

## EVIDENCE FROM AMINO ACID ANALYSIS FOR A RELATIONSHIP IN THE BIOSYNTHESIS OF $\gamma$ - AND $\beta$ -CASEINS

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### SUMMARY

$\gamma$ -Casein, like  $\beta$ -casein, is polymorphic as shown by gel electrophoresis. Two variants of  $\gamma$ -casein, A<sup>2</sup> and B, have been isolated from samples of bovine casein genetically typed  $\beta$ -casein A<sup>2</sup> and B. The  $\beta$ -caseins were also isolated from the same samples. By comparison of the composition of the proteins it was shown that  $\gamma$ -A<sup>2</sup> differs from  $\gamma$ -B only in content of single residues of four amino acids and two substitutions, Ser/Arg and His/Pro are postulated.  $\beta$ -A<sup>2</sup> differs from  $\beta$ -B in the same manner, implying a close relationship in the synthesis of these milk proteins. The  $\gamma$ -caseins differ considerably from the  $\beta$ -caseins in content of proline and phosphorus. Comparison of the composition of four polymorphs of  $\beta$ -casein indicated that, in addition to the Ser/Arg and His/Pro substitutions, a third, Glu/Lys, may occur in the  $\beta$ -casein variants.

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### INTRODUCTION

The term,  $\gamma$ -casein, was introduced by MELLANDER<sup>1</sup> in 1939 to designate a component of bovine casein which migrated more slowly than the major components,  $\alpha$ - and  $\beta$ -caseins, in moving-boundary electrophoresis. Subsequently, methods for the preparation of  $\gamma$ -casein were worked out, and its properties and composition were described. Later, ASCHAFFENBURG<sup>2,3</sup> demonstrated by typing experiments of individual milks by paper electrophoresis that genetically controlled polymorphism of  $\gamma$ -casein, as well as of  $\beta$ -casein, probably occurs. These developments have been reviewed<sup>4,5</sup>.

By means of starch-gel electrophoresis, GROVES *et al.*<sup>6</sup> showed that  $\gamma$ -casein prepared by the methods of HIPP *et al.*<sup>7,8</sup> was in all likelihood a mixture of proteins. A very pure  $\gamma$ -casein could, however, be isolated by column chromatography on DEAE-cellulose of an acid extract of casein. As will be reported presently, this can be done as well by starting with whole casein. In either case, a first fraction, designated "temperature-sensitive" (TS) because it is much less soluble at 25 than at 3°, emerges in the starting buffer of 5 mM phosphate (pH 8.3). A second fraction is eluted with

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\* Agricultural Research Service, U. S. Department of Agriculture.

0.02 M phosphate (pH 8.3), and the major portion of this fraction can be readily purified by rechromatography. It is proposed that the name " $\gamma$ -casein" be retained for this purified material, since its electrophoretic mobility, properties and amino acid composition are similar to those of the  $\gamma$ -casein investigated by HIPP *et al.*<sup>7,8</sup> and analyzed by GORDON *et al.*<sup>9</sup>.

That  $\gamma$ -casein is indeed synthesized as genetic polymorphs was verified by GROVES AND KIDDY<sup>10</sup> by disc-gel electrophoresis at alkaline pH's. Two variants, A and B, were distinguished and furthermore their occurrence was shown to be genetically related to the  $\beta$ -casein variants, A and B, described by PETERSON<sup>11</sup> and THOMPSON *et al.*<sup>12</sup> because the A forms of both are found together, as are the B forms. However, a C variant of  $\gamma$ -casein corresponding to the known  $\beta$ -casein C could not be detected in milk typed  $\beta$ -casein C.

In the case of the  $\beta$ -caseins, when typing is done by gel electrophoresis at acid pH's, three allelic forms of  $\beta$ -casein A, in addition to  $\beta$ -caseins B and C, can be differentiated according to PETERSON AND KOPFLER<sup>13</sup> and KIDDY *et al.*<sup>14</sup>. Likewise,  $\gamma$ -caseins isolated from caseins typed  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and A<sup>3</sup> can be distinguished by differences in mobility at acid pH's (disc-gel electrophoresis, 8 M urea, pH 4.3) and are designated  $\gamma$ -A<sup>1</sup>, A<sup>2</sup> and A<sup>3</sup>; A<sup>1</sup> denoting the band of fastest mobility under these conditions (M. L. GROVES and C. A. KIDDY, unpublished data).

In the present report, we describe methods for the preparation and purification of the  $\gamma$ - and  $\beta$ -caseins present in caseins precipitated by acid from individual milk samples typed  $\beta$ -caseins A<sup>2</sup> and B. We then compare the amino acid composition of the purified  $\gamma$ - and  $\beta$ -caseins from the milk of the same cow. For purposes of further comparison we also include results of amino acid analysis of A<sup>1</sup> and C  $\beta$ -caseins isolated by the same methods.

