[Contribution from the Physico-Chemical Laboratories of the University of Upsala and the New York Agricultural Experiment Station]

# THE MOLECULAR WEIGHT OF CASEIN. I[1]

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The molecular weight of casein was first estimated by Van Slyke and Bosworth[3] from the sulfur and phosphorus content. These workers found 0.72% of sulfur and 0.71% of phosphorus in a sample of casein, and assuming one atom of each of these elements in the original protein arrived at the value 4444. For no clear reason they doubled this value and set down the probable molecular weight as 8888. As to their method of preparing casein, which involves heating for some time in alkaline solution, Harden and Macallum[4] have called attention to the fact that no doubt hydrolytic cleavage of part of the sulfur and phosphorus of the protein has taken place during the preparation. Experiments by one of the writers,[5] while not bearing directly on the matter of sulfur and phosphorus cleavage, show that there is considerable cleavage of nitrogen of the casein molecule under the conditions which Van Slyke and Bosworth imposed on their material. This substantiates the claims of Harden and Macallum and shows that Van Slyke and Bosworth's material and calculations based thereon cannot be seriously considered.

Pauli and Matula[6] ingeniously attempted to evaluate the valency of casein through application of the Ostwald dilution rule. Using this rule they concluded that the valency of casein was 3. Since they found the combining weight of casein for base was about 1000, they concluded that the molecular weight was 3000.

Yamakami[7] examined the molecular weight of casein by Barger's method, which compares solutions of equal vapor pressure. He concluded that for the simplest alkali caseinates which he could prepare, the mean weight of the ions was about 2000, which would mean for a caseinate dissociating into only two ions a molecular weight of about 4000. This figure would then necessarily represent only a minimum value for the molecular weight.

More recently Cohn, Hendry and Prentiss[8] have attempted to evaluate

[1] Read before the Meeting of the American Chemical Society at Columbus, Ohio, on May 2, 1929.

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[3] Van Slyke and Bosworth, *J. Biol. Chem.*, **14**, 203, 228 (1913).

[4] Harden and Macallum, *Biochem. J.*, **8**, 90 (1914).

[5] D. C. Carpenter, *J. Biol. Chem.*, **67**, 647 (1926).

[6] Pauli and Matula, *Biochem. Z.*, **99**, 219 (1919).

[7] Yamakami, *Biochem. J.*, **14**, 522 (1920).

[8] Cohn, Hendry and Prentiss, *J. Biol. Chem.*, **63**, 721 (1925).

the molecular weight of several proteins from their amino acid content. With considerable reluctance they placed the molecular weight of casein at 192,000. On the basis of one molecule of cystine per molecule of casein, the minimal molecular weight of casein was calculated to be 96,000. However, largely on account of the difficulty of fitting the tryptophan content to this latter value, they decided in favor of the value 192,000.

Researches on casein in late years have usually started with preparations made either by the method of Hammarsten[9] or by the method of Van Slyke and Baker.[10] We have examined casein made by each of these methods, by means of the ultracentrifuge, and in this paper will report experiments based on Hammarsten casein.

The recent experiments of Kondo,[11] Linderström-Lang and Kodama,[12] and Linderström-Lang[13] seem to show that Hammarsten casein is a mixture of substances, inasmuch as they found the solubility in dilute hydrochloric acid to be dependent on the casein content of the solution.

Gortner[14] criticized these workers on the ground that they had not removed an alcohol-soluble protein from their material, which had previously been separated and identified by Osborne and Wakeman.[15] Although Gortner may have been right in part, Osborne and Wakeman reported a yield of their alcohol-soluble protein entirely too low to account for the results obtained by the workers in the Carlsberg laboratory.

In view of the discordant state of the literature relating to casein, it was considered advisable to undertake an investigation of this important protein by means of the centrifugal methods developed in this Laboratory[16] and already used for the determination of the molecular weights of hemoglobin,[17] egg albumin,[18] phycocyan, phycoerythrin,[19] hemocyanin,[20] serum albumin and serum globulin[21] and edestin.[22]

[9] Hammarsten, "Handbuch der biochemischen Arbeitsmethoden," E. Abderhalden, Berlin und Wien, **1910**, Vol. II, p. 384.

[10] Van Slyke and Baker, *J. Biol. Chem.*, **35**, 127 (1918).

[11] Kondo, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 8 (1925).

[12] Linderström-Lang and Kodama, *ibid.*, **16**, No. 1 (1925).

