Separation and Partial Characterization of Guinea-Pig Caseins

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1. Guinea-pig caseins A, B and C were purified free of each other by a combination of ion-exchange chromatography and gel filtration. 2. Determination of the amino acid composition showed all three caseins to contain a high proportion of proline and glutamic acid, but no cysteine. This apart, the amino acid composition of the three caseins was markedly different, though calculated divergence values suggest that some homology may exist between caseins A and B. Molecular-weight estimates based on amino acid composition were in good agreement with those based on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 3. *N*-Terminal analysis showed lysine, methionine and lysine to be the *N*-terminal residues of caseins A, B and C respectively. 4. Two-dimensional separation of tryptic digests revealed a distinctive pattern for each casein. 5. All caseins were shown to be phosphoproteins. The casein C preparation also contained significant amounts of sialic acid, neutral and amino sugars. 6. The results suggest that each casein represents a separate gene product, and that the low-molecular-weight proteins are not the result of a post-translational cleavage of the largest. All were distinctly different from the whey protein α -lactalbumin.

Until recently, the purification and detailed structural analysis of the major protein components of milk, the acid-precipitable caseins, has been confined to a few species, notably the cow and ewe, although considerable sequence data are now available for the whey proteins α -lactalbumin and β -lactoglobulin from a number of species (for general review see Jenness, 1974).

Our interest in milk proteins derives from the observation that the limited number of major protein constituents present are organ-specific, and that their expression is modulated by a well-defined combination of both peptide and steroid hormones (see Banerjee, 1976; Craig & Campbell, 1978). Such a situation provides an excellent system with which to study the molecular mechanisms involved in the hormonal regulation of specific gene expression.

We have previously (Craig *et al.*, 1976) described the presence in guinea-pig milk of three major casein components of estimated mol.wts. 28000, 25500 and 20500 as determined by SDS/polyacrylamide-gel electrophoresis. We now report the separation and partial characterization of each casein, and suggest on the basis of amino acid composition, tryptic peptide analysis and *N*-terminal sequence analysis, that each probably represents the expression

of a unique piece of genetic information. Furthermore, detailed analysis of phosphate, neutral and amino sugars, sialic acid and amino acid content, of each casein demonstrates similarities between those of the guinea pig and caseins already well characterized from other species.

Experimental

Preparation of solutions and chemicals

All solutions were prepared with double-distilled water. Acrylamide and *NN'*-methylenebisacrylamide were recrystallized in chloroform and acetone respectively as described by Loening (1967). *NNN'*-Tetramethylethylenediamine was redistilled *in vacuo* immediately before use. All buffers containing urea were made from concentrated stock solutions and deionized by passage through a column of Amberlite MB-3 immediately before use.

Materials

Dunkin-Hartley guinea-pig milk was obtained from Porcellus Animal Breeding, Heathfield, Sussex, U.K. Trypsin treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Whatman DEAE-cellulose DE-52 was from Reeve Angel Scientific, London SE1 6BD, U.K., and Sephadex G-100 was from Pharmacia, Uppsala, Sweden. Cellulose thin-layer plates were from East-

Abbreviation used: SDS, sodium dodecyl sulphate.

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man Kodak, Kirkby, Liverpool, U.K., and trimethylchlorosilane, hexamethyldisilazane, and *N*-acetylneuraminic acid from the Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Toluene-*p*-sulphonic acid 'MAR' grade, Cheng-Chin polyamide layer sheets and anhydrous pyridine were from BDH Chemicals, Poole, Dorset, U.K., as were all other chemicals and solvents, which were of A.R. grade.

Methods and Results

Separation of guinea-pig caseins A, B and C

Guinea-pig milk was fractionated into casein and whey by acid precipitation as previously described (Craig *et al.*, 1976), and the caseins were separated by our modification of the method described by Thompson (1966) for bovine caseins, by using a combination of ion-exchange chromatography and gel

filtration, in the presence of 10mM-imidazole/HCl, pH 7.0, containing 3.3M-urea, 1mM-EDTA and 1% (v/v) 2-mercaptoethanol (buffer A). Throughout the preparation the composition of protein fractions was monitored by SDS/polyacrylamide-gel electrophoresis on 10% (w/v) polyacrylamide gels as described previously (Weber *et al.*, 1972; Craig *et al.*, 1976). Before electrophoresis, all samples were dialysed exhaustively against 20mM-NH₄HCO₃ to remove urea, freeze-dried, and a small amount was dissolved in electrophoresis buffer.