## EXPERIMENTAL AND RESULTS

### *Electrophoresis*

The disc-gel electrophoretic method using gels at pH 9.6 in 4 M urea has been described in an earlier publication<sup>10</sup>. Disc-gel electrophoresis (pH 4.3) in 8 M urea is a modification of the method of REISFELD *et al.*<sup>15</sup>. All stock solutions were made to 8 M urea. Stock Solution A had a pH of 4.5 in the presence of urea and 4.3 in its absence. Solution C contained 30 g acrylamide, 0.8 g *N,N'*-methylenebisacrylamide and 48 g urea in 100 ml. The separating gel (5.6% acrylamide) was made with 2 parts A, 3 parts C, 3 parts 8 M urea and 8 parts 8 M urea containing 0.14 g ammonium persulfate per 50 ml. Electrophoresis was run using  $\beta$ -alanine buffer without urea (pH 5.0) for about 2 h at 60 mA with the 12-tube setup.

### *Phosphorus*

The phosphorus content of the proteins was determined by the method of SUMNER<sup>16</sup> scaled down to permit estimation of about 0.005 mg P.

### *Carbohydrate*

Small amounts of hexose in  $\gamma$ -casein were detected by the orcinol reaction described by WINZLER<sup>17</sup>.

*Tryptophan*

We are greatly indebted to Dr. J. R. Spies who determined the tryptophan content of the  $\gamma$ -caseins by Procedure U (hydrolysis in 5 M NaOH) of his method<sup>18</sup>.

*Amino acid composition*

Proteins were hydrolyzed and analyzed automatically for amino acid content by the procedures described by MOORE AND STEIN<sup>19</sup>. The analyzer was coupled to an analog to digital converter<sup>20</sup>, the output of which was processed on a computer by use of an as yet unpublished program. We thank C. R. Eddy, E. A. Talley and W. R. Porter of this Laboratory for adapting the program to our needs, and Mrs. Ruth Zabarsky for processing the data.

*Preparation and purification of proteins*

The casein in skim milk from an individual cow homozygous for the respective casein variants was precipitated at 25° by the addition of 1 M HCl to pH 4.6. After the precipitate was collected in a cloth bag, it was washed several times by resuspension in distilled water and by adjusting the pH to 4.6–4.7. The wet casein was then lyophilized and stored at –20°.

A first chromatographic fractionation of whole casein was made with Whatman's\* fibrous DEAE-cellulose (DE23) while final purifications of  $\beta$ - and  $\gamma$ -caseins were made with the microgranular form of DEAE-cellulose (DE32). The casein, 12 g, was suspended in 250 ml of 5 mM sodium phosphate (pH 8.3) and dissolved by adding 0.5 M NaOH dropwise with stirring to a final pH of 8.3. The sample was chromatographed at 3–4° on a fibrous DEAE-cellulose column (3.8 cm  $\times$  50 cm) previously equilibrated with 5 mM phosphate (pH 8.3). All phosphate buffers used subsequently, regardless of molarity, were at this pH. Elution was at a rate of about 160 ml/h (20 ml of eluate collected per tube). After the first fraction, "temperature-sensitive" (TS), was eluted, the buffer was changed to 0.02 M phosphate for the elution of the  $\gamma$ -casein fractions (I, II and III) and finally to 0.10 M phosphate, for the  $\beta$ -casein fractions (IV, V and VI). The caseins, including  $\alpha_{s1}$ -casein, remaining on the column were not recovered.

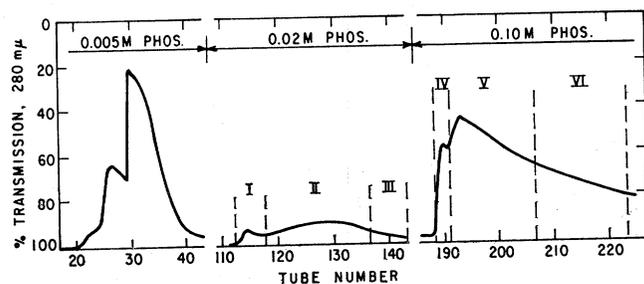


Fig. 1. Stepwise chromatography (pH 8.3) of casein (12 g dissolved in 5 mM phosphate) on DEAE-cellulose column, 3.8 cm  $\times$  50 cm, equilibrated with 5 mM phosphate. The TS fraction is eluted with the 5 mM phosphate starting buffer;  $\gamma$ -casein fractions with 0.02 M phosphate and  $\beta$ -casein fractions with 0.10 M phosphate; percent transmission recorded on LKB Uvicord.

\* Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Fig. 1 shows a typical chromatogram. Contents of the tubes between the dashed lines were combined and dialyzed at 3° until free of salt, and the protein was recovered by lyophilization. The 0.10 M phosphate gives a considerable amount of "trailing" under these conditions. The remainder of the protein eluted by 0.10 M phosphate (contained in Tubes 223-341 and not shown in the chromatogram) was designated Fraction VII. Fractions V, VI and VII all gave similar disc-gel electrophoretic patterns showing a heavy band for  $\beta$ -casein and several other minor, slower-moving bands. The  $\beta$ -casein fractions were further fractionated before rechromatography by dissolving the protein and then precipitating the  $\beta$ -casein at pH 4.6. The precipitate was washed with water and recovered by lyophilization. Yields of TS,  $\gamma$ -casein, and  $\beta$ -casein fractions were as follows: for the A<sup>2</sup> type, 7, 2 and 27%, respectively, and for the B type, 13, 2 and 22% of the original casein.

