CHROM. 4190

CHROMATOGRAPHY AND CHARACTERIZATION OF γ-CASEIN*

KAMALA K. TRIPATHI** AND CHARLES W. GEHRKE***
University of Missouri, Columbia, Mo. 65201 (U.S.A.)

(Received May 27th, 1969)

SUMMARY

A method for the isolation of γ-casein has been developed. The method involves the separation of γ-casein from chemically enriched β- and K-caseins by soft gradient DEAE-cellulose column chromatography. Both of these γ-caseins were eluted by an 0.03 *M* glycine–sodium hydroxide 2 *M* urea buffer system at pH 8.0 using a gradient of 0.05 to 0.30 *M* in sodium chloride. The γ-casein from β-casein was characterized by polyacrylamide gel-urea electrophoresis (PGUE), and contains three major component bands and some minor bands. The isolate from K-casein contains one major component band and some minor bands. The γ-casein from β-casein showed two peaks in 0.076 *M* Tris–citrate buffer pH 8.6, having a sedimentation coefficient of the slow moving peak of 1.35 S and of the fast moving peak of 23.20 S. The γ-casein from K-casein showed a single sharp peak having a sedimentation coefficient of 0.82 S. A remarkable difference was found in the amino acid composition of the γ- isolated from β- as compared to the γ- isolated from K-casein.

INTRODUCTION

The heterogenity and complexity of casein has been known for some time. MELLANDER[1] was first to show by means of free boundary electrophoresis that whole casein separates into three components. He named these α-, β-, and γ-casein in decreasing order of mobility. With the advancements in chromatographic techniques and high resolving power of gel zone electrophoresis, the heterogenity of casein has been studied in greater detail. In recent years the major caseins α_{s1}-, β-, and K- have been investigated and well characterized.

HIPP *et al.*[2] separated γ-casein from isoelectric casein. The method described for the separation of γ-casein was based in part on its solubility at room temperature in a 50% alcohol solution having a pH of 5.7 and containing low concentrations of

* Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 5264. Approved by the Director. Supported in part by funds from the National Institutes of Health, Grant No. GM 08525-06.

** Experimental data taken in part from Ph. D. thesis, the University of Missouri, 1968.

*** Professor, Experiment Station Chemical Laboratories.

salt. Under these conditions, α- and β-casein were insoluble. Later the same authors[3] described two methods for the fractionation of γ-casein from isoelectric casein. In the first method, the separation of α-, β-, and γ-casein was accomplished by precipitation of α- and β-casein from 50% alcohol solutions of casein by means of variations in temperature, pH, and ionic strength, and by isoelectric precipitation from water. The second method was based on the solubility of the casein components in aqueous urea at various concentrations. The urea method was found to be relatively simple and gave products with the same composition as those obtained by the pH and the 50% alcohol methods.

WAKE AND BALDWIN[4] analyzed by zone electrophoresis in 7 *M* urea the γ-casein fraction prepared by the alcohol method of HIPP *et al.*[3]. They found five slow moving bands, which they considered as probably related to γ-casein. NEELIN *et al.*[5] also investigated by starch gel electrophoresis the γ-casein isolated by HIPP *et al.* using the urea method and reported one and two zones for γ-casein at pH 7.0 and 8.0, respectively. GROVES *et al.*[6] described the isolation of β- and γ-casein by DEAE-cellulose column chromatography using a phosphate buffer of pH 8.3. They reported that γ-casein contained one homogeneous band obtained from eluate fraction II. Some minor protein components found in the first eluate fraction were temperature

SCHEME I

CHEMICAL FRACTIONATION OF β- AND γ-CASEIN

Isoelectric casein (15 g) from pooled milk, twice precipitated at pH 4.6 and washed

1 | Disperse in 400 ml H_2O. Dissolve in 6.6 *M* urea, dilute to 4.6 *M* urea. Allow to stand for 8 h at 2°, pH 4.6. Centrifuge at 3000 r.p.m. for 30 min.

Precipitate (α_{s1}-K-casein gel). Use for K-casein preparation.

Supernatant (β- and γ-casein)

2 | Dilute to 3.3 *M* urea. Hold at 2° for 8 h, decant.

Precipitate (α_{s1}-K-casein gel).

Supernatant (β- and γ-casein)

3 | Dilute to 1.7 *M* urea. Add solid $(NH_4)_2SO_4$ to 1.6 *M*. Centrifuge at 2500 × *g* for 15 min, or allow to stand for 6–8 h at 2°.

