

1369

## The Separation of the Components of $\alpha$ -Casein. I. The Preparation of $\alpha_1$ -Casein

T. L. McMeekin, N. J. Hipp and M. L. Groves

*From the Animal Proteins Pioneering Laboratory, Eastern Regional  
Research Laboratory,<sup>1</sup> Philadelphia, Pennsylvania*

Received January 30, 1959

### INTRODUCTION

Casein was shown by Mellander (1) to be composed of three electrophoretic components, which he designated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -casein in the order of their decreasing mobilities in alkaline solutions. Warner (2) devised the first chemical method for separating  $\alpha$ -casein and  $\beta$ -casein and demonstrated that the separated components were heterogeneous as determined by electrophoresis in acid solutions. Waugh and von Hippel (3) described the separation of casein from milk by high-speed centrifugation and its fractionation with calcium chloride. They designated the purified fraction, insoluble in calcium chloride,  $\alpha_s$ -casein, and the purified fractions, soluble in calcium chloride, as  $\kappa$ -casein and  $m$ -casein. Long, Van Winkle, and Gould (4) have separated a casein fraction, soluble in calcium chloride, from  $\alpha$ -casein which they designated  $\lambda$ -casein.

The purpose of the present paper is to describe an improved method of preparing  $\alpha$ -casein and the separation and characterization of its principal component as shown by electrophoresis in acid solutions, designated  $\alpha_1$ -casein because it is the fastest moving component under these conditions.

### EXPERIMENTAL

#### *Preparation of $\alpha$ -Casein*

$\alpha$ -Casein was prepared by a modification of the method described by Warner (2). In the present method,  $\alpha$ -casein was precipitated by adding acid to an alkaline solution of casein at 4° instead of adding alkali to an acid solution of casein as described by Warner.

The casein from 15 gal. of fresh unpasteurized skim milk was precipitated at 30°C. by the addition of 1 *N* HCl to pH 4.6. After filtration through a cloth bag, the casein was washed with distilled water by suspending in a volume of 20 gal. The supernatant

<sup>1</sup> Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

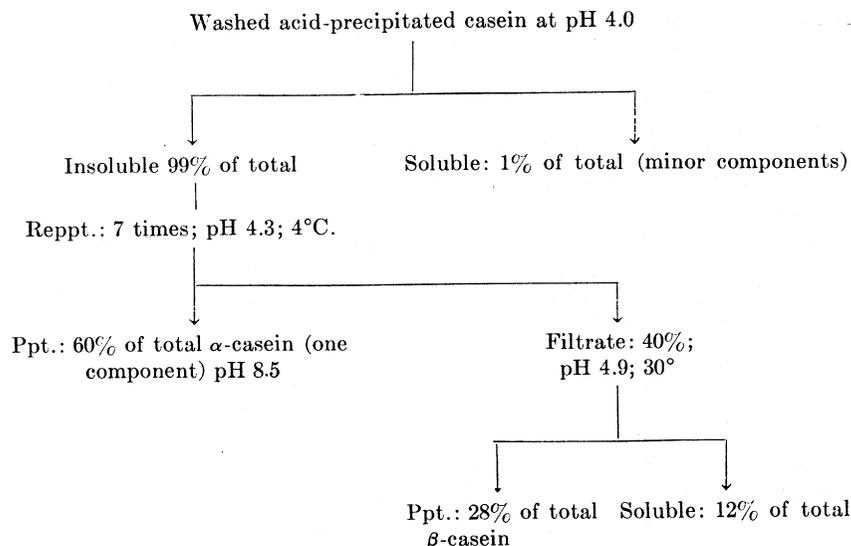


FIG. 1. Preparation of  $\alpha$ -casein.

was removed by decantation. Resuspension of the acid-precipitated casein in distilled water was always accompanied by a drop in the pH which was adjusted to 4.7. The drop in the pH decreases on successive washings, and five washings are usually required before a negligible change in pH is obtained.

The washed casein (Fig. 1) was then suspended in 16 l. water, and the pH was adjusted to 4.0 with 1 *N* acetic acid. After several hours of vigorous stirring, the supernatant was removed by filtration on a large Büchner funnel. The material extracted at pH 4.0, amounting to 1% of the total, containing phosphatase, proteolytic enzyme, red protein, and another casein component, will be described elsewhere.

