[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE MOLECULAR WEIGHT OF LACTALBUMIN

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RECEIVED MAY 27, 1930 PUBLISHED SEPTEMBER 5, 1930

A first attempt to study the dispersity of the proteins of milk by means of ultracentrifugal methods was made by Fåhraeus and one of the present writers in 1924.[1] Cows' milk was submitted to the action of a centrifugal field about 1500 times the force of gravity and exposures were taken at intervals of one hour. From the rate of sedimentation and the spreading of the boundary it was concluded that the natural casein—or calcium casein compound—of milk is a polydisperse colloid of comparatively low degree of dispersity, containing particles of radius ranging from about 10 to 70$\mu\mu$. The behavior of purified casein in centrifugal fields up to 100,000 times the force of gravity was studied by Carpenter.[2] The latter investigation showed that casein is a very unstable protein and that the molecular weight and degree of homogeneity of the product depend on the method of preparation which has been used. The sedimentation constant for casein isolated by the method of Van Slyke indicates a molecular weight around 100,000 for the bulk of this protein material—a value which is in the neighborhood of the molecular weight of serum globulin. Heating to 40° for one hour caused an increase in molecular weight to about double, and treatment with acidified 70% alcohol at 40° after the manner of Linderström-Lang gave a product which possessed a molecular weight nearly four times that of the original casein.

Besides the casein the chief protein constituent of cows' milk is the lactalbumin. It occurs to about 10% of the amount of casein. In view of the complicated behavior of casein it was thought to be of interest to collect some data concerning the dispersity of lactalbumin in order to see whether the very marked instability observed for casein also occurs in the lactalbumin and, therefore, is to be considered as a characteristic of the milk proteins.

Preparation of Lactalbumin.—Seven thousand cc. of fresh cows' milk of *P*H 6.7 and containing some toluene was run through a separator to remove the fat. Saturated ammonium sulfate solution was added to 50% saturation and the *P*H of the mixture brought to 5.2 by means of acetic acid. After standing for twenty-four hours in the ice box the precipitate of casein and globulin was centrifuged off and saturated ammonium sulfate solution added to 80% saturation. After twenty-four hours in the ice box the albumin precipitate was centrifuged off and washed with a weak solution of ammonium sulfate. The precipitate was dissolved in water and brought to crystalliza-

[1] T. Svedberg, *Kolloid-Z.*, **51**, 10 (1930).

[2] T. Svedberg, L. M. Carpenter and D. C. Carpenter, THIS JOURNAL, **52**, 241, 701 (1930).

tion at room temperature by the addition of ammonium sulfate and sulfuric acid.[3] The crystals were dissolved in water, dialyzed against toluene-saturated water at 5° until the sulfate reaction was negative and finally electrodialyzed with a current density of 0.6 milliampere per sq. cm. for thirty hours. No precipitation occurred during the electrodialysis; volume of solution 175 cc.; concentration 0.74% (Material I).

Another sample of lactalbumin was made up from an equal quantity of milk proceeding as described above but omitting the crystallization process. The volume of the final solution was 200 cc. and the concentration 3.14% (Material II).

Specific Volume, Isoelectric Point and Light Absorption of Lactalbumin.—The partial specific volume was measured pycnometrically at 20°. Material II was used and determinations were carried out in electrolyte-free solutions at 3.14 and 2.04%. The values agreed within experimental error and gave $V = 0.750$.

The isoelectric point of Material II was determined by means of the photographic cataphoresis method worked out in this Laboratory by A. Tiselius.[4] The protein was found to be electrochemically inhomogeneous and to possess an isoelectric point of approximately P_H 5.2.

The light absorption was measured on Material II by means of the Judd-Lewis spectrophotometer. The solution was electrolyte-free, of concentration 0.1% and thickness of layer of 2.0 cm. The specific extinction coefficient $\epsilon/c = 1/cd \times \log I_0/I$ (where c is the concentration, d the thickness of the solution, I_0 the intensity of the light beam after passing through the solvent and I the intensity after passing through the same thickness of solution) had a maximum value of 10.6 at a wave length of 279$\mu\mu$ and a minimum value of 3.6 at 255$\mu\mu$. In Fig. 1 the absorption curve is given.

