When ammonium bromide is added to a solution of sodium trimethyl stannide and sodium amide, hydrogen is evolved. For the most part, the ammonium bromide reacts with the stannide to form trimethylstannane, which in turn reacts with sodium amide, a portion to form sodium trimethyl stannide directly and another portion to form sodium trimethyl stannide, sodium trimethylstannylamine and sodium amide.

When di-trimethylstannylmethane is reduced with sodium in liquid ammonia, sodium trimethyl stannide is produced and methane is evolved.

PROVIDENCE, RHODE ISLAND

[CONTRIBUTION FROM THE PHYSICO-CHEMICAL LABORATORIES OF THE UNIVERSITY OF UPSALA AND THE NEW YORK AGRICULTURAL EXPERIMENT STATION]

THE MOLECULAR WEIGHT OF CASEIN. II

BY T. SVEDBERG, L. M. CARPENTER AND D. C. CARPENTER[1]

RECEIVED AUGUST 5, 1929 PUBLISHED FEBRUARY 6, 1930

In a former publication[2] the writers have described their experiments dealing with the molecular weight of casein prepared by the method of Hammarsten and that portion of Hammarsten casein which is soluble in hot acidified 70% alcohol. The first mentioned material was found by the ultracentrifuge to be a mixture of proteins of different molecular weights, while the latter was found probably to be a distinct chemical entity having a molecular weight of 375,000.

It was pointed out in the first paper that the casein that has furnished the starting point for experimental studies in late years has been usually prepared either by the precipitation method of Hammarsten[3] or by the method of Van Slyke and Baker.[4] In the former method the precipitation was effected by the slow addition of dilute acetic acid to milk, while in the latter method normal lactic acid or a mixture of normal acetic and hydrochloric acids was recommended. The latter workers also introduced rapid agitation as a means of distributing the added acid throughout the milk.

One of the principal differences between the two methods seems to lie in the time interval during which the milk is held in a state of partial coagulation. The other principal difference is that Hammarsten redissolves the precipitated casein in dilute alkali and reprecipitates with acid several times, with the hope of removing other substances carried down during the initial precipitation; while on the other hand, Van Slyke and Baker make no attempt to remove organic impurities by reprecipitation

[1] Fellow of the International Education Board.

[2] Svedberg, Carpenter and Carpenter, THIS JOURNAL, **52**, 241 (1930).

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

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

of the casein. The latter workers seem to be more concerned with reducing the ash content than in separating one protein from another in pure form. These authors claim to produce a pure protein by a single precipitation from the very complex mixture of substances that are known to occur in milk. The work described in this paper will deal with casein prepared from cow's milk by the method described by Van Slyke and Baker.

Experimental Part

Preparation of Material.—We have carried out the precipitation process as described by Van Slyke and Baker using the acetic–hydrochloric acid mixture as precipitant, but have not attempted as yet to dry the material by their method, preferring for the present to make progress with material which had not been subjected to any drying process.

Freshly drawn milk was handled as described in our first paper[2] up to the point where it had passed through the separator. It was then, without diluting, precipitated by Van Slyke and Baker's method. After separating the precipitated casein centrifugally from the solution, the casein was beaten up with water, first to a thin paste by means of an electrically driven stirrer, and then more water was added and the stirring continued until the casein was well distributed throughout the whole of the wash water. After allowing to stand for an hour or so, the casein was separated centrifugally from the wash water. The washing was repeated in this manner five or six times, the washed casein finally being stored under water with toluene at 5°. Several such preparations were made at various times and used during the course of the work.

The same technique of preparing the casein solutions was used as was given in the first paper, namely, dissolving two grams of casein (dry basis) in 25 cc. of $M/15$ mixed phosphate buffer solution at P_H 6.8 and diluting this to 100 cc., thereby producing a $M/60$ buffer salt concentration. The solution was prepared in the cold, slowly passed through the milk separator driven at a high speed and stored at 5° with toluene as preservative. The protein content of this solution was determined by evaporating a sample to dryness at 105° and correcting the weight of residue for the buffer salts present. Dilutions were made from the stock solution as required for the centrifuging work. No material changes were made in the centrifuging set-up or technique, which has already been described.