Routinely, total casein was dissolved at 10–15mg/ml in buffer A containing 150mM-NaCl. Caseins A and B were then separated from casein C and minor protein species by ion-exchange chromatography on a column of DEAE-cellulose equilibrated in the same buffer. Under these conditions (see Fig. 1a) only caseins A and B were retained by the column, and casein C was recovered exclusively in the un-

Fig. 1. Separation of caseins A and B, from C by ion-exchange chromatography

(a) Total guinea-pig casein (1g) was dissolved in buffer A containing 150mM-NaCl, and applied to a column (2.5 cm $\times$ 30.0 cm) of DEAE-cellulose (I) equilibrated in the same buffer. The unbound fraction, containing casein C, was retained, dialysed and freeze-dried, and caseins A and B were eluted together with a 300 ml 150–300 mM-NaCl linear gradient in buffer A (----), at 25 ml/h. The peak absorbance fractions (A_{280}) were pooled, dialysed, freeze-dried and stored at 4°C. (b) To separate casein C from residual casein A and B, the unbound fraction from the first DEAE-cellulose column was dissolved in buffer A and applied to a second column (2.5 cm $\times$ 40.0 cm) of DEAE-cellulose (II) equilibrated in the same buffer. Casein C was eluted in multiple peaks with a 300 ml 0–200 mM-NaCl linear gradient in buffer A (----), at 30 ml/h. Individual peaks were separately dialysed, freeze-dried and stored at 4°C.

Fig. 2. Separation of caseins A and B from each other, and casein C from high-molecular-weight proteins, by gel filtration

(a) Caseins A and B (150mg) from DEAE-cellulose column I were dissolved in buffer A and loaded on to a 2.3 cm $\times$ 120 cm column of Sephadex G-100 equilibrated in the same buffer. The column was eluted with buffer A under gravity at 20 ml/h, and the protein-containing fractions were identified by their A_{280} . (b) Casein C (80mg) from DEAE-cellulose column II was dissolved in buffer A, and gel filtration on Sephadex G-100 performed as described above.

bound fraction. Caseins A and B were recovered by using a linear gradient of 150–300 mM-NaCl in buffer A. Both were eluted together in a single peak free of other major contaminants (see Fig. 1a). In order to separate caseins A and B the peak protein fractions were pooled, dialysed against 20 mM-NH₄HCO₃, freeze-dried, redissolved in buffer A at 5–10 mg/ml, and loaded on to a Sephadex G-100 column equilibrated in buffer A. Gel filtration resulted in the separation of caseins A and B into two distinct but overlapping peaks (Fig. 2a), one consisting of casein A and the other casein B. In order to obtain a complete separation, each peak was separately dialysed, freeze-dried, redissolved in buffer A, and the gel-filtration step repeated. Subsequent SDS/polyacrylamide-gel

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified guinea-pig milk caseins

Electrophoresis was performed by using 10% acrylamide in 10 cm tubes as described by Weber *et al.* (1972). Protein bands were identified by staining with Coomassie Brilliant Blue. (a) Total casein fraction (10 μ g); (b) casein A (2.5 μ g); (c) casein B (2.5 μ g); (d) casein C (2.5 μ g).

electrophoresis demonstrated that each contained a single protein band (see Fig. 3).

Casein C was separated from residual casein A, B and other minor species by further ion-exchange chromatography and gel filtration. The unbound fraction from the initial DEAE-cellulose column (I) was dialysed, freeze-dried, redissolved in buffer A at 10 mg/ml, and loaded on to a second DEAE-cellulose column (II) equilibrated in the same buffer. Under these conditions, all casein C bound, and could be eluted in multiple peaks by application of a 0–200 mM-NaCl linear gradient in buffer A (Fig. 1b). Polyacrylamide-gel analysis of each peak showed that, although casein C was always the predominant species, peaks 5 and 6 contained detectable concentrations of casein A and B, and peak 1 contained low-molecular-weight species. Consequently, these were discarded, and the remainder were pooled, and the residual higher- and lower-molecular-weight species

were removed by gel filtration on Sephadex G-100 as described for the separation of caseins A and B (see Fig. 2b). Analysis of the final preparation by SDS/polyacrylamide-gel electrophoresis showed (Fig. 3) that, although casein C was clearly the major constituent, there was in addition a lower-molecular weight species representing perhaps 5–10% (w/w) of the final preparation. However, in spite of this persistent contaminant, we considered that a preparation of casein C, 90–95% pure, should provide reliable information for subsequent comparative sequence and structural analyses with caseins A and B, and the whey protein α -lactalbumin.