[13] Linderström-Lang, *Z. physiol. Chem.*, **176**, 76 (1928); "Dissertation," Copenhagen, **1929.**

[14] Gortner, "Casein and Its Industrial Applications," Sutermeister, New York, **1927**, p. 14.

[15] Osborne and Wakeman, *J. Biol. Chem.*, **33**, 243 (1917).

[16] Svedberg, *Z. physik. Chem.*, **121**, 65 (1926); *ibid.*, **127**, 51 (1927).

[17] Svedberg and Fåhraeus, THIS JOURNAL, **48**, 430 (1926); Svedberg and Nichols, *ibid.*, **49**, 2920 (1927).

[18] Svedberg and Nichols, *ibid.*, **48**, 3081 (1926).

[19] Svedberg and Lewis, *ibid.*, **50**, 525 (1928).

[20] Svedberg and Chirnoaga, *ibid.*, **50**, 1399 (1928); Svedberg and Heyroth, *ibid.*, **51**, 539, 550 (1929).

[21] Svedberg and Sjögren, *ibid.*, **50**, 3318 (1928).

[22] Svedberg and Stamm, *ibid.*, **51**, 2170 (1929).

## Experimental

The casein used in this investigation has in every instance been prepared from cow's milk and in so far as possible was obtained from the same dairy herd. As casein is practically insoluble at its isoelectric point, *P*H 4.7, we have carried out our centrifuging experiments in phosphate buffer solutions of *P*H 6.8, this *P*H value closely approximating that of freshly drawn milk.

**Preparation of Material.**—Milk was drawn from the animal into flasks containing toluene, brought to the laboratory at once and cooled to 5° as rapidly as possible. Butter fat was removed from the cold milk by slow passage through a milk separator operated at 13,200 r.p.m. In this operation practically all the fat was removed from the milk and clung firmly to the center shaft and disks of the separator. Some "separator slime" collected on the sides of the bowl and was discarded.

The separated milk was diluted with 4 volumes of cold water and the casein precipitated by the Hammarsten method[9] through the slow addition of diluted (1:100) acetic acid, the diluted milk being mechanically stirred meanwhile. This operation required the equivalent of 7.5 cc. of glacial acetic acid per liter of milk. The precipitated casein settled rapidly and after washing by decantation several times with water, the precipitate was centrifuged from the wash water and several additional washings made, separating the casein each time centrifugally.

The moist precipitate was then dissolved in dilute ammonia, adding the ammonia slowly during mechanical agitation so as to have no pronounced alkalinity at any time in any part of the solution. (The *P*H of the solution was kept below 7.0.) The ammonium caseinate solution was then passed slowly through the separator and after diluting with water the casein was precipitated by the calculated amount of dilute acetic acid as before. The precipitate was washed with water, redissolved in ammonia and again reprecipitated with dilute acetic acid and washed repeatedly. Hammarsten[23] showed that further reprecipitation does not improve the purity of the product.

A portion of the moist casein (2 g., dry basis) was then dissolved in 25 cc. of $M/15$ mixed phosphate buffer solution (*P*H 6.8) in the cold and diluted to 100 cc., thereby making the buffer salt concentration $M/60$. The solution was stored at 5° with toluene as preservative. This is hereinafter referred to as Hammarsten casein.

Another portion of the moist casein (13 g., dry basis) was extracted at 40° for one hour with two liters of 70% ethyl alcohol containing 1 cc. of normal hydrochloric acid per liter, after the manner of Linderström-Lang.[12,13] The solution was separated from undissolved casein in a centrifuge. (The extraction was continued with a second two-liter portion of acidified alcohol which was separated again in the centrifuge from undissolved material.) From the first extraction with 2 liters of acidified alcohol, 2.15 g. of material (dry basis) was reclaimed by precipitation with dilute sodium hydroxide and from the second extraction 2.19 g. was reclaimed in the same manner. These fractions were dissolved separately in 25-cc. portions of $M/15$ mixed phosphate buffer solution (*P*H 6.8) in the cold, diluted to 100 cc. and stored at 5° with toluene. This is hereinafter referred to as acid–alcohol soluble casein, and would probably correspond to fractions $K_3$, $K_4$ and $K_5$ of Linderström-Lang's nomenclature. The undissolved casein residue from the above treatment was not further extracted or examined.

