical due to scattering from residual colloidal particles of CaC_2O_4 sometimes present in samples. Casein concentrations were therefore measured by means of a colorimetric biuret reaction. The procedure employed is that of Gornall, Bardawill and David,[19] as modified by Gallop.[20] A tartrate-stabilized biuret reagent is used which contains per liter: 1.50 g. of $CuSO_4 \cdot 5H_2O$, 6.00 g. of NaK tartrate·$4H_2O$ and 28.0 g. of carbonate-free KOH. The reagent is stored in a polyethylene bottle and is stable for several months at room temperature.

Concentrations are determined as follows: to 1 ml. of the sample in a test-tube is added 5 ml. of the biuret reagent, the tube is covered and the solution is mixed by five or six inversions. After standing for 15 minutes at room temperature the optical density at λ 5440 Å. is determined against

(14) H. Nitschmann and H. Zurcher, *Helv. Chim. Acta*, **33**, 1698 (1950).

(15) G. E. Perlmann, THIS JOURNAL, **74**, 3191 (1952).

(16) M. Halwer, *Arch. Biochem. Biophys.*, **51**, 79 (1954).

(17) R. Sullivan, M. Fitzpatrick, E. Stanton, R. Annino, G. Kissel and F. Palermiti, *ibid.*, in press.

(18) D. F. Waugh, L. Varga and R. Senderi, unpublished.

(19) A. Gornall, C. Bardawill and M. David, *J. Biol. Chem.*, **177**, 751 (1949).

(20) P. Gallop, Ph.D. Thesis, Dept. of Biology, Mass. Inst. of Tech., August 21, 1953.

a blank of 1 ml. of buffer and 5 ml. of biuret reagent. A calibration curve, based on dry weights at 110°, is the reference standard. Casein concentrations may be determined to ±5%, using this procedure.

Reagents and Buffers.—Analytical reagent grade chemicals were used throughout.

The electrophoresis experiments at neutral *p*H were carried out in phosphate buffer containing per liter: 2.92 g. of NaCl, 1.06 g. of $NaH_2PO_4 \cdot H_2O$, and 2.02 g. of Na_2HPO_4. This buffer has a *p*H of 6.98 and an ionic strength, $\Gamma/2$, of 0.10.

The electrophoresis, viscosity and sedimentation experiments performed at elevated *p*H were carried out in a buffer containing per liter: 8.9 g. of $Na_2HPO_4 \cdot 2H_2O$ and 1.33 g. of NaOH (see Clark[21]). This buffer has a *p*H of 12.0 and an ionic strength of 0.19. Higher ionic strengths were obtained using this buffer with NaCl added.

*p*H, in every case, was determined at the temperature at which the experiment was performed.

Ultracentrifugation.—All analytical ultracentrifugations were carried out in the Model E Analytical Ultracentrifuge manufactured by the Specialized Instruments Corporation of Belmont, California. All runs were made at 59,780 r.p.m. (260,000 × *g*). The runs at *p*H values below *p*H 8 were made with a metal centerpiece and those above *p*H 8 with a plastic (Kel-F) centerpiece.

The temperature of the rotor was measured and controlled using the technique devised by Waugh and Yphantis.[22]

Electrophoresis.—The electrophoresis unit was supplied by Frank Pearson Associates. It is equipped with a Longsworth scanning optical system. The runs were carried out in a Tiselius-type electrophoresis cell (cross-sectional area = 0.75 cm.²), produced by the Pyrocell Company. Boundaries were formed by shearing and moved into position by mechanical compensation. The water-bath was regulated at 1.50 ± 0.05°.

Translational Diffusion.—The diffusion experiments were also performed in the Pearson Electrophoresis apparatus, employing the Tiselius cell without electrodes.

Viscosity.—Viscosity determinations were made in a constant temperature bath regulated at 1.50 ± 0.05° and cooled by circulating ethylene glycol. Ostwald–Cannon–Fenske capillary viscometers were used (size 50), having an outflow time for distilled water at this temperature of approximately seven minutes.

Results

The preparative method involves repetition of a cycle in which casein is first precipitated as centrifugable calcium caseinate and subsequently solubilized by effectively removing the calcium as calcium oxalate. The object of each cycle is to remove, in the supernatant, whey protein and other substances non-precipitable by calcium. Material recovered after the first cycle was examined in the ultracentrifuge at room temperature. The pattern indicated considerable polydispersity, due in part to whey protein presumably entrapped by the micellar precipitate during centrifugation. It was suspected that a number of cycles might remove whey protein completely and yield a homogeneous preparation.

The ultracentrifuge pattern, our criterion of homogeneity, was improved considerably by a second cycle, but not subsequently. The improvement appeared as a decrease in the skewness of the main peak and the effective disappearance of materials giving abnormal gradients near the meniscus and the base of the cell. Since each cycle was attended by a 50% loss, material recovered after the second cycle was used in the studies which follow. That this material is satisfactory will become evident. The casein obtained in this way is called "soluble casein," a name which associates the casein studied here with the non-centrifugable casein found in equilibrium with casein micelles in milk. Further, in the absence of calcium a dried preparation of soluble casein goes into solution easily at neutral *p*H.

Electrophoretic Comparison at *p*H 7.—An electrophoretic examination of soluble casein was given impetus by the fact that electrophoretic patterns obtained under standard conditions by several investigators,[6,7,14,23] have constituted the most reproducible physical characterization of caseins prepared from acid precipitates. Runs were made in phosphate buffer at *p*H 6.98, approximating closely the conditions used by Warner.[7] The results are compared in the first two lines of Table I. It is clear that the components of Warner's unfractionated casein and those of soluble casein at *p*H 7.0 have closely similar electrophoretic mobilities and are present in the same relative concentrations.

