CXXXVIII. ULTRACENTRIFUGAL AND ELECTROPHORETIC STUDIES ON THE MILK PROTEINS.

I. INTRODUCTION AND PRELIMINARY RESULTS WITH FRACTIONS FROM SKIM MILK.

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FEW ultracentrifugal studies on milk proteins have been carried out since the ultracentrifuge was constructed by Svedberg.¹ In 1924 Svedberg and Fåhræus [*v. Svedberg, 1930*] estimated the degree of dispersion of calcium caseinogenate in cow's milk and concluded that the Ca caseinogenate formed a very polydisperse suspension in the milk serum, that the distribution curve was very flat and that the diameters of the particles were of the order of magnitude of $20\text{--}140m\mu$ ($m\mu = 10^{-7}$ cm.). The experiments were carried out in relatively low centrifugal fields of about 1500 times gravity.

Svedberg *et al.* [1930, 1, 2] studied purified caseinogen in centrifugal fields up to 100,000 times gravity. They concluded that caseinogen is a very unstable protein and that the molecular weight and the homogeneity of caseinogen depend on the way in which it has been prepared. The bulk of the crude caseinogen prepared by the method of Van Slyke and Baker had a mol. wt. between 75,000 and 100,000. The caseinogen prepared by the Hammarsten method was still more heterogeneous, but it was possible by means of acidified 70% alcohol to extract from the Hammarsten caseinogen a homogeneous protein with mol. wt. about 375,000.

Svedberg and Sjögren [1930] studied the behaviour of lactalbumin in the ultracentrifuge in fields up to 100,000 times gravity. They found that lactalbumin prepared according to the method of Wichmann was heterogeneous with regard to mol. wt. (12,000–25,000); they mentioned that Tiselius had found it to be electrochemically heterogeneous with isoelectric point near p_H 5.2. Furthermore, they made some experiments in order to ascertain whether the heterogeneity was caused by the procedure of isolation and concluded that the "purified" lactalbumin does not exist in the milk but is formed during the process of "purification", especially by the action of ammonium sulphate in high concentration. According to these authors lactalbumin is formed by successive aggregation from a substance with a mol. wt. not exceeding 1000.

Nichols *et al.* [1931] studied the influence of preheating on the dispersity of Ca caseinogenate in skim milk. They concluded that the maximum on the distribution curve is somewhat more pronounced in the case of preheated skim milk. Most of the particles are less than $200m\mu$ in diameter and the mean size is about $90m\mu$.

In the autumn of 1934 the author started to investigate the crystalline globulin prepared by Palmer [1934] from the albumin fraction of cow's milk. It was immediately evident that this lactoglobulin was a quite uniform substance

¹ For recent review of the method, see Svedberg [1934, 1, 2, 3, 4].

having a definite mol. wt. and a definite isoelectric point. Prof. Svedberg suggested that the problem of lactalbumin should be taken up again, as the efficiency of the centrifuge had been increased very much since the earlier work in 1930.

In the meantime (spring 1935) Philippi¹ made some experiments in order to find the substance of low mol. wt. expected to form the pre-lactalbumin and in order to investigate details of the association process. Contrary to expectation he found that the proteins which are generally called "lactalbumin" are present in the milk at least in a very early stage of the preparation.

The first part of Philippi's investigation was carried out using the light absorption method. However, this method was found to be unsatisfactory for solving this special problem and the refractometric method worked out by Lamm [1928; 1929] was applied.

By this technique it was shown that skim milk from which caseinogen has been removed by isoelectric precipitation contains three distinct proteins, α , β and γ , which are characterised by different sedimentation constants. The magnitude of these sedimentation constants increases in the order from α to γ . With the available resolving power of the ultracentrifuge it was not possible to detect other components in any appreciable amount.

Values of the sedimentation constant of the α -component between 1 and $2 \cdot 10^{-13}$ were observed, very low values for a protein. The sedimentation constant of the β -component was found to be $3 \cdot 13 \cdot 10^{-13}$ in the p_H range 5.5–7.0, identical with that of Palmer's lactoglobulin [Pedersen, 1936] within the limits of error. Hence Philippi concluded that the β -component is identical with the Palmer protein. For the sedimentation constant of the γ -component values between 7.0 and $7 \cdot 4 \cdot 10^{-13}$ were observed.

In the autumn of 1935 Kekwick¹ succeeded in isolating the protein with the low sedimentation constant. The experiments show that it is an electrochemically homogeneous substance; from centrifugal and diffusion experiments which have been carried out so far it seems to be of uniform size.

About the same time the author began to study the sedimentation of skim milk and fractions obtained from it by treatment in different ways. The results of these experiments serve as an introduction to a more detailed study of the high molecular substances present in milk. These experiments serve to show how the Svedberg ultracentrifuge can be used for studying biochemical solutions in their native state, and the changes caused by the different fractionation procedures can be followed.