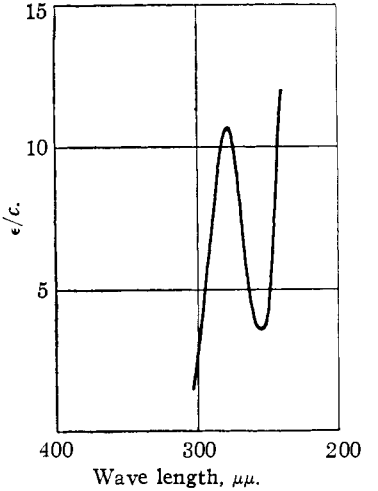

Fig. 1.

Molecular Weight and Sedimentation Constant of Lactalbumin.—A sedimentation equilibrium measurement was carried out on the crystallized Material I at a speed of 11,200 r. p. m. The protein concentration was 0.10% at the start and the solution 0.025 M in KH_2PO_4 and 0.025 M in Na_2HPO_4, giving a P_H of 6.8. The molecular weight was calculated from the relation

$$M = \frac{2\,RT \ln (c_2/c_1)}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)}$$

where R is the gas constant, T the absolute temperature, V the partial specific volume of the solute, ρ the density of the solvent, ω the angular velocity and c_2 and c_1 are the concentrations at the distances x_2 and x_1 from the center of rotation.

The values of the molecular weight were not independent of the distance

[3] A. Wichmann, *Z. physiol. Chem.*, **27**, 575 (1899).

[4] A. Tiselius, "Dissertation," Upsala, 1930.

from the center of rotation but varied from 12,000 at a distance of 5.50 cm. to 25,000 at a distance of 5.90 cm.

In Table I are given the results of two sedimentation velocity runs made on Materials I and II.

TABLE I

LACTALBUMIN SEDIMENTATION VELOCITY RUNS

Material	Solvent KH_2PO_4, M	Solvent Na_2HPO_4, M	P_H of soln.	Concn. of protein, %	$S_{20°} \times 10^{13}$	Remarks
I	0.019	0.001	5.5	0.27	2.22	*a*
II	.025	.025	6.8	.63	2.95	*b*

[a] Strong drift in diffusion constant; trace of non-centrifugible material.

[b] Drift in diffusion constant; no non-centrifugible material.

Material II was again tested after having been left for three months in the ice box. The sedimentation constant was approximately the same but about 15% of the protein was broken up into a non-centrifugible substance.

The drift in the values of the molecular weight with distance from the center of rotation as well as the drift observed in the apparent diffusion constant shows that lactalbumin is not homogeneous with regard to molecular weight. In this respect therefore it resembles casein.

Direct Ultracentrifugal Analysis of Milk and of Lactalbumin at Different Stages of Purification.—The fact brought out by the above measurements that lactalbumin is not homogeneous with regard to molecular weight made it desirable to study the dispersity of this protein at different stages during the process of purification in order to ascertain whether the inhomogeneity is caused by the procedure of isolation.

Sedimentation velocity runs at high speed (centrifugal force about 100,000 times the force of gravity) during three to four hours at a temperature of 20–23° were used for testing the material.

Fresh milk (P_H 6.8) was first centrifuged without any treatment at all. At the high speed used the casein is thrown down in a few minutes. After that period only a very slight sedimentation occurred (Fig. 2A, time between exposures, one hour). The bulk of the light-absorbing material (65–80%) was non-centrifugible. Addition of acetic acid until a molarity of 0.02 was reached with a P_H of 5.8 had no effect on the sedimentation. The P_H of the milk was then raised to 10.3 by the addition of sodium hydroxide until the molarity was 0.02. The sedimentation remained the same. Dilution of the milk with water or with a 2% solution of ammonium sulfate to two-ninths of the original concentration slightly increased the sedimentation.

In order to eliminate the influence of viscosity caused by the presence of the lactose, a sample of milk containing toluene was dialyzed against water for three days in the ice box. Only a slight increase in sedimentation

occurred. Addition of 5% ammonium sulfate to the dialyzed milk had no effect.

These experiments show that untreated milk and milk the P_H of which has been lowered to 5.8 or raised to 10.3 and milk treated with ammonium sulfate up to 5%, or milk which has been freed from lactose and salts by dialysis contain but a small quantity of lactalbumin of the degree of dispersity observed in the product isolated by the usual method. In the milk the lactalbumin is present in a very much higher degree of dispersity than in the finished laboratory product.

A lowering of the P_H of the milk to 4.1 either by the addition of hydrochloric acid or acetic acid raised the sedimentation constant slightly above 2×10^{-13} and converted part of the non-centrifugible material into centrifugible substance.