In the procedure for the preparation of  $\alpha$ -casein described below, every effort was made to isolate all of the  $\alpha$ -casein in casein. One hundred and seventy-two grams acid-extracted casein was dissolved in about 3 l. water at room temperature with sufficient sodium hydroxide to give a pH of about 7.0. The casein solution was then divided equally between two jars in the cold room each containing 44 l. of cold water. Then 0.1 *N* hydrochloric acid was added slowly with stirring to each jar until a pH of 4.3–4.4 was reached. The final volume in this and subsequent reprecipitations was 92 l. After standing overnight at 4°, the precipitate was removed by filtration through a No. 12 Whatman fluted filter paper in the cold room and then dissolved with sodium hydroxide, and the above procedure was repeated. In this case, seven precipitations were required to remove the  $\beta$ -casein completely, which was demonstrated by electrophoresis in Veronal buffer at pH 8.5. After the last precipitation, the  $\alpha$ -casein was dissolved with sodium hydroxide, and the suspended filter paper was removed by centrifugation and filtration. The  $\alpha$ -casein was then precipitated with hydrochloric acid at pH 4.7 and washed free of chloride ion by decantation and centrifugation. All fractions were dried by lyophilization rather than with organic solvents. A yield of 100 g.  $\alpha$ -casein was obtained. The filtrates containing the extracted  $\beta$ -casein fraction were warmed to about 30°, and the pH was adjusted to 4.9, requiring about 15–35 ml. of

0.6% *N* sodium hydroxide/10 l. Usually the  $\beta$ -casein precipitate does not coagulate at this point due to the low salt concentration. A small amount of sodium chloride was added to coagulate the precipitate. After filtering, the precipitate was removed from the filter paper with dilute alkali and then reprecipitated at pH 4.9 with hydrochloric acid. The product was washed free of chloride ions and lyophilized. An outline of the procedure for the preparation of  $\alpha$ -casein is given in Fig. 1, and a summary is given in Table I showing the amount of protein at each stage. The protein content of the filtrates shown in col. 3 was calculated from the *N* content of an aliquot using the usual factor of 6.25 for the conversion to protein. The amount of protein in the filtrate after each precipitation decreased gradually until the sixth precipitation when the amount of protein extracted is greatly reduced. The amount of protein accounted for on the basis of the isolated  $\alpha$ -casein (100 g.) and the amount extracted as determined by the *N* content of the filtrates of 98.4% is good considering the number of operations and volumes involved. In view of the solubility data reported by Hipp *et al.* (5), the loss of a total of 20.7 g. in the recovery of the extracted protein can be due largely to the solubility of  $\beta$ - and  $\gamma$ -casein. Electrophoretic experiments, run on the two main extracts of 21 and 25 g., showed that they were essentially  $\beta$ -casein and were remarkably free of  $\alpha$ -casein. The presence of small amounts of  $\alpha$ -casein could be detected after electrophoresis for 1 hr. but not after 3 hr. at pH 8.5. Whether this efficiency in the separation of  $\beta$ -casein from  $\alpha$ -casein is due to the method used for the precipitation of  $\alpha$ -casein in the cold room or is due to the extraction of the casein with acetic acid at pH 4.0 is not known. However, the removal of proteolytic enzyme and other minor protein components by the extraction procedure with acetic acid before fractionation is obviously desirable. As expected,  $\gamma$ -casein was found to be present in the first fraction (21 g., Table I) by electrophoresis.

TABLE I  
Preparation of  $\alpha$ -Casein

Precipitation No. (1)	Protein in precipitate <sup>a</sup> (2)	Protein in filtrates		Not recovered Col. (5) (3) - (4)
		Calcd. from N content <sup>c</sup> (3)	Recovered (4)	
0	172			
1, 2	144.4	27.6	21.0	6.6
3, 4, 5	108.7	35.7	25.0	10.7
6	104.8	3.9	2.2	1.7
7	102.7	2.1	0.4	1.7 <sup>d</sup>
Total	<sup>b</sup>	69.3	48.6	20.7

<sup>a</sup> Values were obtained by difference.

<sup>b</sup> One hundred grams of dry  $\alpha$ -casein was obtained.

<sup>c</sup> Total protein calculated from the *N* content of each filtrate.

<sup>d</sup> The protein content in the filtrate (92 l.) at room temperature was 1.4 g. calculated from the *N* content of an aliquot and is equivalent to only 0.0015% protein which does not give a precipitate with trichloroacetic acid.