Temperature Effects.—Figure 1 presents line tracings of ultracentrifuge patterns observed at various temperatures. These particular traces were obtained at a *p*H of 6.65, a protein concentration of 0.60%, and an ionic strength of 0.17, (0.09 due to sodium chloride and 0.08 due to potassium oxalate; the latter added as a precautionary measure to remove any calcium or similar ions).

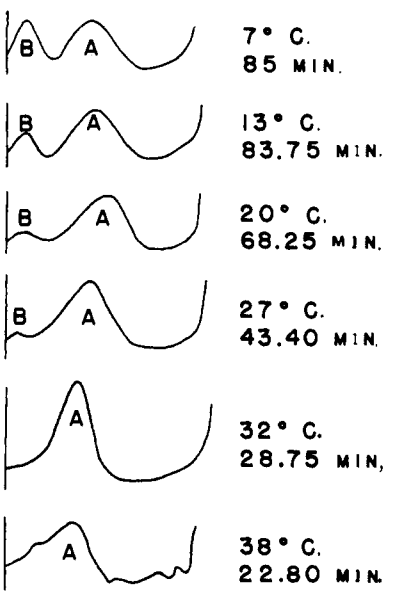

Fig. 1.—The effect of increasing temperature on the sedimentation pattern of soluble casein at *p*H 7. Time is in minutes at 260,000 × *g*.

The trace obtained at 27°, representative of the type of pattern observed during the development of the preparative procedure, reveals a certain amount of heterogeneity. It was felt that this might be due to the ubiquitous interactions so characteristic of casein.[7,14,16] As indicated in Fig. 1, variations in temperature produced marked effects on the sedimentation pattern. Values of the sedimentation constant corrected to water at 20.0°

(21) W. M. Clark, "The Determination of Hydrogen Ions," 3rd Ed., Williams and Wilkins Co., Baltimore, Md., 1928, p. 216.

(22) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instr.*, **23**, 609 (1952).

(23) E. Cherbuliez and P. Baudet, *Helv. Chim. Acta*, **33**, 398 (1950).

(S_{20}, see ref. 24), are plotted against temperature in Fig. 2.

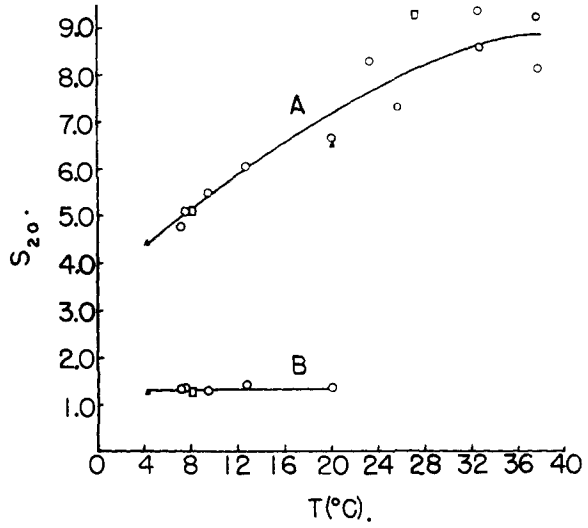

Fig. 2.—The effect of temperature on the S_{20} of the A and B components of soluble casein at *p*H 7: O for $\Gamma/2$ = 0.19 and C = 0.60%; □ for $\Gamma/2$ = 0.32 and C = 0.60%; Δ for $\Gamma/2$ = 0.19 and C = 0.30%.

All the patterns of Fig. 1 show a component with an S_{20} greater than 4.0 S, which will be referred to as the A component. The S_{20} of the A component increases with temperature from approximately 4.4 S at 4° to 9.3 S at 32°. Figure 1 shows that within this temperature range the A component is represented by a single skewed peak, the degree of skewness of which appears to depend upon the temperature. A second peak, corresponding to the B component, can be resolved up to a temperature of ~20°. The S_{20} of the B peak remains constant at ~1.30 S. With increasing temperature its area decreases and that of the A peak increases correspondingly. At temperatures beyond ~32°, in addition to the main peak there appear a number of small auxiliary peaks representing large aggregates (Fig. 1). Further experiments indicated that 30–32° represents a temperature range beyond which there is a general but reproducible deterioration of the ultracentrifuge pattern.

At 4°, the distribution of material between the A and B peaks is similar to that between the α- and β-casein peaks in electrophoretic patterns obtained at the same temperature. This clearly suggests, as will become more apparent, that the A component at 4° is α-casein and that the B component is β-casein. The interactions of α- and β-caseins, revealed by the ultracentrifuge, were also observed in an electrophoretic pattern obtained at 30°. This pattern showed a group of about five overlapping, unresolved peaks, the mobility of the slowest corresponding approximately to that of α-casein (-7.3×10^{-5} cm.^{2} per volt per sec.) while the fastest moving member of the group had a mobility near −9.4. There was no evidence of material having a mobility close to that of β-casein.

(24) Since no information on the change of $\bar{v}$ (partial specific volume) with temperature is available for casein, the singular values given in the Discussion are used in calculations of S_{20} and M.

The upper line of Fig. 2 indicates the marked trend of the A component toward larger particle size with increasing temperature. An examination of the tracings of Fig. 1 reveals that at any particular temperature the polymers produced cluster around a preferred size. Heterogeneity is indicated, since the peaks are skewed, the velocity of the maximum ordinate increases with centrifugation time, and the boundary spreading of the A peak is considerably greater than that accompanying the B peak or that expected from uniform particles having the observed sedimentation constants. The polymerization exhibited by the A peak is known to be sensitive to small variations in preparative procedure and to the age of the preparation used.