A sample of milk was half saturated with ammonium sulfate, the casein–globulin precipitate removed and the solution dialyzed against water in the ice box for two days. The sedimentation constant was identical with that for lactalbumin as determined above. The non-centrifugible material had decreased considerably and now only amounted to about 20% (Fig. 2 B, time between exposures one-half hour).

A similar experiment was performed with milk from which the casein had been removed by precipitation with acetic acid. The product showed the same properties as the one just described.

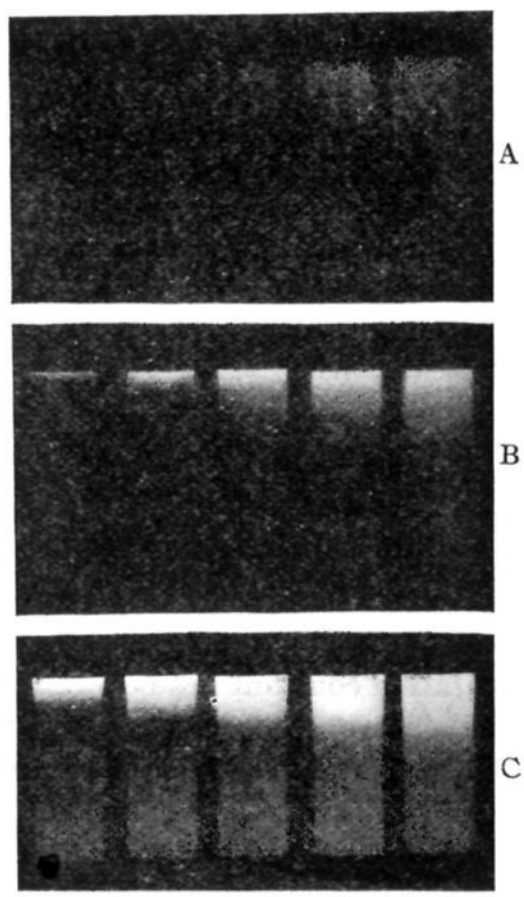

Fig. 2.

To another sample of milk was added ammonium sulfate to saturation. After standing for twenty-four hours in the ice box the precipitate was dialyzed against water until the sulfate reaction was negative (five days). The casein–globulin precipitate was removed and the solution tested in the ultracentrifuge; the sedimentation constant and amount of non-centrifugible material were as before.

The treatment to which the milk was subjected in the last three experiments is identical with the chief processes of isolation used for the preparation of lactalbumin. As a matter of fact this treatment brought about a considerable change in the properties of the original albumin of the milk

toward the properties found in the "purified" lactalbumin. The sedimentation constant was practically the same but the material still contained about 20% of non-centrifugible substance.

For the sake of comparison a picture of the sedimentation of the purified lactalbumin (Material II) is given in Fig. 2 C (time between exposures forty min.).

From the above experiments it is obvious that the lactalbumin is an artificial product built up during the process of "purification" from some material of low molecular weight present in the milk. The degree of dispersity or molecular weight of this non-centrifugible material we have not as yet made any attempt to determine. We have ascertained that it is almost completely precipitated by saturation with ammonium sulfate and that it does not diffuse through collodion bags. From the centrifuging tests it follows that the molecular weight cannot be higher than about 1000. The molecular weight of the "purified" lactalbumin was found to range from 12,000 to 25,000, which shows that the molecular weight of the original lactalbumin of the milk has been raised many times in the "purification" process.

The expenses connected with these experiments have been defrayed by a grant from the foundation "Therese och Johan Anderssons Minne."

Summary

1. The molecular weight and sedimentation constant of lactalbumin prepared from cows' milk were determined by means of the ultracentrifugal methods.

2. Lactalbumin is not homogeneous with regard to molecular weight. Values ranging from 12,000 to 25,000 were observed. The mean sedimentation constant lies between 2 and 3 times 10^{-13}.

3. The observed inhomogeneity of lactalbumin suggested ultracentrifugal tests at different stages during the process of purification.

4. It was found that lactalbumin with the properties observed in the "purified" product does not exist in the milk, but is formed during the process of "purification," especially by the action of ammonium sulfate of high concentration.

5. The bulk of the material from which the lactalbumin is formed has a low molecular weight not exceeding 1000. The comparatively high molecular weight of the final product is a result of gradual aggregation of the material of low molecular weight originally present in the milk.

6. The pronounced instability of casein with regard to molecular weight as found by Carpenter also occurs in lactalbumin and this property is, therefore, probably a characteristic of the milk proteins and may be of considerable physiological importance in nutrition.

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