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Isolation and Polyacrylamide Gel-Urea Electrophoretic Characterization of α_{s_1}-Casein*

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Summary

An improved method for the isolation of pure α_{s_1}-casein from pooled milk is described. The method involves chemical isolation followed by DEAE-cellulose column-chromatographic fractionation.

Alpha$_{s_1}$-casein was characterized by SGUE, PGUE, chemical, and sedimentation analyses. The chromatographically pure α_{s_1}-casein was found to contain α_{s_1}-B and α_{s_1}-C casein variants. These variants cannot be separated by DEAE-cellulose column chromatography.

The preparation contained 1.10%*P*, equivalent to 10 phosphorus atoms per mole of 28,500. The molecular weight by approach to sedimentation equilibrium was 25,500. Alpha$_{s_1}$-casein in 0.1 *M* KCl, pH 5.6, showed random aggregation of 14*S*, while in tris-citrate buffer, pH 8.6, it showed 1.5 *S*, and in Na-PO$_4$ buffer, pH 7.0, 4.2 *S* species.

Isolation in pure form and characterization of the major components of casein α_{s_1}-, β-, and K- are necessary to the understanding of casein–casein, and casein–other protein interactions, as well as

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being prerequisites to structural examinations. In this respect, much work has been done by many different investigators (*2,5,9,10, 12,19,20,22,24*), using a variety of techniques in attempts to prepare pure samples of the various casein components. Varying degrees of success have been achieved in these endeavors.

The objective of this paper is to describe a method for the isolation of pure α_{s_1}-casein from pooled skim milk and to compare it with the genetic variants obtained by Thompson (*14,16,17*) and others (*6*) from the milk of individual cows. This paper also describes a partial characterization of the isolated pure α_{s_1}-casein by gel electrophoresis, chemical analysis, and sedimentation behavior.

EXPERIMENTAL PROCEDURES

Isolation of Pure α_{s_1}-Casein

Chemical Preparation. The procedure for the chemical preparation of α_{s_1}-casein is a modification of several previously published methods (*5,20,21*). The essentials of chemical fractionation of α_{s_1}-casein include urea fractionation of isoelectric acid casein into α_{s_1}-K casein gel and β- and γ-casein in solution (*4,5*); the α_{s_1}-K casein gel is then fractionated by 0.20 *M* $CaCl_2$ in the presence of urea. The α_{s_1}-casein is precipitated as the calcium-α_{s_1}-caseinate. The α_{s_1}-casein was solubilized by $K_2C_2O_4$ (*12,20*). The soluble α_{s_1}-casein was then fractionated by DEAE-cellulose column chromatography in 0.01 *M* imidazole–HCl buffer, containing 3 *M* urea, pH 7.0. The details follow.

The isoelectric acid casein (15 g) prepared from 500 ml of pooled, raw, skim milk was dispersed in 400 ml of distilled water and dissolved by addition of 6.6 *M* urea; the solution was diluted to 4.5 *M* urea at pH 4.6 and held for 8 hours, or overnight, and centrifuged at 3000 rpm for 30 min to remove the α_{s_1}-K complex. This procedure was repeated once.

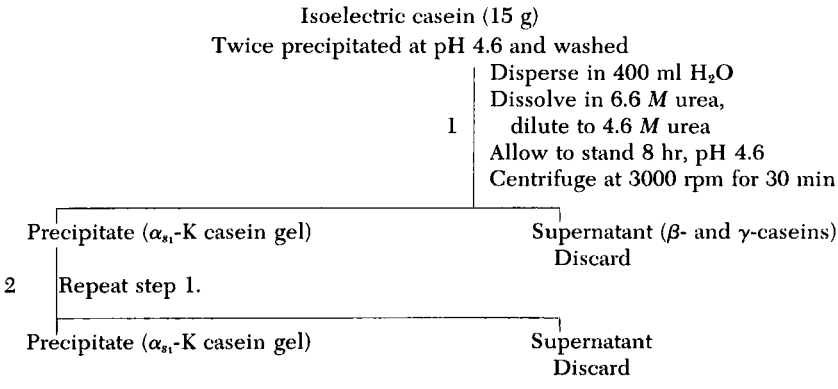
The gelatinous α_{s_1}-K-casein precipitate was dispersed in 250 ml of distilled water and dissolved by dropwise addition of 0.1 *N* NaOH, with constant stirring, to pH 8.5, which was not exceeded. The solution was readjusted to pH 7.0 with 0.1 *N* HCl, and sufficient 3 *M* $CaCl_2$ was added to make the resulting solution 0.2 *M* in $CaCl_2$. The pH was maintained at 7.0 by addition of 0.1 *N* NaOH. The solution was then centrifuged at 3000 rpm for 30 min. This procedure was repeated once.