All purified proteins were stored either in a freeze-dried state at 4°C, or if in solution at –20°C.

Amino acid composition, N-terminal amino acid determination and tryptic peptide 'mapping' of guinea-pig caseins A, B and C

The amino acid composition of each casein was determined on a Rank-Hilger automatic amino acid analyser, after hydrolysis under vacuum of individual samples for various times at 110°C in 6M-HCl containing 1% (w/v) phenol. Tryptophan was determined after hydrolysis in 3M-toluene-*p*-sulphonic acid at 110°C for 22h as described by Allen & Neuberger (1975). Except for tryptophan, the amino acid composition of each protein was based on the average of the 48 and 96h hydrolyses.

Estimations of the number of individual amino acid residues present are based on the mol/100mol of each recovered as determined by using a norleucine internal standard, and the assumption that caseins A, B and C contain one, two and one methionine residues respectively. The methionine contents were based on the apparent molecular weight of each protein as determined by previous estimations by SDS/polyacrylamide-gel electrophoresis (Craig *et al.*, 1976). *N*-Terminal amino acid analysis was performed by dansylation in the presence of urea, as described by Gros & Labouesse (1969), followed by hydrolysis under N₂, and separation on polyamide layer sheets, as described by Weiner *et al.* (1972). All data for guinea-pig α -lactalbumin are from Brew (1972).

The results (Table 1) show that, in common with other caseins, guinea-pig caseins contain no cysteine, but have exceptionally high concentrations of glutamic acid and proline. These general features apart, each had a distinctive amino acid composition, all of which were markedly different from that of α -lactalbumin. In common with α -lactalbumin, both caseins A and C had lysine residues at the *N*-terminus, whereas casein B, in contrast, had an *N*-terminal methionine. *N*-Terminal histidine was also detectable in the casein C preparation. We assume that this is attributable to the minor contaminating protein, as recent sequence analysis has confirmed lysine as the

true *N*-terminus of the secreted protein (F. Northrop & J. I. Harris, unpublished work).

Molecular-weight determination of each casein on the basis of the amino acid composition essentially confirmed our previous estimations using SDS/polyacrylamide-gel electrophoresis, except for casein C which appeared to be somewhat higher. This agreement between molecular-weight estimations confirms that our calculated amino acid compositions are indeed representative of each guinea-pig casein.

For tryptic peptide analysis, all protein samples were reduced, then carboxyaminoethylated with iodoacetamide in the presence of urea, by the procedure described by Crestfield *et al.* (1963), except that iodoacetamide was used as the alkylating agent. A portion of each protein (1 mg) was then suspended in 1 ml of 0.5M-NH₄HCO₃, pH 8.6, and incubated overnight at 37°C in the presence of 20 μ g of trypsin/mg of protein. The reaction was stopped by the addition of acetic acid to pH 3.0, and the samples were freeze-dried. Each was then dissolved and immediately freeze-dried, first in 0.25 ml of pyridine/acetic acid and acid/water (25:1:225, by vol.) and then in 0.25 ml of pyridine/acetic acid/water (1:10:189, by vol.) The final freeze-dried peptides were dissolved in 50 μ l of water and stored at –20°C. Peptides were separated in two dimensions on a 20cm $\times$ 10cm cellulose-coated flexible poly(ethylene terephthalate) support, essentially as described by Inglis *et al.* (1976). Separation in the first dimension was by electrophoresis at pH 3.5 in pyridine/acetic acid/water (1:10:189, by vol.) for 90 min at 250 V in a Shandon low-voltage flat-bed electrophoresis apparatus. Each sample (100–200 μ g) was applied in 5–10 μ l of water after pre-equilibration of the cellulose sheets in the apparatus for 30 min. After electrophoresis the sheets were dried at 70°C for 20 min, and the peptides separated in the second dimension by ascending chromatography in freshly prepared butan-1-ol/acetic acid/water (4:1:5, by vol.; this results in a biphasic mixture, the upper phase was used) at 20°C for 45–60 min. Sheets were again dried at 70°C for 20 min and the peptides made visible by cadmium/ninhydrin staining overnight at 25°C as described by Dreyer & Bynum (1967), and photographed within 48 h. Comparison of the peptide 'maps' (Fig. 4) demonstrates that all the caseins are markedly different from each other and from α -lactalbumin.