First there will be given a description of some of the recent modifications of the refractometric method in the sedimentation velocity and the sedimentation equilibrium methods. Finally the experiments carried out on milk will be described.

EXPERIMENTAL TECHNIQUE.

The sedimentation velocities were determined in the same ultracentrifuge as described previously by McFarlane [1935]. For recording sedimentation two different methods have been used, *viz.* the light absorption method and the refractive index method of Lamm [1928; 1929].

The light absorption method. Details of this method may be found in some of the earlier papers of Svedberg *et al.* [*e.g.* 1926 and 1932]. Throughout this work the short wave ultraviolet light absorption of the proteins was used. In order to reduce the influence of the change in the latent image between exposure and

¹ Results not yet published.

development, the photographic plates were stored 1 day before being developed, since the change in the latent image is most pronounced during the first hours after exposure.

Dilute solutions of potassium chromate in 0.05 *M* sodium carbonate solution were used to give the relationship between concentration and blackening on the photographic plate [Svedberg and Eriksson, 1932].

The refractive index method. This method, described some time ago [McFarlane, 1935], was used with the modifications given below.

The projection system. The projection system was improved to give a more linear projection of the scale. In the first projection apparatus (I) used by McFarlane the projection lens was a single Zeiss Tessar with a focal length of 13.5 cm. and an aperture of $F/4.5$; this lens however is not symmetrical, it does not give a linear scale image. By substituting two special Zeiss Tessars as selected for stereoscopic photography ($f=26$ cm. and an aperture of $F/6.3$) one gets a symmetrical projection lens that gives a much more uniform scale. This projection system is called (II). But even this projection system was found to be unsatisfactory, especially when used with some of the new centrifuge cells having a high sedimentation column. Therefore a new projection system (III) has just been constructed. In this case the projection lens is composed of two Zeiss Tessar lenses with focal lengths 36 cm. and an aperture $F/6.3$. This system seems to give as good delineation as is necessary.

The reference scale. In the work of McFarlane [1935] the standard undeviated scale was obtained before the actual experiment by bringing the centrifuge to the desired speed with the cell filled with water and a photograph was taken shortly after this speed was reached. In this work the cells were filled to exactly the same height with the same "solvent" as the one in which the protein was dissolved. By "solvent" is meant the buffer solution of the same concentration as that in the protein solution (or else the ultrafiltrate from this solution). It was found [Pedersen, 1934] that the sedimentation of the small inorganic molecules gives rise to a refractive index gradient and therefore to a deviated scale. Considering the time for establishment of the equilibrium under the conditions used, which is generally from 8 hours to 1 day, it means that even in the absence of a high-molecular substance from the cell one obtains a different displacement of the lines at different times from the start. Therefore throughout this work special reference scale experiments have always been carried out; exposures are taken at short intervals for all the scale distances used; the experiments are extended over the same time interval as used in the actual protein experiment. When the same "solvent" and the same cell are used in all experiments as far as possible it is found that very often the same reference scale can be used for several experiments.

Since the displacements of the lines are proportional to the scale distance and the thickness of the cell, it can be seen that the necessity of taking reference scales becomes greater when thicker cells and longer scale distances are used. In this work nearly all the experiments were carried out with 6 and 12 mm. cells and with scale distances up to 120 mm. (160 mm. in a few special cases). McFarlane generally worked with a 2 mm. cell and scale distances from 20 to 40 mm., so that in his case it was not so necessary to take the same precautions with the reference scale. Some of his "base line" troubles may have been caused by the fact that he used pure water for reference scale experiments instead of the proper salt solution.

The concentration of the sedimenting substance. McFarlane [1935] generally found that the concentration of the sedimenting substance calculated from the

area below the curve was always lower by 10–15% than the analytical value. Some experiments done by the author have shown the same to be the case for horse serum albumin. But for lactoglobulin the value from the sedimentation diagram agreed very well with the analytical value. We therefore believe that the concentration calculated from the curves gives the correct value for the sedimenting substance [Pedersen, 1936].

Sedimentation equilibrium. The concentration gradient in the centrifuge cell was in this case determined in most of the experiments by means of the refractive index method [Lamm, 1928; 1929]. In a few experiments the new refractive index method of Lamm [1933] was used.

CALCULATIONS OF RESULTS.

Sedimentation equilibrium. According to Svedberg [1925; 1926] the molecular weight of a solute may be obtained from its sedimentation equilibrium by means of the equation

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

where M = molecular weight, R = gas constant, T = absolute temperature, c_2 and c_1 are the concentrations of the solute at the distances x_2 and x_1 from the axis of rotation, ρ the density of the solution, V the partial specific volume of the solute and ω = angular velocity.

