

Fractionation of Casein by Distribution in a Liquid Two-Phase System

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The distribution of casein between a two-phase system of collidine, ethanol, and water has been studied. α -Casein was found to distribute in favour of the water-rich phase and β - and γ -casein in favour of the collidine-rich. Electrophoretically pure α -casein was prepared by extracting a neutralized casein solution with the collidine-rich phase of the collidine-ethanol-water system.

Until recently it has generally been assumed that casein is a pure protein, although there was some evidence to the contrary. Linderström-Lang¹ first demonstrated that casein was a mixture of several distinct proteins. Later on Mellander² showed that casein is composed of at least three components which he denoted α -, β -, and γ -casein in order of decreasing mobility.

It was apparent from previous attempts to fractionate casein that the separation of the different electrophoretic components would be difficult. Warner³ devised the first chemical method for the separation of α - and β -casein, a repeated isoelectric precipitation at low temperature. This technique was further developed by Cherbuliez and Baudet⁴. Hipp *et al.*⁵ separated the different electrophoretic fractions of casein by precipitation from 50 % alcohol by means of variations in temperature, pH, and ionic strength. Another method, developed by the same authors, was based on the solubility of the different casein components in aqueous urea. Walter⁶ succeeded in fractionating α - and β -casein between a liquid two-phase system containing phenol. This system was found to be highly specific to casein.

Fractionation of casein by means of distribution in a two-phase system containing collidine is studied in the present paper.

METHODS

Description of the three-component system of collidine-ethanol-water (C-E-W). A three-component system is usually described in a triangular diagram. The composition of any ternary system can be represented by a point in the diagram, when the side of the equi-

lateral triangle is taken as unity and the amounts of the three components are expressed as fractions of the whole. The corners of the triangle represent the pure component. The distance from one point to any side, measured parallel to either of the others, gives the proportion of the component occupying the opposite corner. Any point within the triangle represents three components, but a point situated on one of the sides indicates two components only.

To determine the diagram, 10 g of collidine were titrated in an Erlenmeyer flask with distilled water under thorough mixing to a permanent turbidity showing the presence of two phases. 1 ml of ethanol (96 %) was then added, and the titration was again carried out with water to slight turbidity. By repeating this procedure the ascending part of the curve could be constructed to its vertex. The descending part of the curve was determined in a similar way by titrating 10 g of distilled water with collidine and adding ethanol after the endpoint of the titration was shown by a slight turbidity. Any point outside the constructed curve represents one liquid layer only. Inside the curve two liquid layers are in equilibrium, the components being given by the appropriate points on the curve. One known concentration term allows the position of one layer to be fixed on the constructed curve and defines the system completely. In order to determine the composition of the layers an analysis of collidine was made.

Determination of collidine. Collidine was determined on the basis of the absorption at 280 μ , using a Beckman spectrophotometer. For the analysis the samples were diluted with distilled water. The amount of collidine was read from a curve constructed by diluting a known amount of collidine with distilled water.

Electrophoresis. Moving boundary electrophoresis was carried out with a Klett electrophoresis apparatus. The optical system was that of Philpot-Svensson. In the runs a 15 ml analytical cell was used. All samples were diluted with veronal buffer of pH 8.6 and of ionic strength 0.12 and dialyzed against 2 l of the same buffer for a week. In all experiments so much buffer was added to the samples as to make the total protein concentration 1.0 %. The runs were carried out with a potential gradient of 9 volt/cm at 4°C.

Since the area enclosed by the electrophoresis curve of the gradient and the basis line is proportional to the concentration of the gradient, the relative amounts of the different protein components can be determined by calculating their corresponding areas. This was done by enlarging the films on millimeter paper and drawing the diagrams on the paper. From the drawings the areas were easily determined by means of a planimeter.

Determination of the pH. The pH of the solutions was measured with a glass electrode (Beckman).