The yield of 100 g. of  $\alpha$ -casein from 172 g. of casein, amounting to 58%  $\alpha$ -casein in whole casein is in good agreement with the value of 60% recently reported by MacRae and Baker (6) who determined quantitatively the components of casein by a method based on paper electrophoresis and dye combining capacities. Values reported previously from this laboratory (7, 2) of 75 and 80% were obtained from electrophoretic data and as shown by Warner (2), such values are subject to error due to interaction of  $\alpha$ - and  $\beta$ -casein.

### *Preparation of $\alpha_1$ -Casein*

$\alpha$ -Casein prepared as described gives only one electrophoretic component in Veronal at pH 8.5; however, at pH 2.35, it gives at least three electrophoretic components as shown in Fig. 2a.  $\alpha$ -Casein was dissolved with a minimum amount of 0.1 *N* sodium hydroxide to give a 2% solution. The solution was then made to 0.20 *M* CaCl<sub>2</sub> and pH 7.0 by the slow addition with stirring of 1 *M* CaCl<sub>2</sub> and 0.1 *N* sodium hydroxide. The solution was kept at room temperature for 2 hr. and then placed in the cold room overnight. After warming to room temperature, the precipitate was removed by centrifugation at 3500  $\times g$  for 20 min. Calcium ion was removed from the products with potassium oxalate or more conveniently by dialysis from the acid side of the isoelectric point which will be described later. The electrophoretic patterns of the calcium-insoluble and calcium-soluble fractions of  $\alpha$ -casein at pH 2.35 are shown in Figs. 2b and 2c.

Electrophoretically homogenous preparations of  $\alpha_1$ -casein were prepared by reprecipitation with 0.1 *M* CaCl<sub>2</sub> at 37°. However, such preparations contained 1.1% phosphorus, and it was demonstrated that extraction of the calcium-insoluble fraction with 0.4 *M* ammonium sulfate removed a soluble fraction containing a greater amount of phosphorus than the insoluble portion. Therefore, the calcium-insoluble material was next extracted four times with 0.4 *M* ammonium sulfate. For the first extraction, the precipitate was suspended in 0.4 *M* ammonium sulfate to give a concentration of about 4% protein, and the pH was adjusted to 6.0 with 0.1 *N* sodium hydroxide. Sufficient alkali must be used to maintain a pH of 6.0 after stirring for about 15 min., and then an equivalent volume of 0.8 *M* ammonium sulfate is added to maintain the molarity at 0.4. After 1–2 hr. of stirring, the oily insoluble material is sedimented by centrifugation and the supernatant is decanted. The next three extracts are made by suspending the precipitate in the same volume of 0.4 *M* ammonium sulfate used previously or by dissolving in water and then making to 0.4 *M* with a concentrated ammonium sulfate solution. The final precipitation of  $\alpha_1$ -casein was made from a 0.2 *M* ammonium sulfate solution. This was done by adding an equal volume of 0.4 *M* ammonium sulfate at pH 6 to a 2% solution of the precipitate at pH 7 giving a final pH of 6.5. The precipitated  $\alpha_1$ -casein was removed by centrifugation. The electrophoretic pattern of  $\alpha_1$ -casein at pH 2.35 is shown in Fig. 2d, and its composition is given in Table II.

An outline of the procedure showing the yields and phosphorus content of the fraction are given in Fig. 3. The changes in the phosphorus content were used to follow the course of the fraction. The values are relative since they were based on air-dried samples.

In Table III, the mobilities and approximate amounts of components of  $\alpha$ -casein fractions are shown. Variations in mobilities of the components are probably the result of interactions. The amount of the minor components in  $\alpha$ -casein agree reasonably well with the portion soluble in calcium chloride as shown in Fig. 3, but the resolution of these components in Fig. 2c is not reflected in Fig. 2a.

The isolation of the salt-free products was best accomplished in a simple and satis-