Supernatant traces of α_{s1}- and *K*-casein

Precipitate (β- and γ-casein)

4 | Disperse in enough 4.6 *M* urea to make about a 3% (w/v) protein solution. Hold for 2 h at 2°, decant.

Precipitate (α_{s1}-K-casein). Discard.

Supernatant (β- and γ-casein)

5 | Repeat Step 2

Supernatant (β- and γ-casein)

Precipitate (α_{s1}-K-casein). Discard.

6 | Repeat step 3

Precipitate (β- and γ-casein)

Supernatant. Discard.

7 | Dissolve in 0.1 *N* NaOH to make *ca.* 3% (w/v) protein solution, pH <8.0.

Solution (β- and γ-casein)

8 | Dialyze *vs.* H_2O at 2°, using 4 changes at 4–8 h each.

Solution chemically enriched β- and γ-casein
Follow with DEAE-cellulose chromatography for the isolation of γ-casein.

SCHEME II

CHEMICAL FRACTIONATION OF K- AND γ-CASEIN

Isoelectric casein (15 g) from pooled milk, twice precipitated at pH 4.6 and washed.

1 | Disperse in 400 ml H_2O. Dissolve in 6.6 *M* urea, dilute to 4.6 *M* urea. Allow to stand for 8 h at 2°, pH 4.6. Centrifuge at 3000 r.p.m. for 30 min.

Supernatant (β- and γ-casein). Use for preparation of β- and γ-casein.

Precipitate (α_{s_1}-K-casein gel)

2 | Repeat step I.

Supernatant. Discard.

Precipitate (α_{s_1}-K-casein gel)

3 | Disperse in 250 ml H_2O. Add 0.1 *N* NaOH dropwise with stirring to pH 8.5. Adjust pH to 7.0 with 0.1 *N* HCl. Make 0.25 *M* in $CaCl_2$ with 3 *M* $CaCl_2$. Maintain at pH 7.0 with 0.1 *N* NaOH. Centrifuge at 3000 r.p.m. for 30 min.

Precipitate (Ca-α_{s_1}-caseinate). Use for α_{s_1}-casein preparation

Supernatant (K- and γ-casein)

4 | Dialyze, lyophilize and repeat step 3.

Precipitate. Discard.

Supernatant (K- and γ-casein)

5 | Centrifuge at 30,000 r.p.m. for 30 min.

Precipitate. Discard (Ca-α_{s_1}) caseinate.

Supernatant(K- and γ-casein)

6 | Dialyze *vs.* H_2O at 2° using 4 changes at 4–8 h each.

Chemically enriched K- and γ-casein.
Follow with DEAE-cellulose chromatography for isolation of γ-casein.

sensitive. A solution of this protein was clear at 2°, pH 8.0, but on warming at 25°, it precipitated. THOMPSON[7], using DEAE-cellulose urea chromatography of casein in the presence of 2-mercaptoethanol, reported that the first eluate fraction contained several casein components; the most concentrated one was similar in behavior to the temperature-sensitive casein, and eluate fraction II was found to be γ-casein as reported by GROVES[6]. Thus, to this time, considerable confusion exists in the literature regarding the γ-casein fraction and its components.

This paper reports on the isolation of γ-casein from chemically enriched β- and K-casein fractions on DEAE-cellulose, characterization by polyacrylamide gel-urea electrophoresis (PGUE), amino acid, and sedimentation analysis.

EXPERIMENTAL

Chemical preparation of β- and K-casein

The β-casein was isolated from acid-precipitated whole casein (pI) from pooled milk by the urea–ammonium sulphate method of GEHRKE *et al.*[8] with the modifications given in Scheme I. The method for the preparation of K-casein is the procedure routinely used in our laboratory and described in Scheme II.

DEAE-cellulose column chromatography

DEAE-cellulose with 0.70 mequiv./g exchange capacity was obtained from Bio-Rad Laboratories. The anion exchanger was regenerated with 0.5 *N* NaOH,

TABLE I

STEPWISE ELUTION SCHEDULE FOR COLUMN CHROMATOGRAPHIC FRACTIONATION OF ENRICHED β- AND γ-CASEIN ON DEAE-CELLULOSE

Step	*Volume of eluent (ml)*	*Molarity of NaCl in eluent*[a]
1	500	0.00
2	1300	0.05
3	1500	0.10
4	1500	0.15
5	1250	0.20
6	1000	0.25
7	500	0.30
8	1000	0.50 *N* NaOH[b]

[a] All eluents were 0.03 *M* glycine–NaOH buffer containing 2 *M* urea of pH 8.0.
[b] The NaOH eluate fraction was not collected.