RESULTS AND DISCUSSION

Preliminary experiments were carried out with the phase-pair collidine-water. When casein solutions of different concentrations, neutralized to a pH of about 7, were mixed with an equal volume of collidine, stable milk-white emulsions were formed. It is well known that proteins act as emulsifying agents. In order to prevent the formation of these emulsions, some lower alcohols were tested as de-emulsifying agents. Methanol as well as ethanol were found to have strong de-emulsifying effects. A diagram of the three-component system C-E-W can be seen in Fig. 1. The phases obtained at both temperatures are extremely temperature-labile. A mere warming with the hand caused turbidity. In the following experiments the two-phase system was obtained by mixing the three components at 20°C in the following proportion: 30 ml of collidine, 16.5 ml of ethanol, and 60 ml of distilled water. The pH of the mixture was 9.5. The pH value as measured with a glass electrode is not exact because of the high content of organic solvents.

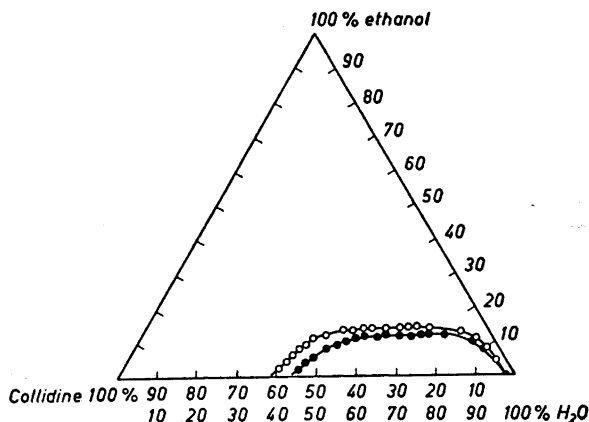


Fig. 1. Phase diagrams of the C-E-W system at 15°C (●) and at 20°C (○).

When the bottom and the top phase had separated completely, their composition was determined and found to be as follows:

Collidine phase (top phase)	Water phase (bottom phase)
41.9 % collidine	13.7 % collidine
46.1 % water	72.3 % water
12.0 % ethanol	14.0 % ethanol

It can be seen from Fig. 1 that these points are situated in the homogenous part of the diagram when the temperature is 15°C.

Acid Hammarsten's casein (Merck) was found to be easily soluble in the top phase without any neutralizing. When in a two-phase system in equilibrium different columns of the top phase were replaced by the corresponding volume of the top phase containing casein, and the two phases were mixed, no separation in two phases occurred, but the solution was completely clear and homogeneous. When, however, the casein in the top phase was neutralized with NaOH, dissolved in the top phase, a separation again occurred. It is evident that casein neutralized by collidine functioned as a homogenizing agent in the liquid two-phase system. In order to avoid this, the casein solutions in the following experiments were neutralized to a slight alkalinity.

Fractionation of casein in the C-E-W system. 1 g of Hammarsten's casein (Merck) was dissolved in water by neutralizing it to pH 8.7 and made up to 60 ml. 30 ml of collidine and 16.5 ml of ethanol were added to this solution, well mixed and kept at room temperature (20°C) until both phases had separated completely. Samples of the two phases were taken and evaporated to dryness *in vacuo*, the temperature of the bath not rising over 45°C. Both these dried fractions were dissolved in veronal buffer, dialyzed against the same buffer and analyzed by electrophoresis. The diagrams of the ascending limbs of the different preparations can be seen in Figs. 2—4.

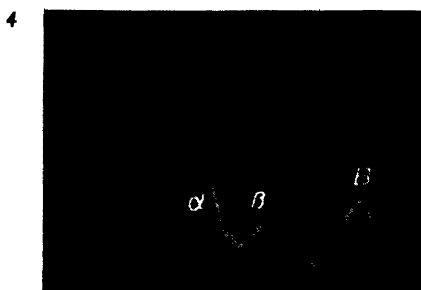
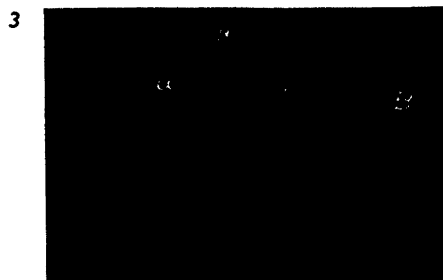
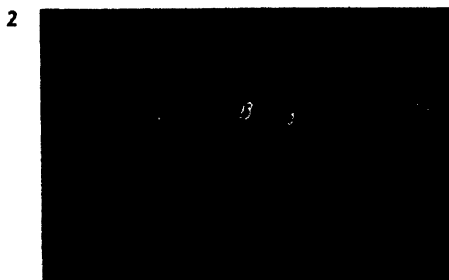


Fig. 2. Electrophoresis diagram of Hammarsten's casein.