COMPONENTS OF  $\alpha$ -CASEIN. I

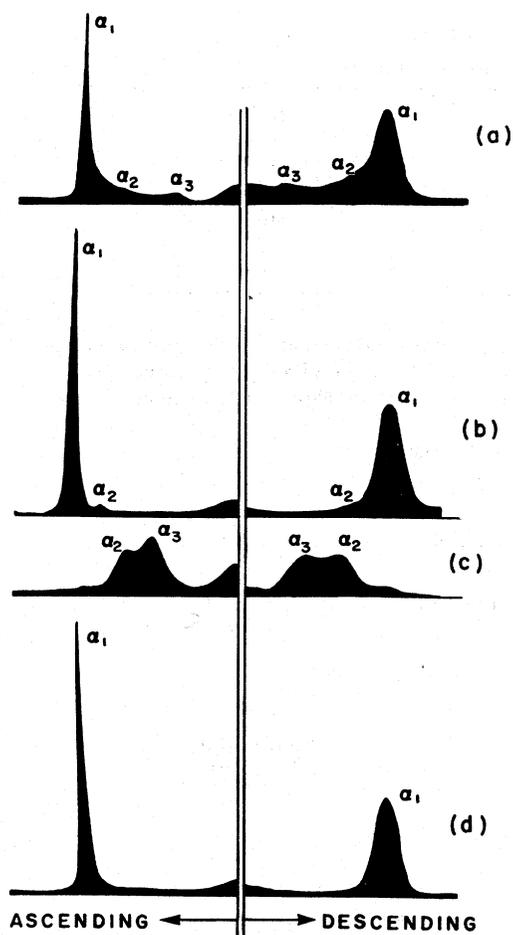


FIG. 2. Electrophoretic patterns obtained in HCl-NaCl buffer at pH 2.35 with an ionic strength of 0.1, protein concentration about 1% at a field strength of 4.70-4.92 v/cm. after electrophoresis for 3 hr. Casein solutions were prepared by first dissolving in alkali and then acidifying and dialyzing against the buffer. (a)  $\alpha$ -casein. (b)  $\alpha$ -casein fraction insoluble in 0.2 M  $\text{CaCl}_2$ . (c)  $\alpha$ -casein fraction soluble in 0.2 M  $\text{CaCl}_2$ . (d)  $\alpha_1$ -casein.

factory manner by adjusting the pH to about 3.6 with hydrochloric acid and dialyzing until tests show that the supernatant is free of the ion to be removed. The dialysis is usually complete within 24 hr., and the pH of the solution will rise to the point of minimum solubility. The precipitate was then removed by centrifugation and lyophilized.

DISCUSSION

The partial separation of  $\alpha$ -casein was reported by Cherbuliez and Baudet (8) by fractionation with ammonium sulfate at pH 6. The fraction soluble

TABLE II  
Composition<sup>a</sup> of  $\alpha_1$ -Casein<sup>d</sup>

	%
N	14.1
P	0.85
S	1.10
Ash <sup>b</sup>	2.35
True ash <sup>c</sup>	0.40

<sup>a</sup> On a moisture-free basis.

<sup>b</sup> Determined after adding magnesium acetate and corrected for magnesium oxide.

<sup>c</sup> Attributed to inorganic contamination. The value given was obtained by subtracting the theoretical ash of phosphorus pentoxide formed from the casein from the determined ash.

<sup>d</sup> No carbohydrate could be detected by the Schiff test.

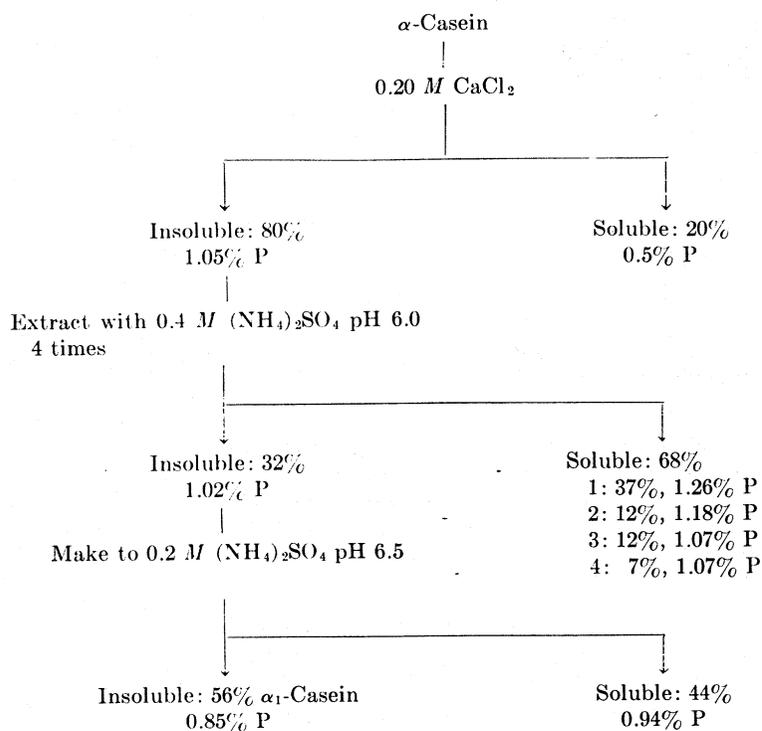


FIG. 3. Preparation of  $\alpha_1$ -casein.