At the time these experiments were performed our interest centered around a determination of the minimum size of α- and β-caseins. It was felt that the S_{20} of the A peak at 4° ($S_{20} \sim 4.4$ S) might not represent a true minimum size for α-casein, but an intermediate value close to that corresponding to the lowest attainable temperature. The sedimentation constants of the A and B peaks were examined, in a preliminary fashion, with respect to their dependence upon ionic strength and concentration. The data are included in Fig. 2 and show that these variables have little effect. A progressive dissociation of the A component was accomplished by working in solutions of increasing alkalinity.

Effect of *p*H.—It was recognized that only *p*H levels consistent with reversibility would yield significant data and only such conditions were used. Caseins were tested by allowing them to stand at the highest *p*H values used for periods up to 48 hours, after which they were returned to *p*H 7. Criteria of reversibility included: (a) a complete restoration of the ultracentrifuge pattern characteristic of 4° and *p*H 7, considered to be the most significant pattern of the group, and (b) the reconstitution at *p*H 7 of casein micelles "clottable" on the addition of rennin. The term "clottable" is used here in a qualitative sense. A study of the reconstitution of casein micelles and their clotting by rennin are phenomena of considerable complexity which will be treated in a future publication.

Figure 3 details the changes in S_{20} of the A and B peaks with *p*H. The measurements were carried out at an ionic strength of 0.23 (0.15 due to potassium chloride and 0.08 due to potassium oxalate) and the *p*H was adjusted with 0.1 M potassium hydroxide. All but one of the runs were made at 8 to 10°, the point at *p*H 10.8 being obtained at 4°. Both the sedimentation coefficient of the B component and the areas of the peaks are relatively constant over the *p*H range from 7 to 10.8 while over the same *p*H range, the S_{20} of the A peak decreases from 5.8 to 3.7 S. These observations suggest that only the subunits of the A component are redistributing as the *p*H rises. As before, the shape and boundary spreading of the A peak reveals some heterogeneity, but the fact that symmetrical peaks are observed indicates that at each *p*H there is distribution of polymers centering closely around an equilibrium size.

Beyond *p*H 10.9 the A peak disappears, the area of the B peak being augmented correspondingly. At

pH values beyond 10.9, and up to the highest pH used, (pH 12.0), all of the protein in the solution is reversibly dissociated into monomeric units with an average S_{20} of 1.25 S, homogeneity being indicated by the shape and behavior of the peak.

Electrophoretic Examination at pH 12.—Reference has been made previously to the close similarity of the electrophoretic patterns given by acid precipitated and soluble caseins at pH 7 and 1.5° (Table I). It should be remembered that two conclusions have been drawn at this point, namely: (a) that the system dissociates into its monomeric constituents only at high pH and (b) that the monomers of α- and β-casein are similar in frictional characteristics in spite of their differences in phosphorus content and amino acid composition.[25,26] Since the mobility of a particle is a function of both its charge and its frictional resistance, an electrophoretic examination of soluble casein at pH 12 was undertaken.

TABLE I

ELECTROPHORETIC PROPERTIES OF ACID-PRECIPITATED[a] AND SOLUBLE CASEINS

Casein	pH[b]	Temp., °C.	α-Casein μ[c]	α-Casein Area,[d] %	β-Casein μ[c]	β-Casein Area,[d] %
Acid-pptd.	6.98	0	−7.52	81	−3.00	19
Soluble	6.98	1.5	−7.29	76	−3.12	24
Soluble	12.0	1.5	−8.92	73	−5.71	27
Soluble	7.0	30.3	Unresolved group, μ ranging from −7.3 to −9.4			

[a] After Warner.[7] [b] Phosphate buffer was used in all cases. Ionic strengths were 0.1 except for pH 12.0 where the ionic strength was 0.19. [c] μ in cm.^{2} volt^{-1} sec.$^{-1}$ × 10^5. [d] Relative area. All areas and mobilities were measured on descending boundaries.

Table I, line 3, compares soluble casein at pH 12 with caseins at pH 7. It is seen that the relative areas in line 3 (pH 12) are not significantly different from those in line 2 (pH 7). The mobilities, of course, are greater at pH 12.

Dependence of the Monomers on Temperature, Ionic Strength and Protein Concentration.—Previous evidence of extensive casein interaction suggested that the behavior of casein at pH 12 be examined with respect to temperature. In phosphate buffer (NaOH–Na_2HPO_4), $\Gamma/2$ = 0.19, the S_{20} shows no significant temperature dependence since it increases only from 1.14 S at 1.6° to 1.17 S at 25.2°, a difference falling within the limits of error of the measurements. In unbuffered solution, (KCl–$K_2C_2O_4$–KOH), $\Gamma/2$ = 0.23, on the other hand, a marked temperature dependence is observed, S_{20} increasing from 1.28 S at 4° to 2.56 S at 34°. These results suggest that the lowest possible temperatures be used in monomer characterization. Buffered solutions appear preferable although it is apparent that unbuffered caseins may be used at low temperatures.

The marked increase of S_{20} with temperature in unbuffered KCl is too large to attribute to reasonable alterations in particle hydration or shape, or to a combination of both. It is therefore likely that this increase in S_{20} is brought about by some type of aggregation which does not occur in buffered solution.