In preliminary experiments, samples of the  $\gamma$ - and  $\beta$ -casein fractions were each rechromatographed on microgranular DEAE-cellulose. Disc-gel electrophoretic patterns of the isolated proteins showed a number of new bands that represented a significant amount of the material put on the column. This indicated degradation, presumably by the milk proteolytic enzyme concentrated in these fractions. Consequently, the  $\gamma$ - and  $\beta$ -casein fractions were dissolved in phosphate buffer (pH 8.3) and heated for 5 min at 100° before rechromatography as follows.

The  $\gamma$ -casein fractions corresponding to II (Fig. 1) were rechromatographed on microgranular DEAE-cellulose columns equilibrated with 5 mM phosphate at 3°. A typical chromatogram for the  $\gamma$ -casein A<sup>2</sup> fraction is shown in Fig. 2. The protein, 200 mg, was dissolved in 20 ml of 0.05 M phosphate buffer and adjusted to pH 8.3 with 0.1 M NaOH. After the solution was heated 5 min at 100°, it was equilibrated by dialysis against several changes of 5 mM phosphate and then chromatographed on a 2 cm  $\times$  35 cm column. In this experiment, 83 mg of purified  $\gamma$ -casein A<sup>2</sup> were obtained as represented in Fig. 2 by the curve enclosed by dashed lines. The chromatographic pattern for the  $\gamma$ -casein B fraction is similar to that shown for  $\gamma$ -casein A<sup>2</sup> except that only one sharp peak is eluted with the starting buffer and the  $\beta$ -casein B is eluted a

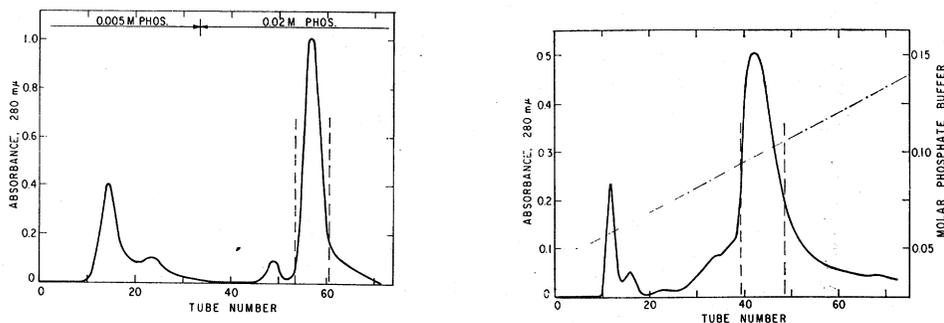


Fig. 2. Stepwise rechromatography of  $\gamma$ -casein A<sup>2</sup>—200 mg of  $\gamma$ -casein fraction (Fig. 1, II) dissolved in phosphate (pH 8.3) and equilibrated with 5 mM phosphate—on a DEAE-cellulose column, 2 cm  $\times$  35 cm, equilibrated with 5 mM phosphate. The purified  $\gamma$ -casein eluted with 0.02 M phosphate buffer (about 9 ml/tube, 27 ml/h) is indicated by the dashed lines.

Fig. 3. Gradient elution of  $\beta$ -casein B fraction (Fig. 1, V)—216 mg in 20 ml 25 mM phosphate (pH 8.3)—on DEAE-cellulose column 2 cm  $\times$  34 cm, equilibrated with 25 mM phosphate buffer. Gradient was 0.05–0.15 M phosphate buffer. Elution was at a rate of 66 ml/h (11 ml/tube). The dashed lines indicate the  $\beta$ -casein fraction used for the final stepwise rechromatography.

little sooner after the 0.02-M phosphate buffer change. In one experiment, the  $\gamma$ -casein fraction from casein typed  $\gamma$ -casein AB was rechromatographed. Two major peaks were eluted with the 0.02 M phosphate buffer; the first contained  $\gamma$ -casein B and the second  $\gamma$ -casein A<sup>2</sup>.