When the light absorption method is used, the *concentrations* at all points along the cell are obtained and this equation may be used directly to calculate the molecular weight of the solute. By the refractive index method the values for the *concentration gradient* at all points along the cell are obtained. According to Lamm [1928; 1929] the scale displacement Z is determined by the equation

$$Z = Gab \frac{dn}{dx} = Gab \alpha \frac{dc}{dx} \quad \text{or} \quad \frac{dx}{dc} = \frac{Z}{Gab\alpha} \quad \dots\dots(2),$$

where G = camera magnification of the displacement, a = the cell thickness, b = optical distance between the scale and the middle of the cell, $\frac{dn}{dx}$ = the refractive index gradient in the cell and $\alpha = \frac{dn}{dc}$ or the refractive increment of the solute.

When the concentration gradient is known at any point in the cell, it is possible to calculate the concentrations throughout the cell, if we assume that the whole material present at the start is still present in solution in the cell when equilibrium is established. That means in the case of a rectangular cell that $\int_{x_0}^{x_n} c_0 dx = \int_{x_0}^{x_n} c_i dx$ where c_0 is the original concentration of the solution and c_i the concentration in the level x_i from the centre of rotation after equilibrium is established. In order to find the values for c_i we may use the following procedure.

If the values for $\frac{dc}{dx}$ vary only slightly with x , then $\frac{dc}{dx}$ is calculated for every 0.05 cm. in the cell and as a first approximation it is assumed that at the point x_m (somewhere in the middle of the cell) the concentration $c_m = c_0$ and at the next point $x_{m+1} = x_m + 0.05$ cm., the concentration is (Fig. 1)

$$\begin{aligned} c_{m+1} &= c_0 + \frac{0.05}{2} \left[\left(\frac{dc}{dx} \right)_m + \left(\frac{dc}{dx} \right)_{m+1} \right] \\ c_{m+2} &= c_{m+1} + \frac{0.05}{2} \left[\left(\frac{dc}{dx} \right)_{m+1} + \left(\frac{dc}{dx} \right)_{m+2} \right] \\ c_{m-1} &= c_0 - \frac{0.05}{2} \left[\left(\frac{dc}{dx} \right)_m + \left(\frac{dc}{dx} \right)_{m-1} \right] \text{ etc.} \end{aligned} \quad \dots\dots(3),$$

If the values for $\frac{dc}{dx}$ vary greatly with x the following calculations have generally been used:

$$c_{m+1} = c_0 + \frac{0.01}{2} \left[\left(\frac{dc}{dx} \right)_m + 2 \left(\frac{dc}{dx} \right)_{m+1/5} + 2 \left(\frac{dc}{dx} \right)_{m+2/5} + \dots + \left(\frac{dc}{dx} \right)_{m+1} \right] \text{ etc. } \dots (4).$$

Fig. 1.

When all the values for c are calculated and plotted, the value of $\int_{x_0}^{x_n} c_i dx$ is determined. If its value is equal to $\int_{x_0}^{x_n} c_0 dx$, then the calculated values for c give the actual values in the cell. If the two integrals are not equal then all the c values should be changed by the same amount Δc , where

$$\Delta c = \frac{\int_{x_0}^{x_n} c_0 dx - \int_{x_0}^{x_n} c_i dx}{x_n - x_0} \dots (5).$$

If the cell is not rectangular in shape but sectorial, then the following expression must be fulfilled:

$$\int_{x_0}^{x_n} c_0 \frac{x_i}{x_0} dx = \int_{x_0}^{x_n} c_i \frac{x_i}{x_0} dx \dots (6).$$

If these two integrals are not equal all the calculated values of c should be changed by the amount Δc and

$$\Delta c = \frac{\int_{x_0}^{x_n} c_0 \frac{x_i}{x_0} dx - \int_{x_0}^{x_n} c_i \frac{x_i}{x_0} dx}{\frac{1}{2} \left(1 + \frac{x_n}{x_0} \right) (x_n - x_0)} \dots (7).$$

If the cell has another shape then the expression becomes more generally

$$\int_{x_0}^{x_n} c_0 f(x) dx = \int_{x_0}^{x_n} c_i f(x) dx \dots (8),$$

where $f(x)$ gives the geometrical shape of the cell, and

$$\Delta c = \frac{\int_{x_0}^{x_n} c_0 f(x) dx - \int_{x_0}^{x_n} c_i f(x) dx}{\int_{x_0}^{x_n} f(x) dx} \dots (9).$$

The calculation of the (c, x) curve from the $\left(\frac{dc}{dx}, x \right)$ curve could be made in other ways than described here, but other methods generally require two successive integrations instead of the one necessary in the method just described.

If the value for α or for some reason that for c_0 is difficult to obtain it is still possible to use the method just described if a refractive index diffusion experiment or sedimentation velocity experiment is carried out on the same solution used for the sedimentation equilibrium experiment. From either diffusion or sedimentation curve one can calculate the value for the difference $(n_1 - n_0)$ in refractive index due to the sedimenting substance.¹ It is only necessary to substitute $(n_1 - n_0)$ for c_0 , $(n_i - n_0)$ for c_i and $\frac{dn}{dx} = \frac{Z}{G\alpha b}$ for $\left(\frac{dc}{dx}\right)$ to get the $(n_i - n_0)$, x curve.