Fig. 3. Electrophoresis diagram of casein isolated from the collidine-rich phase after distribution in the C-E-W system.

Fig. 4. Electrophoresis diagram of casein isolated from the water-rich phase after distribution in the C-E-W system.

(Ascending boundaries, pH 8.6, $\mu = 0.12$, $F = 9$ Volt/cm, $t = 90-120$ min, α , β , and γ denote the different casein fractions and B the buffer peak.)

It is apparent from these diagrams that α -, β -, and γ -casein vary considerably, and that a distinct enrichment of the β -component occurs in the collidine phase after only one distribution. By quantitative evaluation of the electrophoretic diagrams the following values were obtained for α -, β -, and γ -casein (Table 1). The diagrams of the ascending limbs were used in the calculations, because it was found that the ascending limbs showed values for β -casein more closely to the real value of 25 % than those of the descending limbs. Nitschmann and Zürcher⁷ have shown that the concentration of β -casein, shown in electrophoresis diagrams of descending limbs, decreases, continuously with increasing casein concentration, a fact which is due to a formation of a reversible complex or association between α - and β -casein. At infinite dilution the complex was found to be completely dissociated, the amount of

Table 1. Distribution of α -, β -, and γ -casein in the C-E-W system at 20°C.

Fraction	Relative amount present in %		
	α -	β -	γ -casein
Hammarsten's casein	76	22	2
Collidine phase	46	37	17
Water phase	91.5	8.5	—

Table 2. Concentrations and distribution constants of α - and β -casein in the C-E-W system at 20°C.

Fraction	Collidine phase c_{coll} %	Water phase c_w %	Distribution factor $K = c_w/c_{\text{coll}}$
α -Casein	0.294	1.35	$K_\alpha = 4.6$
β -Casein	0.236	0.117	$K_\beta = 0.5$

β -casein being 25 %, whereas in a 1 % casein solution the amount of β -casein was 18 % and in a 2 % solution only 14 %.

In order to evaluate the distribution constants of α - and β -casein, the protein concentrations of the two phases were determined on a dry weight basis. It was found that the protein concentration of the collidine-rich phase was 0.64 % and that of the water-rich phase 1.48 %. Compared with the amount of casein added, the sum of the protein concentrations in the two phases was a little too high, but, owing to the semiquantitative nature of the calculation, it was assumed to be satisfactory. The α - and β -casein concentrations of the two phases, calculated from the electrophoretic diagrams, and the distribution constants of the two casein fractions in the C-E-W system can be seen in Table 2. The distribution coefficient of α -casein is high compared with that of β -casein. The separation factor, β , defined as the ratio of the partition coefficients, was found to be:

$$\beta = K_\beta/K_\alpha = 9.2$$

Preparation of electrophoretically pure α -casein. On the basis of the above findings electrophoretically pure α -casein was prepared in the following way: 1 g of casein was dissolved in 60 ml as earlier, and the same amounts of collidine and ethanol were added, mixed and kept at room temperature until both phases separated completely, after which the top phase was carefully removed. The remaining bottom phase was now treated three times with fresh top phase (50 ml each time), the top phase being removed after each treatment. At the end of the experiment the bottom phase was evaporated to dryness

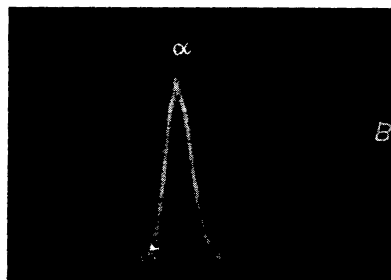


Fig. 5. Electrophoresis diagram of the bottom fraction from a four step distribution of casein in the C-E-W system at 20°C. (Conditions as in Figs. 2-4).

in vacuo, dissolved in veronal buffer as earlier and analyzed by electrophoresis in the usual way. The diagrams of the ascending and descending limbs can be seen in Fig. 5. Since both boundaries are completely symmetric it is assumed that the concentration of β -casein is very low. It is, however, to be assumed that the tendency for complex formation between α - and β -casein is greater at a low β -casein concentration when the concentration of α -casein is high.

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