

## TS-A<sup>2</sup>, TS-B, R-, and S-Caseins: Their Isolation, Composition, and Relationship to the $\beta$ - and $\gamma$ -Casein Polymorphs A<sup>2</sup> and B

M. L. GROVES, W. G. GORDON, E. B. KALAN, and S. B. JONES  
Eastern Regional Research Laboratory,<sup>1</sup> Philadelphia, Pennsylvania 19118

### Abstract

TS-A<sup>2</sup>, R-, S-, and TS-B caseins are minor components of micellar caseins typed  $\beta$ - and  $\gamma$ -A<sup>2</sup> and B. Based on amino acid analysis, TS-A<sup>2</sup> and S-caseins form one pair of polymorphs and R- and TS-B another. The polymorphs in each pair differ only by a single amino acid substitution (Arg  $\rightarrow$  Ser) also found in the  $\beta$ - and  $\gamma$ -variants. R- and TS-B are smaller than TS-A<sup>2</sup> and S-casein by two amino acids in the N-terminal portion of the molecule. These proteins have a molecular weight of about 12,000 and contain no phosphorus or cystine but have one residue of tryptophan. These findings, together with results of partial sequencing, support the hypothesis that the proteins have the same structure as the C-terminal portions of the  $\beta$ - and  $\gamma$ -caseins.

### Introduction

Temperature-sensitive (TS), R-, and S-caseins are minor components of acid-precipitated bovine casein and can be identified as constituents of micellar casein by gel electrophoresis. They can be isolated by chromatographic methods described in this paper. Although they do not contain phosphorus, they have been designated caseins because of their presence in whole casein and their similarity to other components of casein, namely in high content of proline, glutamic acid, valine, leucine, and absence of cystine (5).

Gamma and  $\beta$ -caseins occur in several genetic types differentiated by specific amino acid substitutions. These types are linked; i.e., for a given milk sample the same genetic type is found for both the  $\gamma$ - and  $\beta$ -caseins. The TS-, R-, and S-caseins also occur as genetic variants which are related to the  $\gamma$ -,  $\beta$ -casein polymorphs. Thus, the pair, TS-A<sup>2</sup> and R-caseins, is always associated with samples typed A<sup>2</sup>

with respect to  $\gamma$ - and  $\beta$ -caseins whereas another pair, S- and TS-B caseins, invariably accompanies  $\gamma$ - and  $\beta$ -caseins B (4, 5, 6, 7, 8).

We have proposed (2) that bovine  $\gamma$ -casein is the same as a major fragment of  $\beta$ -casein and that TS-, R-, and S-caseins have the same structures as large segments of  $\gamma$ -casein. In the present paper we describe the isolation, composition, and partial structure of TS-, R-, and S-caseins and some evidence supporting our hypothesis.

### Experimental Procedures

*Isolation of the caseins.* Separation and purification procedures were monitored by disc gel electrophoresis at pH 9.6 in 4 M urea and at pH 4.3 in 8 M urea as described in earlier publications (5, 7).

The methods for preparing and fractionating whole caseins from individual milks typed  $\gamma$ -,  $\beta$ -caseins A<sup>2</sup> or B by chromatography on DEAE-cellulose columns were those used previously (5). Protein eluted with the starting buffer (.005 M phosphate, pH 8.3) was called the temperature-sensitive or TS-fraction. TS-fraction from casein typed  $\beta$ -A<sup>2</sup> contains TS-A<sup>2</sup> and R-caseins, whereas TS-fraction from casein typed  $\beta$ -B contains S- and TS-B caseins. Also present in the TS-fraction in considerable amounts are  $\kappa$ -casein and other proteins with electrophoretic mobilities similar to  $\beta$ - and  $\alpha_{s1}$ -caseins. The TS-fraction eluted as an irregular peak (5) but by suitable cuts of the peak, subfractions highly enriched in TS-A<sup>2</sup> and R-caseins (or S- and TS-B caseins) were obtained.

In some procedures for purifying components of whole casein, milk protease activity presents serious problems (5). Such activity also has been observed in the TS-fractions. Accordingly, before further purification, samples of TS-fractions were suspended in .005 or .05 M phosphate, pH 8.3, and heated for 5 min at 100 C to inactivate any proteolytic enzyme. The heated protein solutions were turbid and some contained insoluble material. They were dialyzed at 3 C against several changes of starting buffer before rechromatography.