Determination of Molecular Weight.—In the experiments immediately following, the sedimentation velocity method was applied. Owing, however, to the fact that no constant diffusion value was obtainable from Van Slyke and Baker's casein, on account of its being a mixture, we are unable to use the usual molecular weight equation $M = RTS/D(1 - V\rho)$, where R is the gas constant, T the absolute temperature, S the specific sedimentation velocity or $1/\omega^2 x \cdot dx/dt$, D the diffusion constant, V the partial specific volume of the protein, ρ the density of the solvent, x the distance from the axis of rotation, ω the angular velocity and t the time. We must, therefore, rely upon sedimentation velocity measurements entirely for a solution of the problem.

According to Stokes' Law for a spherical particle of radius r_1

$$4/3\pi r_1^3 (\rho_1 - \rho_2) = 6\pi h r_1 s_1 \qquad (1)$$

and for any other spherical particle of radius r_2

$$^4/_3\pi r_2^3 (\rho_1 - \rho_2) = 6\pi h r_2 s_2 \quad (2)$$

where $(\rho_1 - \rho_2)$ represents the difference in density between the particle and solvent and s_1 and s_2 represent the specific sedimentation velocities of the two particles of radius r_1 and r_2, respectively.

If we assume that the two particles have the same partial specific volume, then dividing (1) by (2) we obtain

$$\frac{r_1}{r_2} = \left(\frac{s_1}{s_2}\right)^{1/2} \quad (3)$$

However, where M_1 and M_2 represent the molecular weight of the respective species

$$\frac{M_1}{M_2} = \left(\frac{r_1}{r_2}\right)^3 \quad (4)$$

from which

$$\frac{M_1}{M_2} = \left(\frac{S_1}{S_2}\right)^{3/2} \quad (5)$$

In Tables I and II are given data on specific sedimentation velocity and diffusion, respectively, for a typical run at 1.0% protein concentration.

TABLE I

VAN SLYKE AND BAKER CASEIN. SEDIMENTATION VELOCITY RUN

Initial protein concentration, 1.00%; solvent, $M/60$ mixed phosphate buffer at P_H 6.8; speed, 42,000 r.p.m. (ω = 1399.8 π); interval between exposures, 20 min.; T, 298.4°; illumination, mercury lamp; light filters, Cl_2 and Br_2; Imperial Process photographic plates; exposure time, 45 sec.; metol–hydroquinone developer; development time, 2 min.

Sedimentation curves used in calcn.[a]	Δx, cm.	x, mean, cm.	$S_{20°}$, cm. $\times 10^{13}$
3–4	0.070	4.775	5.60
4–5	.078	4.849	6.15
5–6	.076	4.926	5.89
6–7	.077	5.002	5.88
7–8	.075	5.078	5.64
8–9	.076	5.154	5.63
9–10	.078	5.228	5.70
		Mean	5.78

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

In Fig. 1 is shown a photographic record of a sedimentation series at 1.0% initial concentration. The top row of exposures represents the progress of the sedimentation, beginning at the left side, with a centrifuge speed of 42,000 r.p.m. The lower row of exposures represents the concentration scale by regular increments from pure buffer solution on the left to the initial protein concentration on the right. In Fig. 2 are shown the sedimentation curves, showing the distribution within the cell during the progress of the sedimenting experiment. To arrive at an approximation of the molecular weight of the main bulk of the protein in this material

TABLE II

VAN SLYKE AND BAKER CASEIN. DIFFUSION MEASUREMENTS

Initial protein concentration, 1.00%; same experimental details as given in Table I

Reading no.[a]	Diffusion time, sec.	Mean[b] Z, cm.	"Apparent" $D_{20°}$, cm.^{2}/sec. $\times 10^7$
3	1500	0.0170	1.84
4	2700	.0295	3.08
5	3900	.0395	3.83
6	5100	.0465	4.06
7	6300	.0565	4.85
8	7500	.0690	6.07
9	8700	.0820	7.40
10	9900	.0950	8.72

[a] Reading numbers refer to corresponding sedimentation curves 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)).

we have considered the break in the curves of this series to occur at the point of intersection with the line AB.