0.5 *N* HCl, then 0.5 *N* NaOH, with thorough washing with distilled water after each regeneration cycle. Two columns were prepared with beds of 6.5 × 25 cm, and 4.5 × 25 cm, for the β- and K-casein isolation, respectively. The columns were equilibrated with 2 l of 0.03 *M* glycine–NaOH buffer containing 2 *M* urea, pH 8.0, at a flow rate of 300 ml/h, and 250 ml/h, respectively.

Five grams and 1.5 g of chemically prepared β- and K-casein were separately dissolved and made 1% w/v in solution in 0.03 *M* glycine NaOH, 2 *M* urea, pH 8.0 buffer and placed on the columns. A soft gradient stepwise elution was accomplished with successively higher concentration of NaCl in the buffer following the schedules given in Tables I and II. The effluent was continuously monitored and recorded by a Vanguard automatic UV analyzer, Model 1056. The effluent fractions were dialyzed against distilled water for 24 h with changes each 4 h, then lyophilized. All of the steps in the isolation were carried out at 2–4°.

TABLE II

STEPWISE ELUTION SCHEDULE FOR COLUMN CHROMATOGRAPHIC FRACTIONATION OF ENRICHED K- AND γ-CASEIN ON DEAE-CELLULOSE

Step	*Volume of eluent (ml)*	*Molarity of NaCl in eluent*[a]
1	500	0.00
2	750	0.05
3	750	0.10
4	1000	0.15
5	750	0.20
6	500	0.25
7	500	0.50 *N* NaOH[b]

[a] All eluents were 0.03 *M* glycine–NaOH buffer containing 2 *M* urea of pH 8.0.
[b] The NaOH eluate fraction was not collected.

PGUE characterization

The eluate DEAE fractions were characterized by means of vertical polyacrylamide gel electrophoresis using an EC apparatus. The stock buffer was made from 121 g of reagent grade Tris (hydroxymethyl)aminomethane, 15.6 g Na_2EDTA and 9.2 g H_3BO_3 diluted to 4 l with distilled water. The bridge solution was prepared by mixing one part of the above mentioned stock solution with two parts of distilled water (final pH 9.2). The gel solution was prepared with 70 g of cyanogum, 270 g of urea, 1 ml of TMED (N,N,N',N'-tetramethylethylenediamine), 1/3 l of stock buffer, and made to 1 l with distilled water (pH 9.2 and 4.5 *M* in urea). Twenty microliters of the 1% protein sample solution prepared with the bridge buffer containing 4.5 *M* urea and 8% sucrose were placed in the wedge-shaped slots. The run was carried out at 210 V for 5 h with tap water cooling at about 15 to 18°.

Amino acid analysis

The amino acid composition of the γ-casein was determined with a Technicon amino acid analyzer using a modification of the Piez and Morris method. The separations were made on a 0.6 × 133 cm column of 22 μ spherical polynuclear sulfonic acid resin beads (Chromosorb A) at 60° using citrate buffers of pH 2.875, 3.80, and 5.00 for the gradient elution. Thirty milligrams of sample, after drying over P_2O_5 at room temperature, were hydrolyzed for 24 h with 6 *N* HCl at 110° under conditions rigidly excluding oxygen. The data are given in Table III.

Sedimentation analysis

The sedimentation studies were made using a Spinco model E analytical ultra-

TABLE III

AMINO ACID COMPOSITION OF γ-CASEIN ISOLATED FROM β- AND K-CASEINS

Amino acid	*Observed value*[a]		
	GORDON *et al.*[b]	*γ- from β-casein*	*γ- from K-casein*
Aspartic acid	4.0	3.99	9.82
Threonine	4.4	4.45	13.08
Serine	5.5	5.49	9.30
Glutamic acid	22.9	19.22	22.45
Proline	17.0	16.32	10.34
Glycine	1.5	1.49	1.66
Alanine	2.3	2.03	4.95
Valine	10.5	5.55	7.58
Methionine	4.1	4.02	1.71
Isoleucine	4.4	2.88	5.80
Leucine	12.0	14.19	6.89
Tryosine	3.7	4.08	2.10
Phenylalanine	5.8	6.97	3.87
Lysine	6.2	4.95	8.14
Histidine	3.7	3.86	3.31
Arginine	1.9	1.83	4.19
	109.9	101.32	117.94

[a] Each value is an average of analyses on different preparations.

