will clot on the addition of rennin. According to Fig. 1 and Table II, α_s-casein fractions contain, in addition to κ-casein and $\alpha_{s1,2}$-caseins, five components. Which, if any, of these components in the pure state can combine with κ-casein and form micelles is not known. If caseins of appropriate properties are isolated from α_s-casein fractions it is suggested that they be termed α_{s3}-casein, etc.

The authors wish to express their appreciation to Dr. V. M. Ingram and Dr. M. A. Naughton for their advice and assistance in the determination of C-terminal groups.

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

Light Scattering Studies of $\alpha_{s1,2}$-Caseins[1]

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Light scattering studies have been carried out on $\alpha_{s1,2}$-caseins, a preparation which accounts for 45% of the optical density of whole casein and consists of two similar components.[13] The components exist in monomeric state at pH 12, ionic strength 0.3 to 1.2 and room temperature. Under these conditions the refractive index increment is 0.181 ml. per g. The molecular weight of protein dialyzed at pH 12 using protein concentrations above 4 mg. per ml. was found to be 27,300 ± 1500 (95% confidence limits). The second virial coefficient was approximated by the equation $B = [17 - 17\Gamma/2] \times 10^{-4}$ mole ml./g.^{2}. Protein dissolved directly at pH 12 gave a molecular weight of 27,000 ± 1000. Unpredictable, usually irreversible, aggregation occurs under these conditions: (a) at ionic strengths below 0.3 at pH 12; (b) on interaction with a factor released by dialysis membranes at high pH, acting at protein concentrations below 3 mg. per ml.; (c) when a rapid pH–ionic strength increase is imposed on protein in solution at pH 7; (d) on surface denaturation caused by bubbling or foaming.

Introduction

Whole casein has been treated by a variety of preparative techniques each of which produces a major fraction easily precipitable with calcium ion and having a greater electrophoretic mobility than the remaining proteins. These major fractions have been referred to as α-caseins.[4–8] Wake and Baldwin, using tris-citrate starch gels, have demonstrated that the α-casein preparations so far described are multicomponent.[9]

Physico-chemical studies of molecular weight have been carried out on such preparations. In the recent analysis of McKenzie and Wake[10] on a material prepared by calcium and alcohol precipitation[11] and known to contain several components,[9] molecular weights from ultracentrifugation studies were reported to be between 24,000 and 27,600. Earlier studies by Gillespie and Waugh, reported by Waugh,[12] on an α_s-casein prepared by calcium precipitation and also known to be impure gave a molecular weight of 23,300.

Recently, a casein preparation, termed $\alpha_{s1,2}$-caseins, has been obtained in this Laboratory.[13] On either acetic acid or tris-citrate starch gel electrophoresis, two sharply defined bands of nearly the same electrophoretic mobility are observed. These bands have been associated with two components, each having the same C-terminal groups (tryptophan, leucine, and probably tyrosine), the same N-terminal group (arginine[14]) and, so far, identical adsorption characteristics on DEAE-cellulose. That the two components, which are present in nearly equal amounts, are similar and are probably small variations of the same fundamental structure is clear. The $\alpha_{s1,2}$-caseins account for 45% of the optical density of the total casein. They exist as polymers at pH 7. The $\alpha_{s1,2}$-caseins interact with κ-casein and the resulting complexes in the presence of divalent cations form micelles which clot on the addition of rennin.

In this paper are described light scattering studies intended to determine the monomer molecular weight of $\alpha_{s1,2}$-caseins. Reduction to monomers of the polymers that occur at pH 7 requires either the introduction of charge as at high pH or a dissociating solvent system such as urea. Under these conditions ultracentrifugal methods necessitate the measurement of partial specific volume for the solvent environment,[15] and either correction for charge effects at high pH[16] or for possible preferential solvent interactions in urea solution.[17] These difficulties suggested the use of light scattering in solutions at high pH. By so doing preferential solvent interactions are avoided and the technique inherently yields a molecular weight and an interaction coefficient.[18]

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(2) Postdoctoral fellow of the Donner Foundation, National Research Council.