**Specific Volume.**—The partial specific volume was determined pycnometrically at 19.8°. The amount of protein in solution was found by drying a sample of the stock solution to constant weight at 105° and corrected for the amount of residue due to the

[23] Hammarsten, *Z. physiol. Chem.*, **7**, 227 (1883).

buffer salts themselves. This method has been shown to give results identical with those obtained by removing the protein by some precipitation process and weighing directly.

The partial specific volume was found to be 0.750 and was independent of concentration, within the limits of experimental error, for protein concentrations between 1.0 and 2.0%. This value closely approximates similar values for several proteins already examined.

**Light Absorption.**—Experiments were carried out by means of the Judd-Lewis spectrophotometer to determine the absorption in the short-waved ultraviolet part of the spectrum. The toluene used as preservative in the protein solutions was first removed by bubbling moist nitrogen gas through the solution before measuring the light absorption. The solutions were examined at PH 5.9, 6.8 and 8.0 in 20-mm. cells with protein contents, respectively, of 0.02, 0.05 and 0.10%. The specific extinction coefficient $\epsilon/C = 1/Cd \log I_0/I$, where $C$ is the protein concentration in per cent., $I_0$ the intensity of the light beam after passing a layer of solvent $d$ cm. in thickness and $I$ the intensity after passing the same thickness of protein solution. The extinction coefficient is plotted against wave length in Fig. 1.

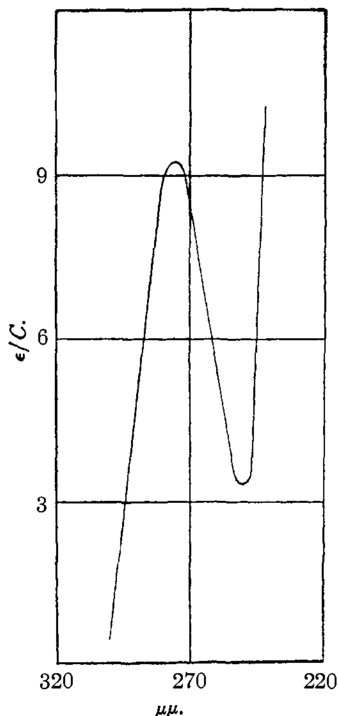


Fig. 1.

The absorption curve has a maximum at 276μμ and a minimum at 250μμ. The values of the extinction coefficient fall somewhat below those reported for serum globulin but are higher than for the other proteins thus far examined in this Laboratory. This order is to be expected if the extinction coefficient in this region is due to the tryptophan content of the protein molecule. Differences in extinction coefficient due to differences in PH of the solutions fell within the experimental error.

**Hammarsten Casein.**—Several runs were made by the sedimentation velocity method with solutions containing various concentrations of Hammarsten casein. In Tables I and II are recorded data of a representative sedimentation series at an initial protein content of 0.75%. The sedimentation curves for this run are shown in Fig. 2.

The unusual shape of the sedimentation curves (Fig. 2) suggests at once that we are not dealing with a monomolecular substance, but rather with a substance containing several different kinds of molecules. Similar curves have been obtained for other proteins which were in a state of change. To arrive at an approximation, we have assumed that the upper break in the successive curves occurs at about their respective points of intersection with the line AB. On this basis we have calculated the specific sedimentation velocity and the "apparent" diffusion constant as shown in Tables I and II.

Table I

Hammarsten Casein. Sedimentation Velocity Run

Initial protein concentration, 0.75%; solvent, $M/60$ mixed phosphate buffer at $P_H$ 6.8; speed, 43,500 r.p.m. ($\omega = 1450\pi$); interval between exposures, 15 min.; $T$ = 295.2°; illumination, mercury lamp; light filters, $Cl_2$ and $Br_2$; Imperial Process photographic plates; exposure time, 70 sec.; metol-hydroquinone developer; development time, 2 min.

| Sedimentation curves used[a] in calcn. | $\Delta X$, cm. | $X$, mean, cm. | $S_{20°}$, cm. × $10^{-13}$ |
|---|---|---|---|
| 3–4 | 0.064 | 4.787 | 6.83 |
| 4–5 | .062 | 4.850 | 6.53 |
| 5–6 | .058 | 4.910 | 6.04 |
| 6–7 | .053 | 4.965 | 5.46 |
| 7–8 | .060 | 5.022 | 6.11 |
| 8–9 | .057 | 5.080 | 5.74 |
| 9–10 | .056 | 5.137 | 5.58 |
| | | Mean | 5.97 |

[a] Sedimentation curves bear the same number in Fig. 2 as in these data.