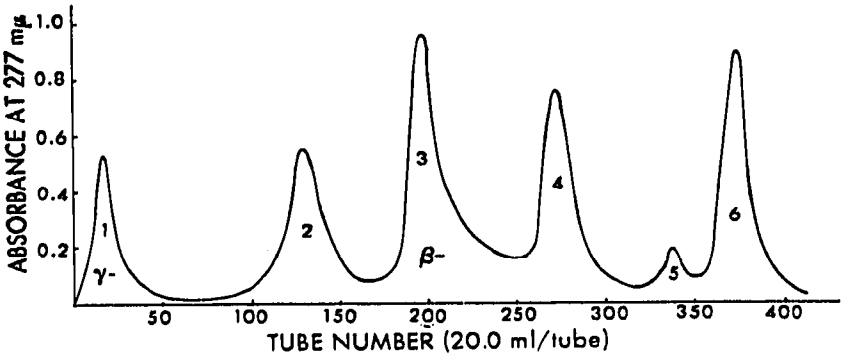

Fig. 1. DEAE-cellulose chromatography of chemically enriched β- and γ-casein. Column (6.5 × 25 cm) equilibrated with 2 l of 0.03 *M* glycine–NaOH buffer containing 2 *M* urea (pH 8.0). Sample load approx. 5.0 g. Stepwise elution as indicated in Table I. Flow rate 300 ml/h. Absorbance of the effluent was measured at 277 mμ.

centrifuge at 20° with a rotor speed of 59,780 r.p.m. A valve-type 4°–12 mm synthetic boundary cell was used. A Gaertner coordinate plate and film comparator was used to measure the photographic plates. Calculations were made from the following formula:

$$S = \frac{2.303 \text{ d log } \bar{x}}{\omega^2 \cdot \text{d}t}$$

where S is the sedimentation coefficient, $\bar{x}$ is the distance of the boundary from the axis of rotation, ω is the angular velocity (radians/sec), and d log $\bar{x}$/dt was obtained from the graph of log $\bar{x}$ *vs.* time.

RESULTS AND DISCUSSION

The PGUE patterns of the DEAE-cellulose eluate fractions obtained from chromatographic separations (Figs. 1 and 2), show that the first eluate peak is γ-casein as evidenced in Slot No. 4 (Figs. 3 and 4) and Slot No. 3 (Fig. 7). This casein was found to be the slowest moving component of whole casein as was also reported by other research workers[3–5]. The chromatographically isolated γ-casein was found to contain three major and some minor component bands when isolated from chemi-

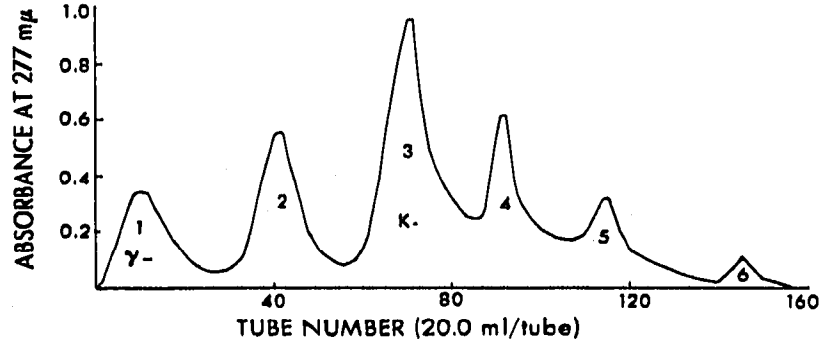

Fig. 2. DEAE-cellulose chromatography of chemically enriched K- and γ-casein. Column (4.5 × 25 cm) equilibrated with 2 l of 0.03 *M* glycine–NaOH buffer containing 2 *M* urea (pH 8.0). Sample load approx. 1.5 g. Stepwise elution as indicated in Table II. Flow rate 250 ml/h. Absorbance of the effluent was measured at 277 mμ.