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Materials and Methods

Protein, Standard Buffers and Reagents.—Purified samples of $\alpha_{s1,2}$-caseins were obtained by means of a procedure described elsewhere.[13] Samples of protein which had undergone freeze-drying and are termed "dry protein" were routinely dissolved in buffers at pH 12. The buffers contained 0.01 M Na_2HPO_4, usually 0.004 M Na_2–EDTA, NaCl sufficient to attain the desired ionic strength, and NaOH sufficient to titrate to pH 12. Analytical reagent grade chemicals were used throughout.

Protein samples contained variable amounts of salts as a result of the preparative procedure[13] and for this reason were dialyzed at pH 12 for the initial experiments. Dialysis membranes were cut from $^{18}/_{32}$ Visking tubing (Visking Co., Union Carbide Corp., Chicago, Ill.).

Turbidity.—Turbidity was measured using a Brice–Phoenix light scattering photometer, model 1000-DM (Phoenix Precision Instrument Co., Philadelphia, Penna.), on solutions in T-101 square cells or in D-101 semioctagonal cells. Data were obtained using light at a wave length of 5460 Å., with simultaneous recording of intensities at 0 and 90°. An opal glass diffusor calibrated by the Phoenix Precision Instrument Co. was utilized as an absolute standard of known turbidity. For repeated use during the course of an experiment, a working standard was obtained by calibrating a solution of Ludox (E. I. du Pont de Nemours and Co., Wilmington, Del.) against the opal glass diffusor. We have equipped the light scattering photometer with a rotatable magnet just beneath the cell table, which operated a small stirring bar within the cell. This was helpful in studying time changes in turbidity, checking for the appearance of particulate matter during a run, and in bringing the solution to uniform concentration after making additions to the cell.

The removal of dust from solutions used for measurement of turbidity required special care. The following procedure was satisfactory: scattering cells were washed successively in Lakeseal detergent, chromic acid cleaning solution, and distilled water which had been filtered through GS Millipore filters (pore size 2200 Å., Millipore Filter Corp., Bedford, Mass.). The cells were then dried and stored in a chamber through which room air flowed continuously after passing through an HA Millipore filter (pore size 4500 Å.). A solution on which scattering measurements were to be obtained was introduced directly into a cell after passing through a GS Millipore filter, 47 mm. diameter, which was enclosed in a Lucite chamber connected to a high-pressure tank of nitrogen gas. Rates of flow in excess of 10 ml. per min. were obtained at pressures from two to four pounds per square inch. Filtration was repeated if extraneous dust particles were found, as indicated by direct examination of forward scattering from the solution, or by marked fluctuations in the recorded intensity at a scattering angle of 90°. Although this procedure yielded solutions relatively free from dust, a slight decrease in scattering often was noted in solutions which were left unstirred for about 10 minutes. Hence, definitive measurements were obtained routinely on solutions which had stood quiescent for periods of 10 to 20 minutes.

Since depolarization was less than 1 per cent., no correction for fluorescence was made.

Protein Concentration.—Protein in solution was determined by measurement of optical density at 2800 Å. on a Beckman DU spectrophotometer. The extinction coefficient of $\alpha_{s1,2}$-caseins in phosphate buffer at pH 12.0 was E 1% = 9.31. This was based on an extinction coefficient of 10.1 in imidazole buffer at pH 6.0, a value calculated from measurements of dry weight of protein after extensive dialysis.[13]

Refractive Index Increment.—Refractive indices in solutions of protein at pH 12 were measured at a wave length of 5460 Å., by means of a Brice–Phoenix differential refractometer, with aqueous solutions of sucrose utilized as a reference standard. Measurements were performed on serial dilutions obtained using dialysate as a diluent.

Analysis of Data.—Simplifications of the general expression for light scattering by a solution of macromolecules was possible, since angular dissymmetry is sensibly unity for globular proteins of molecular weight less than 50,000. In the experiments measuring monomer molecular weight, HC/τ was related linearly to the concentration of protein in the range examined. Hence, the well-known expression of Debye[19] was employed

(19) P. Debye, *J. Appl. Phys.*, **15**, 338 (1944).

$$\frac{HC}{\tau} = \frac{1}{M} + 2BC$$

where M = molecular weight; B = second virial coefficient; C = protein concentration; $H = 32\pi^3 n_0^2 (\mathrm{d}n/\mathrm{d}C)^2/N_0\lambda^4$; and τ = turbidity.