The supernatant was discarded and the gelatinous precipitate of Ca-α_{s_1}-caseinate was dispersed in 250 ml of distilled water, and the dispersed solution was brought to pH 8.5 by dropwise addition of 0.1 *N* NaOH with stirring. Sufficient 1.5 *M* $K_2C_2O_4$ was added to make the resulting solution 0.5 *M* in $K_2C_2O_4$. The pH was maintained at 8.5 and the solution was centrifuged at 20,000 rpm for 20 min. The supernatant was saved and this step was repeated on the precipitate. The resulting supernatants were combined and exhaustively dialyzed against distilled water, until $C_2O_4^=$ free, then lyophilized. All operations were carried out at 2 to 4°C.

DEAE-Cellulose Chromatographic Purification. The chemically prepared α_{s_1}-casein was further purified by stepwise elution chromatography on a 6.5 × 12 cm DEAE-cellulose column at 25°C. The column was packed to give a flow rate of 300 to 400 ml/hr and was equilibrated with at least 2 liters of 0.01 *M* imidazole-HCl buffer, containing 3 *M* urea, pH 7.0 (*20,21*). Approximately 1.5 g of chemically prepared α_{s_1}-casein in 200 ml of 0.01 *M* imidazole-HCl and 3 *M* urea solution (pH 7.0) were placed on the column. Stepwise elution was performed according to the schedule in Table 1 (see also scheme 1). The effluent was continuously monitored at 277 mμ by a Vanguard automatic UV analyzer, model 1056. The α_{s_1}-casein fraction was eluted by 0.25 *M* NaCl and dialyzed against distilled water at 4°C until salt-free, then lyophilized.

Chemical Analyses. Calcium stability tests, as described by Zittle

SCHEME 1
Chemical Preparation of α_{s_1}-Casein



SCHEME 1 (Continued)

Disperse in 250 ml H_2O
Add 0.1 *N* NaOH dropwise w/stirring, to pH 8.5
3 Adjust to pH 7.0 w/0.1 *N* HCl
Make 0.2 *M* in $CaCl_2$ w/3 *M* $CaCl_2$
Maintain at pH 7.0 w/0.1 *N* NaOH
Centrifuge at 3000 rpm for 30 min

Precipitate (Ca-α_{s_1}-caseinate) | Supernatant (K-casein) Discard

4 Repeat step 3

Precipitate (Ca-α_{s_1}-caseinate) | Supernatant Discard

Disperse in 250 ml H_2O
5 Adjust to pH 8.5 w/0.1 *N* NaOH w/stirring
Add 1.5 *M* $K_2C_2O_4$ to make a 0.5 *M* solution in $K_2C_2O_4$
Centrifuge at 20,000 rpm for 20 min

Precipitate | Supernatant (α_{s_1}-casein) No. 1 Save

6 Repeat step 5

Precipitate Discard | Supernatant (α_{s_1}-casein) No. 2

Combine supernatants No. 1 and 2
7 Dialyze against d. H_2O until $C_2O_4^=$-free
Freeze-dry

Chemically prepared α_{s_1}-casein
Yield: (at least 4.5 g)

All operations were carried out at 2 to 4°C

Stepwise elution column chromatography on DEAE-cellulose (refer to Table 1 and text)

Pure α_{s_1}-casein

TABLE 1
Stepwise Elution Schedule for Column-Chromatographic Purification of α_{s_1}-Casein on DEAE-Cellulose

Step	Volume of eluent, ml	Molarity of NaCl in eluent[a]
1	300	0.00
2	300	0.10
3	600	0.20
4	600	0.25
5	300	0.35
6	300	1 *N* NaOH[b]

[a] All eluents were 0.01 *M* imidazole-HCl buffer containing 3 *M* urea at pH 7.0.
[b] Added to remove remaining adsorbed proteins.

(*23,24*), Kjeldahl nitrogen, and phosphorus analyses were performed on samples of the purified α_{s_1}-casein.