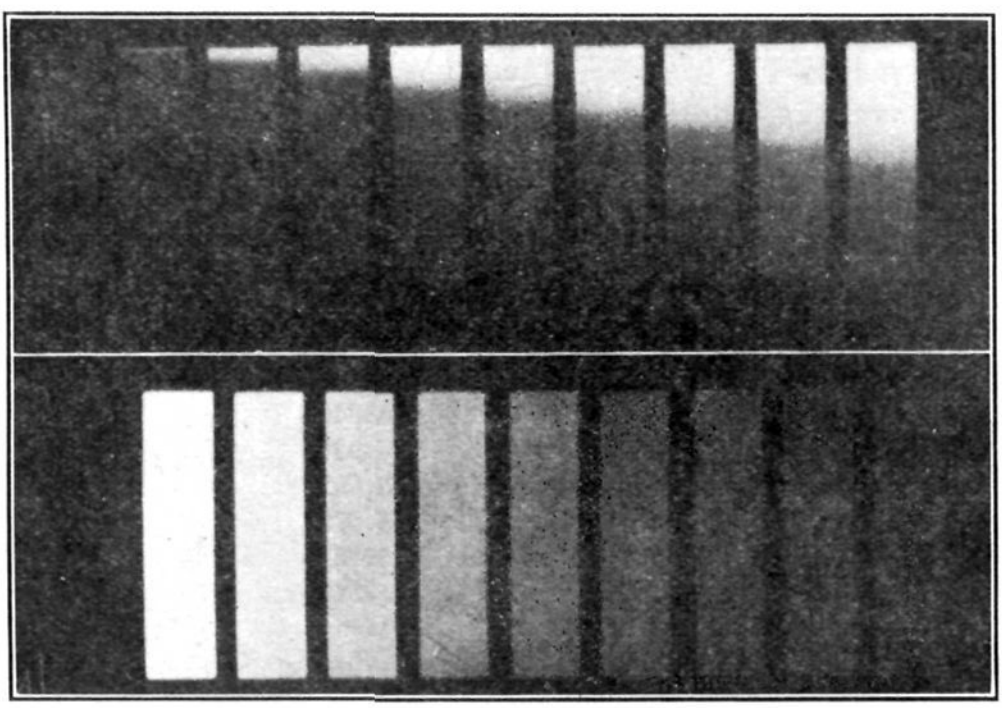

Fig. 1.

The shape of the sedimentation curves in Fig. 2 indicates that casein prepared by the method of Van Slyke and Baker is little or no improvement over the Hammarsten method. The material is definitely not one single protein substance but contains several kinds of molecules of different weights. The sedimentation values as shown in Table I are fairly constant, but the "apparent" diffusion values given in Table II show that as the experiment proceeds these values increase and are not constants such

as we would have were we dealing with a homogeneous substance. The increase in the "apparent" diffusion is attributed to a spreading out of the boundary due to the effect of the centrifugal force on particles of different size. On the assumption that we are dealing with a spherical particle, we have calculated the theoretical distribution at the boundary after two hours of centrifuging of a monomolecular system having the specific sedimentation velocity we have found experimentally, and represented it as curve C in Fig. 2. As the above assumption involves the condition of minimum frictional resistance of the particle and conse-

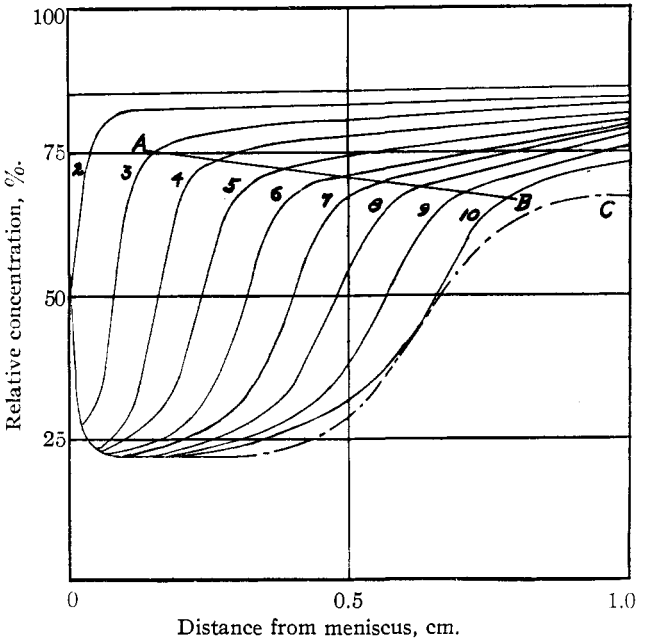

Fig. 2.

quently the maximum possible diffusion, we must conclude that there are protein molecules of at least three different sizes in Van Slyke and Baker's casein, one being larger and one considerably smaller than the main bulk of the material.