Subfractions from TS-fractions A<sup>2</sup> and B

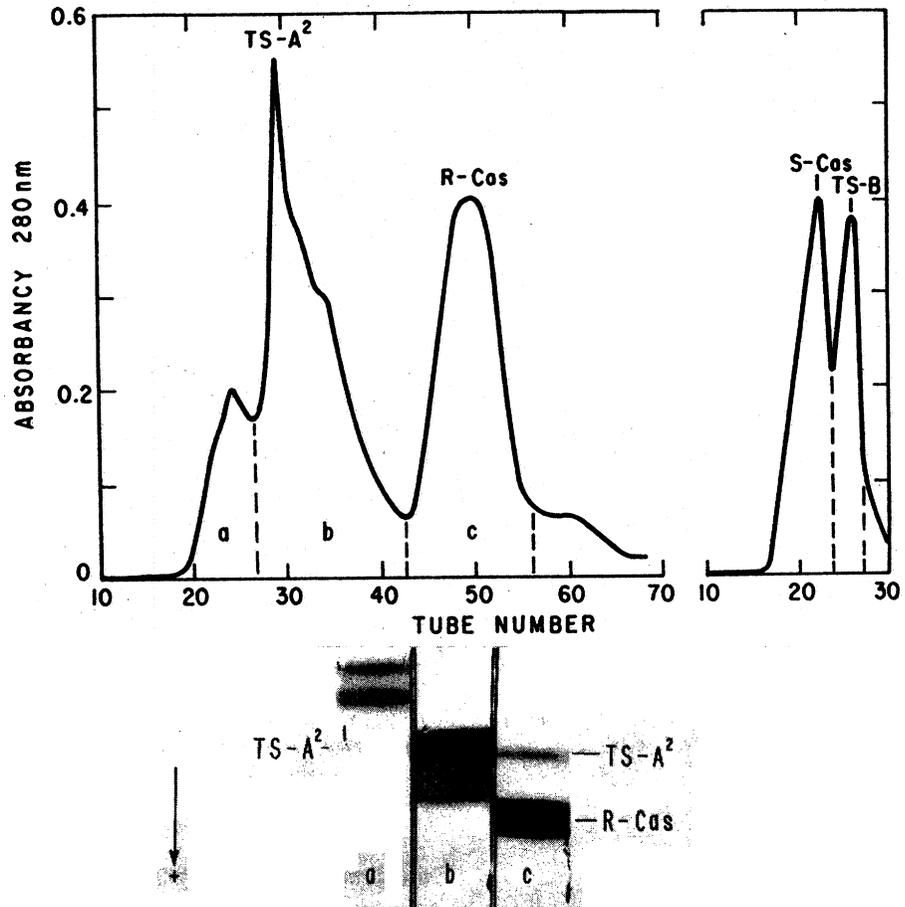


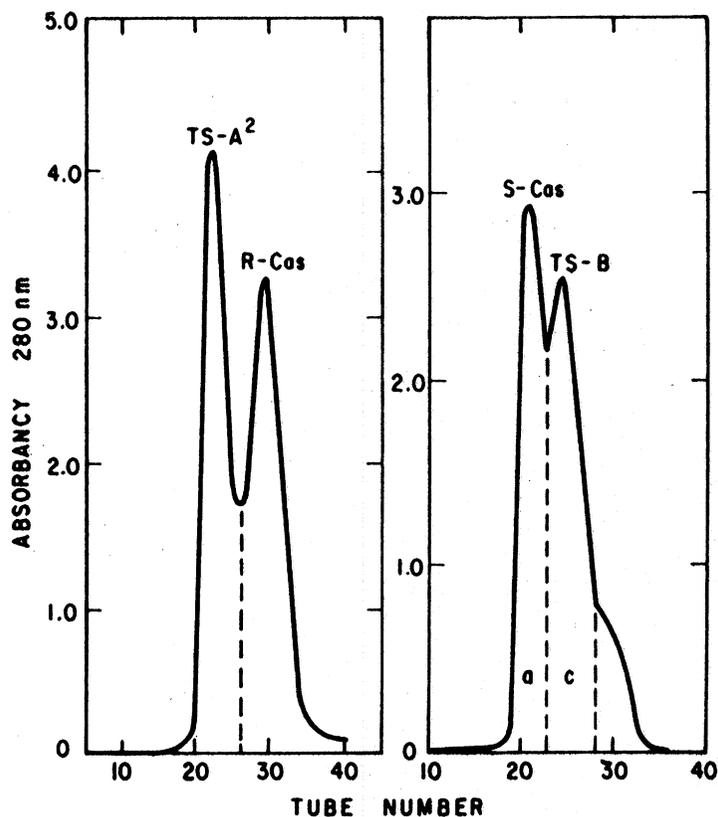
FIG. 1. Elution patterns of the TS-subfractions A<sup>2</sup> and B. Two hundred and fifty milligrams protein in 30 ml .005 M phosphate, pH 8.3, was applied to a DEAE-cellulose column 2 x 70 cm at 3 C. Fractions of 10 ml were collected every 20 min. Disc-gel electrophoretic patterns, pH 9.6, 4 M urea, of the eluted TS-A<sup>2</sup> and R-caseins also are shown.

were rechromatographed on Whatman<sup>2</sup> microgranular DEAE-cellulose columns, equilibrated with .005 M sodium phosphate, pH 8.3, with or without 3 M urea. Elution patterns from rechromatography (without urea) of typical TS-A<sup>2</sup> and B subfractions were devoid of  $\kappa$ - and other caseins (Fig. 1). The TS-A<sup>2</sup>, R- and S-, TS-B caseins were concentrated in the areas separated by dashed lines. The purified TS-A<sup>2</sup>, TS-B, and S-casein solutions were clear at 3 C but became turbid on warming to 25 C while the R-casein solution was clear at both temperatures.<sup>3</sup> In these experiments without urea the relatively pure TS-subfractions were

<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the USDA over others of a similar nature not mentioned.

difficult to dissolve in starting buffer. About 25% of the S- and TS-B caseins in this TS-subfraction B was insoluble in the buffer at 3 C whereas those TS-subfractions containing  $\kappa$ - and other caseins dissolved readily even at 25 C.

<sup>3</sup> TS-A<sup>2</sup> and S-caseins became blue in color when subfractions containing these proteins were rechromatographed at pH 8.3 on microgranular DEAE-cellulose. Solutions of the eluted proteins remained blue on dialysis. Presumably, the proteins combine with copper ions present as impurities in the buffers. TS-A<sup>2</sup> and S-caseins lose their blue color when rechromatographed at pH 5 to 6 on phosphocellulose probably because of the lower pH. R- and TS-B caseins were colorless in all fractionations. Studies of the copper binding of these caseins will be reported elsewhere.



TUBE NUMBER

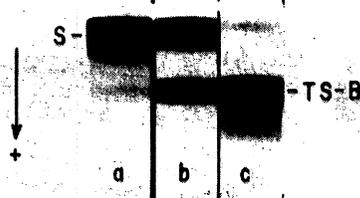


FIG. 2. Elution patterns of the TS-subfractions A<sup>2</sup> and B. Conditions were similar to those in Fig. 1 except that the buffer was 3 M in urea. Two hundred and fifteen milligrams of the TS-A<sup>2</sup> subfraction (no  $\kappa$ -casein present) in 5 ml and 740 mg of the TS-B subfraction (containing  $\kappa$ -casein)

in 17 ml were applied to the DEAE-cellulose column and fractions of 6 ml were collected every 20 min. Disc-gel electrophoretic patterns (pH 9.6, 4 M urea) of the eluted TS-B and S-caseins also are shown, pattern b showing the fraction between the main peaks.