Electrophoretic Characterization of α_{s_1}-Casein. The homogeneity of the chemically prepared α_{s_1}-casein and of the chromatographically purified α_{s_1}-casein were determined by means of vertical SGUE using the techniques of Gehrke et al. (*3,4*). Homogeneity was further verified by PGUE, using methods similar to those of Thompson et al. (*15*). A commercial, E-C No. 470, vertical gel electrophoresis apparatus was employed. The polyacrylamide gel contained 7% cyanogum and was 4.5 *M* in urea, 0.3 *M* in tris(hydroxymethyl) aminomethane, 0.014 *M* in Na_2EDTA, and 0.05 *M* in H_3BO_3 buffer at pH 9.1 to 9.2. Twenty-five microliters of 1% protein solution in the pH 9.2 buffer described above with 8% added sucrose was placed in the gel slot and electrophoresis was carried out with tap water cooling at 15 to 18°C. The most satisfactory results were obtained with 200 volts for $4\frac{1}{2}$ to 5 hours (start of run 75 ma, end of run ca. 30 ma).

Analytical Ultracentrifuge Studies. The isolated pure α_{s_1}-casein was also studied by means of a Spinco Model *E* analytical ultracentrifuge. Sedimentation velocity experiments were performed at 20°C using a valve-type, 4°–12 mm synthetic boundary cell at 59,780 rpm. The sedimentation coefficients were calculated from the second moment of the total gradient curve. A plot was made of the logarithm of the second moment against time having a slope of sw^2. Measurements of the photographic plates were made with

a two-coordinate Gaertner microscope comparator. The intrinsic sedimentation coefficient, $s^0_{20,w}$, was corrected for the viscosity and buoyancy of water at 20°C in the customary manner.

For molecular-weight estimations the Klainer and Kegeles (*7*) modification of the Archibald (*1*) procedure was employed: Measurements were made only at the meniscus. A value of 0.73 ml/gm was determined for the partial specific volume of α_{s_1}-casein using a 25-ml pycnometer in a temperature-controlled water bath at 20° ± 0.01°C (*8*).

RESULTS AND DISCUSSION

Figure 1 shows the elution-curve absorbance vs. effluent volume from the chromatography of a sample of chemically prepared α_{s_1}-casein from pooled milk. A single major absorbance peak for α_{s_1}-casein appears in the elution curve and represents a total

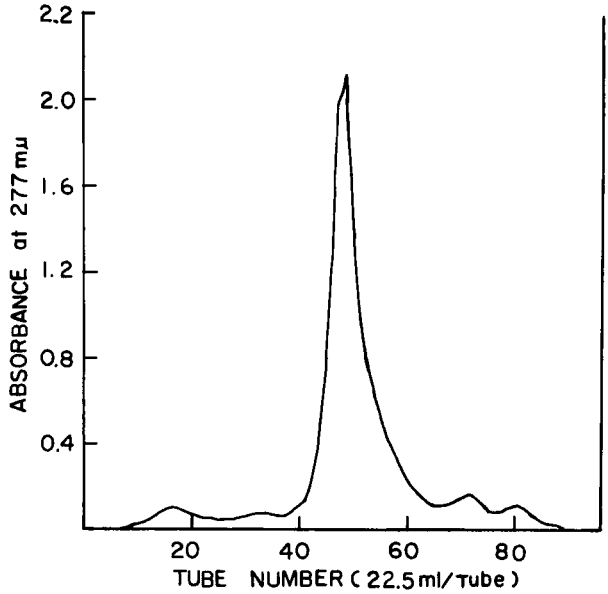


FIG. 1. DEAE-cellulose chromatographic purification of chemically prepared α_{s_1}-casein. Column (6.5 × 12 cm) equilibrated with 2 liters of 0.01 *M* imidazole-HCl buffer containing 3 *M* urea (pH 7.0). Sample load approximately 1.5 g of protein. Stepwise elution as indicated in Table 1. Flow rate approximately 300 ml/hr. Absorbance of the effluent was measured at 277 mμ. Pure α_{s_1}-casein was eluted at 0.25 *M* NaCl.

TABLE 2
Phosphorus Content and Calcium Stability of α_{s_1}-Casein[a]

Casein	%P	%N	P/N ratio	Absorbance (24)			
				Conc. mg/ml	Initial	Final	% decrease due to interaction with Ca^{++}
α_{s_1}	1.10	15.2	0.0724	30	2.40	0.92	62

[a] Each value is an average of several independent analyses.

recovery of about 90% of the protein originally applied to the column.