TABLE III  
 Mobilities of  $\alpha$ -Casein Components at pH 2.35<sup>a</sup>

$\alpha$ -Casein	2.1 (11)	5.2 (15)	6.8 (74)
$\alpha$ -Casein, insoluble in CaCl <sub>2</sub>	—	5.4 (10)	7.3 (90)
$\alpha$ -Casein, soluble in CaCl <sub>2</sub>	3.1 (42)	4.6 (45)	7.0 (3)
$\alpha_1$ -Casein	—	—	6.9 (100)

<sup>a</sup> In HCl-NaCl buffer ionic strength 0.1 as calculated from the descending electrophoretic patterns shown in Fig. 2. Values in parentheses are approximate amounts of each component as calculated from the electrophoretic patterns of the descending boundary.

in ammonium sulfate, designated by them  $\alpha_1$ -casein, contained a greater amount of phosphorus than the insoluble portion called  $\alpha_2$ -casein. No electrophoretic results were reported on these fractions; consequently, the designation of the fractions was arbitrary. We have designated the principal component of the  $\alpha$ -casein fraction which has the greatest mobility at pH 2.3,  $\alpha_1$ -casein (Fig. 2*d*), which appears to be the reverse of the nomenclature used by Cherbuliez and Baudet. The use of ammonium sulfate at pH 6 as described was effective in obtaining an  $\alpha_1$ -casein preparation of constant phosphorus value; however, the yield obtained was low. This raises the question whether the product obtained by exhaustive fractionation represents the major component of  $\alpha$ -casein as shown by the electrophoresis in acid. It was demonstrated by refractionation and electrophoretic experiments that the material soluble in ammonium sulfate was largely  $\alpha_1$ -casein. A small amount of a component with a high phosphorus content was isolated from the fraction soluble in ammonium sulfate which accounts for the greater phosphorus content of this casein fraction.

Waugh (3*c*) used calcium chloride to fractionate casein to obtain a fraction designated  $\alpha_s$ -casein which is stated to be about 95% pure. The properties of  $\alpha_1$ -casein are similar to  $\alpha_s$ -casein in that both are easily coagulated by small amounts of calcium ions and are stabilized to calcium ions by the calcium-soluble fraction; however, the phosphorus content of  $\alpha_1$ -casein is only 0.85% as compared to 1.1% reported for  $\alpha_s$ -casein.  $\alpha_s$ -Casein is reported to have a sedimentation  $S_{20, w} = 1.59$ , though the pH of the solution is not stated. The sedimentation constant of  $\alpha_1$ -casein was found to vary greatly with pH; consequently, no comparison of the sedimentation data on these caseins is possible at present. Long *et al.* (4) reported a sedimentation constant of 4.5 at pH 7 and a phosphorus content of 1.1% for the fraction of  $\alpha$ -casein which is insoluble in calcium chloride differing in these respects from  $\alpha_1$ -casein.

It is of interest to compare the sedimentation data on  $\alpha_1$ -casein with that reported by Sullivan *et al.* (9) on unfractionated  $\alpha$ -casein. They report

a value of  $S_{20} = 3.99$  for  $\alpha$ -casein at pH 7.8 in Veronal, and in phosphate at pH 7 the presence of a major and two minor components. As shown in Fig. 4,  $\alpha_1$ -casein, in phosphate at pH 7.1, has only one component with a value of  $S_{20} = 3.0$ . A further point of interest is that temperature does not affect the sedimentation value of  $\alpha_1$ -casein at pH 7.1 or 2.35. However, at pH 2.35 there is a marked aggregation at both temperatures studied as compared to the result obtained at pH 7.1.