Rechromatography in the presence of urea is illustrated in Fig. 2. A TS-A<sup>2</sup> subfraction containing only TS-A<sup>2</sup> and R-caseins is compared with a TS-B subfraction made up of S-, TS-B,  $\kappa$ -, and other caseins. The  $\kappa$ - and other caseins remain on the column under these conditions. Although resolution is somewhat poorer with urea present, recoveries for TS-A<sup>2</sup>, TS-B, and S-caseins are better because of their greater solubility in urea solutions.

R-casein, partially purified by these proce-

dures (Fig. 1 and 2), was obtained completely pure by rechromatography without urea as described for Fig. 1. Partially purified TS-A<sup>2</sup>, TS-B, and S-caseins were purified further by rechromatography in the presence of urea as described for Fig. 2, by chromatography on phosphocellulose columns, or by both methods. Chromatography on phosphocellulose columns was performed at 3 C with starting buffer .05 M sodium phosphate, pH 5.0 or 6.0, 3 M urea. Under these conditions using pH 5.0 buffer,

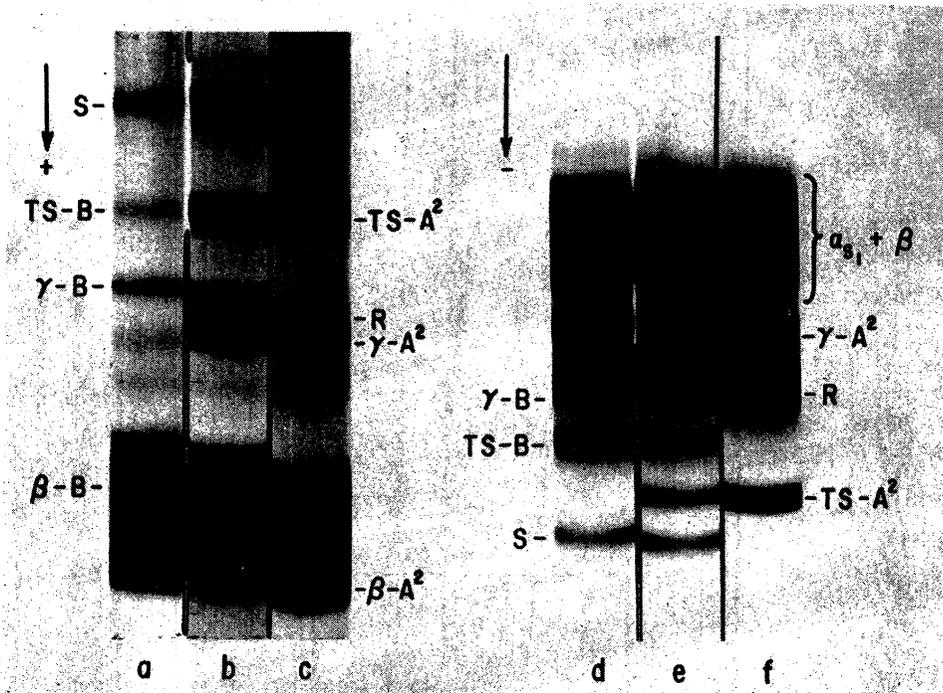


FIG. 3. Disc-gel electrophoretic patterns of caseins typed  $\gamma$ -,  $\beta$ -caseins B,  $A^2B$ , and  $A^2$ . Gels a, b, and c were run at pH 9.6, 4 M urea and d, e, and f at pH 4.3, 8 M urea. Gels a, d represent the homozygous casein B; gels b, e represent the heterozygous casein  $A^2B$ ; and gels c, f represent the homozygous casein  $A^2$ . For the gels a, b, and c the  $\alpha_s$ -casein, with a mobility greater than that of  $\beta$ -casein, is not seen.  $\kappa$ -Casein remains at the origin (not shown).

TS-B casein was retained beyond the hold up volume but was eluted later by the same buffer. TS- $A^2$  casein applied in the same starting buffer required a two-step increase in molarity of buffer for elution: .1 M and .2 M phosphate, pH 5.0, 3 M urea. S-casein was applied in starting buffer, .05 M phosphate, pH 6.0, 3 M urea, and was eluted at the same pH and urea concentration after a two-step increase in molarity of phosphate to .075 and .1 M.

The yield of each protein, TS- $A^2$ , TS-B, R, and S-casein, is estimated to be between 1 and 2%, based on original acid-precipitated casein. Purified proteins used for analysis were dissolved in .1 M acetic acid, centrifuged to remove debris, dialyzed at 3 C until free of phosphate, and lyophilized.

**Other methods.** The methods used for amino acid analysis, determination of phosphorus and tryptophan, mapping of chymotryptic peptides, identification of N- and C-terminal amino acids, and estimation of molecular weight by gel electrophoresis in the presence of sodium dodecyl sulfate have been described (6).

N-terminal amino acids of the proteins also were determined by the Edman<sup>®</sup> PTH procedure as modified by Schroeder et al. (17).

## Results

**Electrophoretic patterns.** The disc gel electrophoretic patterns at pH 9.6 and 4.3 for acid-precipitated caseins typed  $\beta$ - and  $\gamma$ -caseins B,  $A^2B$ , and  $A^2$  (Fig. 3) show good resolution of TS- $A^2$ , TS-B, R, and S-caseins. S-casein, associated with the B type casein, is present in caseins typed B and  $A^2B$  but absent from the homozygous  $A^2$  casein. S-casein has the slowest mobility at alkaline pH (gels a, b) and the fastest mobility at acid pH (gels d, e). The TS-caseins have the same mobility at pH 9.6 (gels a, b, c) but are clearly resolved at pH 4.3. Gels d and e show TS-B for caseins typed B and  $A^2B$ , respectively, and gels e and f show TS- $A^2$  bands for the caseins typed  $A^2B$  and  $A^2$ . R-casein, associated with caseins typed  $A^2$ , may be seen at alkaline pH in gels b, c. It is absent from caseins typed B, gel a. R-casein is not clearly resolved from  $\gamma$ -casein B at acid pH.