When the (c, x) or $(n_i - n_0)$, x curve is found, it may be used in the same way as in the absorption method to calculate the molecular weight from the formula

$$M = \frac{2RT \ln \frac{c_2}{c_1}}{(1 - V\rho) \omega^2 (x_2^2 - x_1^2)} \text{ or } M = \frac{2RT \ln \frac{n_2 - n_0}{n_1 - n_0}}{(1 - V\rho) \omega^2 (x_2^2 - x_1^2)} \quad \dots (10)$$

or we may use the differential formula from which the above integrated expression was originally derived:

$$M = \frac{RT d \ln c}{(1 - V\rho) \omega^2 x} = \frac{RT \frac{dc}{dx}}{(1 - V\rho) \omega^2 x} \quad \dots (11)^2 \quad \text{or} \quad M = \frac{RT \frac{dn}{dx}}{(1 - V\rho) \omega^2 (n_i - n_0) x} \quad \dots (12),$$

or

$$M = \frac{RT Z_i}{(1 - V\rho) \omega^2 x_i c_i G\alpha b} \quad \dots (13)^2 \quad \text{or} \quad M = \frac{RT Z_i}{(1 - V\rho) \omega^2 x_i (n_i - n_0) G\alpha b} \quad \dots (14).$$

If the solute is monodisperse and obeys the gas laws, it is generally easier to use another formula for the calculation of M , since it has been shown by Lamm [1929] (see also Pedersen [1934]) that $\frac{Z_2 x_1}{Z_1 x_2}$ could be substituted for $\frac{c_2}{c_1}$ and the expression for the molecular weight becomes accordingly

$$M = \frac{2RT \ln \frac{Z_2 x_1}{Z_1 x_2}}{(1 - V\rho) \omega^2 (x_2^2 - x_1^2)} \quad \dots (15).$$

If the solute is polydisperse, then $\frac{Z_2 x_1}{Z_1 x_2}$ differs from $\frac{c_2}{c_1}$ and the equation (15) does not give the mol. wt.; but Lansing and Kraemer [1935] have shown that equation (15) gives M_{zx} , which is of some value for finding out the degree of

¹ It may be of interest to have both the value for the area of the diffusion curve and for the area of the sedimentation curve. If the values of the two areas are the same, then the diffusing substances are equal to the sedimenting ones; if they are different, then either the solution contains some coarse disperse substances which sediment to the bottom of the cell before the measurements are started or the solution contains a low-molecular substance that does not give rise to a measurable refractive index gradient in the cell. In such a case it is not justifiable to use the area from the diffusion curve (or the analytical concentration), since in this case the refractive index gradient in the equilibrium cell will be determined only by the amount of substance corresponding to the area of the sedimentation curve (from the velocity experiment). In some experiments of the author with serum albumin (results not published) it was found that there was a strong drift in the calculated values for M as long as the analytical values for the concentrations were used; if the values calculated from the sedimentation velocity diagram were used, the drift in the molecular weight became rather small and M_W was not much different from M_D (the value calculated from diffusion and sedimentation constants).

² These formulae have been given before [Heidelberger and Pedersen, 1935] and their use was first mentioned by Pedersen [1935].

polydispersity. According to these authors one can calculate three different average molecular weights from the ultracentrifuge data, *viz.*:

$$\text{number-average } M_n = \frac{\sum n_i M_i}{\sum n_i} = \sum \frac{w_i}{M_i} \quad \text{.....(16)}$$

$$\text{weight-average } M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} = \frac{\sum w_i M_i}{\sum w_i} \quad \text{.....(17)}$$

$$z\text{-average } M_z = \frac{\sum n_i M_i^3}{\sum n_i M_i^2} = \frac{\sum w_i M_i^2}{\sum w_i M_i} \quad \text{.....(18),}$$

where n_i is the number of molecules of weight M_i , while w_i is the total weight of that molecular species. The usual physico-chemical methods (*e.g.* osmotic pressure measurements) generally give M_n values, whilst M_w and M_z may be obtained from ultracentrifugal data. For a monodisperse solute we have $M_n = M_w = M_z$, but for a polydisperse solute we have $M_n < M_w < M_z$, at least in very dilute solutions where the gas laws are valid.

There is often a small drift in the M_{wx} values; sometimes the values increase towards the bottom of the cell (which would indicate that the substance was heterogeneous) and sometimes they decrease towards the bottom (which ordinarily means departure from the gas laws). But since the drift is very small and sometimes in one direction and sometimes in another, it is assumed that the drift is caused by small experimental errors in the Z values or in computing the c values from the Z values.