The electrophoretic data as shown in Fig. 5 suggest that the isoelectric point of  $\alpha_1$ -casein is between pH 4.3 and 4.7. Its insolubility between pH 3.0 and 5.7 prevents the determination of this point more accurately. The

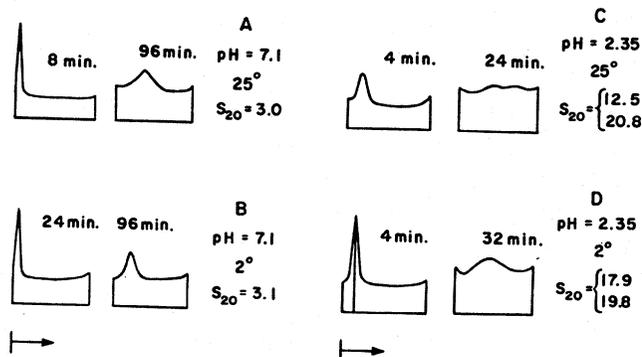


FIG. 4. Sedimentation patterns of solutions of  $\alpha_1$ -casein, ionic strength 0.1, 59,780 r.p.m. ( $259,700 \times g$ ): pH 7.1 phosphate buffer; A at  $25^\circ$ , B at  $2^\circ$ ; pH 2.35 HCl-NaCl buffer: C at  $25^\circ$ , D at  $2^\circ$ .

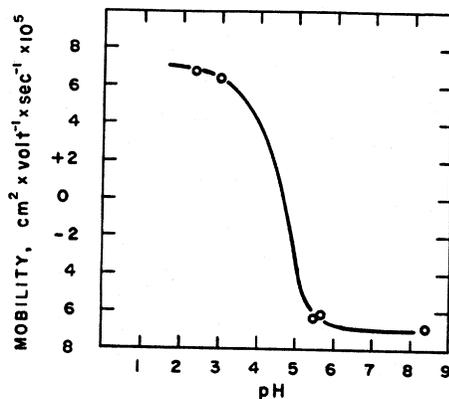


FIG. 5. Effect of pH on mobility of  $\alpha_1$ -casein at  $1.0^\circ$  at an ionic strength of 0.1 at a protein concentration of about 1.0% except at pH 5.5. Veronal buffer was used at pH 8.45, sodium acetate at pH's 5.7 and 5.5 (maximum solubility 0.5%), and HCl-NaCl at pH's 3.0 and 2.35.

lack of change in net charge as indicated by the mobility curve from pH 5.5 to 8.5 is unexpected and is in contrast with the titration data reported by Hipp *et al.* (10) for unfractionated  $\alpha$ -casein.

#### SUMMARY

An improved method is described for the separation of the  $\alpha$ -casein fraction from unfractionated casein. The principal component of the  $\alpha$ -casein fraction as shown by electrophoresis in acid solutions, designated  $\alpha_1$ -casein, has been prepared in pure form as shown by electrophoresis.  $\alpha_1$ -Casein contains 0.85% phosphorus and 1.1% sulfur. At 25°, it has a sedimentation constant of 3.0 at pH 7.1; at pH 2.3 it has two components with sedimentation constants of 12 and 21.

#### REFERENCES

1. MELLANDER, O., *Biochem. Z.* **300**, 240 (1939).
2. WARNER, R. C., *J. Am. Chem. Soc.* **66**, 1725 (1944).
- 3a. VON HIPPEL, P. H., AND WAUGH, D. F., *J. Am. Chem. Soc.* **77**, 4311 (1955).
- 3b. WAUGH, D. F., AND VON HIPPEL, P. H., *J. Am. Chem. Soc.* **78**, 4576 (1956).
- 3c. WAUGH, D. F., *Discussions Faraday Soc. No.* **25**, 186 (1958).
4. LONG, J., VAN WINKLE, Q., AND GOULD, I. A., *J. Dairy Sci.* **41**, 317 (1958).
5. HIPPI, N. J., GROVES, M. L., CUSTER, J. H., AND McMEEKIN, T. L., *J. Am. Chem. Soc.* **72**, 4928 (1950).
6. MACRAE, H. F., AND BAKER, B. E., *J. Dairy Sci.* **41**, 233 (1958).
7. HIPPI, N. J., GROVES, M. L., CUSTER, J. H., AND McMEEKIN, T. L., *J. Dairy Sci.* **35**, 272 (1952).
8. CHERBULIEZ, E., AND BAUDET, P., *Helv. Chim. Acta* **33**, 398 (1950).
9. SULLIVAN, R. A., FITZPATRICK, M. M., STANTON, E. K., ANNINO, R., KISSEL, G., AND PALERMITI, F., *Arch. Biochem. Biophys.* **55**, 455 (1955).
10. HIPPI, N. J., GROVES, M. L., AND McMEEKIN, T. L., *J. Am. Chem. Soc.* **74**, 4822 (1952).