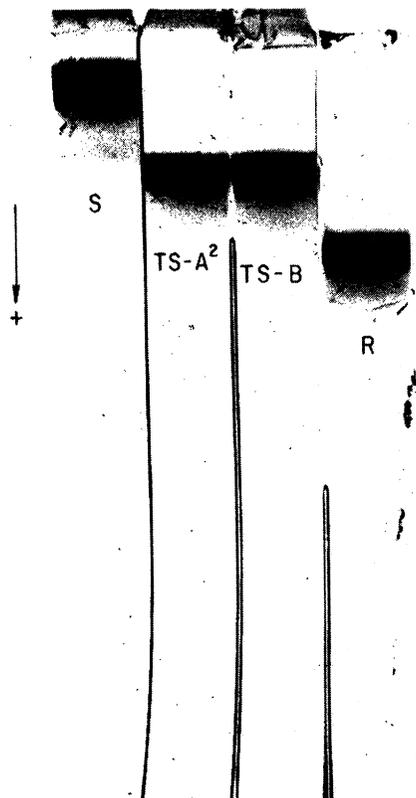


FIG. 4. Disc-gel electrophoretic patterns of the purified caseins, TS-A<sup>2</sup>, TS-B, R-, and S-caseins, at pH 9.6, 4 M urea.

The disc-gel electrophoretic patterns in Fig. 4 and 5 indicate the purity of the isolated TS-A<sup>2</sup>, TS-B, R-, and S-caseins at both alkaline and acid pH. These were the protein samples used in the present study.

*Molecular weights and compositional analysis.* In an earlier paper the molecular weights of TS-B, R-, and S-caseins, as determined by sedimentation-equilibrium in low ionic strength buffers, were reported to be 12,900, 13,250, and 16,150 (9).

Molecular weight estimates based on the relative electrophoretic mobilities of sodium dodecyl sulfate complexes of TS-A<sup>2</sup>, TS-B, R-, and S-caseins in polyacrylamide gels are shown in Fig. 6. Several determinations by this method demonstrated that these proteins all have molecular weights of 12,000 to 13,000. Since these estimates of molecular weights for all four caseins agree with values for the TS-B and R-caseins by the method of sedimentation-equilibrium, we conclude that the value of 16,150

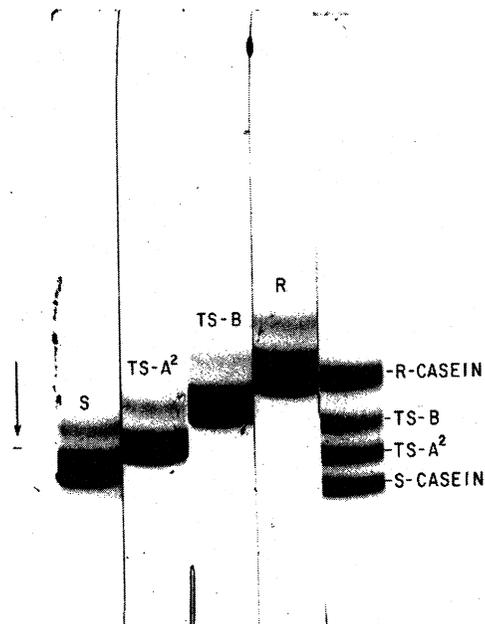


FIG. 5. Disc-gel electrophoretic patterns of the purified caseins, TS-A<sup>2</sup>, TS-B, R-, and S-casein, at pH 4.8, 8 M urea. The pattern for all four caseins in one gel also is shown.

reported previously for S-casein is too high.

Previously published tryptophan values for TS-A<sup>2</sup>, TS-B, R-, and S-caseins were 1.31, 1.52, 1.49, and 1.24% (9). Calculations of minimum molecular weights based on these values indicate that the two pairs, TS-A<sup>2</sup>, S- and TS-B, R-caseins, have molecular weights of about 16,000 and 13,000. Caseins in this study had tryptophan contents: TS-A<sup>2</sup>, 1.66, 1.61, 1.42, 1.55, 1.62%; TS-B, 1.67, 1.57, 1.49%; R-casein, 1.65, 1.69%; and S-casein, 1.63, 1.41, 1.47%. Minimum molecular weights calculated from these values indicate a range of 12,000 to 14,000. Apparently, the earlier reported tryptophan values, especially those for TS-A<sup>2</sup> and S-caseins, are too low. From these experiments it appears that the TS-A<sup>2</sup>, TS-B, R-, and S-caseins all have similar molecular weights and that each contains one residue of tryptophan per molecule.

The amino acid compositions, derived from mean molar ratios and based on the presence of 2 glycine and 2 alanine residues per molecule, together with estimates of amide NH<sub>3</sub> are in Table 1. Whole-number residues for comparisons are in Table 2. The molecular weights of TS-A<sup>2</sup>, TS-B, R-, and S-caseins calculated

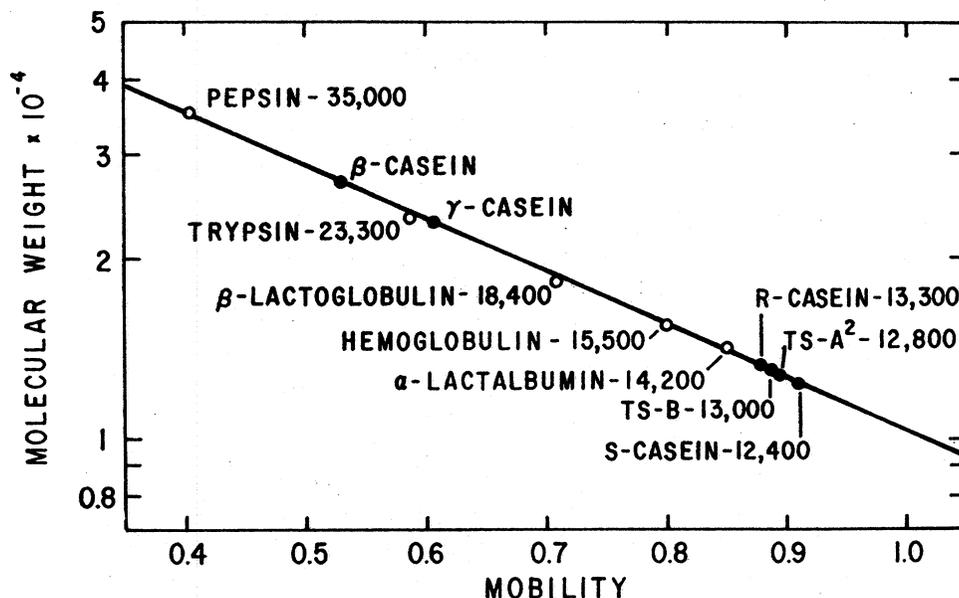


FIG. 6. Molecular weights of caseins and standard proteins as determined by their relative mobilities on polyacrylamide gels containing sodium dodecyl sulfate. The caseins are indicated by the filled circles.