In Table III is given a summary of a large number of sedimentation velocity measurements on two different samples of Van Slyke and Baker casein at various protein concentrations. In Fig. 3 these data are shown graphically. It is to be noted that the influence of protein concentration on the sedimentation velocity is practically constant down to a concentration of about 0.50%, below which value the sedimentation velocity drops off rather rapidly. As the region between 0.50 and 2.00% seems to indicate a reasonably constant condition of the protein molecules, we

will confine our attention largely to this region. Within this concentration range the main component of this protein mixture behaves very differently from the acid–alcohol soluble casein which we described in a former publication.[2] The latter protein was more stable in dilute solutions (below 0.45%), while this protein appears to be more stable at concentrations

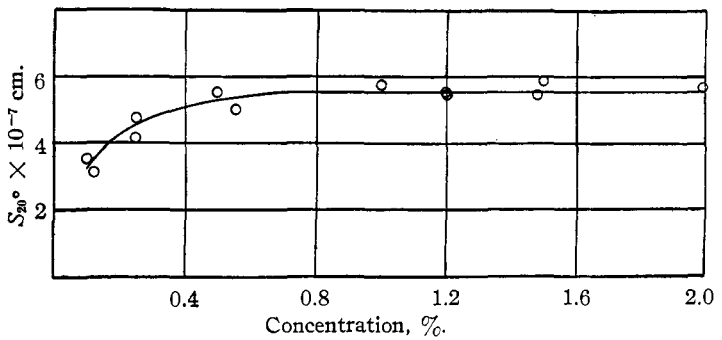

Fig. 3.

above 0.50%. The buffer salt, the concentration and P_H have been exactly alike with respect to both proteins, and identical procedures were used in dissolving the moist proteins in this buffer solution.

TABLE III

VAN SLYKE AND BAKER CASEIN. SUMMARY OF SEDIMENTATION VELOCITY MEASUREMENTS AT VARIOUS CONCENTRATIONS

Init. concn., %	$S_{20} \times 10^{13}$ cm./sec.								Mean
2.00	5.18	5.05	5.43	5.79	6.01	6.24	6.05	6.21	5.74
1.50		5.85	5.72	6.10	5.92	5.88	5.92	6.20	5.94
1.48		5.32	5.63	5.46	5.39	5.17	5.71		5.47
1.20				5.39	5.13	5.73			5.42
1.20			5.44	4.96	5.60	5.88	5.86		5.55
1.00		5.60	6.15	5.89	5.88	5.64	5.63	5.70	5.78
0.55			5.12	4.81	4.83	5.07	5.02		4.97
.50		5.60	5.13	5.06	5.38	5.30	5.46		5.32
.25			4.63	4.79	4.94	4.88			4.81
.25				4.02	4.40	3.99			4.10
.12			3.06	3.18	3.07	2.95			3.09
.10			3.69	3.24	3.24	3.69			3.47

The two samples of Van Slyke and Baker casein shown in Fig. 3 show differences between themselves, one apparently being a purer specimen than the other. Some preliminary results which we have obtained by fractionating Van Slyke and Baker casein with potassium oxalate indicate that the purer of the two samples is the one having the smaller sedimentation velocity. For the purposes of the calculations of this paper, however, we have taken a mean of the 47 specific sedimentation velocity values recorded in Table III at concentrations of 0.50% and above, where the

molecule is comparatively stable. This mean value is $S_{20^\circ} = 5.56 \times 10^{-13}$ and is probably somewhat greater than the true value representing the pure protein, on account of the fact that it represents an average obtained with specimens both of which contained a protein of rather high molecular weight as impurity.