The M_{zx} values often fluctuate widely, but the M_w and M_z values are not so very different. By a simple comparison of the determining factors in the two cases we get

$$\frac{c_2}{c_1} = \frac{z_2 x_1}{z_1 x_2} = \frac{x_1}{x_2} \left(1 + \frac{z_2 - z_1}{z_1} \right) \quad \text{for } M_{zx},$$

and

$$\frac{c_2}{c_1} = \frac{c_1 + \Delta c}{c_1} = 1 + k \frac{z_1 + z_2}{2c_1} \quad \text{for } M_{wx}.$$

From this it is quite evident that the error in $\frac{z_2 - z_1}{z_1}$ must be much larger than the error in $\frac{z_1 + z_2}{2c_1}$, especially when Δx is very small.

PRELIMINARY ULTRACENTRIFUGAL STUDY OF THE MILK PROTEINS.

Material. All the experiments here described were carried out on cow's milk. The fresh milk was drawn into Jena flasks and immediately brought to the laboratory, where the bulk of the butter fat was removed by skimming the milk in one of the ordinary laboratory centrifuges for a short time, after which the skim milk was spun in a closed separator bowl at high speed for about 10 min. By this procedure some fat went to the central part of the bowl and some "slime" to the periphery; these two fractions were generally thrown away and the solution, the milk plasma, was used for the experiments. Toluene was added as preservative to all solutions that were dialysed.

The first experiments were carried out on milk serum which was spun directly in the ultracentrifuge, since the large caseinogen particles sedimented to the bottom of the centrifuge-cell before the centrifuge had reached the speed necessary for the measurements on the milk serum. In two successive experiments 1.6 ml. of milk plasma were placed in the largest ultracentrifuge-cells and submitted to the action of a centrifugal field of about 100,000 times gravity for some minutes. By this procedure about 3 ml. of milk serum were obtained, an amount sufficient for carrying out four different experiments (*M* 1–*M* 4). As found by Svedberg and Fåhræus [Svedberg, 1930] and by Nichols *et al.*

[1931] it was evident that the suspended caseinogen (or calcium caseinogenate) was present in a quite polydisperse condition; at the start the solution was quite non-transparent, but as the speed increased the solution became more and more transparent and finally water-clear. By following the caseinogen separation by means of the scale method it was not possible to detect any measurable sedimentation of monodisperse substances during these milk plasma experiments.

Exp. M 1. A portion of the milk serum was investigated in a centrifugal field of about 230,000 times gravity by means of the scale method. The reference scale was made with the ultrafiltrate from the milk. The sedimentation diagram showed peaks corresponding to the molecules $\alpha + \beta$ and γ (for nomenclature see later); besides these there were probably present to a very small extent a few more species. γ was present only in a very low concentration (less than 10% of $\alpha + \beta$) and was rather difficult to identify owing to the refractive index gradient caused by the sedimentation of the large amount of lactose. During the experiment an attempt was made to take some light absorption pictures using chlorine and bromine light filters, but even with exposure times as long as 10 min. it was impossible to get any blackening on the photographic plate (the usual exposure time is 30 sec.); the thickness of the cell was 12 mm. The large light absorption is due to lactose and lactoflavin and perhaps to some other low molecular substances.

Exp. M 2. A second portion of the milk serum was dialysed in the ice-box against frequently changed buffer solution (0.06 *M* NaCl + 0.023 *M* KH_2PO_4 + 0.017 *M* Na_2HPO_4 , p_{H} about 6.7). When the lactose had disappeared the solution was investigated in the centrifuge. (In all the following experiments the reference scale was made with the corresponding buffer in the cell.) The sedimentation diagram showed the same peaks as in M 1, that is $\alpha + \beta$ and γ plus some less distinct peaks. Light absorption pictures were easily taken during the experiment and showed a fairly "uniform" curve (due to $\alpha + \beta$), when γ had sedimented down to the bottom of the cell. The sedimentation constants were for β $s_{20} = 3.2 \cdot 10^{-13}$ and for γ $s_{20} = 7.1 \cdot 10^{-13}$; the constant for α could not be determined; its presence was just evident from the curve.

Exp. M 3. A third portion of the milk serum (about 1.5 ml.) was dialysed until it was in equilibrium against a very frequently changed acetate buffer (0.2 *M* NaCl + 0.075 *M* sodium acetate + 0.025 *M* acetic acid; p_{H} 5.0). The sedimentation diagram showed the same peaks as in the preceding experiments. For β $s_{20} = 3.04 \cdot 10^{-13}$ and for γ $s_{20} = 7.4 \cdot 10^{-13}$. In this experiment it was just possible to get an estimate of the sedimentation constant for α ($s_{20} = 1.8 \cdot 10^{-13}$).

Exp. M 4. A fourth portion of the milk serum was used as in M 3, but the light absorption method was employed. On the microphotometer record the presence of α could hardly be detected, so that values calculated as the sedimentation constant for β are rather a mean of the sedimentation constants for α and β , namely $s_{20} = 2.7 \cdot 10^{-13}$. The presence of γ was quite evident from the curve, but it was not possible to calculate its sedimentation constant.