TABLE 1. Amino acid composition of TS-A<sup>2</sup>, S-, R-, and TS-B caseins.

Amino acid	Residues <sup>a</sup> amino acid per molecule calculated from mean molar ratios based on Gly = 2, Ala = 2							
	TS-A <sup>2</sup>	SD	S-casein	SD	R-casein	SD	TS-B	SD
Lys	3.85	.11	3.84	.08	2.89	.05	2.83	.11
His	3.74	.08	3.74	.08	2.89	.09	2.85	.09
Amide NH <sub>2</sub>	11.65	.30	11.60	.22	12.05	.74	11.68	.44
Arg	1.85	.04	2.82	.06	1.89	.10	2.79	.04
Asp	2.93	.05	2.91	.04	2.96	.04	2.94	.07
Thr	3.71	.04	3.75	.03	3.80	.03	3.68	.04
Ser	6.55	.07	5.60	.05	6.79	.05	5.62	.07
Glu	14.34	.18	14.27	.20	14.49	.13	14.19	.15
Pro	19.77	.28	19.74	.31	19.93	.37	19.48	.24
Gly	1.95	...	1.97	...	1.99	...	2.00	...
Ala	2.06	...	2.04	...	2.02	...	2.00	...
Cys	0		0		0		0	
Val	9.40	.13	9.32	.14	9.39	.14	9.22	.15
Met	3.86	.11	3.84	.05	3.84	.08	3.71	.08
Ile	2.78	.09	2.82	.09	2.84	.03	2.81	.06
Leu	13.45	.11	13.33	.16	13.58	.18	13.33	.18
Tyr	2.76	.06	2.73	.06	2.80	.05	2.76	.09
Phe	4.80	.07	4.78	.04	4.80	.10	4.67	.08
Trp	1		1		1		1	

<sup>a</sup>Residue numbers are averages of, or extrapolated values from, nine determinations. Triplicate analyses were made on samples hydrolyzed 24, 72, and 96 h. For isoleucine the figures are averages of six determinations made on 72 and 96-h hydrolysates. The threonine, serine, and amide NH<sub>2</sub> numbers were obtained by linear regression analysis, and for these the standard error is shown rather than the standard deviation. Tryptophan results from which these values are derived are shown in text.

TABLE 2. Comparison of composition of TS-A<sup>2</sup>, S-, R-, and TS-B caseins.

Amino acid	Whole number residues per molecule containing 2 Gly and 2 Ala			
	TS-A <sup>2</sup>	S-casein	R-casein	TS-B
Lys	4	4	3	3
His	4	4	3	3
Arg	2	3	2	3
Asp	3	3	3	3
Thr	4	4	4	4
Ser	7	6	7	6
Glu	14-15	14-15	14-15	14-15
Pro	19-20	19-20	19-20	19-20
Gly	2	2	2	2
Ala	2	2	2	2
Val	9-10	9-10	9-10	9-10
Met	4	4	4	4
Ile	3	3	3	3
Leu	13-14	13-14	13-14	13-14
Tyr	3	3	3	3
Phe	5	5	5	5
Trp	1	1	1	1
Total amino acids (maximum numbers)	103	103	101	101
Mol wt <sup>a</sup>	11,737	11,807	11,472	11,542

<sup>a</sup> Calculated from the maximum number of amino acids shown; amide groups not considered but one molecule of water added.

from these data all are slightly less than 12,000.

The phosphorus content of TS-A<sup>2</sup>, TS-B, R-, and S-caseins was .06, .05, .06, and .04%. These values yield calculated minimum molecular weights of between 52,000 and 78,000 based on one atom of phosphorus per

molecule. Since TS-A<sup>2</sup>, TS-B, R-, and S-caseins all have molecular weights of about 12,000, the small amounts of phosphorus are most likely impurities.

*Peptide patterns and specific staining.* The high voltage electrophoretic patterns for chymotrypsin digests of TS-A<sup>2</sup>, TS-B, R-, and

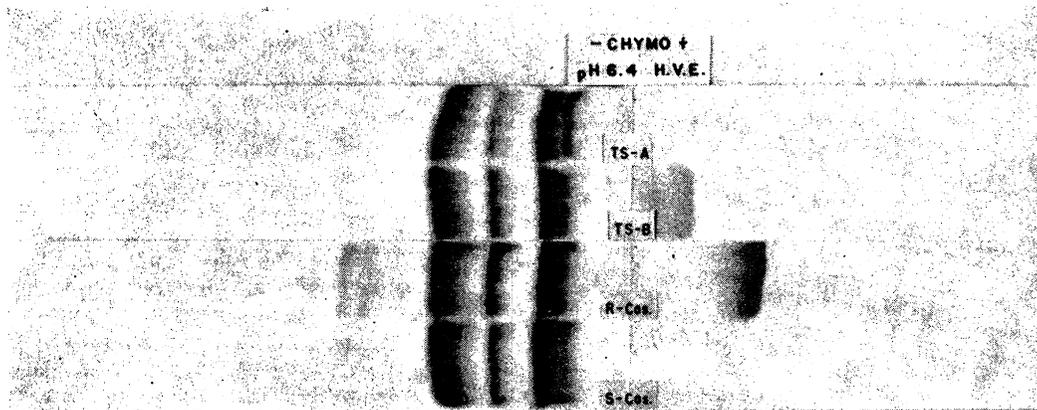


FIG. 7. Peptide pattern from chymotryptic digests of TS-A<sup>2</sup> (TS-A in Fig.), TS-B, R-, and S-caseins after one-dimensional, high-voltage paper electrophoresis.