The results obtained from calcium stability tests and chemical analyses are presented in Table 2 and are in accord with previously reported data (*3,16,21,24*). Several independent preparations of pure α_{s_1}-casein contained an average phosphorus content of 1.10%, or the equivalent of 10 g atoms/mole of 28,500. Thompson et al. (*16*), Waugh et al. (*21*), and Zittle and Custer (*24*) report 9 g atoms/mole of 27,500 based on a phosphorus content of 1.01%. Schmidt

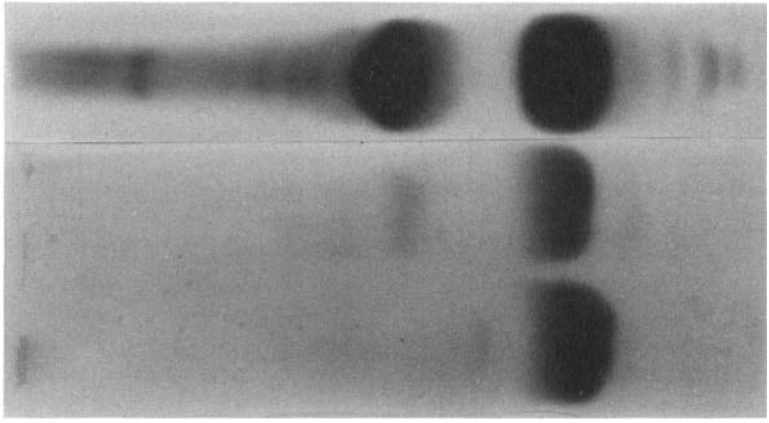


FIG. 2. SGUE patterns of α_{s_1}-casein. Electrophoretic conditions: vertical SGUE with 0.076 *M* tris–0.005 *M* citric acid buffer, pH 8.6, 7 *M* urea; 1 mg protein; 4.0 volts/cm; 20 hr at 5°C; bridge solution, sodium borate buffer (0.3 *M* H_3BO_3 and 0.06 *N* NaOH), pH 8.6. The positive electrode compartment contained 10% aqueous NaCl. These conditions are a combination of the methods of Wake et al. (*19*), Smithies (*13*), and Poulik (*11*). Top, Isoelectric acid casein (pI); middle, chemically prepared α_{s_1}-casein; bottom, alpha$_{s_1}$-casein purified by DEAE-cellulose column chromatography.