Table II

Hammarsten Casein. Diffusion Measurements

Initial protein concentration, 0.75%: same experimental details as given in Table I

| Reading[a] | Diffusion time, sec. | Mean[b] $Z$, cm. | "Apparent" $D_{20°}$, cm.$^2$/sec. × $10^{-7}$ |
|---|---|---|---|
| 3 | 1080 | 0.027 | 7.03 |
| 4 | 1980 | .041 | 8.84 |
| 5 | 2880 | .052 | 9.78 |
| 6 | 3780 | .0705 | 13.69 |
| 7 | 4680 | .0835 | 15.51 |
| 8 | 5580 | .0950 | 16.84 |
| 9 | 6480 | .1150 | 21.26 |
| 10 | 7380 | .1345 | 25.53 |

[a] Reading number refers to corresponding sedimentation curve in Fig. 2.

[b] "Mean $Z$" represents the mean of the distances from $C$ = 25% to $C$ = 50% and from $C$ = 50% to $C$ = 75%, see This Journal, **49**, 2922 (1927).

It will be noted that the "apparent" diffusion constant is not at all constant and that the value increases as the experiment proceeds. This is evidence that we are dealing with a mixture of substances.

If we assume a spherical particle and apply Stokes' Law

$$r = \sqrt{\frac{9\eta s}{2(\rho_1 - \rho_2)}}$$

in which $r$ is the radius of the particle, $\eta$ the viscosity of the solvent, $s$ the specific sedimentation velocity and $(\rho_1 - \rho_2)$ the difference in density between particle and solvent, we find that the particle radius is $2.85 \times 10^{-7}$ cm. From Einstein's equation

$$D = \frac{RT}{N} \times \frac{1}{6\pi r\eta}$$

we calculate that a spherical particle of this radius will have a diffusion constant equal to $7.48 \times 10^{-7}$. This will distinctly be a limiting case and one in which the frictional force opposing the motion of the sphere will be a minimum. In other words, the maximum diffusion constant we can possibly expect would be $7.48 \times 10^{-7}$. That we find higher values than this shows that the "apparent" diffusion must be due to a spreading out of the boundary on account of the presence of molecules of more than one size.

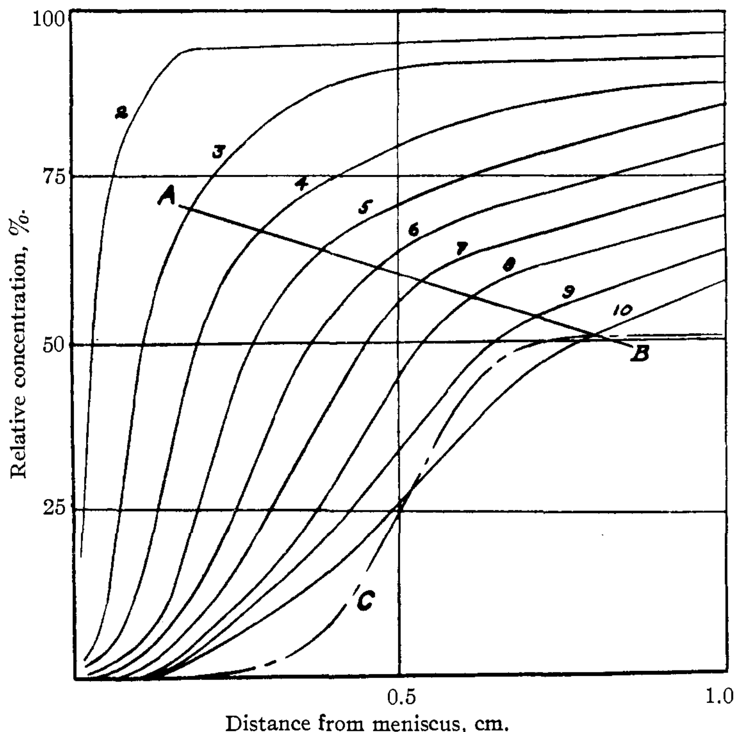



Fig. 2.

The broken line C in Fig. 2 represents how a monomolecular system having $S_{20°} = 5.97 \times 10^{-13}$ and $D_{20°} = 7.48 \times 10^{-7}$ would have been distributed under the same conditions as in Curve 10.

From these experiments we are forced to conclude that Hammarsten casein is a mixture of protein molecules of different weight.