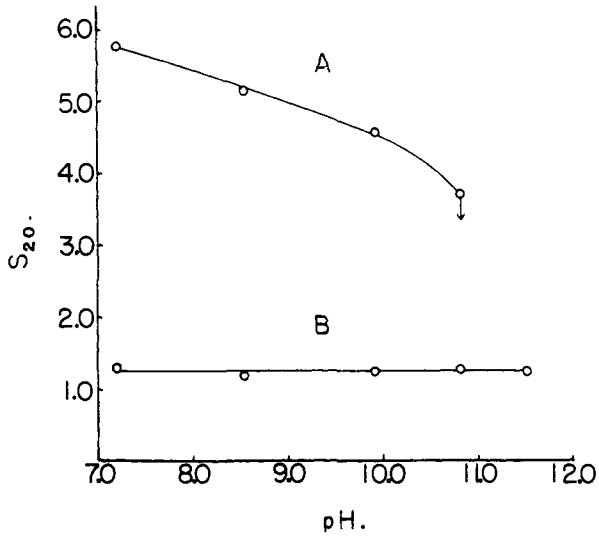

Fig. 3.—The effect of pH on the S_{20} of the A and B components of soluble casein at 8–10° and $\Gamma/2$ = 0.23; KCl–$K_2C_2O_4$ solution.

Values of S_{20} at pH 12 and several different protein concentrations were measured at two different levels of ionic strength, and at each level an S_{20} at infinite dilution was determined by extrapolation. At $\Gamma/2$ = 0.19, (NaOH–Na_2HPO_4) the extrapolated S_{20} is 1.18 S, while at $\Gamma/2$ = 0.68, (NaOH–Na_2HPO_4–NaCl) it is 1.43 S. At both ionic strengths S_{20} exhibits a small concentration dependence, increasing by about 4% as C decreases from 0.60 g./100 ml. to zero. At C = 0.60 g./100 ml. the value of S_{20} at $\Gamma/2$ = 0.19 is 1.14 S and at $\Gamma/2$ = 0.68 it is 1.38 S.

The 20% change in S_{20} found on increasing the ionic strength from 0.19 to 0.68 could be due to decreases in hydration, axial ratio or electroviscous effects, or to the appearance of aggregates. It is clear that the higher ionic strength has not reversed the dissociation obtained by increasing the pH. Since small changes of the first three types should affect S and D proportionally, and since measurements on partially aggregated systems are to be avoided, calculations made in the Discussion are based on properties determined at $\Gamma/2$ = 0.19.

Diffusion Coefficient.—Figure 4 presents the results of a series of diffusion experiments performed at pH 11.5, an ionic strength of 0.15, (KCl–KOH), and a temperature of 1.50°. The ordinate gives the diffusion coefficient corrected to a solvent having the viscosity of water at 20°. The straight line in Fig. 4 is drawn according to calculations made using the method of least squares. The value of D^0_{20} found by extrapolation to infinite dilution in this way is 7.11 ± 0.10 × 10^{-7} cm.^{2}/sec.

The fact that a mixture of α- and β-caseins is involved led to an examination of the characteristics of the experimental diffusion curves. To this end three representative diffusion curves were analyzed by the method of second moments, diffusion constants were calculated, and normalized curves were drawn. It was found that the maximum ordinate passed through the centroid of the enclosed area in

(25) W. Gordon, W. Semmett, R. Cable and M. Morris, THIS JOURNAL, **71**, 3293 (1949).

(26) W. Gordon, W. Semmett and W. Bender, *ibid.*, **72**, 4282 (1950).

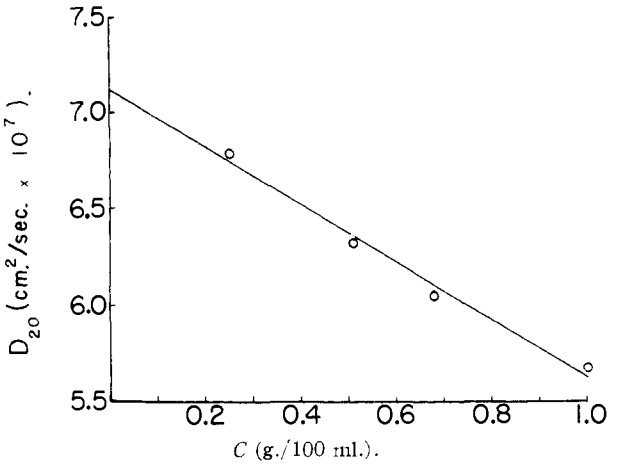

Fig. 4.—The dependence of D_{20} on concentration at pH 11.5, T = 1.50° and $\Gamma/2$ = 0.15, (KCl–KOH solution).

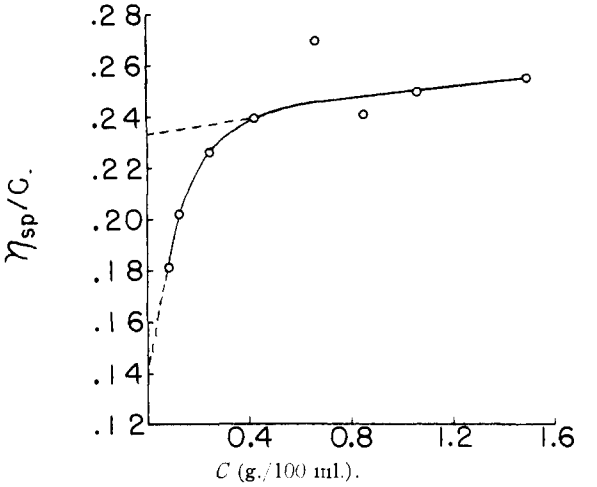

Fig. 5.—Dependence of η_{sp}/C on concentration at pH 12.0, T = 1.50° and $\Gamma/2$ = 0.19 (Na_2HPO_4–NaOH buffer).