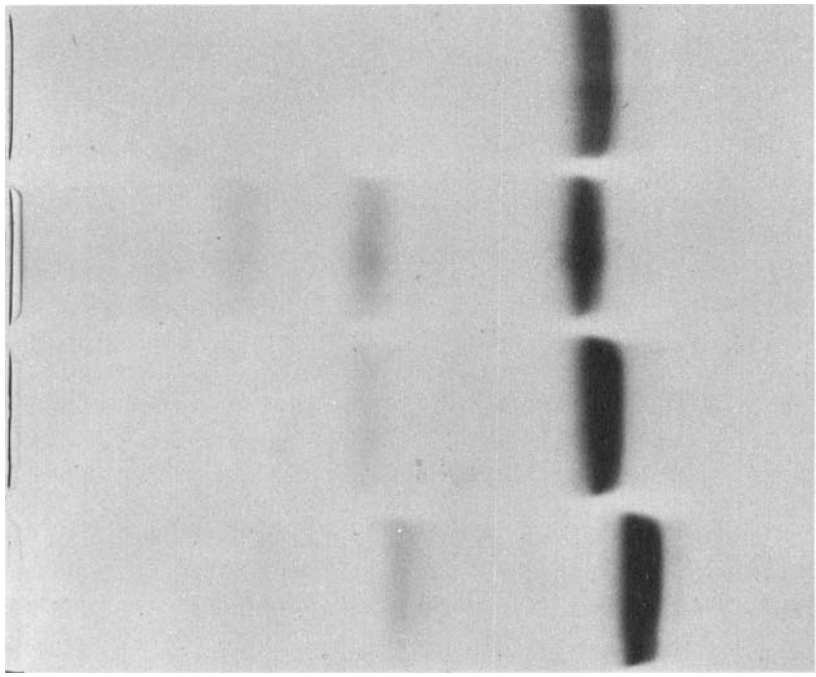


FIG. 3. Comparison of PGUE patterns of pure α_{s_1}-casein from pooled milk and genetic variants isolated by Thompson (*16*) (not chromatographically purified). PGUE conditions: vertical PGUE with 7% cyanogum, 4.5 *M* urea, 0.3 *M* tris–0.014 *M* Na_2EDTA, and 0.05 *M* H_3BO_3 buffer, pH 9.2; 0.60 mg protein; 200 volts for 4½ hr at 15 to 18°C. Top, α_{s_1} (Gehrke et al.); second from top, α_{s_1}-C (Thompson); second from bottom, α_{s_1}-B (Thompson); bottom, α_{s_1}-A (Thompson).

and Payens, however, report a phosphorus value of 1.12% or 6 g atoms/mole of 16,500 molecular weight (*12*).

The degree of homogeneity of the pure α_{s_1}-casein preparations was determined by starch gel-urea and polyacrylamide gel-urea electrophoresis, as shown in Figs. 2 and 3. Comparison of our preparations has been made largely on the basis of electrophoretic mobilities. Starch gel-urea patterns of Schmidt and Payens (*12*) designated as 1.10 and 1.07 by Wake and Baldwin (*19*) are similar to our preparation. These are the α_{s_1}-B and α_{s_1}-C variants reported by Thompson et al. (*16*). Direct PGUE electrophoretic comparison of our α_{s_1}-casein preparation with samples of Thompson's genetic

variants is shown in Figs. 3 and 4. It is clear that our chromatographic preparation of α_{s_1}-casein is primarily composed of α_{s_1}-B and α_{s_1}-C (Figs. 3 and 4). On further electrophoretic comparison of genetic variants at low protein concentration (Fig. 4), it may be seen that the α_{s_1}-casein definitely is composed of two components.

In a number of experiments we were unsuccessful in separating α_{s_1}-BC caseins into single variants in this laboratory utilizing differential gradient gel (3 to 12% cyanogum) (*2a*) and two-dimensional gel electrophoresis or DEAE-cellulose chromatography. Perhaps electrophoresis at acid pH is an approach to the separation of genetic variants (*10a*).

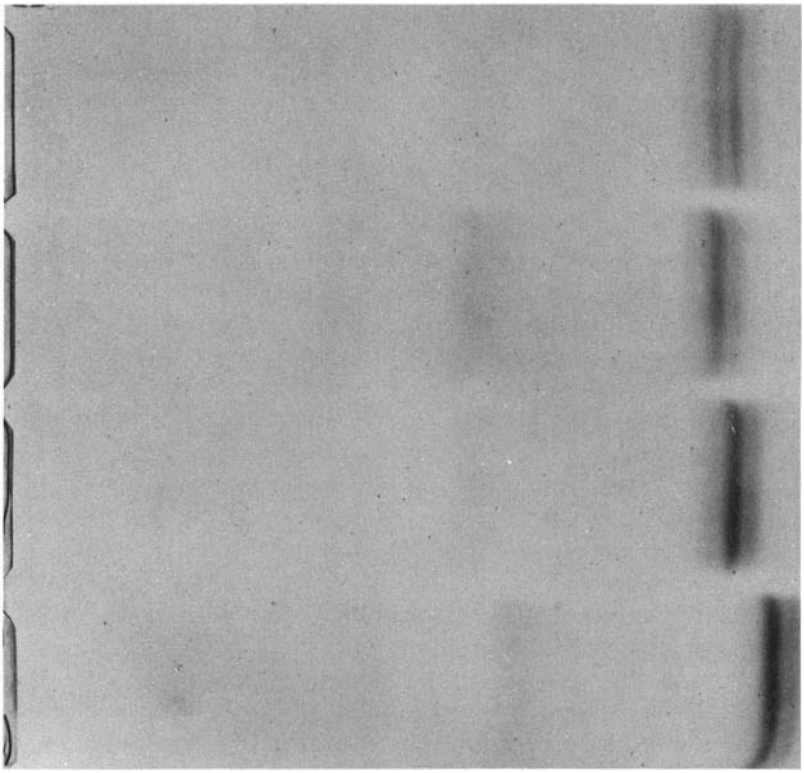


FIG. 4. Comparison of PGUE patterns of pure α_{s_1}-casein from pooled milk and genetic variants isolated by Thompson (*16*) (not chromatographically purified). PGUE conditions: same as Fig. 3, except using 0.30 mg of protein. Top, α_{s_1} (Gehrke et al.); second from top, α_{s_1}-C (Thompson); second from bottom, α_{s_1}-B (Thompson); bottom, α_{s_1}-A (Thompson).