During the course of a dilution experiment, the variation in repeated readings of turbidity was found to be constant, irrespective of protein concentration. Consequently the usual condition of homoscedadicity[20] did not apply to these data, and linear regression could not be employed for statistical analysis. Instead, weighted linear regression was utilized, where the weighting factor, $1/C$, was derived in this way: By the usual method of error analysis

$$\Delta\left(\frac{HC}{\tau}\right) = \frac{HC}{\tau^2}(\Delta\tau) + \frac{H}{\tau}(\Delta C)$$

where ΔX indicates error in X. Since the error in the measurement of concentration by optical density was small compared to the error in turbidity

$$\Delta\left(\frac{HC}{\tau}\right) \cong \frac{HC}{\tau^2}(\Delta\tau) \cong \frac{1}{HCM^2}(\Delta\tau)$$

Since H, M, and $\Delta\tau$ are constants

$$\Delta\left(\frac{HC}{\tau}\right) = (\text{constant})\,\frac{1}{C}$$

The pertinent formulas including the weighting factor are derived by the method of maximum likelihood.[21] Equation 1 may be expressed as

$$Y_i = M^{-1} + 2BC_i$$

$$\text{where } Y_i = \left(\frac{HC}{\tau}\right)_{C_i}$$

The estimators, $\hat{B}$ and $\hat{M}^{-1}$, are found to be

$$2\hat{B} = \frac{\sum_i w_i \sum_i w_iC_iY_i - \sum_i w_iC_i \sum_i w_iY_i}{\sum_i w_i \sum_i w_iC_i^2 - \left(\sum_i w_iC_i\right)^2}$$

and

$$\hat{M}^{-1} = \frac{\sum_i w_iY_i - 2B\sum_i w_iC_i}{\sum_i w_i}$$

where the weighting factor $w_i = 1/C_i$, and all summations are from 1 to n, where n is the number of determinations.

Equations for variances of $\hat{M}^{-1}$ and $\hat{B}$, written in forms suitable for calculation, are

$$S_{\hat{B}}^2 = \frac{1}{n-2}\left\{\frac{\sum_i w_i \sum_i w_iY_i^2 - \left(\sum_i w_iY_i\right)^2}{\sum_i w_i \sum_i w_iC_i^2 - \left(\sum_i w_iC_i\right)^2} - \hat{B}^2\right\}$$

$$S_{\hat{M}^{-1}}^2 = S_{\hat{B}}^2\,\frac{\sum_i w_iC_i^2}{\sum_i w_i}$$

It may be shown that

$$t_{\hat{B}} = (\hat{B} - B)/S_{\hat{B}}$$

$$t_{\hat{M}^{-1}} = (\hat{M}^{-1} - M^{-1})/S_{\hat{M}^{-1}}$$

follow "Students t"-distribution, with $(n-2)$ degrees of freedom.[21] Calculations were performed on the IBM 709 Computer at the M.I.T. Computation Laboratory.

Results

Light scattering measurements have been obtained on solutions of $\alpha_{s1,2}$-caseins using a variety of experimental conditions at pH 12. The conditions under which a single consistent molecular weight was observed will be discussed first. Under other conditions, increases in apparent molecular weight were observed, often unpredictable in extent, and therefore attributed to uncontrolled aggregation.

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(21) A. M. Mood, "Introduction to the Theory of Statistics," McGraw-Hill Book Co., Inc., New York, N. Y., 1960.

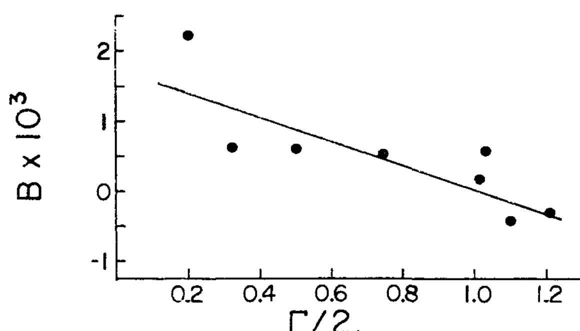

Fig. 1.—Second virial coefficient of $\alpha_{s1,2}$-caseins, plotted against ionic strength, for experiments where dialyses were performed at room temperature at protein concentrations greater than 4 mg. per ml.