Carbohydrate, sialic acid and phosphate content of guinea-pig caseins

Quantitative analysis of the individual monosaccharides present, was performed by separation of the trimethylsilyl derivatives of methyl glycosides by g.l.c. chromatography. The carbohydrate derivatives were prepared from 4 mg of each casein as described

Table 1. *Amino acid composition of guinea-pig milk proteins*

The amino acid content of each casein was determined as described in the text. Data for α -lactalbumin are from Brew (1972). Values for aspartic acid and glutamic acid are compound values, representing both dicarboxylic acid and amide. No adjustments were required to allow for the destruction of amino acids during acid hydrolysis, as analysis of threonine and serine contents after 24h, 48h and 96h hydrolysis failed to reveal any trend that might indicate significant destruction of amino acids during hydrolysis.

	Casein A		Casein B		Casein C		α -Lactalbumin	
	Mol/100mol	Estimated residues	Mol/100mol	Estimated residues	Mol/100mol	Estimated residues	Mol/100mol	Known residues
Asp	12.08	30	8.03	16	2.42	6	16.3	20
Thr	8.96	22	4.29	9	1.55	4	4.9	6
Ser	10.67	27	9.56	20	7.95	18	6.5	8
Glu	13.28	33	14.71	41	15.12	34	9.8	12
Pro	6.14	15	9.14	19	15.23	35	1.6	2
Gly	0.51	1	1.80	4	2.54	6	3.3	4
Ala	3.72	9	4.98	10	15.23	35	4.1	5
$\frac{1}{2}$ CyS	0.00	0	0.00	0	0.00	0	6.5	8
Val	4.93	12	5.82	12	9.60	22	2.4	3
Met	0.40	1	0.97	2	0.44	1	0.8	1
Ile	5.83	15	6.23	13	1.10	3	9.8	12
Leu	5.45	14	7.04	13	12.47	28	11.4	14
Tyr	6.70	17	3.87	8	1.98	5	4.1	5
Phe	4.03	10	6.17	13	1.88	4	2.4	3
Trp	0.60	2	0.27	1	0.22	1	2.4	3
Lys	11.48	29	8.17	17	2.87	7	8.9	11
His	2.41	6	3.74	8	1.88	4	3.3	4
Arg	2.82	7	5.27	11	7.51	17	1.6	2
Total residues		250		217		230		123
Apparent mol.wt. (amino acid content)		29438		25511		24955		14500 (known)
Apparent mol.wt. ('SDS electrophoresis')		28000		25500		20500		
N-Terminal amino acid		Lys		Met		Lys		Lys

Fig. 4. *Two-dimensional analysis of tryptic peptides derived from purified guinea-pig milk proteins*

Each tryptic digest (100 μ g) was applied in 10 μ l of water, as described in the text. In practice, caseins A and B (a and b) were spotted in parallel, one on the edge, and the other in the centre of a 20cm $\times$ 20cm cellulose thin-layer sheet, and electrophoresis was then performed simultaneously on both samples. The sheets were then divided into two, dried and separated in the second dimension by ascending chromatography performed again simultaneously in the same tank. This procedure was also followed for casein C and α -lactalbumin (c and d).

Table 2. *Phosphate and sugar content of guinea-pig caseins*

Casein	Estimated mol.wt.	Phosphate (% w/w)	Sialic acid (% w/w)	Amino sugar (% w/w)	Neutral sugar (% w/w)
A	28000	1.43 (4 mol/mol of protein)	< 0.1	Trace	Galactose < 0.05
B	25500	1.38 (3 mol/mol of protein)	< 0.1	Trace	—
C	20500	3.06 (6 mol/mol of protein)	0.20	Glucosamine < 0.55	Fucose 0.07 Mannose 0.35 Galactose 0.17

by Kamerling *et al.* (1975) by methanolysis. Mannitol was used as an internal standard.

The trimethylsilyl derivatives were then prepared by using the trimethylsilyl reagent (pyridine/hexamethyldisilazane/trimethylchlorosilane; 5:1:1, by vol.), and then separated in a Pye 104 gas chromatograph fitted with a dual-flame ionization detector precisely as described by Hall & Wood (1976). The carbohydrate contents of the caseins, estimated from the areas under the recorder trace by comparison with known monosaccharides, demonstrated that only casein C (see Table 2) contained significant concentrations of neutral sugars (fucose, mannose and galactose), representing a carbohydrate content of about 0.6% (w/w), though galactose was also detected in the casein A preparation.