S-caseins are in Fig. 7. The peptide patterns for all four caseins are similar except that TS-B and R-caseins each contained a peptide migrating toward the anode. This peptide was absent in the TS-A<sup>2</sup> and S-caseins. The two dimensional peptide maps of these caseins (not shown) also indicated that most of the peptides of TS-A<sup>2</sup>, TS-B, R-, and S-caseins are identical. Only one peptide from each of the caseins stained specifically for tryptophan. This peptide corresponds in mobility to the single tryptophan-containing peptide found in the  $\gamma$ - and  $\beta$ -casein digests (6).

**Amino- and carboxyl-terminal amino acid determinations.** Results of the Edman and dansylation procedures were similar. Histidine is N-terminal in TS-A<sup>2</sup> and S-casein, and glutamic acid is N-terminal in R- and TS-B caseins.

The TS-A<sup>2</sup>, TS-B, R-, and S-caseins all gave the same C-terminal sequence (-Ile-Ile-Val·OH) on hydrolysis with carboxypeptidase. The release of valine was rapid after short times of incubation with the enzyme. Isoleucine was released at a slightly later time and became predominant in total quantity of amino acids recovered. In a typical experiment on TS-A<sup>2</sup>, the release of valine and isoleucine leveled off after about 22 h at 1.4 and 2.5 moles, respectively, per mole of protein. No other amino acids were released except in trace quantities. The rate of release of valine and isoleucine was slower for S-casein than for the others, but the total amino acids released were comparable.

**The digestion of  $\gamma$ - and  $\beta$ -caseins B with trypsin.** Disc gel electrophoretic patterns of tryptic digests of  $\gamma$ - and  $\beta$ -caseins B are in Fig. 9. Bands corresponding in mobility to  $\gamma$ -B, TS-B, and S-caseins were produced from  $\beta$ -casein B, while  $\gamma$ -casein B produced bands corresponding to TS-B and S-caseins.

#### Discussion

On the basis of our earlier studies and because of the observed differences in mobilities in disc gel electrophoresis, we believed that TS-A<sup>2</sup> and TS-B caseins constituted one set of genetic polymorphs and R- and S-caseins a second set. The proteins were named accordingly. However, the results of N-terminal group identification and amino acid analysis reported here show that the pairs are, in fact, TS-A<sup>2</sup> and S and R and TS-B. It is not clear why the operational definition of temperature-sensitive can be applied only to the TS- and S-caseins but not to R-casein. Revision of these names seems desirable.

These caseins are simple proteins with mo-

lecular weights about 12,000 and contain one residue of tryptophan per molecule but lack cysteine. In composition TS-A<sup>2</sup> differs from S, and R from TS-B, only in a single amino acid substitution, Ser  $\rightarrow$  Arg. The same substitution occurs in the A<sup>2</sup> and B polymorphs of both  $\gamma$ - and  $\beta$ -caseins (6). TS-A<sup>2</sup> is larger than R, and S is larger than TS-B by two residues, one histidine plus one lysine. Otherwise, all four proteins are identical in composition. Peptide maps suggest close similarity in composition and primary structure. End-group analysis discloses the same C-terminal sequence in all, namely, -Ile-Ile-Val·OH, also found in  $\gamma$ - and  $\beta$ -caseins (6). The N-terminal amino acid is different, however: H·His- is found in TS-A<sup>2</sup> and S, H·Glu- in R and TS-B. H·Arg- is N-terminal in  $\beta$ -casein, H·Lys- in  $\gamma$ -casein (6).

Considering these facts in conjunction with the partial structure of  $\beta$ -casein A<sup>2</sup> worked out by Ribadeau Dumas et al. (13, 14, 15), we thought it possible that TS-, R-, and S-caseins represent large C-terminal segments common to the sequence of  $\gamma$ - and  $\beta$ -caseins (2). Subsequently, Ribadeau Dumas et al. (12) reported the complete sequence of  $\beta$ -casein A<sup>2</sup>. This is reproduced as Fig. 8, for it incorporates our suggested positioning (2) of  $\gamma$ -, TS-A<sup>2</sup>, S-, R-,

TABLE 3. Comparisons of amino acid composition of  $\gamma$ -A<sup>2</sup> and TS-A<sup>2</sup> caseins and a portion of  $\beta$ -A<sup>2</sup> casein.

Amino acid	Amino acid residues			
	$\gamma$ -casein A <sup>2a</sup>	TS-A <sup>2</sup>	Difference	B-casein A <sup>2b</sup> residues 29 through 105
Lys	10	4	6	6
His	5	4	1	1
Arg	2	2	0	0
Asp	7	3	4	4
Thr	8	4	4	4
Ser	11	7	4	4
Glu	32	15	17	17
Pro	33	20	13	13
Gly	4	2	2	2
Ala	5	2	3	3
Val	17	10	7	7
Met	6	4	2	2
Ile	7	3	4	4
Leu	19	14	5	5
Tyr	4	3	1	1
Phe	9	5	4	4
Trp	1	1	0	0
Total	180	103	77	77

<sup>a</sup> Groves et al. (6).

<sup>b</sup> Ribadeau Dumas (12).



FIG. 8. The primary structure of bovine  $\beta$ -casein A<sup>2</sup> according to Ribadeau Dumas et al. (12). (Reproduced by permission.)

and TS-B caseins as segments of  $\beta$ -casein.

The schematic diagram in our previous paper (2) was based on a  $\beta$ -casein A<sup>2</sup> with 208 residues (15) and the other caseins positioned accordingly. When the complete sequence of  $\beta$ -casein A<sup>2</sup> was published (12), an extra serine was found in the phosphopeptide portion (11) preceding  $\gamma$ -casein, which necessitates changes incorporated in Fig. 8. Thus,  $\beta$ -casein A<sup>2</sup> has 209 residues,  $\gamma$ -casein begins with Residue 29 of  $\beta$ -casein, S and TS-A<sup>2</sup> with 106, and R and TS-B with 108. Conclusions drawn from previous data are unaffected.