It has been shown that for spherical particles having the same partial specific volume $M_1/M_2 = (S_1/S_2)^{3/2}$, where M_1 and S_1 represent the molecular weight and specific sedimentation velocity, respectively, of one chemical species and M_2 and S_2 the corresponding values of some other species. The egg albumin molecule has been deduced to be spherical in shape, to have a molecular weight of 34,500 and S_{20° equal to 3.32×10^{-13} and a partial specific volume of 0.748, almost identical with the value of 0.750 for casein.[5] Using this as a reference substance, from the above relationship we calculate that the molecular weight of Van Slyke and Baker casein cannot be less than 75,000.

Assuming a spherical particle and applying Stokes' Law $r = \sqrt{\frac{9}{2}\frac{hS}{(\rho_1 - \rho_2)}}$, where r equals the particle radius, h the viscosity of the solvent, S the specific sedimentation velocity and $(\rho_1 - \rho_2)$ the difference in density between particle and solvent, we calculate the molecular radius to be 2.75×10^{-7} cm. The majority of the proteins which have been examined to date, however, do not seem to have spherical shaped molecules, but are of some different shape which operates to increase the frictional resistance and the effective radius of the particle. We have calculated this in the case of acid-alcohol soluble casein and have found the ratio r_E/r_S to be 1.43. If we assume a similar ratio for the Van Slyke and Baker casein, we will have a molecular radius of 3.94×10^{-7} cm. and a corresponding diffusion constant, as calculated from Einstein's Law $D = \frac{RT}{N}\frac{1}{6\pi hr}$, of 5.41×10^{-7} cm. Solving the molecular weight equation with this diffusion value, we find the molecular weight of the bulk of the protein material of Van Slyke and Baker casein to be 100,300. This value corresponds very well with the molecular weight values 103,600 and 105,500, respectively, for serum globulin and phycocyan, which have been found to have S_{20° values of 5.57×10^{-13} and 5.59×10^{-13}, respectively. The S_{20° value we have found for the main protein component of Van Slyke and Baker casein is 5.56×10^{-13}, practically identical with the former of these last values. This S_{20° value, however, is no doubt somewhat high, as is also the molecular weight figure, due to the presence of the impurity of higher molecular weight, as we have mentioned before. There can be no doubt that the bulk of the casein prepared by Van Slyke and

[5] Svedberg, "Colloid Chemistry," 2d ed., Chemical Catalog Co., New York, 1928, p. 164,

Baker's method has a molecular weight between 75,000 and 100,000. Attention is again called to the fact that Van Slyke and Baker casein does not consist of a single molecular species.

Effect of Heat on the Casein Molecule.—Inasmuch as other investigators have paid little or no attention to the temperature conditions that have been employed in the preparation of their protein solutions, we have thought it advisable to compare casein solutions that had been heated to 40° during their process of solution in phosphate buffer with casein solutions that had been prepared by dissolving in the same buffer in the cold and which have just been described.

In these experiments casein prepared by the method of Van Slyke and Baker was dissolved in the same phosphate buffer solution and with the same technique as previously described, except that the protein and solvent were, in the present case, subjected to a temperature of 40° for one hour while the protein was going into solution. This solution was then passed slowly through the milk separator, which was operated at a high speed, and the solution stored at 5° with a few drops of toluene as preservative.

In order to show clearly the range of size of the protein particles existing in this solution and their respective molecular weights after the above treatment, we have examined the solution by the sedimentation equilibrium method. This method has been described in detail elsewhere and it is sufficient here to state that the molecular weight is given by the relationship

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

where M represents molecular weight, R the gas constant, T the absolute temperature, C_2 and C_1 the protein concentrations at the distances X_2 and X_1, respectively, from the center of rotation after equilibrium is attained, V the partial specific volume of the protein, ρ the density of the solvent and ω the angular velocity.

The results of a run with Van Slyke and Baker casein which had been heated for one hour at 40° are given in Table IV. It will be seen that the bulk of the protein in the cell corresponds closely to a molecular weight of 188,000 and at the bottom of the cell we have a heavier constituent of molecular weight 370,000. This latter appears to be the acid–alcohol soluble casein described in our former communication. There is no sign of any protein material in this solution having a molecular weight around 100,000, such as we find the bulk of Van Slyke and Baker casein to consist of when it has not been subjected to the action of heat. From this we must conclude that the protein of molecular weight between 75,000 and 100,000 has been entirely converted into a different and a larger molecule by the action of the applied heat. The data indicate that the molecule after the