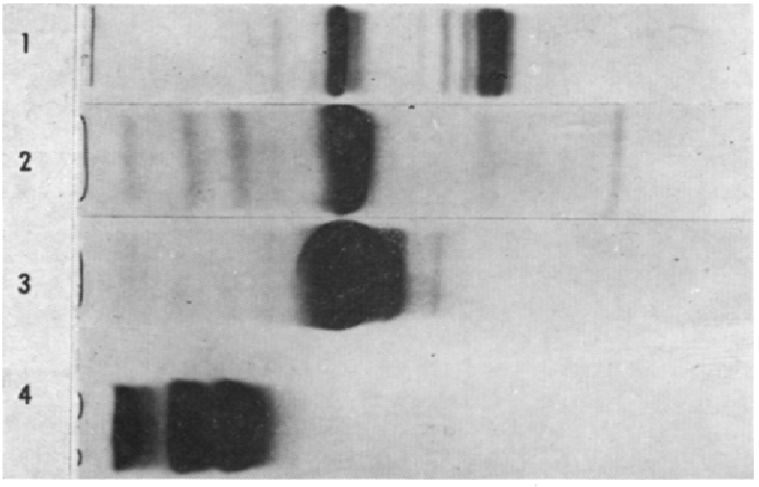

Fig. 3. PGUE patterns of β- and γ-casein. Effect of high loading on gel pattern. (1) P^1 casein (200 μg); (2) chemically enriched β-casein (600 μg); (3) chromatographically purified β-casein from (2) (600 μg); (4) chromatographically purified γ-casein from (2) (600 μg).

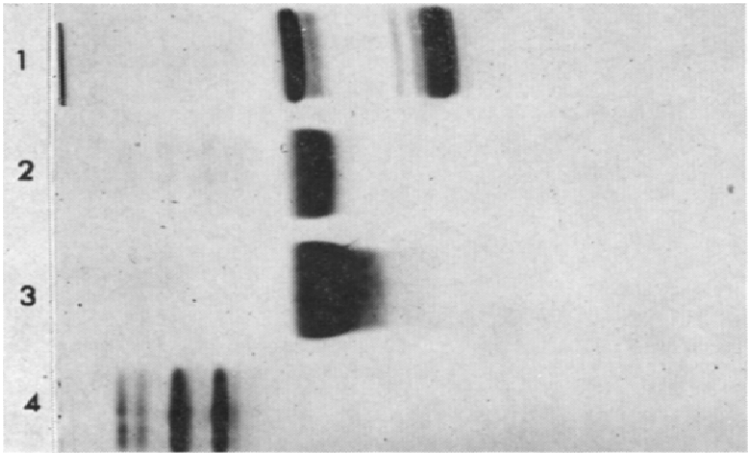

Fig. 4. PGUE patterns of β- and γ-casein. Effect of moderate loading on gel pattern. (1) P^1 casein (200 μg); (2) chemically enriched β-casein (200 μg); (3) chromatographically purified β-casein from (2) (200 μg); (4) chromatographically purified γ-casein from (2) (200 μg).

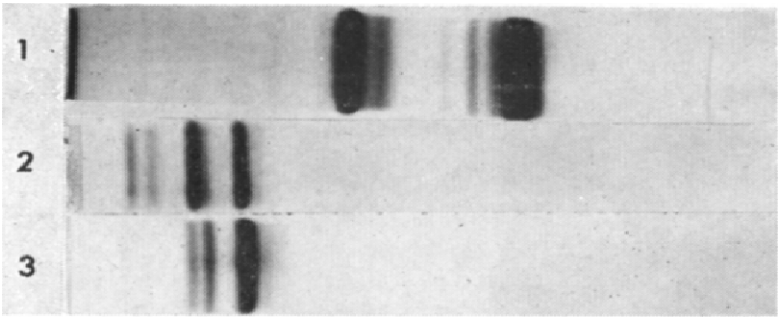

Fig. 5. PGUE patterns of γ-casein from enriched β-casein. Effect of temperature. (1) P^1 casein; (2) γ-casein, in solution at 2–4°; (3) γ-casein, in solution at room temperature. (Slowest moving components precipitated at room temperature.)