It will be evident that monomer aggregation is produced by the use of low ionic strengths, by a factor released from dialysis membranes, by an increase in the pH of protein solutions from 7 to 12, or by surface denaturation.

Refractive Index Increment.—In Table I are shown data on $(dn/dc)_{5460\text{Å}}$ for $\alpha_{s1,2}$-caseins in buffer at pH 12. The average value was 0.181 ml. per g., and the results were independent of the ionic strength of the buffer. The experimental procedure was checked by measuring $(dn/dc)_{5460\text{ Å}}$ for bovine serum albumin in aqueous solution at pH 7.0; the value of 0.186 ml. per g. so obtained is in agreement with published data.[22,23]

TABLE I

REFRACTIVE INDEX INCREMENT AT WAVE LENGTH OF 5460 Å., FOR $\alpha_{s1,2}$-CASEINS DIALYZED IN PHOSPHATE BUFFER AT pH 12.0

Ionic strength	Dialysis: Temp., °C.	Dialysis: Protein concn., mg./ml.	dn/dC, ml./g.[a]
0.246	7	6.80	0.1895 ± 0.0055
0.234	7	7.54	.1774 ± .0045
1.06	25	7.30	.1765 ± .0049
1.02	25	6.74	.1819 ± .0071
		Av.	.181 ± .006

[a] Mean ± 95% confidence limit.

Molecular Weight Following Dialysis at Room Temperature and pH 12.—At pH 12, ionic environment is of primary concern because of coulombic interactions and of salting-in and salting-out characteristics of proteins. Ionic environment was maintained sensibly unchanged during an experiment by measuring turbidity and concentration in serial dilutions of protein with dialysate. Table II summarizes the results obtained following dialysis of protein against twenty-fold volumes of buffer at room temperature for ten hours.

As discussed below, the apparent molecular weight after dialysis is affected by the concentration of protein within the dialysis sack. In experiments where the protein dialysis concentration exceeded 4 mg. per ml. (lines 1 to 8 in Table II), the molecular weight was essentially independent of ionic strength for values greater than 0.3.

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(23) M. Halwer, G. C. Nutting and B. A. Brice, *ibid.*, **73**, 2786 (1951).

TABLE II

LIGHT SCATTERING DATA FOR $\alpha_{s1,2}$-CASEINS DIALYZED AT ROOM TEMPERATURE IN PHOSPHATE BUFFER AT pH 12.0

Ionic strength	Protein dialysis concn., mg./ml.	$(HC/\tau)_C \to 0$ mole/g.[a]	M, g./mole	B, mole-ml./g.^{2} $\times 10^{-4a}$
0.20	5.49	2.72 ± 0.41	36,820	22.21 ± 6.85
0.32	5.42	3.71 ± .37	26,930	6.30 ± 6.44
0.50	4.47	3.94 ± .36	25,370	6.14 ± 6.85
0.74	6.35[b]	3.31 ± .28	30,190	5.34 ± 5.45
1.01	4.51	3.90 ± .34	25,630	1.72 ± 8.38
1.03	4.96	3.57 ± .42	28,050	5.65 ± 6.37
1.10	8.56	3.72 ± .27	26,880	−4.38 ± 4.34
1.21	4.27	3.63 ± .26	27,570	−3.04 ± 5.03
1.02	1.89[c]	2.66 ± .13	37,630	−10.19 ± 5.21
1.02	1.74[b,c]	3.52 ± .64	28,420	−8.27 ± 27.75
1.08	1.92	2.63 ± .28	38,040	7.45 ± 10.82
0.92	1.88[d]	2.83 ± .39	35,350	31.51 ± 14.22
1.25	2.05[d]	3.56 ± .40	28,070	−0.20 ± 18.82
1.11	1.19[e]	[f]		

[a] Mean ± 95% confidence limits. [b] Dialysis membrane boiled in 5% Na_2CO_3. [c] Ratio of membrane area to dialysand volume increased by a factor of 1.5. [d] Dialysate volume increased by a factor of 10. [e] Ratio of membrane area to dialysand volume increased by a factor of 5. [f] The apparent molecular weight, τ/HC, varied from 62,300 at a concentration of 1.1 mg. per ml. to 35,300 at 0.48 mg. per ml. The data did not appear to be linear, and a statistical analysis was not performed.