Exps. M 5, 13, 16 and 17. In these experiments the milk plasmata from two different samples of milk were dialysed for about a week in the cold (3–5°) against frequently changed phosphate buffers (in M 5: 0.06 *M* NaCl + 0.017 *M* KH_2PO_4 + 0.023 *M* Na_2HPO_4 , in the others: 0.2 *M* NaCl + 0.02 *M* KH_2PO_4 + 0.03 *M* Na_2HPO_4 , p_{H} 6.8). During this time most of the suspended caseinogen went into solution and the appearance changed from milky to opalescent. When the solution was spun in the centrifuge, it became clear at a low speed and the amount of precipitate was very small. The sedimentation diagrams from these four experiments were on the whole quite similar but entirely different from the diagrams in M 1–4. The peaks belonging to the molecules α , β and γ were likewise present here, but there were at least 7 more peaks apparent (see Fig. 2 a–d). For the 4 slowest sedimenting peaks the mean sedimentation constants were: α , 1.8; β , 3.18; γ , 6.8 and δ , $10.4 \cdot 10^{-13}$. For the other molecules the sedimentation velocity was too high to allow an accurate determination of the sedimentation constant; it ranged from $13\text{--}30 \cdot 10^{-13}$ (in order to get a proper determination of these high sedimentation constants the centrifugal field should be less than 100,000 times gravity). The total protein concentration in Exps. M 16 and M 17 was 1.4%.

The concentrations of α and β were not the same in the different experiments; in M 5 the concentration of α was about 1/5 of that for β , while in M 16 and

M 17 it was about twice that of β . Whether this difference in the concentration was due to differences in the two samples of milk or to different permeability of the cellophane membranes to the α molecules cannot be decided at present.

Fig. 2 A-D. Ultracentrifugal sedimentation diagrams from M 16 obtained by the refraction method. The abscissae (X in cm.) represent distances from the centre of rotation and the ordinates (Z in mm.) represent the scale line displacements which are proportional to the concentration gradient. Each peak on the diagram corresponds to the sedimentation of molecules of a certain size. The faster the peak moves toward the right side of the diagram, the larger the molecule: the area below the peak is proportional to the concentration of the molecule which gives rise to the peak. Exp. M 16 was carried out with a dialysed (in phosphate buffer) sample of skim milk and the solution contained most of the proteins present in milk. Centrifugal force: 260,000 times gravity. Cell thickness: 12 mm. A. Exposure taken 13 min. after reaching full speed. B. Exposure taken 49 min. after reaching full speed. C. Exposure taken 115 min. after reaching full speed. D. Exposure taken 185 min. after reaching full speed.

In the description of the experiments the different peaks of the sedimentation diagrams were named by Greek letters in such a way that the one with the smallest sedimentation constant was called α , the next smallest β and so on.

The new milk protein isolated by Kekwick has in the purified condition a sedimentation constant of about $2.10 \cdot 10^{-13}$, a value very close to that of the component α , so we may assume that α is the Kekwick protein.

In the same way the sedimentation constant found for β agrees very well with that found for Palmer's lactoglobulin (see Pedersen [1936]). Probably the so-called lactalbumin is a mixture of the two molecules α and β and the molecule usually referred to as lactoglobulin is γ , but this has so far not been proved.

Since all peaks from δ on appeared when the caseinogen was dissolved, we must suppose that at least some of these peaks originate from the caseinogen. Owing to its size the bulk of the caseinogen must be present in the δ peak and its sedimentation constant therefore must be $10.4 \cdot 10^{-13}$.

Some experiments were carried out to follow a fractionation process. Equal amounts of neutral saturated ammonium sulphate solution and milk plasma were mixed together. The caseinogen precipitate was centrifuged off and suspended in a solution of $0.2 M$ NaCl + $0.02 M$ KH_2PO_4 + $0.03 M$ Na_2HPO_4 and dialysed against the same buffer (frequently changed). The filtrate from the ammonium sulphate precipitation was dialysed under pressure against a solution of $0.06 M$ NaCl + $0.017 M$ KH_2PO_4 + $0.023 M$ Na_2HPO_4 until it was free from sulphate. The volume was reduced to 1.2 times the volume of milk plasma (Exp. M 6). Later the dialysis was continued against a solution of $0.2 M$ NaCl + $0.02 M$ KH_2PO_4 + $0.03 M$ Na_2HPO_4 until the volume was reduced to 0.4 times the volume of milk plasma (Exp. M 8).

Exp. M 6. The experiment was carried out on the filtrate from the ammonium sulphate precipitation. The bulk of the material belonged to the molecules α and β , giving sedimentation constants of 1.9 and $2.6 \cdot 10^{-13}$ respectively (an extraordinarily low value for β). Very small amounts of faster-sedimenting molecules could be observed.