each case, indicating little concentration dependent interaction between the components. The ratio of the diffusion constant calculated by second moments to that calculated from measurements of maximum ordinate and area is generally considered to be a measure of homogeneity, a homogeneous system resulting in a ratio of unity. In general, paucidisperse systems are expected to give ratios greater than unity. In the present case ratios of approximately 0.8, 0.9 and 0.9 were obtained and it was noted that as diffusion time increased the ratio tended toward unity. These properties of the diffusion curves are due to a combination of two effects: (a) underestimation of the height of the diffusion tracing (5 to 10% would account for the entire deviation from unity) and (b) diffusion from the slightly truncated concentration gradient curve characteristic of an unsharpened boundary. As diffusion progresses this anomaly is smoothed out and the ratio approaches unity.

Intrinsic Viscosity.—Viscosity determinations were carried out at pH 12.0, 1.50°, and an ionic strength of 0.19 ($NaOH$–Na_2HPO_4 buffer). We have examined the casein system using Ostwald–Cannon–Fenske viscometers of standard design.[27] Even with these relatively simple viscometers considerable care had to be exercised in preparing solutions. For example, all were centrifuged at 25,000 × g for 30 minutes and only the top half of the supernatant was then carefully withdrawn. Buffers were filtered through hardened filter paper until found optically free of foreign matter.

Figure 5 presents the results obtained with the procedure just described. Here η_{sp}/C (ordinate) is plotted against C (g. protein per 100 ml.). The resulting curve suggests a concentration dependence which is non-linear in form. This dependence is examined by making the two extrapolations indicated in the figure. The extrapolated values obtained are $[\eta]$ = 0.23 and 0.15. The lower extrapolation is not precise and could fall between 0.13 and 0.16.

Caseins Prepared from the Isoelectric Precipitate.—α- and β-casein fractions are prepared from skim milk by carrying out acidification at ~2°.[7] The observations reported here indicate that such should be the case, for it is at temperatures near 0° (pH 7) that α- and β-caseins occur in solution in separate physical states; β-casein as the monomer and α-casein in a polymerized form. The system behaves as though acidification preserves the essential aspects of this physical separation and allows β-casein to be removed subsequently by fractional precipitation. At more acid pH values, ~ pH 1.5, extensive polymerization is still evident, for preliminary experiments reveal components with S_{20} ranging from 15 to 20 S and no evidence of the presence of casein monomers.

The isoelectric precipitate requires the addition of alkali to effect solution.[5,7,9] We have noted the coincidence in electrophoretic patterns of products derived from acid precipitated casein and soluble casein at pH 7 and have also noted that molecular weight studies carried out on the former give divergent results. On the basis of these facts we felt it likely that isoelectric precipitation produces changes leading to aggregates of marked stability and that under ordinary conditions complete dissociation might not occur during subsequent manipulation. We have found this to be the case.

A commercially available isoelectric precipitate was dissolved in phosphate buffer ($\Gamma/2$ =0.20, pH 7), a cloudy solution resulting. At 1°, the ultracentrifuge pattern obtained from this material revealed a β-peak of customary area with an S_{20} of 1.42 S, a markedly diminished and polydisperse α-peak, the maximum ordinate of which sedimented with an S_{20} of ~4.5, and the presence of larger polydispersed aggregates. The same type of pattern was obtained after solution at pH 8.0. Evidently α-casein is more susceptible to stable aggregation during isoelectric precipitation than β-casein.

When dissolved at pH 12, acid-precipitated casein dissociates completely, resulting in a single peak with an S_{20} of 1.14 S (1°, 0.60 g. protein/100 ml.). After dialysis to pH 7.0, the resulting clear solution yields an ultracentrifuge pattern (1°) comparable to that obtained with soluble casein under the same conditions (see Fig. 1, 7° C.) except that the α-

(27) M. Cannon and M. Fenske, *Ind. Eng. Chem., Anal. Ed.*, **10**, 297 (1938).

peak sediments more rapidly ($S_{20,\alpha} = 6.0\ S$ instead of ~4.4 S). The S_{20} of the β-peak is 1.37 S. It is clear that the properties of soluble casein have been largely restored.

The $S_{20,\beta}$ values obtained from isoelectric casein dissolved at *p*H 7 and after treatment at *p*H 12 are close to those determined by Sullivan, *et al.*,[17] who found that β-casein fractionated from the isoelectric precipitate gave $S_{20,\beta} = 1.36\ S$ in phosphate buffer. We feel that $S_{20,\beta} = 1.36\ S$ is the maximum value consistent with all available experimental evidence.

Ultracentrifuge runs at 25° on acid precipitated casein dissolved at *p*H 12 and returned to *p*H 7 result in patterns comparable again to those of soluble casein (Fig. 1, 27°). At the same temperature acid-precipitated casein dissolved directly at *p*H 8.0 yields patterns indicating the presence of highly polydisperse aggregates.

From the information presented here concerning the association of α- and β-caseins, it is clear that the experimental conditions used by previous investigators could not have revealed α-casein monomers irrespective of the effects of acid precipitation. It is clear that acid-precipitated casein dissolved at *p*H 7 contains large stable aggregates. If this material is brought to a *p*H in excess of 12 it dissociates into characteristic monomers. It is also apparent that solubilization at some intermediate *p*H leads to a solution containing physically separate α- and β-caseins, with polymers having characteristics different from those observed in soluble casein.