In seven such experiments, the average molecular weight was 27,150. A statistical analysis of these data was done, where for each experiment the value of the zero-intercept of HC/τ was weighted by a term inversely proportional to the variance of the estimate, as calculated by the method of weighted linear regression described above. Corrections were small. The analysis indicated an average molecular weight of 27,300 with 95% confidence limits at 25,900 and 28,900. In each of these seven experiments and the experiment at ionic strength 0.2 (first line of Table II), a plot of HC/τ *vs.* C was linear. The second virial coefficients (B) varied from 22.2×10^{-4} to -4.38×10^{-4} mole ml./g.^{2} In Fig. 1 the line drawn through the data for the second virial coefficient plotted against ionic strength was calculated by means of linear regression, weighting each value of B by a term inversely proportional to the variance of the estimate. This line is given by the equation $B = [17 - 17\ \tau/2] \times 10^{-4}$ mole ml./g.^{2} The standard error of the slope is 0.5 ml.^{2}/g.^{2} The slope is different from zero at the 98% level of significance.

Molecular Weight in the Absence of Dialysis.—Two circumstances prompted examination of protein solutions without prior dialysis at pH 12. The first was aggregation of protein related to dialysis at low protein concentration, discussed below, and the second was the fact that B values were low at higher ionic strengths (Fig. 1) which suggested that the salt introduced by dissolving the protein directly could be neglected. Table III summarizes the results, the separate experiments being indicated by the ionic strength values in the first column. Since single concentrations were used (col. 2) the observed τ/HC values (col. 3) were corrected to mo-

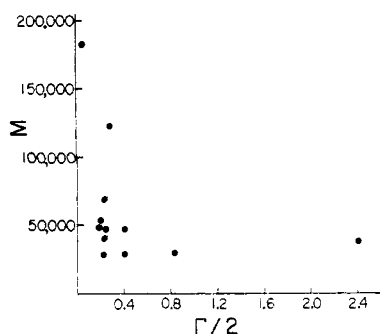

Fig. 2.—Apparent molecular weight of $\alpha_{s1,2}$-caseins, plotted against ionic strength, for experiments where dialyses were performed at a temperature of 4 to 8° at protein concentrations greater than 4 mg. per ml.

lecular weights at infinite dilution by using B values characteristic of each ionic strength from the line of Fig. 1. For ionic strengths above 0.6, the correction is seen to be small.

TABLE III

LIGHT SCATTERING DATA FOR $\alpha_{s1,2}$-CASEINS DISSOLVED IN PHOSPHATE BUFFER AT pH 12 WITHOUT SUBSEQUENT DIALYSIS

Ionic strength	Protein concn., mg./ml.	τ/HC	M
0.107	1.44	24,400	27,600
	0.945	25,400	27,580
.146	1.50	31,370	36,850
	1.56	33,090	39,540
	1.69	28,960	34,310
.408	1.04	28,790	30,860
.66	1.05	27,240	28,340
.943	1.08	27,590	27,900
	1.14	27,160	27,480
1.01	1.62	28,530	28,720
	1.56	27,070	27,230
	1.73	26,040	26,210
1.03	0.928	27,700	27,750
	0.885	27,300	27,350
1.07	1.07	24,000	23,960
	0.723	25,300	25,270

The average molecular weight for ionic strengths above 0.6 is 27,000 with 95% confidence limits at 26,000 and 28,000. This is to be compared with the value of 27,300 obtained after room temperature dialysis at protein dialysis concentrations above 4 mg. per ml.

Protein Aggregation

Aggregation Related to Low Ionic Strengths.— Early experiments were performed with protein solutions dialyzed at pH 12 for 16 hours at 4 to 8°. This temperature was chosen because casein association at pH 7 is known to have a negative temperature coefficient[24] and also so that denaturation and hydrolysis might be avoided. In Fig. 2 apparent M is plotted against ionic strengths for experiments with protein dialysis concentrations between 4 and 7 mg. per ml. The data reveal unpredictable aggregation with a particularly large scatter in apparent M at ionic strengths less than 0.4, where the values ranged from 28,800 to 181,000.

(24) N. Burk and D. Greenburg, *J. Biol. Chem.*, **87**, 197 (1930).