Exp. M 8. The same solution was used as in M 6, but concentrated by dialysis. The concentration was 3 times that in M 6. α and β molecules were present, but the amount of α was considerably decreased; s could only be measured for β and was $3.03 \cdot 10^{-13}$. There were indications of molecules with higher sedimentation constants.

Exp. M 7. This experiment was on the precipitate from the ammonium sulphate precipitation. The centrifugal field was in this case only 180,000 times gravity. The molecules α , γ and δ were present. For δ $s_{20} = 11.1 \cdot 10^{-13}$ and for γ $6.2 \cdot 10^{-13}$. s could not be determined accurately for α owing to the low speed and the short time of the experiment. The calculated value was $1.1 \cdot 10^{-13}$, an extremely low value for α . The δ peak predominated.

The rest of the solution from M 7 was dialysed against a frequently changed solution containing $0.1 M$ sodium acetate + $0.1 M$ acetic acid. The heavy precipitate was centrifuged off, washed several times with water, and suspended in a solution containing $0.2 M$ NaCl + $0.02 M$ KH_2PO_4 + $0.03 M$ Na_2HPO_4 and dialysed against the same buffer (Exp. M 9). The solution from the caseinogen precipitation was dialysed under pressure against a solution containing $0.2 M$ NaCl + $0.075 M$ sodium acetate + $0.025 M$ acetic acid (exp. M 11).

Exp. M 9. The caseinogen fraction. The concentration of the caseinogen was 1.2% . The sedimentation diagram showed the movement of just one single peak with a sedimentation constant of $11.6 \cdot 10^{-13}$. The peak was not quite symmetrical but was somewhat broader towards the meniscus, perhaps indicating some dissociation of the molecule. At the top of the cell there was an indication of a small peak sedimenting very slowly, but the concentration was too small to allow determination of the sedimentation velocity. The solution was also investigated electrophoretically by means of the scale method. It appeared quite heterogeneous with regard to electrophoretic migration showing several peaks with different migrations.

Exp. M 10. For this experiment an aliquot of the solution used in M 9 was boiled for a few minutes in order to study the influence of boiling on the sedimentation constant. The sedimentation diagram had exactly the same appearance as the one from the preceding experiment, but the sedimentation constant showed a slight increase ($s_{20} = 12.8 \cdot 10^{-13}$).

Exp. M 11. The filtrate from the caseinogen precipitation. The concentration of the proteins in this solution was considerably increased by means of pressure dialysis. The sedimentation diagrams showed the presence of α , γ and some faster-sedimenting molecules in very low concentration. For the sedimentation constant the values were: for γ $s_{20} = 6.9 \cdot 10^{-13}$ and for α $s_{20} = 1.2 \cdot 10^{-13}$ (again a very low value), but this time the experimental conditions were favourable for determining s , since α was the predominant peak.

For the next experiments the skim milk was spun for 20 min. at high speed in the closed separator bowl. By this procedure a rather large amount of material collected at the periphery of the separator bowl. This "separator slime" was suspended in a solution of $0.2\text{ }M$ $\text{NaCl} + 0.02\text{ }M$ $\text{KH}_2\text{PO}_4 + 0.03\text{ }M$ Na_2HPO_4 and dialysed against the same buffer (Exp. M 12). The milk plasma was first dialysed against $0.4\text{ }M$ acetic acid + $0.07\text{ }M$ sodium acetate for a short time and then against a solution of $0.2\text{ }M$ $\text{NaCl} + 0.075\text{ }M$ sodium acetate + $0.025\text{ }M$ acetic acid. The caseinogen precipitated very well and could be easily washed with the same buffer solution; it was suspended afterwards in a solution of $0.2\text{ }M$ $\text{NaCl} + 0.02\text{ }M$ $\text{KH}_2\text{PO}_4 + 0.03\text{ }M$ Na_2HPO_4 and dialysed against the same buffer (Exps. M 14 and M 15).

Exp. M 12. The "separator slime". On dialysing the separator slime not all of the suspended material went into solution; its appearance after several days of continuous dialysis was still quite milky, even after it had been spun in an ordinary laboratory centrifuge at high speed. "Separator slime" evidently consists of what is usually called separator slime *plus* the more coarsely dispersed part of the Ca caseinogenate. The centrifugal field was 180,000 times gravity. The sedimentation diagram was of a very complex nature showing a main peak with a sedimentation constant of $13.1 \cdot 10^{-13}$ and a number of small peaks with sedimentation constants ranging from 19 to $35 \cdot 10^{-13}$ (see Fig. 3). Later exposures showed indications of a very slowly sedimenting substance (α ?). Evidently the main peak must be due to caseinogen (δ).

Fig. 3. Sedimentation diagram of "separator slime" + coarsely disperse part of Ca caseinogenate dissolved in phosphate buffer p_H 6.8. Centrifugal force: 180,000 times gravity. Cell thickness: 12 mm. Exposure taken 20 min. after reaching full speed. Abscissa, X in cm.; ordinate, Z in mm.