The  $\beta$ -casein was purified by rechromatographing the  $\beta$ -casein fraction (reprecipitated Fraction V or VI) on microgranular DEAE-cellulose twice, the first time using gradient elution and the second using stepwise elution. The gradient elution removed all but a few minor impurities while the final stepwise procedure gave the pure protein as indicated by disc-gel electrophoresis. In a typical experiment (Figs. 3 and 4) the 216-mg  $\beta$ -casein B fraction was dissolved in 20 ml of 25 mM phosphate buffer and adjusted to pH 8.3 with 0.1 M NaOH. The solution was heated 5 min at 100°, cooled and then applied to a microgranular DEAE-cellulose column, 2 cm  $\times$  34 cm, previously equilibrated with 25 mM phosphate buffer. A 5-chambered Varigrad was used for elution of the protein. Each chamber contained 150 ml of buffer (pH 8.3) in the following sequence: 0.05, 0.075, 0.10, 0.125 and 0.150 M phosphate buffer. The major peak enclosed by dashed lines (Fig. 3) represents the  $\beta$ -casein fraction that was used for the final stepwise rechromatography. The elution of the major peak of  $\beta$ -casein B was calculated to occur at about 0.10 M phosphate buffer concentration. A similar elution pattern was obtained for  $\beta$ -casein A<sup>2</sup> but the first peak was somewhat higher.

For the final purification of  $\beta$ -casein, the sample in a typical experiment, 138 mg, was dissolved in 10 ml of 25 mM phosphate buffer and adjusted to pH 8.3 with NaOH. It was applied to a 2 cm  $\times$  34 cm column equilibrated with 0.05 M phosphate buffer. The  $\beta$ -casein was eluted after the stepwise increase in buffer strength to 75 mM as shown in Fig. 4. In this experiment,  $\beta$ -casein A<sup>2</sup> was used and the purified protein is indicated by the dashed lines.  $\beta$ -Casein B gave a similar elution pattern except that the  $\beta$ -casein peak was a little broader.

By the same procedures, pure samples of  $\beta$ -caseins A<sup>1</sup> and C were prepared from milk of cows homozygous for these variants.

#### Electrophoretic patterns

Fig. 5 shows disc-gel electrophoretic patterns (pH 9.6) in 4 M urea of the original caseins typed  $\gamma$ -,  $\beta$ -caseins A<sup>2</sup> and B (Gels a, b) together with the corresponding TS

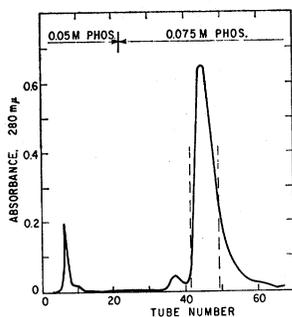


Fig. 4. Stepwise rechromatography of  $\beta$ -casein A<sup>2</sup>—138 mg in dilute phosphate (pH 8.3)—on a DEAE-cellulose column equilibrated with 0.05 M phosphate; elution at the rate of 27 ml/h, 9 ml/tube. The purified  $\beta$ -casein indicated by dashed lines was eluted after the 75 mM phosphate buffer change.

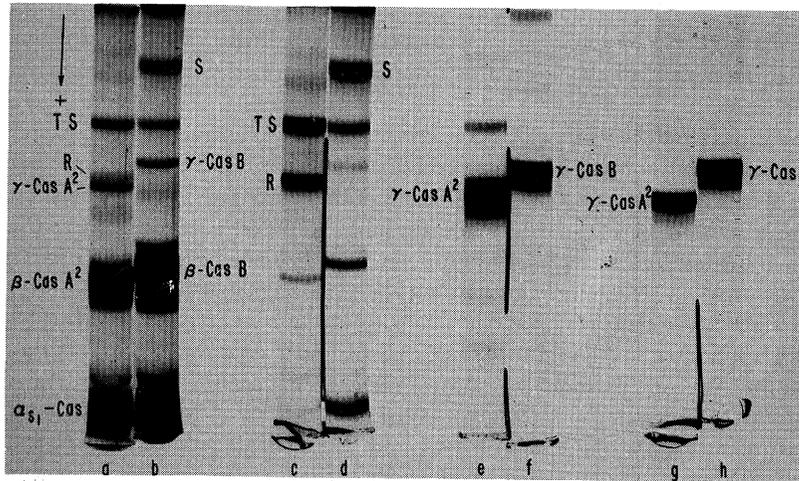


Fig. 5. Disc-gel electrophoresis (pH 9.6, 4 M urea) of casein typed  $\gamma$ ,  $\beta$ -casein A<sup>2</sup> and B and the corresponding TS fractions,  $\gamma$ -casein fractions, and purified  $\gamma$ -caseins. a, A<sup>2</sup> type casein; b, B type casein; c, TS fraction, A<sup>2</sup> type; d, TS fraction, B type; e,  $\gamma$ -casein fraction, A<sup>2</sup> type; f,  $\gamma$ -casein fraction, B type; g, purified  $\gamma$ -casein A<sup>2</sup>; h, purified  $\gamma$ -casein B.