**Acid–Alcohol Soluble Casein.**—The protein material extracted from Hammarsten casein by warm 70% ethyl alcohol containing 1 cc. of hydrochloric acid per liter[12,13] was examined in the ultracentrifuge by both the sedimentation velocity and the equilibrium methods.

**Sedimentation Velocity Method.**—Sedimentation velocity runs were made at various protein concentrations between 0.32 and 1.50%. A

summary of these runs is given in Table III. A graph showing the relationship between concentration and specific sedimentation velocity is to be found in Fig. 3. It is to be noted that as the protein solution is progressively diluted with $M/60$ phosphate buffer the specific sedimentation velocity is found to increase rapidly until the concentration 0.45% is

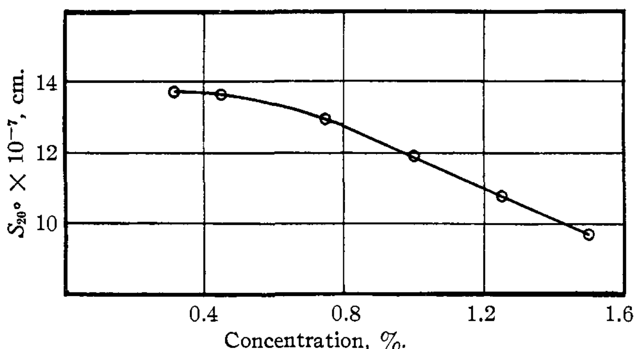



Fig. 3.

reached, below which concentration the sedimentation velocity remains practically constant. It is inferred that in concentrations of this protein above 0.45% the protein particles in the solutions are sufficiently close together to have an interfering effect on the free movement of one another and hence the normal diffusion and sedimentation rates are lowered.

TABLE III

ACID-ALCOHOL SOLUBLE CASEIN. SUMMARY OF SPECIFIC SEDIMENTATION VELOCITY VALUES

| Concn., % | 1.50 | 1.25 | 1.00 | 0.75 | 0.45 | 0.32 |
|---|---|---|---|---|---|---|
| | Mean $S_{20°}$ cm. $\times 10^{-13}$ | | | | | |
| | 8.62[a] | 10.14 | 11.30 | 12.02[a] | 12.40 | 13.95 |
| | 8.90[a] | 10.33 | 11.56 | 12.95 | 13.84 | 13.73 |
| | 9.39 | 10.80 | 12.00 | 12.85 | 13.57 | 13.70 |
| | 9.74 | 11.05 | 12.22 | 12.66 | 13.06 | 13.32 |
| | 9.76 | 11.64 | 12.51 | 13.10 | 14.38 | 13.88 |
| | 9.79 | 12.01[a] | 13.60[a] | 12.59 | 14.60 | 13.68 |
| | 9.70 | | | | 13.62 | |
| Mean $S_{20°}$ | 9.68 | 10.79 | 11.92 | 12.83 | 13.64 | 13.72 |

[a] Value discarded in calculating mean.

As we are primarily interested in the more dilute solutions, for this reason we will direct attention to those solutions in which the protein concentration is sufficiently low to eliminate inter-particle forces tending to disturb the free motion of the individual particle.

In Fig. 4 is shown the photographic record of a sedimentation run at 43,600 r.p.m. on a solution containing 0.45% of protein. The progress of sedimentation as the experiment proceeds is shown in the upper row of

photographs, while the lower row represents the concentration scale from pure buffer solution (left side) by successive increases to the initial concentration of the protein solution centrifuged (right side). In Fig. 5 are shown the sedimentation curves representing the distribution in a series of photographs obtained in a duplicate run to Fig. 4, at a speed of about 25,000 r.p.m.