Amino-sugar measurements on a Biocal acid analyser after hydrolysis of the proteins at 100°C for 22 h in 3M-toluene-*p*-sulphonic acid (Allen & Neuberger, 1975) revealed detectable, but low, concentrations of glucosamine in the casein C preparation. No significant concentrations of amino sugar were identified in the preparations of caseins A and B.

Analysis of the sialic acid content of each casein preparation by the thiobarbituric assay described by Warren (1959), after the release of bound sialic acid by heating the protein samples for 90 min at 80°C in 0.05M-H₂SO₄, showed that all contained detectable concentrations, but that only casein C contained significant amounts, a result consistent with the previous identification of both neutral and amino sugars associated with this protein.

Phosphate was determined by the method of Bartlett (1959). As expected all proved to be phosphoproteins, with a calculated average phosphate content of 4, 3 and 6 mol/mol of protein, for caseins A, B and C respectively.

Discussion

Apart from a general analysis of guinea-pig milk (Nelson *et al.*, 1951), and our own brief description of the major acid-precipitable proteins present in guinea-pig milk (Craig *et al.*, 1976), no detailed characterization has been performed on guinea-pig

caseins. Moreover, characterization of caseins in general, particularly sequence analysis, has been limited to those isolated from the larger mammals; for example, the bovine α_s -, β - and κ -caseins (see Mercier *et al.*, 1971; Ribadeau-Dumas *et al.*, 1972; Mercier *et al.*, 1973). As the cow is not a convenient laboratory animal, we have instead chosen the guinea pig, as its mammary tissue is particularly amenable to subcellular fractionation procedures, and one of its milk proteins, α -lactalbumin, has already been sequenced (Brew, 1972).

We have now identified, and separated free of each other, three guinea-pig caseins, as determined by SDS/polyacrylamide-gel electrophoresis, and the identification of a single *N*-terminal amino acid in each preparation. Furthermore, comparison of the amino acid composition and tryptic peptide 'maps' of each casein suggests that, although the caseins share common features, such as high concentrations of proline and glutamic acid, and the total absence of cysteine, each contains a unique amino acid sequence, though this does not exclude the possibility that regions of sequence homology may exist. As expected, α -lactalbumin contained no features in common with the caseins.

Our initial conclusion from these observations was that all four guinea-pig milk proteins were derived from discrete regions of genetic information. The data seem to exclude the possibility that one or more of the lower-molecular-weight milk proteins might have resulted from a post-translational cleavage of an *N*- or *C*-terminal peptide from one of the larger milk proteins, a process known to result in the production of certain minor bovine caseins from the larger β -casein (Gordon *et al.*, 1972; Ribadeau-Dumas *et al.*, 1972). However, the data do not exclude the possibility that all the guinea-pig milk proteins might be synthesized as a single macromolecule (see Thompson & Farrell, 1974) subsequently cleaved into functional proteins by specific endopeptidases.

This is extremely unlikely on two counts. Firstly, we have already demonstrated by isolation and characterization of the milk-protein mRNA species (Craig *et al.*, 1976) that no single mRNA has the

Fig. 5. Analysis of guinea-pig caseins by SDS/polyacrylamide-gel electrophoresis in a discontinuous buffer system

Electrophoresis was performed at pH 8.9 on a 12.5% polyacrylamide slab gel (14.0 cm $\times$ 13.0 cm $\times$ 0.15 cm) with a 5.0% polyacrylamide stacking gel as described by Maizel (1972), for 3.5 h at 150 V. Protein bands were identified by staining with Coomassie Brilliant Blue. (a) Casein A (5 μ g); (b) casein B (5 μ g); (c) casein C (5 μ g); (d) total casein fraction (15 μ g).

coding capacity for more than two guinea-pig milk proteins. Secondly, recent experiments *in vitro* using an mRNA-directed protein-synthesizing system in the presence of *N*-formyl[35 S]methionyl-tRNA demonstrate that each casein and α -lactalbumin is initiated *de novo* (P. A. J. Perera, A. Mellor, A. Smith & R. Craig, unpublished work). Thus the evidence taken *in toto* suggests that guinea-pig caseins A, B and C, and α -lactalbumin each result from the transcription and subsequent translation of discrete units of genetic information, and are not the result of post-translational cleavage events.