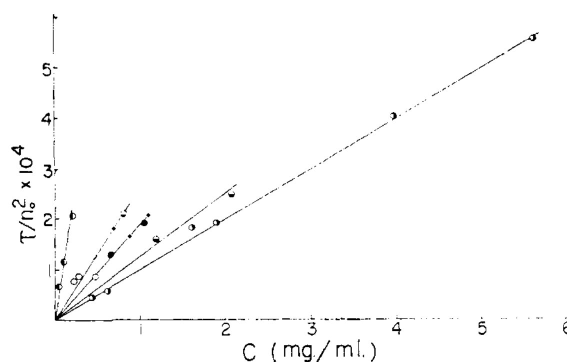

Fig. 3.—Light scattering data from an experiment where five samples of $\alpha_{s1,2}$-caseins were dialyzed in a common reservoir at a temperature of 8° and ionic strength 0.23. Data were obtained on dilution series of protein dialyzed at concentrations of 6.96 mg. per ml., ◑; 4.21 mg. per ml., ⊖; 1.98 mg. per ml., ●; 1.12 mg. per ml., O; 0.26 mg. per ml., ◐.

The second virial coefficients ranged between 0 and 30×10^{-4} mole ml./g.^{2}, indicating that extensive aggregate dissociation was unlikely. Aggregation was observed in the presence of EDTA or oxalate, suggesting that traces of heavy metal ions were not responsible for the phenomenon.

In subsequent experiments, aggregation was observed at low ionic strengths following dialysis at room temperature (Table II) and also in the absence of dialysis (Table III).

Low temperature alone does not cause this effect since protein dissolved directly at room temperature and ionic strengths 0.15 to 1.0 did not change in turbidity after refrigeration at 4° for 16 hours. It should be noted that the lowest values of molecular weight in Fig. 2 are comparable to the molecular weight of 27,300 described above.

Aggregation Related to Dialysis Membrane.— A surprising phenomenon was first observed in one of the low temperature dialysis experiments. Protein solutions at concentrations between 0.26 and 6.96 mg. per ml. were dialyzed for 16 hours in a common reservoir at pH 12, 4 to 8°, and ionic strength 0.23. The results are shown in Fig. 3. The slopes of the plots of τ/n_0^2 *vs.* C reveal a marked increase in apparent molecular weight, from 28,800 to 258,000, as the protein dialysis concentration is diminished. There is no significant change in τ/HC on dilution of the individual dialysands. In other experiments where protein was dialyzed at high ionic strengths, either at room temperature or at 4 to 8°, apparent M was increased for the solutions where protein dialysis concentrations were less than 2 mg. per ml. These experiments indicated that in addition to effects related to low ionic strength at pH 12, there was an increase in apparent M related to the dialysis of protein at low concentrations.

Possible explanations for this phenomenon include the shedding of high molecular weight particles from the dialysis membrane or protein aggregation mediated by polyvalent cations, a freely dialyzable buffer component, or a factor extracted from the dialysis membrane. These possibilities were explored as follows:

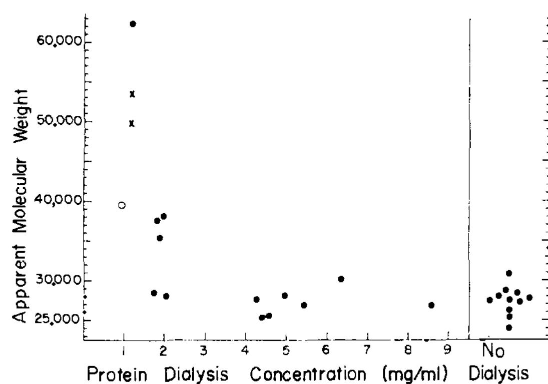

Fig. 4.—On the left, apparent molecular weight of $\alpha_{s1,2}$-caseins is plotted against protein dialysis concentration. Solid circles ● represent data from Table 2; crosses X and open circles O represent data from special experiments described in the text. Points on the right, labeled "No dialysis," represent data from Table III at ionic strength greater than 0.4.

Turbidity measurements on buffer dialysands demonstrated that high molecular weight particles were not shed from the membranes.