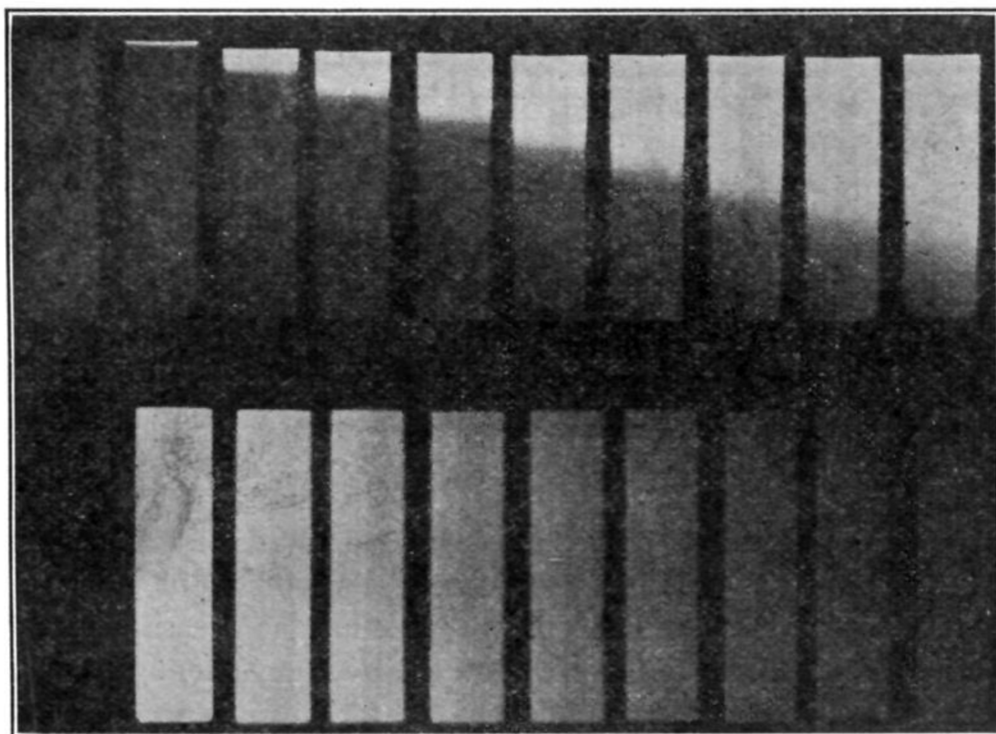


Fig. 4.

The diffusion constant for this protein was found to be $3.56 \times 10^{-7}$ at concentrations below 0.45%. This value represents a mean of several well-agreeing determinations. In Fig. 5 the circles represent the theoretical distribution after three hours at the boundary of a monomolecular substance having a diffusion constant of $3.56 \times 10^{-7}$. The excellent agreement between theory and experiment is all that can be asked of the experimental method.

In Table IV are recorded the sedimentation data on the above run, showing that the mean specific sedimentation velocity at 20° equals $13.64 \times 10^{-13}$ when the concentration is 0.45% protein. Another run at 0.32% protein concentration gives $S_{20°} = 13.72 \times 10^{-13}$.

Using the above data for $S_{20°}$ and $D_{20°}$ we have calculated the molecular weight of this protein from the equation $M = RTS/D(1 - V_{\rho})$ where $M$ = molecular weight, $S$ = specific sedimentation velocity, $V$ = partial specific volume of the protein, $\rho$ = density of solvent, and $R$, $T$ and $D$ have the usual significance. In the solutions of 0.32% and 0.45% the molecular weight was found to be 375,000 and 373,420, respectively, the former number being perhaps preferable to the latter.

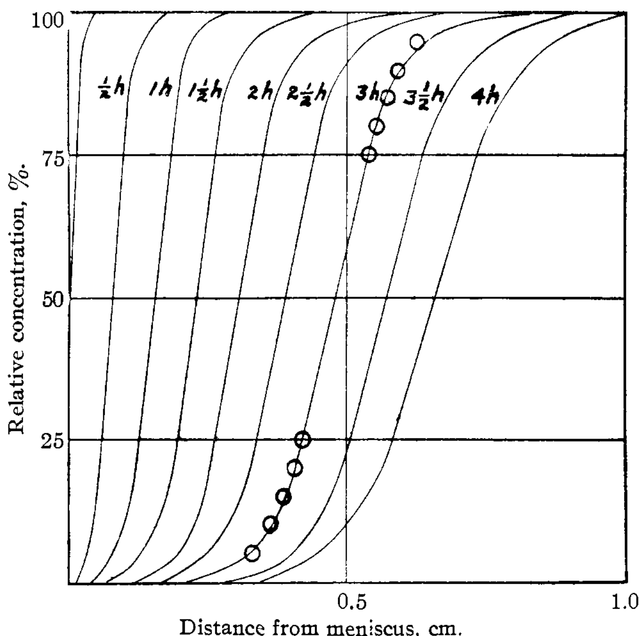
TABLE IV

ACID–ALCOHOL SOLUBLE CASEIN. SPECIFIC SEDIMENTATION VELOCITY METHOD

Concentration, 0.45%; PH, 6.8; thickness of cell, 0.60 cm.; interval between exposures, 30 min.; mean $T$, 294.2°; illumination, Hg lamp with $Br_2$ and $Cl_2$ filters; Imperial Process photographic plates