Discussion

The Casein Monomers.—At temperatures near 0° and *p*H 12, unfractionated soluble casein dissociates into monomeric units having an average S°_{20} of $1.18 \pm 0.02 \times 10^{-13}$ sec. and D°_{20} of $7.11 \pm 0.10 \times 10^{-7}$ cm.²/sec. These values and the partial specific volume of unfractionated casein, $\bar{v} = 0.731$,[28] lead to an average molecular weight of 15,000.

The ratio of the frictional resistance of the hydrated molecule to that of the anhydrous equivalent sphere, (f/f_0), may be calculated from values of S, D and $\bar{v}$ (see Svedberg and Pedersen[29]). An f/f_0 of 1.84 is obtained for the casein monomers.

Oncley[30] postulates that f/f_0 has two components: f/f_e which represents the contribution to the frictional resistance of the kinetic water and f_e/f_0 which represents the effect of particle shape. If the extent of hydration is known or assumed, f/f_e may be calculated by means of equation 1

$$\frac{f}{f_e} = \left[1 + \frac{w\bar{v}_w}{\bar{v}}\right]^{1/3} \qquad (1)$$

Here w is the kinetic water (the amount of water, in grams, which moves with one gram of anhydrous particles), and $\bar{v}_w$ is the partial specific volume of this water, taken as unity.

The ratio f_e/f_0 has been related to the particle shape by Perrin,[31] for cases where the particles may be represented by equivalent ellipsoids of revolution. It will be assumed that casein molecules are best represented by equivalent prolate ellipsoids.

The increase in the viscosity of a solution of appropriate large molecules over that of the solvent alone is given physical interpretation through the viscosity increment, ν, defined as the limit of η_{sp}/Φ as Φ approaches zero. η_{sp} is the specific viscosity and Φ is the fraction of the total volume occupied by the particles, including kinetic water. Simha[32] has related ν to the axial ratios of either prolate or oblate equivalent ellipsoids. The experimentally obtained intrinsic viscosity is used to calculate a preliminary viscosity increment, ν', as in equation 2

$$\nu' = \frac{100[\eta]}{\bar{v}} \qquad (2)$$

Since $[\eta]$ is based on dry-weight concentrations, ν', which is termed a viscosity coefficient by Edsall,[33] has no direct physical interpretation. By means of equation 3, the physically significant entity, ν, may be calculated.

$$\nu = \frac{\nu'}{(1 + w/\bar{v}\rho)} \qquad (3)$$

where ρ is the solvent density. Edsall[33] presents a more extensive treatment of equations 2 and 3.

Intrinsic viscosities of 0.23 and 0.15 lead, through equation 2, to viscosity coefficients, ν', of 31.5 and 20.5, respectively. By means of equation 3 corresponding values of the viscosity increment, ν, are calculated assuming various levels of w. These calculated viscosity increments lead to axial ratios of the hydrated particles *via* an equation of Simha for prolate ellipsoids. In Fig. 6 axial ratios, a/b, are plotted against w for $\nu' = 31.5$ (curve 1) and $\nu' = 20.5$ (curve 2).

Axial ratios obtained from sedimentation and diffusion constants may also be related to kinetic water. Paired values of a/b and w are obtained as follows. A value of w is chosen and an f/f_e obtained through equation 1. The corresponding f_e/f_0 inserted into the appropriate equation of Perrin, leads to an axial ratio. Thus, $\bar{v}$ and M lead to the dimensions of the anhydrous particle. We assume that the kinetic water may be represented by a shell of uniform thickness, and calculate the dimensions and the axial ratio of the hydrated particle. These axial ratios, plotted against the assumed value of w, result in curve 3 of Fig. 6.

Figure 6 shows that $\nu' = 31.5$ cannot be reconciled with data derived from S°_{20} and D°_{20} and is probably due to particle interaction. An axial ratio of 10 to 11 for the hydrated particle and a water of hydration of 0.3 g./g. protein are consistent with the sedimentation and diffusion data and with the viscosity data obtained at $\nu' = 20.5$. From equation 1 we obtain $f/f_e = 1.12$; therefore $f_e/f_0 = 1.64$. According to Perrin's equation, an f_e/f_0 of 1.64 corresponds to an axial ratio of 12.0 for the anhydrous particle. The latter and a molecular

(28) T. McMeekin, M. Groves and N. Hipp, THIS JOURNAL, **71**, 3298 (1949).

(29) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," The Clarendon Press, New York, N. Y., 1940, pp. 38–44.

(30) J. L. Oncley, *Ann. N. Y. Acad. Sci.*, **41**, 121 (1941).

(31) F. Perrin, *J. phys. radium*, **7**, 1 (1936).

(32) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(33) J. T. Edsall, "The Proteins," H. Neurath and K. Bailey, Ed., Vol. I, Part B, Academic Press, New York, N. Y., 1953, pp. 686–693.

weight of 15,000 lead to an ellipsoid with a major axis of 210 Å. and minor axes of 17.6 Å.[34]

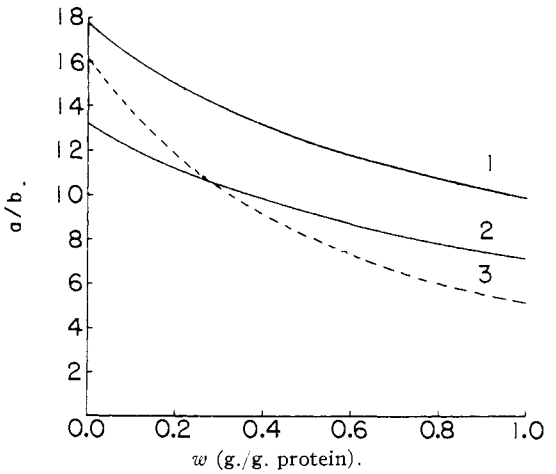

Fig. 6.—Calculated axial ratios of the hydrated particle (ordinates) are plotted against assumed values of kinetic water (abscissa). Curves 1 and 2 are derived from viscosity data, (1) from $\nu' = 31.5$ and (2) from $\nu' = 20.5$. Curve 3 is derived from measured sedimentation and diffusion constants. For details see text.