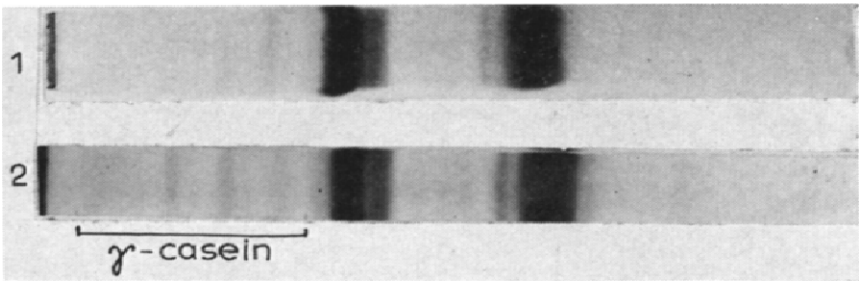

Fig. 6. PGUE patterns of pl casein showing γ-casein. (1) gel stained with Amido Black; (2) gel stained with Coomassie Blue.

cally enriched β-casein (Figs. 3 and 4), and one major and some minor component bands when isolated from chemically enriched K-casein (Fig. 7).

WAKE AND BALDWIN[4] have reported five bands by starch gel electrophoresis using the γ-casein of HIPP *et al.*[3]. NEELIN *et al.*[5] have reported one major component by using starch gel electrophoresis at pH 7.0 and two major component bands at pH 8.0. They also used the γ-casein of HIPP *et al.*[3]. However, one observation occurred in common in all of the reported studies, including this investigation, that γ-casein was found to occupy the same slowest region in gel zone electrophoresis.

GEHRKE *et al.*[10] studied the behavior of synthetic mixtures of different weight ratios of α_{s1}-, β-, and K-casein components by gel electrophoresis. It was observed that the presence of 1% of any casein was detectible in 200 μg mixtures of the others. Thus, Fig. 3, Slot No. 4, shows that the γ-casein, chromatographically isolated from chemically enriched β-casein, was free from any of the major casein components. The three major component bands of γ-casein appear in exactly the same positions as the slowest moving components in the chemically enriched β-casein (Fig. 3, Slot No. 2). It was also observed that these three components appear in the PGUE pattern for pI casein (Fig. 6).

The moderate loadings (Fig. 4) of 200 μg (20 μl of 1 w/v % protein solution) illustrate the resolution of the γ-casein components. The slowest migrating component contained two bands, and the other components were well resolved. The gel pattern

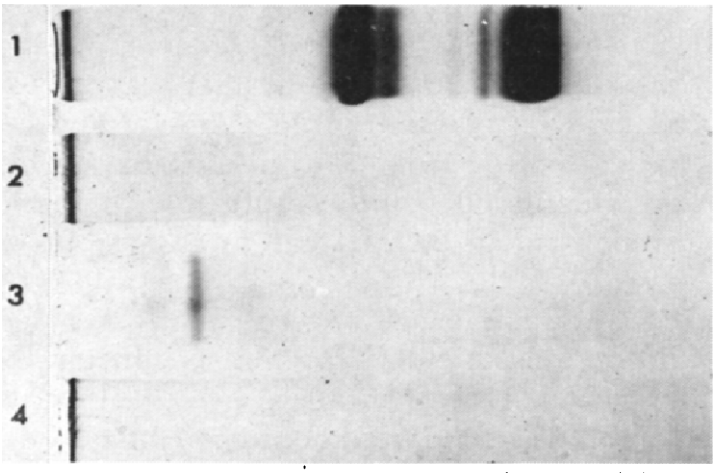

Fig. 7. PGUE patterns of γ-casein from enriched K-casein. (1) P^1 casein; (2) chemically enriched K-casein; (3) chromatographically purified γ-casein from (2); (4) chromatographically purified K-casein from (2).

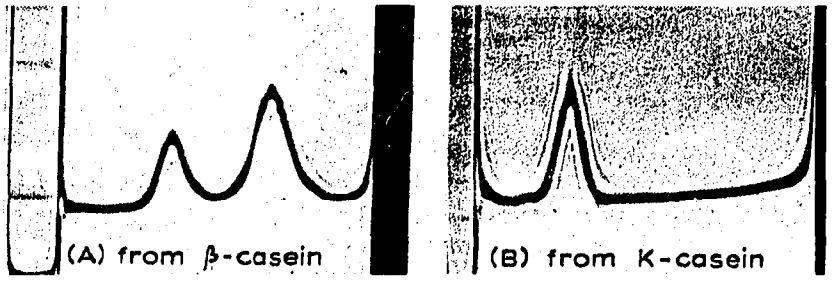

Fig. 8. Ultracentrifuge pattern of γ-casein from enriched β- and K-casein. Sedimentation of γ-casein after approx. 450 sec (A) and 300 sec (B) at 59,780 r.p.m., using a valve type synthetic boundary cell (4°–12 mm sector). 1.0% γ-casein in 0.076 *M* Tris–citrate buffer, pH 8.6; bar angle 60°.

for the same sample weight of γ-casein from K-casein shows one major component; the minor components were present in very low concentration (Fig. 7, Slot No. 3).