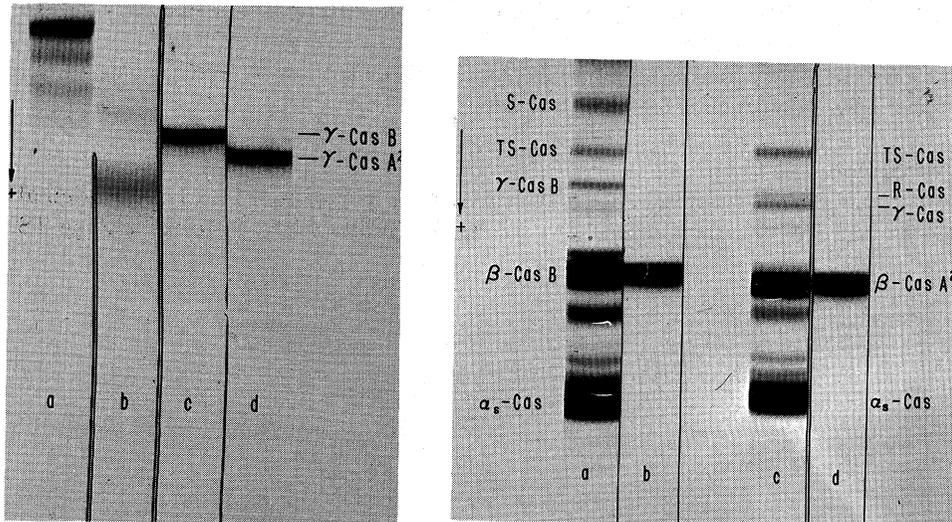


Fig. 6. Disc-gel electrophoresis (pH 9.6, 4 M urea) of  $\gamma$ -casein A<sup>2</sup> and  $\gamma$ -casein B isolated from a casein type A<sup>2</sup>B. a, protein eluted with 5 mM phosphate, pH 8.3; b, protein eluted with 0.02 M phosphate in peak just before the  $\gamma$ -caseins; c,  $\gamma$ -casein B peak; d,  $\gamma$ -casein A<sup>2</sup> peak.

Fig. 7. Disc-gel electrophoresis (pH 9.6, 4 M urea) of the purified  $\beta$ -casein A<sup>2</sup> and B together with the unfractionated caseins. a, casein B type; b, purified  $\beta$ -casein B; c, casein A<sup>2</sup> type; d, purified  $\beta$ -casein A<sup>2</sup>.

fractions (Gels c, d), the  $\gamma$ -casein fractions from the initial chromatographic separation (Gels e, f), and the rechromatographed  $\gamma$ -caseins A<sup>2</sup> and B (Gels g, h) that were used for amino acid analysis. The R-, S- and TS-proteins are caseins\* that show an electrophoretic mobility less than that of the  $\beta$ -caseins at alkaline pH's<sup>10</sup>. The R-casein in the casein sample (Gel a) has a mobility between the  $\gamma$ -caseins A<sup>2</sup> and B and is found in caseins of the  $\beta$ -casein types A, AB or AC, but is absent in the homozygous B type caseins. The S-casein (Gel b) is found in samples typed B or AB but is absent in the homozygous A-type samples. Both the R- and S-caseins, together with the TS-caseins are concentrated in the TS fraction (Gels c, d).

Results of rechromatography of the  $\gamma$ -casein fraction obtained from the heterozygous casein AB are shown in Fig. 6. Gels a and b represent unidentified fractions eluted before the  $\gamma$ -caseins, while the purified  $\gamma$ -caseins were eluted in two consecutive peaks: the first one was  $\gamma$ -casein B (Gel c) and the second  $\gamma$ -casein A<sup>2</sup> (Gel d).

Fig. 7 shows the purified  $\beta$ -caseins B and A<sup>2</sup> (Gels b, d) together with the original caseins (Gels a, c). These  $\beta$ -casein samples were used for amino acid analysis. The  $\beta$ -caseins A<sup>1</sup> and C analyzed were of equal purity.

Comparisons of the mobilities of the purified  $\gamma$ - and  $\beta$ -caseins A<sup>2</sup> and B at pH 9.6, 4 M urea, are shown in Fig. 8. Disc-gel electrophoresis of these proteins at pH 4.3 in 8 M urea is shown in Fig. 9. In contrast to the run at alkaline pH, the  $\beta$ -caseins at an acid pH value have a slower mobility than the  $\gamma$ -caseins. Also, the mobilities of the B types of both  $\gamma$ - and  $\beta$ -caseins are faster than the A<sup>2</sup> types at pH 4.3.