| Sedimentation time, hrs.[a] | $\Delta X$, cm. | $X$, med., cm. | $\omega$ med. | $S_{20°}$, cm./sec. $\times 10^{-13}$ |
|---|---|---|---|---|
| 1 | 0.072 | 4.754 | 2573.0 | 12.40 |
| 1.5 | .076 | 4.828 | 2481.8 | 13.84 |
| 2 | .075 | 4.903 | 2471.7 | 13.57 |
| 2.5 | .081 | 4.981 | 2597.1 | 13.06 |
| 3 | .090 | 5.067 | 2586.6 | 14.38 |
| 3.5 | .090 | 5.156 | 2544.7 | 14.60 |
| 4 | .084 | 5.244 | 2523.7 | 13.62 |
| | | | Mean | $13.64 \times 10^{-13}$ |

[a] Sedimentation curves bear the same number in Fig. 5 as in these data.




$S = 13.64 \times 10^{-13}$; $D = 3.56 \times 10^{-7}$; $M = 373{,}400$.

Fig. 5.

**Sedimentation Equilibrium Method.**—In this method the molecular weight is given by the relation

$$M = \frac{2\,RT \ln (C_2/C_1)}{(1 - V_\rho)\omega^2(X_2^2 - X_1^2)}$$

where $C_1$ and $C_2$ represent the protein concentrations at the distances $X_1$ and $X_2$, respectively, from the center of rotation. The other symbols have the usual significance.

The optical system as regards light source, filters, photographic plates, developer, etc., was the same as previously used. Photographs were taken with three different lengths of exposure time in order to obtain suitable photographic densities at all concentration levels within the cell for comparisons in the microphotometer. Changes in the intensity of illumination were corrected for by photographing a potassium chromate solution of suitable concentration at the same time as the protein solution. The potassium chromate solution was contained in a cell counterbalancing the cell containing the protein solution, and was masked off from the light source by a brass disk, except for a small window cut in the disk at an appropriate height above the level of the protein solution in the opposite cell.

In Table V are given data on a typical sedimentation equilibrium run with the acid–alcohol soluble protein. The mean molecular weight as shown by this run was 374,210. A duplicate run gave a mean value of 377,795.

Table V

Acid–Alcohol Soluble Casein. Sedimentation Equilibrium Method

Initial concentration, 0.50%; $P_H$, 6.8; thickness of cell, 0.80 cm.; $T$, 293°; speed, 2986.6 r.p.m. ($\omega$ = 99.557 $\pi$); equilibrium time, 66 hrs.; illumination. Hg lamp with $Br_2$ and $Cl_2$ filters; Imperial Process photographic plates

| $X_2$, cm. | $X_1$ cm. | $C_2$, % | $C_1$, % | $M$ |
|---|---|---|---|---|
| 5.81 | 5.76 | 0.8050 | 0.7190 | 389,200 |
| 5.76 | 5.71 | .7190 | .6465 | 369,300 |
| 5.71 | 5.66 | .6465 | .5800 | 380,460 |
| 5.66 | 5.61 | .5800 | .5225 | 369,190 |
| 5.61 | 5.56 | .5225 | .4715 | 366,490 |
| 5.56 | 5.51 | .4715 | .4250 | 373,770 |
| 5.51 | 5.46 | .4250 | .3830 | 378,050 |
| 5.46 | 5.41 | .3830 | .3465 | 367,230 |
| | | | Mean mol. wt. | 374,210 |

The value of the molecular weight calculated from the protein concentrations at different distances from the axis of rotation was identical within the limits of error with those calculated from the sedimentation velocity method. These results are summarized in Table VI. The mean value of the molecular weight was found to be 375,256 ± 11,000.

Table VI

Summary of Molecular Weight Values for Acid–Alcohol Soluble Casein

| Method | Init. concn., % | Mean speed, r.p.m. | Mol. wt. |
|---|---|---|---|
| Sedimentation velocity | 0.45 | 25,000 | 373,420 |
| Sedimentation velocity | .32 | 43,950 | 375,600 |
| Sedimentation equilibrium | .50 | 2,986 | 374,210 |
| Sedimentation equilibrium | .50 | 2,972 | 377,795 |
| | | Grand mean | 375,256 |