That polyvalent cations were not responsible was suggested by the fact that addition of EDTA up to 0.026 M prior to or following dialysis did not affect the turbidity. Also, in a solution containing protein at 1.2 mg. per ml., the addition of manganese ion at 8×10^{-5} M did not increase the turbidity, but doubling the concentration of manganese produced a fifty-fold increase in turbidity, probably due to hydroxide formation, which was reversed by the subsequent addition of EDTA (0.025 M).

In order to test whether some other freely dialyzable trace material in the buffer produced aggregation, protein at concentrations of approximately 2 mg. per ml. were dialyzed against 250 to 300-fold volumes of buffer. The molecular weights in these experiments (lines 12 and 13 of Table II) did not significantly exceed those found for solutions of similar concentration dialyzed against the usual 20-fold volume of buffer.

That a factor may be extracted from the dialysis membrane was shown by allowing buffer at pH 12 and ionic strength 1.0 to be in contact with sacks for 12 to 17 hours at room temperature, removing the buffer and using it to dissolve dry protein. The resulting τ/HC values for two such experiments, the crosses of Fig. 4, were 53,500 and 49,800. One of these sacks was used later to dialyze protein for 16 hours at room temperature. The resulting value of 39,500, the open circle on Fig. 4, indicated that the prior extraction was not complete.

It is of interest to summarize the experiments of Tables II and III for ionic strengths greater than 0.3, and those on membrane washings; the pertinent results of which are plotted in Fig. 4. To the left, the solid circles represent data from Table II. The origins of the open circle and crosses have been given in the previous paragraph. To the right are recorded points from Table III. It is clear that the aggregation related to dialysis membrane factor is only evident at low protein dialysis concentrations, and that the molecular weights determined following dialysis at concentrations greater than 4 mg. per ml. are comparable to those determined without any contact with membrane at pH 12.

In the experiments where full dilution series were obtained for the aggregated protein, namely, the last five experiments of Table II, the B values indicate that there was no consistent change in τ/HC on dilution.

Protein Dissolved at pH 7.—In the experiments presented so far, dry protein was dissolved directly at pH 12. Under these conditions dialysis membranes release a substance which produces aggregation. It seemed reasonable that this factor would not be released at pH 7. The ionic environment then could be controlled by dialysis at pH 7, followed by adjustment of ionic strength and pH in the absence of the sack.

Four experiments were carried out in which protein was dialyzed for 10 hours at 4°, pH 7, and ionic strength 0.1. The dialysand was diluted to duplicate standard buffer at pH 12 and ionic strength 1.0. Dilution curves were obtained. Unexpectedly, the HC/τ *vs.* C plots were not comparable among the experiments, were non-linear, and appeared to have maximum values. Individual τ/HC values ranged between 29,000 and 56,000.

The possibility that dialysis at pH 7 contributed to these high values *via* a dialysis membrane factor was disturbing in view of the fact that all samples were dialyzed at pH 7 during the preparative procedure.[13] Protein was dissolved at 45 mg. per ml., pH 7, 4° and 0.1 ionic strength. After standing 17 hours the conditions were altered rapidly to pH 12 and 1.0 ionic strength as above. Turbidity measurements showed that extensive, frequently time dependent, aggregation took place. These results are comparable to those obtained after dialysis at pH 7, are related to the presence of protein in solution when the increases in pH and ionic strength are effected, and differ strikingly from the results obtained on dissolving dry protein in buffer at pH 12 (Table III).

Aggregation Related to Surface Denaturation.—Aggregation followed foaming or the presence of bubbles in the delivery tube of the Millipore assembly but not vigorous stirring or repeated filtration.

Discussion

The experimental data indicate that $\alpha_{s1,2}$-caseins are in monomeric state with molecular weight 27,300 + 1,500 in phosphate buffer at pH 12, ionic strengths from 0.3 to 1.2 and at room temperature. Solutions of protein are stable over periods of time well in excess of those required for experimental measurements. Selection of the value of 27,300 is based on the equivalence of molecular weight values obtained with dialysis (Table II) or without dialysis (Table III) on protein dissolved at pH 12.