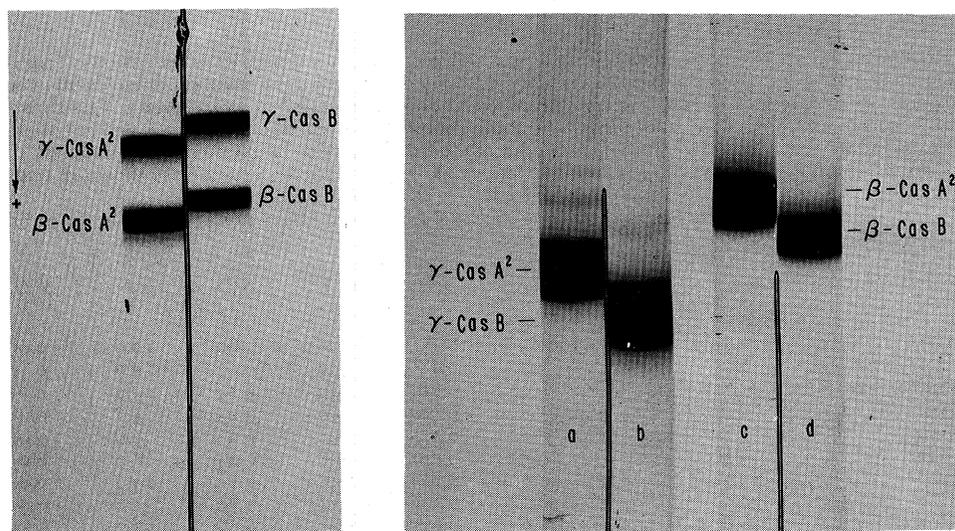


Fig. 8. Disc-gel electrophoretic comparisons (pH 9.6, 4 M urea) of mobilities of purified  $\gamma$ - and  $\beta$ -caseins A<sup>2</sup> and B.

Fig. 9. Disc-gel electrophoresis (pH 4.3, 8 M urea) of purified  $\gamma$ - and  $\beta$ -caseins A<sup>2</sup> and B. a,  $\gamma$ -casein A<sup>2</sup>; b,  $\gamma$ -casein B; c,  $\beta$ -casein A<sup>2</sup>; d,  $\beta$ -casein B.

\* We designate these proteins as caseins because they occur in acid-precipitated casein, can be identified in micellar casein by gel electrophoresis and are similar to other caseins in content of high percentages of proline, glutamic acid, valine and leucine and in lack of cystine. It is not yet known, however, that they are phosphoproteins.

*Results of compositional analyses*

Because of the low concentration of phosphorus in the  $\gamma$ -caseins and the limited amounts of purified proteins available, only single analyses for phosphorus were made. Values of 0.17 and 0.16%, respectively, were obtained for  $\gamma$ -caseins A<sup>2</sup> and B. Analyses previously reported are 0.11% for an older preparation of  $\gamma$ -casein<sup>7</sup> and 0.14% for  $\gamma$ -casein<sup>6</sup> purified by column chromatography.  $\beta$ -Caseins A<sup>1</sup>, A<sup>2</sup>, B and C contained 0.64, 0.62, 0.61 and 0.48% phosphorus, respectively, values in agreement with those of THOMPSON AND PEPPER<sup>21</sup>: 0.59, 0.57 and 0.50% for  $\beta$ -caseins A, B and C.

A small amount of hexose, 0.5%, was found in the  $\gamma$ -caseins by the orcinol reaction.

Duplicate determinations of the tryptophan content of each of the  $\gamma$ -caseins gave identical results of  $0.82 \pm 0.01\%$  for both A<sup>2</sup> and B. By the same method SPIES<sup>18</sup> found  $\beta$ -caseins A and B to contain  $0.876 \pm 0.008$  and  $0.832 \pm 0.001\%$  tryptophan, respectively.

Analytical results for other amino acids and amide NH<sub>3</sub> together with estimates of the precision of the analyses are summarized in Table I. Similar data for  $\beta$ -caseins A<sup>1</sup> and C will be reported elsewhere.

TABLE I

AMINO ACID COMPOSITION OF  $\gamma$ - AND  $\beta$ -CASEINS

Residue numbers listed are averages of or extrapolated values from nine determinations. Triplicate analyses were made on samples hydrolyzed 24, 72 and 96 h. For valine and isoleucine the figures are averages of the six determinations made on the longer-term hydrolysates. The threonine, serine and amide NH<sub>3</sub> numbers were obtained by linear regression analysis, and for these the standard error is shown rather than the standard deviation. Amino acid substitutions in variants are in italics. Tryptophan results from which these values are derived are shown in text.

Amino acid	<i>Residues amino acid per molecule calculated from mean molar ratios based on Gly = 5, Ala = 6</i>							
	<i>Gly = 5, Ala = 6</i>				<i>Gly = 5, Ala = 5</i>			
	$\gamma$ -A <sup>2</sup>	S.D.	$\gamma$ -B	S.D.	$\beta$ -A <sup>2</sup>	S.D.	$\beta$ -B	S.D.
Lys	12.1	0.14	12.3	0.12	11.0	0.06	10.9	0.09
His	6.1	0.08	7.3	0.14	5.0	0.04	5.9	0.10
Amide NH <sub>3</sub>	25.5	0.68	26.4	0.76	24.0	0.54	24.7	0.91
Arg	2.4	0.04	3.6	0.06	3.9	0.05	4.8	0.08
Asp	8.7	0.05	8.7	0.13	9.0	0.09	9.0	0.08
Thr	9.5	0.07	9.5	0.06	8.8	0.04	8.6	0.03
Ser	12.7	0.08	11.6	0.08	14.8	0.15	13.6	0.08
Glu	39.0	0.67	39.1	0.51	39.1	0.58	38.6	0.60
Pro	40.8	0.32	39.6	0.63	34.1	0.69	32.7	0.47
Gly	5.0	0.02	5.0	0.03	5.1	0.03	5.1	0.04
Ala	6.0	0.02	6.0	0.02	4.9	0.02	4.9	0.04
Cys	0		0		0		0	
Val	20.0	0.57	20.0	0.28	18.4	0.28	18.1	0.34
Met	7.1	0.13	7.1	0.15	5.8	0.08	5.7	0.06
Ile	8.4	0.04	8.4	0.09	9.8	0.07	9.7	0.04
Leu	22.9	0.24	23.0	0.12	21.5	0.18	21.2	0.15
Tyr	4.7	0.16	4.6	0.09	3.8	0.07	3.7	0.08
Phe	10.8	0.13	10.9	0.06	8.8	0.06	8.7	0.07
Trp	1.0	—	1.0	—	1.0	—	1.0	—