Amino acid analyses (Table 1) were rounded to give the whole numbers in Table 2. It was impossible to assign with confidence a single number to certain amino acids. This also was true for the  $\gamma$ - and  $\beta$ -caseins (6). A summation of the maximum number of amino acids for each gives 207 and 180 residues, respectively, for  $\beta$ -A<sup>2</sup>, B and  $\gamma$ -A<sup>2</sup>, B (6); 103 and 101 for TS-A<sup>2</sup>, S- and R-, TS-B caseins (Table 2). Table 3 lists the upper estimates of the

amino acid residues in  $\gamma$ -A<sup>2</sup> and TS-A<sup>2</sup> caseins. A comparison of the last two columns of Table 3 indicates that the differences in number of residues for each amino acid agree exactly with the number of those amino acids in the sequence of  $\beta$ -casein from Residue 29 through 105. This segment is presumably the N-terminal portion of  $\gamma$ -casein A<sup>2</sup>. Further, R-casein is smaller than TS-A<sup>2</sup> by histidine plus lysine. The sequence shows that Residue 106 is His and 107 is Lys. Together with the end-group data, this kind of fitting of amino acid numbers led to the positioning of the smaller proteins in the sequence of  $\beta$ -casein. There are two residues in the sequence of  $\beta$ -casein A<sup>2</sup> (Fig. 8) unaccounted for by our data, Ser 22 and one Pro in the region 118 to 209. These inconsistencies cannot be explained at present.

Regarding the amino acid substitutions which differentiate the A<sup>2</sup> and B genetic polymorphs under discussion, they now may be located in the sequence. Grosclaude et al. (3) found the Pro  $\rightarrow$  His substitution in the  $\beta$ - and

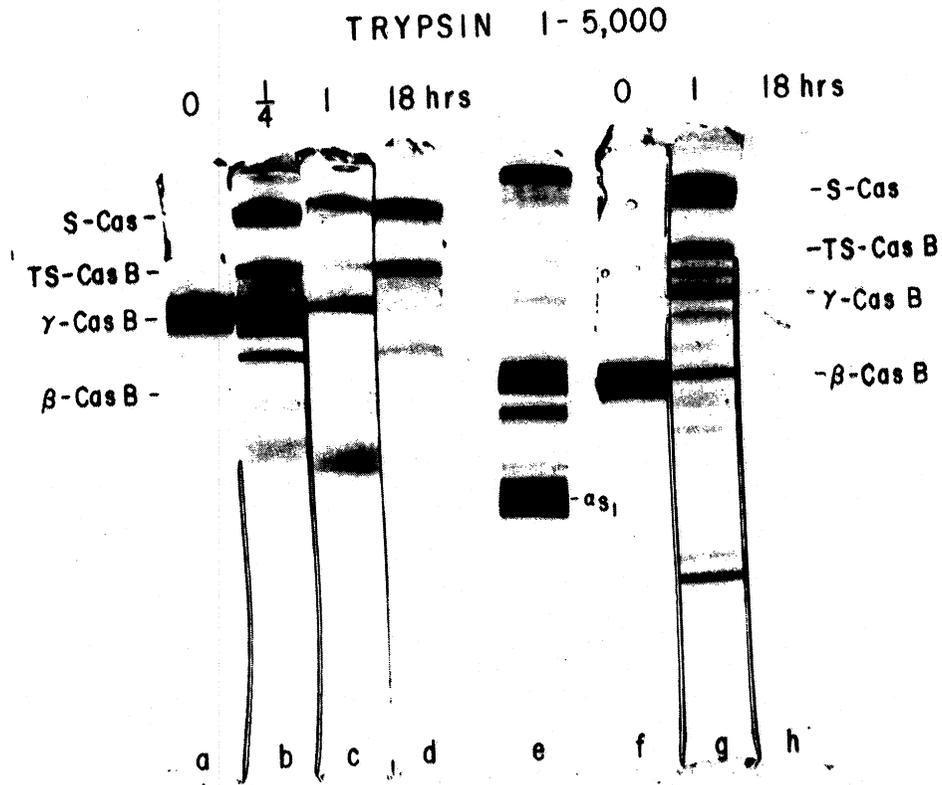


FIG. 9. Disc-gel electrophoretic patterns (pH 9.6, 4 M urea) of  $\gamma$ -casein B (gels a, b, c, d) and of  $\beta$ -casein B (gels f, g, h) digested by trypsin for different times; gel e shows untreated casein typed  $\gamma$ -,  $\beta$ -B.

$\gamma$ -caseins at Residue 67. Brignon et al. (1) showed the Ser  $\rightarrow$  Arg substitution common to the  $\beta$ -A<sup>2</sup> vs. B,  $\gamma$ -A<sup>2</sup> vs. B, TS-A<sup>2</sup> vs. S, and R vs. TS-B pairs to occur at Residue 122. The actual sequencing, of course, has been done only with the  $\beta$ -caseins. Likewise, the single Trp in all these caseins is Residue 143. The location of the one phosphoserine residue in  $\gamma$ -casein not known before (2) now can be specified to be at Residue 35 (1, and unpublished data).

The sequencing of N-terminal positions of  $\gamma$ -A<sup>2</sup>, TS-A<sup>2</sup>, and TS-B caseins reported previously (2) gave results in excellent agreement with appropriate regions of the sequence in Fig. 8 except for one discrepancy, i.e., Residue 13 in  $\gamma$ -casein A<sup>2</sup> is Glx. In Fig. 8 Thr is in this position (Residue 41).

One of the hypotheses previously proposed to explain the origin of  $\gamma$ -casein in milk was a limited, highly specific proteolysis of  $\beta$ -casein (6). Identification of bands corresponding in

mobility to TS-B and S-caseins, as well as to  $\gamma$ -casein B, in the tryptic digest of  $\beta$ -casein B (Fig. 9) suggests that these caseins may arise in the same way. Further support for the hypothesis is the identification of bands corresponding in mobility to TS-B and S-caseins in the tryptic digest of  $\gamma$ -casein B (Fig. 9).