TABLE 3
The Distribution of α_{s_1}-Casein Aggregates in Two Different Buffer Systems[a]

	α_{s_1}-casein[b]				α_{s_1}-casein[c]			
		Concentration, g/100 ml				Concentration, g/100 ml		
rpm	Time	Theory[a]	Exptl.	ΔC_A	Time	Theory[a]	Exptl.	ΔC_B
12,590	550	1.000	1.000		530	1.000	1.000	
12,590	750	0.999	0.998		750	0.998	0.990	0.008
59,780	1430	0.985	0.983	0.002	1433	0.955	0.943	0.012
59,780	1750	0.973	0.970	0.003	1758	0.943	0.931	0.012
59,780	2250	0.970	0.965	0.005	2255	0.940	0.930	0.010
59,780	3125	0.965	0.962	0.003	3005	0.939	0.928	0.011

[a] Concentrations were corrected for radial dilution and stretching of the rotor.
[b] Same system as Fig. 5B.
[c] Same system as Fig. 5C.

The degree of homogeneity of the pure α_{s_1}-casein preparations was further determined by sedimentation boundary analysis. In this study the estimate of aggregates present in the preparation and deviation of schlieren area from the area of a normal distribution curve were obtained from enlarged tracings of each schlieren pattern at low speed (12,590 rpm) and at high speed from the centrical ordinate to the base line, then subtracting each area about this perpendicular from the total gradient area. The area so obtained was converted to concentration on a gram per 100 ml scale, as shown in Table 3. It is clearly evident that the values for ΔC_A show a small difference (Fig. 5B) as a function of time. The ratio of areas symmetrical about the perpendicular axis was found to be 1:1 for α_{s_1}-casein in a tris-citrate buffer at pH 8.6. The theoretically calculated area of a normal distribution curve is in excellent agreement with the experimental area (Fig. 5B). However, when α_{s_1}-casein is in phosphate buffer, pH 7.0, this is no longer the case, because there is less symmetry about the perpendicular axis, as shown in Table 3 and Fig. 5C, thus showing the presence of aggregates.

The molecular weight of α_{s_1}-casein in 0.076 *M* tris–0.005 *M* citric acid buffer obtained by approach to sedimentation equilibrium was found to be 25,500, which is in good agreement with values reported by other investigators (*18,19,21,24*).

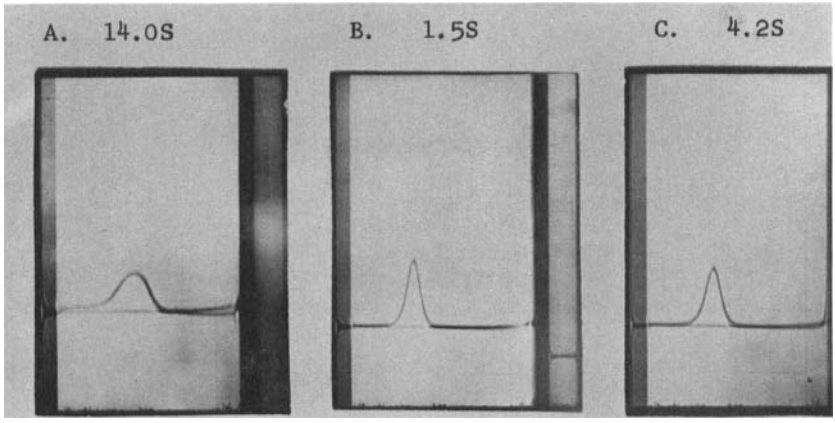


FIG. 5. Ultracentrifuge patterns of pure α_{s_1}-casein in various solvents at 59,780 rpm, using a synthetic boundary cell 4°–12 mm sector cell. A, 0.83% α_{s_1}-casein in 0.1 *M* KCl, pH 5.6, bar angle 65°; 1800 sec; B, solution A exhaustively dialyzed against 0.076 *M* tris-0.005 *M* citric acid buffer, pH 8.6; bar angle 65°; 1800 sec; C, solution A exhaustively dialyzed against 0.034 *M* Na-PO_4 buffer, pH 7.0; bar angle 65°; 1800 sec.

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