$\gamma$ - AND  $\beta$ -CASEINS

## DISCUSSION

There can be little doubt that  $\gamma$ -casein prepared by older methods using alcohol or urea fractionation is a mixture of proteins. The relatively pure material isolated by GROVES *et al.*<sup>6</sup> using column chromatography could quite properly be called  $\gamma$ -casein, but it was prepared from mixed herd milk and, in all probability, was polymorphic. The  $\gamma$ -caseins prepared from typed milks by chromatographic methods herein described appear by the criterion of disc-gel electrophoresis to be entirely suitable for compositional analysis and comparison with other caseins. These methods also provide similarly purified  $\beta$ -caseins and, in this investigation, we have emphasized comparison of casein components prepared from the same sample of homozygous milk.

In comparing the amino acid composition of  $\gamma$ - and  $\beta$ -caseins it should be pointed out that the values in Table I are based on the presence of five glycine residues per molecule of protein. For the  $\beta$ -caseins a molecular weight of 24 000–25 000 for the monomer is generally accepted<sup>22</sup>; this is consistent with the numbers of residues shown. For purified  $\gamma$ -casein, however, measurements of molecular weight have not yet been made. Nevertheless, a simple calculation of minimal molecular weight from the accurately determined tryptophan percentage of 0.82 gives 24 900 per tryptophan residue. A molecule of this weight with one phosphorus atom would contain 0.12% P. On the basis of these considerations we have assumed that there are five glycine and

TABLE II

COMPARISON OF COMPOSITION OF  $\gamma$ - AND  $\beta$ -CASEINS

Amide NH<sub>3</sub> determinations have been omitted because significant differences could not be demonstrated.

Amino acid	Whole number residues per molecule containing 5 Gly					
	$\gamma$ -A <sup>2</sup>	$\gamma$ -B	$\beta$ -A <sup>1</sup>	$\beta$ -A <sup>2</sup>	$\beta$ -B	$\beta$ -C
Lys	12	12	11	11	11	12
His	6	7	6	5	6	6
Arg	3	4	4	4	5	4
Asp	9	9	9	9	9	9
Thr	10	10	9	9	9	9
Ser	13	12	15	15	14	15
Glu	39	39	39	39	39	37
Pro	41	40	33	34	33	33
Gly	5	5	5	5	5	5
Ala	6	6	5	5	5	5
Val	20	20	18	18	18	18
Met	7	7	6	6	6	6
Ile	8	8	10	10	10	10
Leu	23	23	21	21	21	21
Tyr	5	5	4	4	4	3
Phe	11	11	9	9	9	9
Trp	1	1	1	1	1	1
P	1	1	5	5	5	4
Hexose	1	1	—	—	—	—
Mol. wt.*	25 020	25 130	23 550	23 590	23 700	23 260

\* Calculated from composition shown.

six alanine residues per molecule of  $\gamma$ -casein. When the numbers of residues shown in Table I for the  $\gamma$ -caseins are rounded off (as in Table II), molecular weights calculated for  $\gamma$ -caseins A<sup>2</sup> and B are 25 020 and 25 130, respectively. If this estimate of molecular weight is approximately correct, our single analyses for phosphorus are admittedly of questionable accuracy. The results of the hexose determinations are also inconclusive but, since a protein of this size with one hexose residue would contain 0.7% hexose, it is unlikely that more than one residue per molecule could be present.

Regardless of the accuracy of our estimates of molecular weight, valid comparisons concerning amino acid substitutions and differences in composition can be made from the data in Table I. In the first place, it is immediately apparent that  $\gamma$ -A<sup>2</sup> and  $\gamma$ -B differ only by one residue each of histidine, arginine and serine. The same differences may be seen in  $\beta$ -A<sup>2</sup> and  $\beta$ -B. Furthermore, the *t* test<sup>23</sup> shows that the difference between the two means of the proline determinations is significant at the 0.1% probability level; and this is true for both  $\gamma$ - and  $\beta$ -pairs. No other significant differences are discernible so we suggest that two substitutions involving single base changes in the genetic code, namely Ser/Arg and His/Pro, distinguish the polymorphs A<sup>2</sup> and B of both  $\gamma$ - and  $\beta$ -caseins. The close relationship in the synthesis of these caseins disclosed by the typing experiments of GROVES AND KIDDY<sup>10</sup> is demonstrated again by the analytical results.