The light scattering molecular weight agrees well with the value of 27,000–27,500 obtained by chemical analyses of C-terminal groups and with the value of 27,500 obtained by osmotic pressure.[13]

Molecular weight values of 24,800 and 25,500 were reported by McKenzie and Wake[10] on the basis of sedimentation-diffusion and sedimentation approach to equilibrium at pH 11–12 and ionic

strength 0.19. These and the preliminary value obtained earlier in this laboratory[12] are difficult to compare with the present result for several reasons. First, ionic strength was not varied. At the ionic strength used by McKenzie and Wake light scattering measurements reveal unpredictable aggregation. Also, a charge correction was not attempted for ultracentrifuge data. Correction would increase sedimentation molecular weights to an extent which is at present difficult to estimate.[16,17] Finally, partial specific volume, which has been found to be pH dependent for several proteins,[15] was not determined for the specific conditions employed. It should be noted that using equilibrium centrifugation we have obtained at pH 12 and ionic strength 0.4 reduced molecular weights equivalent to those given by McKenzie and Wake.[10] It is of interest that McKenzie and Wake reported a molecular weight of 27,600 at pH 7.3 in the presence of 6 *M* urea, using sedimentation-diffusion.

The studies of Burk and Greenberg[24] were the first of several[25-27] determinations of the average molecular weight of whole casein. These authors have used osmotic pressure and light scattering measurements and a variety of dissociating solvents. Minimum molecular weights in the range of 24,500 to 33,600 were obtained. Although these molecular weights are interesting by virtue of their being close to the molecular weight of $\alpha_{s1,2}$-caseins, no serious comparison can be made in view of the complexity of whole casein.[9]

In the experiments at pH 12 reported here unexpected aggregation occurred at low ionic strength, with low protein dialysis concentrations, and with a rapid pH-ionic strength increase after the protein was in solution at pH 7. The fact that no particular relationship between size and time has been observed in any of the aggregating systems studied, suggests that first order denaturation followed by higher order aggregation is not part of the fundamental mechanism. The characteristics of casein polymers, including association-dissociation phenomena at extreme pH values, have been investigated for example, by Halwerda,[28] Svedberg and Petersen,[29] Halwer,[30] von Hippel and Waugh,[31] Sullivan, *et al.*[32] and McMeekin and Peterson.[33] The extent to which unpredicable, irreversible aggregation might have influenced the results obtained in some of these studies is of course not known. Aggregation phenomena should be examined critically in future work.

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[CONTRIBUTION FROM THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH, NEW BRUNSWICK, N. J.]

8-L-Citrulline Vasopressin and 8-L-Citrulline Oxytocin[1,2]

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The protected nonapeptides S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide and S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullyl-glycinamide were synthesized stepwise from their C-terminal amino acid by the nitrophenyl ester method. Removal of the protecting groups and conversion to the corresponding cyclic disulfides gave the 8-citrulline analogs of vasopressin and oxytocin. Biological activities of the two hormone analogs are reported.

This paper presents a part of our studies on peptides containing a citrulline moiety.[1] The objectives in undertaking this work were to find methods for the synthesis of citrulline peptides and to study the changes in biological activity that occur when an arginine moiety in a physiologically active peptide is replaced by citrulline. We are dealing here with the synthesis of 8-L-citrulline vasopressin (XII) and 8-L-citrulline oxytocin (XVI). Although oxytocin does not contain an arginine residue, the corresponding hormone of birds and reptiles, vasotocin, has an arginine or lysine in the 8 position; thus the oxytocin analog described here can be called 8-L-citrulline vasotocin as well, and it is of considerable interest to gain information on its biological properties.

The protected nonapeptide S-benzyl-N-benzyloxycarbonyl - L - cysteinyl - L - tyrosyl - L - phenylalanyl - L - glutaminyl - L - asparaginyl - S-benzyl - L - cysteinyl - L - prolyl - L - citrullylglycinamide (XI), the key intermediate in the synthesis of the vasopressin analog, was prepared by the stepwise nitrophenyl ester method[3,4]

(1) For a preliminary report of this work see M. Bodanszky, M. A. Ondetti, B. Rubin, J. Piala, J. Fried, J. T. Sheehan and C. A. Birkhimer, *Nature*, **194**, 485 (1962).

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(3) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959); M. Bodanszky, J. Meienhofer and V. du Vigneaud, *ibid.*, **82**, 3195 (1960).

(4) In the stepwise synthesis a technique somewhat different from those previously used[3] was applied. In a personal communication Dr. J. E. Stouffer and Professor V. du Vigneaud suggested the use of pyridine both as a solvent and as the base necessary to liberate the