TABLE IV

VAN SLYKE AND BAKER CASEIN SUBJECTED TO HEAT. SEDIMENTATION EQUILIBRIUM METHOD. PRELIMINARY DATA

Initial concentration, 0.95%; P_H, 6.8; thickness of cell, 0.20 cm.; T, 293°; speed 4430 r.p.m. ($\omega = 147.7\pi$); equilibrium time, 60 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
5.85	5.80	1.643	1.289	373,700[a]
5.80	5.75	1.289	1.064	298,150[a]
5.75	5.70	1.064	0.9170	233,080[a]
5.70	5.65	0.9170	.8160	184,550
5.65	5.60	.8160	.7265	185,350
5.60	5.55	.7265	.6455	190,310
5.55	5.50	.6455	.5760	185,070
5.50	5.45	.5760	.5100	199,490
5.45	5.40	.5100	.4555	186,960
			Mean mol. wt.	188,620

[a] Excluded in calculating mean value of lighter species.

action of heat has at least twice the molecular weight that it had before such treatment. This suggests a polymerization or association of the molecules of molecular weight betweon 75,000 and 100,000 to form the larger molecules through the influence of heat. Our experiments do not show which of these suggested mechanisms is responsible for the changes in molecular weight which we have recorded. They do show, however, that in the case of casein, other factors remaining constant, the temperature at which the protein material is put into solution has a great influence on the state of the molecule.

It has often been noted that temperature changes and various other factors influenced certain physical properties of protein solutions. Such effects have been due largely to obscure causes and have been referred to as depending on "life history," etc., of the material in question. Evidence is here presented that in the case of casein the heat effect is a very definite one which results in at least doubling the molecular weight.[6]

Discussion of Results.—The experiments described in this paper with casein prepared from cow's milk by the method of Van Slyke and Baker have shown that this material is a mixture of protein substances of different molecular weight. Furthermore, samples from separate casein preparations made by this method of preparation, are shown to be different from each other, showing that not only a mixture of proteins is produced by the Van Slyke and Baker method, but a different mixture is to be found in different individual preparations. That little or no definite conclusions could be arrived at from researches based on such starting materials is entirely obvious.

[6] The authors are indebted to B. Sjögren for repeating and confirming our centrifuging results with the heated casein solutions described above.

There can be no doubt that the molecular weight of the main bulk of the protein separated by Van Slyke and Baker's method lies between 75,000 and 100,000.

In the equilibrium experiments where the casein solutions were subjected to a temperature of 40° while the moist casein was dissolving in the buffer solution, we have found that the protein of molecular weight between 75,000 and 100,000 has been altered to form larger molecules of molecular weight 188,000. That this represents an actual change in the molecules is evidenced from the fact that calculations of the specific sedimentation velocity of the 188,000 molecule yield an S_{20° value of 10.55×10^{-13} on the basis of a spherical molecule, and a similar value of 7.59×10^{-13} on the basis of a molecule of the same shape as the acid-alcohol soluble casein reported in the first paper. Neither of these values can be confused with or mistaken for the S_{20° value of 5.56×10^{-13} which we have obtained for the 75,000 to 100,000 weight molecule. We must be dealing, therefore, with another casein molecule in this casein which has been subjected to the influence of heat. In other words, the 100,000 molecule has been converted into the 188,000 weight type through the agency of heat. This change has been so complete that we do not find any of the 75,000 to 100,000 weight molecule even at the top of the sedimentation cell, where they would most certainly occur were any left unchanged in the solution.

Summary

1. Casein prepared by the method of Van Slyke and Baker has been examined at P_H 6.8 in phosphate buffer solutions at various protein concentrations by the centrifugal sedimentation velocity method, and found to consist of a mixture of protein substances of different molecular weight.

2. Different preparations made by this method were shown to be mixtures of proteins in different proportions. Not even a constant mixture was obtained by the Van Slyke and Baker method.

3. It was found that the bulk of the crude casein prepared by this method had a molecular weight between 75,000 and 100,000.

4. Crude casein subjected to a temperature of 40° during the time of dissolving in buffer solutions was found to have a molecular weight of 188,000 by the sedimentation equilibrium method. No molecules of molecular weight between 75,000 and 100,000 remained in the solution after this heat treatment.

UPSALA, SWEDEN
GENÈVA, NEW YORK