Secondly, it is clear that the outstanding difference in amino acid composition between the  $\gamma$ - and  $\beta$ -caseins is seven proline residues per molecule. This and other smaller differences in amino acid content, notably, in serine, valine, isoleucine, leucine and phenylalanine ( $\pm 2$ ) and in lysine, histidine, arginine, threonine, alanine, methionine and tyrosine ( $\pm 1$ ) are apparent from the rounded-off integer values\* of Table II. Another large and important difference is, of course, in phosphorus content, also shown in Table II. The proteins are similar in lack of cysteine or cystine and in general distribution of most amino acids. In its high proportion of nonpolar to polar side-chain groups,  $\gamma$ -casein is unique among the recognized components of casein, principally because of its high proline and low phosphorus content.

Our amino acid analyses for  $\beta$ -caseins A<sup>2</sup> and B agree in most respects with previously published analyses<sup>13,24</sup>. However, because of the large number of polymorphs of  $\beta$ -casein, the difficulties involved in obtaining them of equal purity, and the analytical problems involved in pinpointing the exact number of residues of amino acids present in high percentages, we have included in Table II rounded results of our analyses of  $\beta$ -casein A<sup>1</sup> and C. The four variants were prepared, purified and analyzed under similar conditions in order to permit direct comparison of amino acid composition. The difference in phosphorus content should be mentioned at once for it has been overlooked by some investigators. In addition to the Ser/Arg and His/Pro substitutions presumed to occur in  $\beta$ -caseins A<sup>2</sup> and B, a third, Glu/Lys, also involving a single base change, is suggested by our data for  $\beta$ -C as well as by the data of PETERSON AND KOPFLER<sup>13</sup>.  $\beta$ -A<sup>1</sup> is distinguishable from  $\beta$ -A<sup>2</sup> by the His/Pro substitution. The amino acid replacements listed in Table III would account for most of the differences in the analytical results. The listing, however, is an oversimplification even for the four  $\beta$ -casein variants considered here. Thus, we cannot explain the finding of only

\* The rounding-off process is obviously arbitrary and open to question, particularly in the numbers for arginine, threonine and isoleucine. Nevertheless, the validity of the conclusions drawn in the preceding paragraph is not affected.

## $\gamma$ - AND $\beta$ -CASEINS

TABLE III

POSSIBLE AMINO ACID SUBSTITUTIONS IN  $\beta$ -CASEINS

<i>Comparison</i>	<i>Substitution</i>
A <sup>1</sup> -A <sup>2</sup>	His/Pro
A <sup>1</sup> -B	Ser/Arg
A <sup>1</sup> -C	Glu/Lys
A <sup>2</sup> -B	Pro/His, Ser/Arg
A <sup>2</sup> -C	Pro/His, Glu/Lys
B-C	Arg/Ser, Glu/Lys

37 rather than 38 glutamic acid and only 3 rather than 4 tyrosine residues in  $\beta$ -casein C. Besides the known  $\beta$ -casein A<sup>3</sup>, other polymorphs of  $\beta$ -casein B and a  $\beta$ -casein D have been detected by ASCHAFFENBURG *et al.*<sup>25</sup> and there is reason to believe that  $\beta$ -casein C also occurs in several forms (unpublished work). It may well be impossible to define all  $\beta$ -casein variants in terms of amino acid substitutions, even multiple substitutions.

ASCHAFFENBURG<sup>26</sup> has recently reviewed comprehensively previous investigations of genetic polymorphism of components of bovine casein and has also summarized evidence for linkage relationships among the  $\alpha_{s1}$ ,  $\beta$ , and  $\kappa$ -casein systems. Thus, there appears to be a close genetic linkage in the biosynthesis of the  $\alpha_{s1}$ - and  $\beta$ -caseins and a somewhat less close linkage between the  $\kappa$ -caseins and the  $\alpha_{s1}$ - and  $\beta$ -systems. The present results from amino acid analysis support the finding of GROVES AND KIDDY<sup>10</sup> that there also exists a close association between the  $\gamma$ - and  $\beta$ -casein alleles. As ASCHAFFENBURG<sup>26</sup> has concluded, it would appear that "all the known genetic systems of cow casein are linked to each other, and presumably, located on the same chromosome".

In summary, we have shown that (1)  $\gamma$ -caseins A<sup>2</sup> and B differ in composition only in respect to single residues of four amino acids which may be paired as two substitutions, Ser/Arg and His/Pro, (2) the same differences distinguish  $\beta$ -casein A<sup>2</sup> and B, confirming the close relationship in genetic control of the synthesis of these caseins, (3)  $\gamma$ - and  $\beta$ -caseins are similar in distribution of amino acids but the former are considerably richer in proline and poorer in phosphorus content, and (4) almost all the differences in amino acid composition among  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup>, B and C may be accounted for in terms of three possible amino acid substitutions, Ser/Arg, His/Pro and Glu/Lys.

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