Extensive experiments regarding the origin of minor caseins have been made independently by Kaminogawa and Yamauchi (10, 18). They digested  $\beta$ -casein, presumably type A, with concentrated milk protease. Fragments with electrophoretic mobilities equal to temperature-sensitive and R- (or  $\gamma$ -) casein were produced. Two products, F-II and F-III, were isolated and compared with preparations of TS- and R-caseins. Comparison of amino acid composition, molecular weight, and sedimentation coefficient showed F-II to be essentially identical to TS- and F-III to R-casein. They suggested that TS- and R-caseins are possibly the decomposed products of  $\beta$ -casein by milk

protease. The data of Kaminogawa and Yamauchi agree with those reported here, and apparently their TS- and R-casein preparations were similar to ours.

The unusual temperature-sensitivity of these proteins is an interesting phenomenon, undoubtedly related to their primary structure. A somewhat similar temperature-sensitivity has been described by Schade and Reinhardt (16) for galactothermin, a protein isolated from human milk. Galactothermin also is a relatively small protein rich in hydrophobic amino acids, free of cystine and phosphorus, and containing one residue of tryptophan per molecule. One may speculate that galactothermin might be closely related to the main component of human casein, just as the proteins under discussion are related to bovine  $\beta$ -casein.

#### Acknowledgment

We thank Dr. B. Ribadeau Dumas for permission to reproduce the primary sequence of  $\beta$ -casein, Fig. 8, and Gregory Hemighaus for technical assistance.

#### References

- (1) Brignon, G., B. Ribadeau Dumas, F. Grosclaude, and J.-C. Mercier. 1971. Structure primaire de la caséine  $\beta$  bovine. Séquence partielle. *European J. Biochem.* 22:179.
- (2) Gordon, W. G., M. L. Groves, R. Greenberg, S. B. Jones, E. B. Kalan, R. F. Peterson, and R. E. Townend. 1972. Probable identification of  $\gamma$ -, TS-, R- and S-caseins as fragments of  $\beta$ -casein. *J. Dairy Sci.* 55:261.
- (3) Grosclaude, F., M.-F. Mahé, J.-C. Mercier, and B. Ribadeau Dumas. 1972. Caractérisation des variants génétiques des caséines  $\alpha_{s1}$  et  $\beta$  bovines. *European J. Biochem.* 26:328.
- (4) Groves, M. L. 1969. Some minor components of casein and other phosphoproteins in milk. A review. *J. Dairy Sci.* 52:1155.
- (5) Groves, M. L., and W. G. Gordon. 1969. Evidence from amino acid analysis for a relationship in the biosynthesis of  $\gamma$ - and  $\beta$ -caseins. *Biochim. Biophys. Acta* 194:421.
- (6) Groves, M. L., W. G. Gordon, E. B. Kalan, and S. B. Jones. 1972. Composition of bovine  $\gamma$ -caseins A<sup>1</sup> and A<sup>2</sup>, and further evidence for a relationship in biosynthesis of  $\gamma$ - and  $\beta$ -caseins. *J. Dairy Sci.* 55:1041.
- (7) Groves, M. L., and C. A. Kiddy. 1968. Polymorphism of  $\gamma$ -casein in cow's milk. *Arch. Biochem. Biophys.* 126:188.
- (8) Groves, M. L., and C. A. Kiddy. 1970.  $\gamma$ -Caseins isolated from milk samples typed  $\beta$ -casein A<sup>1</sup> and A<sup>2</sup>. *J. Dairy Sci.* 53:931.
- (9) Groves, M. L., and R. E. Townend. 1970. Molecular weight of some human and cow caseins. *Arch. Biochem. Biophys.* 139:406.
- (10) Kaminogawa, S., and K. Yamauchi. 1972. Decomposition of  $\beta$ -casein by milk protease. Similarity of the decomposed products to temperature-sensitive and R-caseins. *Agr. Biol. Chem.* 36:255.
- (11) Ribadeau Dumas, B., G. Brignon, F. Grosclaude, and J.-C. Mercier. 1971. Structure primaire de la caséine  $\beta$  bovine. Enchaînement de 32 résidus d'amino-acides de la partie NH<sub>2</sub>-terminale. *European J. Biochem.* 20:264.
- (12) Ribadeau Dumas, B., G. Brignon, F. Grosclaude, and J.-C. Mercier. 1972. Structure primaire de la caséine  $\beta$  bovine. Séquence complète. *European J. Biochem.* 25:505.
- (13) Ribadeau Dumas, B., F. Grosclaude, and J.-C. Mercier. 1970. Structure primaire de la caséine  $\beta$  bovine. Isolement et composition en amino-acides des peptides tryptiques et des peptides obtenus par action du bromure de cyanogène. *European J. Biochem.* 14:451.
- (14) Ribadeau Dumas, B., F. Grosclaude, and J.-C. Mercier. 1970. Localisation dans la chaîne peptidique de la caséine  $\beta$  bovine de la substitution His/Gln différencient les variants génétique A<sub>2</sub> and A<sub>3</sub>. *C. R. Hebd. Séances Acad. Sci. Paris* 270:2369.
- (15) Ribadeau Dumas, B., F. Grosclaude, and J.-C. Mercier. 1971. Structure primaire de la caséine  $\beta$  bovine. Enchaînement des peptides tryptiques et des peptides obtenus par action du bromure de cyanogène. *European J. Biochem.* 18:252.
- (16) Schade, A. L., and R. W. Reinhardt. 1970. Galactothermin, a reversibly heat-precipitable protein of human milk at neutral pH. *Biochem. J.* 118:181.
- (17) Schroeder, W. A., J. R. Shelton, J. B. Shelton, J. McCormick, and R. T. Jones. 1965. The amino acid sequence of the  $\gamma$  chain of human fetal hemoglobin. *Biochemistry* 2:992.
- (18) Yamauchi, K., and S. Kaminogawa. 1972. Decomposition of milk proteins by milk protease. *Agr. Biol. Chem.* 36:249.