## Discussion of Results

The experiments described in this paper with casein precipitated from cow's milk by Hammarsten's method have shown that this material is a mixture of several protein molecules of different weight. From 13 g. of this mixture we have separated and reclaimed 4.34 g. of protein by extraction with warm acidified 70% alcohol. This represented at least 33% of the original material and was found to be a definite chemical entity having a molecular weight of 375,000. It might be possible to obtain more than 33% of this protein from Hammarsten casein, as we have only performed the extraction twice, each time removing about the same amount of protein. On the other hand, the shape of the curves in Fig. 2, compared with the curves of other proteins studied in this Laboratory, indicates that, provided the extinction coefficient is the same for all of the protein molecules in Hammarsten casein, there can hardly be present as much as 33% of a protein possessing a molecular weight of 375,000 in Hammarsten casein. If this were the case, one would expect to find two distinct breaks in the upper parts of these sedimentation curves.

It might be argued that by the extraction process we have definitely changed the casein into some polymerized substance which was not originally present in the Hammarsten casein. In support of this argument we find the experiments of Robertson,[24] who concluded from ionic mobility experiments that potassium caseinate polymerized in the presence of 70% alcohol. It is quite possible, however, that compounds of casein with acid would behave differently with respect to alcohol than compounds of casein with base.

According to the experiments of Linderström-Lang[12,13] the polymerization argument seems to be untenable, for continued extractions eventually reach a point where no great amount of protein is further dissolved. This would not be the case were we dealing with a progressive polymerization.

Our own experiments were not designed to show which of the above views was correct. Our only aim was to ascertain whether a definite chemical entity was obtained by the extraction process. The matter would seem to hinge on whether, after continued extractions, the remaining product possessed the same solubility in fresh solvent as it exhibited in the beginning.

The particle size of the acid–alcohol soluble casein may be calculated from the specific sedimentation velocity data using Stokes' Law, under the assumption that the particle is spherical. This gives the value $r = 4.177 \times 10^{-7}$ cm. Using the diffusion constant, we can also calculate the particle size from Einstein's Law without making any assumption as to the shape of the particle. This gives the value $r = 5.994 \times 10^{-7}$ cm. From these values we conclude that the protein particle must be of some other shape

[24] Robertson, "Physical Chemistry of the Proteins," T. B. Robertson, New York and London, **1918**, p. 268.

than spherical. The ratio between the two values for the particle radius given by the two laws ($r_E/r_S$) is 1.43. This compares well with similar ratios already obtained in the cases of hemoglobin, serum albumin, serum globulin and phycocyan. This probably means that the departure of the particle from a spherical shape is about the same amount for all of these proteins.

## Summary

1. Casein prepared by the Hammarsten method has been examined at *P*H 6.8 in phosphate buffer solution by the centrifugal sedimentation velocity method and found to consist of a mixture of protein molecules of different weight.

2. Hammarsten casein was extracted with hot acidified 70% alcohol and the soluble protein precipitated out with dilute sodium hydroxide. This protein after washing was dissolved in phosphate buffer solution of *P*H 6.8. Various concentrations were examined by both the sedimentation velocity and sedimentation equilibrium methods.

3. It was found that within the limits of experimental error the acid–alcohol soluble protein was homogeneous with regard to molecular weight and that it therefore probably was a pure chemical individual.

4. The molecular weight was found to be 375,000 ± 11,000.

5. The molecule was not spherical and deviated from the spherical shape by about the same amount that has been found for several other proteins.

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[Contribution from the Chemical Laboratory of the University of Washington]

# THE BENZILIC ACID REARRANGEMENT

By Theodore W. Evans and William M. Dehn

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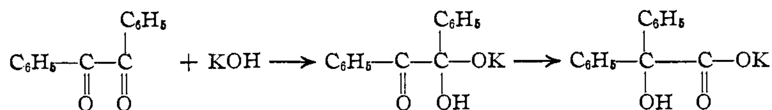
It seems probable that the benzilic acid rearrangement is best formulated as follows




The addition of one molecule of potash was postulated by Hoogewerff and van Dorp;[1] the migration of both $C_6H_5$ and H, by Michael;[2] ex-

[1] Hoogewerff and van Dorp, *Rec. trav. chim.*, **9**, 225 (1890). These authors assumed that Ph and OK migrate from one carbon atom to the other.

[2] Michael, This Journal, **42**, 814 (1920). Michael assumes the addition of one mole of potassium hydroxide, and this forms a system which "may now completely convert itself, by the transmigration of phenyl and hydrogen, into a salt of the relatively strong benzilic acid."