Temperature sensitivity of γ-casein

HIPP *et al.*[3], GROVES *et al.*[6], and THOMPSON[7] have observed a temperature-sensitive casein fraction in the region of γ-casein on gel zone electrophoresis. These studies were made to resolve this problem. The γ-casein was found to remain in solution at 2–4°, pH 7.0, but when the solution was brought to room temperature, and held a few minutes, a precipitate occurred. A study[11] was made of the solubility of γ-casein at 2–4°, and of the proteins in the supernatant obtained at room temperature. The precipitate obtained at room temperature dissolves at 2–4° and reprecipitates at room temperature. This is illustrated on the PGUE pattern (Fig. 5). It was noted that the two slowest moving bands of the three major components of γ-casein from β-casein were temperature sensitive and were precipitated at room temperature.

Sedimentation velocity experiments were performed on both of the γ-caseins obtained from chemically enriched β- and K-casein. The γ-casein from β-casein showed two peaks in 0.076 *M* Tris–citrate buffer pH 8.6, having a sedimentation coefficient of the slow moving peak of 1.35 S and of the fast moving peak of 23.20 S. The γ-casein from K-casein showed a single sharp peak having a sedimentation coefficient of 0.82 S (Fig. 8).

Amino acid analysis

The amino acid analysis of γ-casein from β-casein in this study in general agrees with the composition of γ-casein reported by GORDON *et al.*[9]. A difference was noted with respect to valine, isoleucine, and leucine. However, remarkable differences were found in the amino acid composition of γ-casein isolated from β- and K-caseins. The aspartic acid, serine, alanine, and arginine were much higher; and proline, methionine, leucine, tyrosine, and phenylalanine were lower in the γ- from K-casein as compared to the γ- from β-casein.

The γ-caseins from chemically enriched β- and K-caseins were isolated at the same salt concentration (0.05 *M* in NaCl) and on gel zone electrophoresis constitute the slowest moving components of the whole casein. However, there is a significant difference in their amino acid composition (Table III) and sedimentation behavior, showing them to be different proteins. The yield of γ-casein from β-casein is very significant. Approximately 500 mg/5.0 g of γ-casein were obtained from chemically prepared β-casein.

REFERENCES

1 O. MELLANDER, *Biochem. Z.*, 300 (1939) 240.
2 N. J. HIPP, M. L. GROVES, J. H. CUSTER AND T. L. MCMEEKIN, *J. Am. Chem. Soc.*, 72 (1950) 4928.
3 N. J. HIPP, M. L. GROVES, J. H. CUSTER AND T. L. MCMEEKIN, *J. Dairy Sci.*, 35 (1952) 272.
4 R. G. WAKE AND R. L. BALDWIN, *Biochim. Biophys. Acta*, 47 (1961) 225.
5 J. M. NEELIN, D. ROSE AND H. TESSIER, *J. Dairy Sci.*, 45 (1962) 153.
6 M. L. GROVES, T. L. MCMEEKIN, N. J. HIPP AND W. G. GORDON, *Biochim. Biophys. Acta*, 57 (1962) 197.
7 M. P. THOMPSON, *J. Dairy Sci.*, 49 (1966) 792.
8 C. W. GEHRKE, C. W. FREEARK, Y. H. OH AND P. W. CHUN, *Anal. Biochem.*, 9 (1964) 423.
9 W. G. GORDON, W. F. SEMMETT AND M. BENDER, *J. Am. Chem. Soc.*, 75 (1953) 1678.
10 C. W. GEHRKE, D. A. CRAVEN, J. ABSHEER AND K. K. TRIPATHI, *Separation Sci.*, 2 (1967) 199.
11 K. K. TRIPATHI AND C. W. GEHRKE, *Isolation of Temperature Sensitive γ-casein from Chemical Preparations of β- and K-Caseins*, presented at the 62nd Annual Meeting, American Dairy Science Association, Cornell University, Ithaca, N.Y., June 1967.