Electrophoretic data provide another approach to the subject of frictional resistance. Equation 4 may be used, as a first approximation, to relate the electrostatic and frictional properties of a molecule.

$$\mu = M(Q/M)/f' \qquad (4)$$

Here μ is the mobility, Q is the net charge per particle in electronic charge units and f' is a frictional coefficient related to the frictional resistance of the hydrated particle. Amino acid compositions[25,26] are used to calculate the net charge of α- and β-casein units of molecular weight 15,000.

TABLE II

Casein	pH	μ^a	Q^b	f'^c
α-Casein	7	−7.29	−15	2.06
	12	−8.92	−32	3.59
β-Casein	7	−3.12	−11.5	3.69
	12	−5.71	−21.5	3.77

[a] μ = mobility in cm.^{2} volt^{-1} sec.$^{-1}$ × 10^5. [b] Q = net charge in electronic charge units for M = 15,000. [c] f' = frictional resistance coefficient in terms of Q/μ.

Table II summarizes the relative frictional resistances calculated using equation 4. In calculating Q it was considered that: (a) all carboxyl and phosphate groups are charged at both *p*H values, the latter carrying two charges since substituted phosphates have pK_2 values in the range 6.1 to 6.3 (see ref. 36); (b) arginine residues are charged at both *p*H levels; (c) cysteine and tyrosine residues are uncharged at *p*H 7 and charged at *p*H 12; (d) lysine residues are charged at *p*H 7 and uncharged at *p*H 12; (e) there is one carboxyl and one amino end group per molecule, the latter being uncharged at *p*H 12. Values of Q given in Table II are calculated on the basis of a molecular weight of 15,000. The relative frictional coefficients, f', of β-casein at *p*H 7 and *p*H 12 and of α-casein at *p*H 12 are closely similar, indicating that their frictional resistances per unit weight are much the same. The considerably lower f' of α-casein at *p*H 7 is clearly due to polymerization.

The frictional resistance of a molecule is related to other variables by equation 5

$$M = NSf_e\left(\frac{f/f_e}{1 - \bar{v}\rho}\right) \qquad (5)$$

where N = Avogadro's number, f_e is the frictional resistance of the anhydrous molecule and f/f_e is the hydration factor. Using the values of $\bar{v}$ determined by McMeekin, *et al.*,[28,37] and assuming hydration to be proportional to charge, the term in brackets in equation 5 may be shown to be approximately the same for α- and β-casein. Thus equation 5 now suggests that M_α and M_β vary with the products of the corresponding values of S and f_e. S_α and S_β are close, but probably not identical. At *p*H 12 all protein is in the monomeric form while below *p*H 10.8 α-casein polymerizes preferentially. The removal of ~75% of the total protein changes the sedimentation constant of the remaining monomer from 1.17 ± 0.02 S to 1.25 ± 0.08 S (at C = 0.15%), the latter referring to β-casein.

It is clear that accurate values of the molecular weights of α- and β-caseins must await fractionation and independent determination of D_α and D_β, (and thus of $f_{e,\alpha}$ and $f_{e,\beta}$), since the diffusion behavior of a mixture is largely insensitive to the properties of its components. However, a certain amount of additional information may be obtained from an examination of the system as a whole. To this end we have postulated various molecular weights for β-casein, ranging from 10,000 to 25,000. Using these molecular weights and an S_{20} of 1.25 × 10^{-13} sec., values of $D_{20,\beta}$ have been calculated. Corresponding values of $D_{20,\alpha}$ were then determined by means of equation 6

$$D_{20} = 7.11 \times 10^{-7} = \frac{\Sigma C_i D_i}{\Sigma C_i} \qquad (6)$$

where C_i and D_i represent the concentrations and diffusion constants of the components of the mixture (see ref. 38). The values of $D_{20,\alpha}$ obtained in this way are combined with $S_{20,\alpha}$ (taken as 1.15 × 10^{-13} sec.), to yield values of M_α.

The diffusion constants obtained for the α- and β-casein components lead to diffusion curves which may be combined to give single curves, using relative concentrations of 0.75 and 0.25. A comparison of these curves, the normal curve for a single component with a D_{20} of 7.11 × 10^{-7} cm.^{2}/sec., and the normalized experimental diffusion curves, suggests that $M_\beta \geq M_\alpha$, that a molecular weight of 25,000 is probably the upper limit for β-casein and

(34) The α-helix of Pauling and Corey[35] has a translation of 1.47 Å. per residue. The average number of residues involved in constructing α- and β-casein molecules of M = 15,000 lead to lengths of 182 and 189 Å., respectively. The average diameter is calculated to be 15.4 Å., a value based on the average side chain extension (see 40, Table V) of several proteins and the diameter of the α-helix. The volume of a right circular cylinder 186 Å. in length and 15.4 Å. in diameter is sensibly the same as that of a prolate ellipsoid of revolution with corresponding dimensions of 210 and 17.6 Å.

(35) L. Pauling and R. B. Corey, *Proc. Roy. Soc. (London)*, **B141**, 21 (1953).