Exp. M 14. The caseinogen fraction. Concentration: 2.6%. Centrifugal field: 180,000 times gravity. The sedimentation diagram showed a single peak (besides indications in the last exposures of the sedimentation of α ?) having a sedimentation constant of $23 \cdot 10^{-13}$. The peak was quite unsymmetrical being much broader toward the top part of the cell and the apparent diffusion was much higher than would be expected for a molecule with such high sedimentation constant. It must be concluded that the substance is dissociating during sedimentation and producing new molecules with smaller sedimentation constants in the part of the boundary, where the concentration is lowest. No molecules with higher sedimentation constants than $23 \cdot 10^{-13}$ could be detected in the diagram (Fig. 4).

Exp. M 15. On caseinogen from the same solution as in M 14, but with a protein concentration $1/3$ of the concentration in M 14. Centrifugal field: 180,000 times gravity. The sedimentation picture from this experiment was entirely different from that of M 14 (compare Figs. 4 and 5, which were taken almost at the same time from the start of the centrifuge). In this experiment the main component showed a sedimentation constant of $11.1 \cdot 10^{-13}$, but besides this main

Fig. 4.

Fig. 5.

Fig. 4. Sedimentation diagram of a caseinogen fraction. Concentration: 2.6 %. Centrifugal force: 180,000 times gravity. Exposure taken 24 min. after reaching full speed. Abscissa, X in cm.; ordinate, Z in mm.

Fig. 5. Sedimentation diagram of the same caseinogen fraction as in Fig. 4 after dilution. Concentration: 0.9 %. Centrifugal force: 180,000 times gravity. Exposure taken 20 min. after reaching full speed. Abscissa, X in cm.; ordinate, Z in mm.

component the sedimentation diagram showed several different peaks corresponding to particles with sedimentation constants ranging from 24 to $36 \cdot 10^{-13}$. In the later exposures the peak became very broad and unsymmetrical thus indicating dissociation of the molecule.

DISCUSSION.

From the experiments just described some conclusions may be drawn. In the first experiments (M 1-4), where the suspended Ca caseinogenate was removed from the milk plasma by ultracentrifuging, it was found that practically the whole material was present as molecules α , β and γ , where α is a low-molecular protein isolated by Kekwick, β the lactoglobulin isolated by Palmer and γ corresponds probably to what is generally called lactoglobulin. It may be assumed that what was earlier called lactalbumin is a mixture consisting mainly of $\alpha + \beta$.

The results mentioned are very similar to the earlier results of Philippi. In his experiments however the caseinogen fraction was removed by isoelectric precipitation. Comparison of the two series of experiments shows that it makes little or no difference whether caseinogen is removed by isoelectric precipitation or by ultracentrifuging.

Since only the molecules α , β and γ could be found in the milk serum, we must suppose that the caseinogen is only present in the milk as very coarsely dispersed particles (Ca caseinogenate) and that the lactalbumin and lactoglobulin (α , β and γ) molecules present in the milk are small as compared with the smallest of the caseinogen particles.

The experiments carried out on the caseinogen fraction have shown that this protein is polydisperse and of a very complex nature; it was found that the

sedimentation constant (and therefore the dispersity) of the caseinogen was markedly dependent upon its concentration; s decreased with decreasing protein concentration. Svedberg *et al.* [1930, 1, 2] found in their studies on the Hammarsten and on the Van Slyke and Baker caseinogens a similar concentration effect on the sedimentation constant. The protein prepared by them according to both methods was polydisperse and the sedimentation constants found for this caseinogen were considerably lower than the values found in the present investigation. It is possible that the disagreement may be due to the different salt concentrations used, 0.017 M in the earlier work and 0.25 M in the present paper. Lundgren in Upsala found (results not yet published) that for a protein which dissociates by dilution, the dissociation is much more pronounced in dilute salt solutions than in the 0.2 M solution usually applied. In this work it has not been possible to identify in the caseinogen fractions a protein corresponding to the homogeneous one which Svedberg *et al.* isolated from the Hammarsten caseinogen by means of hot acidified 70 % alcohol.

From the few experiments done in this investigation it could not be ascertained whether the peak named α corresponded to one and always the same substance or whether it corresponded to two different substances.

The work is being continued.

SUMMARY.

1. A description has been given of a modified method for calculating sedimentation equilibrium concentrations from the refractive index data by the method of Lamm.

2. In some preliminary experiments on milk plasma it has been shown that the caseinogen is present in the milk only as a coarse polydisperse suspension.

3. When the caseinogen has been brought into solution in sodium and potassium phosphate buffers it is of a very complex nature, the degree of dispersity being dependent (amongst other factors) on the caseinogen concentration.

4. The milk serum was found to contain at least three different proteins: a substance of low mol. wt. isolated by Kekwick, the lactoglobulin of Palmer and what is usually called lactoglobulin.

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