(36) E. Cohn and J. Edsall, "Proteins, Amino Acids, and Peptides, as Ions and Dipolar Ions," Reinhold Pub. Corp., New York, N. Y., 1943, p. 134.

(37) T. L. McMeekin and K. Marshall, *Science*, **116**, 142 (1952).

(38) H. Neurath, *Chem. Revs.*, **30**, 357 (1942).

that the molecular weight of α-casein lies between 13,000 and 15,000. Thus M_β is probably between 15,000 and 25,000.

Monomer Interactions.—The interactions of the α- and β-casein monomers examined here appear to be largely dependent upon pH and temperature. At pH 12 and $\sim 0°$ both α- and β-caseins are present as monomers, the two components being distinguished electrophoretically. If the pH is gradually decreased at low temperatures, there appears in the ultracentrifuge at $\sim$ pH 10.8, in addition to the diminished peak due to monomer, a second faster peak. The sedimentation constant of this peak increases progressively, indicating a corresponding increase in the degree of polymerization. The material remaining after the formation of this second peak maintains a constant relative area and a sedimentation constant characteristic of casein monomers. At pH 7 and $\sim 0°$ a comparison of ultracentrifuge and electrophoretic patterns reveals that β-casein has remained as a separate component and that the polymers which have appeared consist primarily, if not entirely, of α-casein. The relative areas observed by either technique at this temperature are consistent and the electrophoretic patterns are equivalent to those which have been obtained from acid-precipitated casein.[6,7,14]

If the temperature at pH 7 is increased from 0°, there is a progressive disappearance of the B peak, corresponding to β-casein, until at $\sim 20°$ it is too small to measure. At the same time there is an increase in both the area and sedimentation constant of the A peak, which was due only to α-casein at 0°. At pH 7 and 30° a single peak is observed in the ultracentrifuge (see Fig. 1). Likewise, electrophoretic patterns obtained at this temperature reveal no β-casein component. Instead a single broad separated peak is observed, the minimum mobility of which corresponds to that of α-casein. It is clear that polymers containing both α- and β-casein have been formed, the electrophoretic pattern suggesting polymers which differ in their proportions of α- and β-caseins and/or frictional characteristics. The progressive changes described take place as the temperature increases from 0° to $\sim 32°$. At higher temperatures the ultracentrifuge pattern reveals marked polydispersity.

Conditions have been described for the formation of polymers of varying size, either predominantly from α-casein or through the interaction of α- and β-caseins. In all cases the polymers tend to center about a preferred size, but polymers of α-casein alone appear to approach monodispersity more closely. Clearly there exists in each case a balance of attractive and repulsive forces dependent on the physical conditions. These polymerizations bear a marked resemblance to those responsible for the formation of soap micelles, as described and analyzed by Debye.[39]

A review of the facts given above leads to the conclusion that α-casein polymerizes more readily than β-casein. This is so in spite of its higher net charge at all pH values used here. It appears that forces other than those due to charge interactions are responsible for polymer formation. A number of possibilities in this connection have been examined recently.[40] A comparison of caseins with other proteins (see ref. 40, Table III, p. 343) reveals that casein has an unusual number of non-polar residues which may be involved in casein interactions.

(39) P. Debye, *Ann. N. Y. Acad. Sci.*, **51**, 575 (1949).
(40) D. F. Waugh, *Advances Protein Chem.*, **9**, 325 (1954).

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Synthesis of Tryptamines Related to Serotonin

BY ELLIOTT SHAW

RECEIVED FEBRUARY 28, 1955

Modifications in the serotonin structure have been made by introduction of alkyl groups into the 1- and 2-positions. The Fischer rearrangement of methyl levulinate p-methoxyphenylhydrazone gave high yields (80%) of 2-methyl-5-methoxy-3-indoleacetic acid. With succinaldehydic acid as the carbonyl moiety, comparable yields were obtained with only an *asym*-N-alkyl derivative of the hydrazine. Direct amidification of 3-indoleacetic acids by heating with urea or tetramethylurea provided amides for reduction to tryptamines by means of lithium aluminum hydride. A number of related indoles have also been prepared.

The naturally occurring tryptamine, serotonin (I), has many physiological properties[1,2] in the mammalian circulatory system and apparently represents an important metabolite in tryptophan utilization in man.[3] We initially undertook the synthesis of analogs of serotonin in order to obtain substances which would prevent the pressor response characteristic of this tryptamine by acting as antimetabolites.[4] A number of contributions were subsequently made in this direction.[5,6] Meanwhile from considerations including the occurrence of serotonin in the brain and the evidence that a number of indole alkaloids may act as inhibitors at serotonin receptors[7] and are known to cause mental disturbances in man, the hypothesis was advanced[8] that serotonin is essential for normal brain function; mental disease may thus reflect

(1) I. H. Page, *Physiol. Rev.*, **34**, 563 (1954).
(2) V. Erspamer, *Rend. sci. farmitalia*, **1**, 5 (1954).
(3) E. Titus and S. Udenfriend, *Federation Proc.*, **13**, 411 (1954).
(4) D. W. Woolley, "A Study of Antimetabolites," John Wiley and Sons, Inc., New York, N. Y., 1952.
(5) D. W. Woolley and E. Shaw, *J. Pharm. Exper. Therap.*, **108**, 87 (1953).
(6) E. Shaw and D. W. Woolley, THIS JOURNAL, **75**, 1877 (1953).
(7) E. Shaw and D. W. Woolley, *J. Biol. Chem.*, **203**, 979 (1953).
(8) D. W. Woolley and E. Shaw, *Proc. Nat. Acad. Sci.*, **40**, 228 (1954).