We have already described (Craig *et al.*, 1976) the anomalous mobility on SDS/polyacrylamide gels of guinea-pig milk proteins synthesized *in vitro* when compared with the mature secreted proteins, a difference we presume to be due in part to the inability of the protein-synthesizing systems *in vitro* to either glycosylate or phosphorylate. Unfortunately, this picture has increased in complexity, as analysis of the purified caseins (apparently homogeneous by all the criteria described above), by SDS/polyacrylamide-gel electrophoresis in buffer systems other than phosphate

Fig. 6. Analysis of guinea-pig caseins by urea/polyacrylamide-gel electrophoresis

Electrophoresis was performed using 7.5% acrylamide in 8 cm tubes, in the presence of 4.5 M-urea at pH 8.4 as described by Akroyd (1968), for 60 min at 3 mA/gel. Protein bands were made visible by staining with Coomassie Brilliant Blue. (a) Total casein fraction (50 μ g); (b) casein A (15 μ g); (c) casein B (15 μ g); (d) casein C (15 μ g).

provides evidence for heterogeneity, particularly within casein B.

When analysed on SDS/12.5% polyacrylamide gels using a discontinuous gel buffer, pH 8.9, as described by Maizel (1972), casein B separated into two distinct polypeptide bands. One of these co-electrophoresed with casein A, which itself exhibited a degree of heterogeneity (see Fig. 5). Whether this represents a post-translation cleavage at the C-terminus of the protein, the presence or absence of some prosthetic group(s) or limited amino acid substitution, any of which may affect the mobility of proteins on SDS/polyacrylamide gels, must remain supposition. However, it seems unlikely in the light of the evidence presented, that the casein B preparation contains two polypeptides of distinctly different amino acid composition. Heterogeneity of a human casein on urea/

Table 3. *Matrix of composition divergence values*

All values have been multiplied by 10^4 . Data were taken from *Groves & Gordon (1970), †Travis & Roberts (1969), ‡Walsh & Neurath (1964) and §Johnson (1976). Values of 100–200 indicate that marked similarity exists between the proteins in question, and values greater than 400 indicate dissimilarity.

		Guinea-pig casein			Human casein		Bovine			Human Bovine Rabbit		
		A	B	C	Major	κ -	β -	α_{s1} -	κ -	Trypsin		
Guinea-pig casein	A	—										
	B	322	—									
	C	740	547	—								
*Human casein	Major	580	404	431	—							
	κ -	508	349	462	271	—						
*Bovine casein	β -	614	392	492	218	265	—					
	α_{s1} -	427	271	556	365	225	317	—				
	κ -	432	316	436	301	229	365	308	—			
†Human	Trypsin	511	458	587	569	483	592	467	402	—		
‡Bovine		571	559	695	672	585	690	562	506	171	—	
§Rabbit		554	508	638	633	542	641	527	474	138	106	—

polyacrylamide gels has already been reported (Groves & Gordon, 1970) and shown to be a result of different levels of phosphorylation of the same protein. Analysis of the guinea-pig caseins on similar urea/polyacrylamide gels (Fig. 6) showed that only casein C exhibited marked heterogeneity, a situation similar to that described for its elution profile from DEAE-cellulose (Fig. 1b). However, we have never observed such heterogeneity of casein C on SDS/polyacrylamide gels, suggesting that if multiple bands on urea-containing gels are indicative of various degrees of phosphorylation within a single polypeptide, then the observed heterogeneity, particularly of casein B on SDS/polyacrylamide gels, is not due to this, as analysis of both caseins A and B on urea/polyacrylamide gels results in one prominent band and some minor species in each preparation.

In an attempt to relate the three guinea-pig caseins to those of other species, the amino acid compositions of guinea-pig caseins were compared with those of the major human caseins, bovine β_1 -, α_{s1} - and κ -

caseins (Groves & Gordon, 1970), by use of the composition divergence (D_c) formula of Ghirelli-Magaldi *et al.* (1975):

$$D_c = \sqrt{\sum_{i=1}^{18} \left(\frac{X_{iA} - X_{iB}}{n_i} \right)^2}$$

where X_{iA} and X_{iB} are the molar fractions of the amino acid i in the proteins A and B respectively, and n_i is the number of codons for the same amino acid. This formula is useful for comparison of proteins of similar molecular weight when sequence data are not available. Low values of D_c may indicate similarity between proteins, but high values of D_c correlate with dissimilarity. As a control the composition of divergence was also calculated for human (Travis & Roberts, 1969), bovine (Walsh & Neurath, 1964) and rabbit trypsins (Johnson, 1976), as these are known to retain considerable homology, and are of similar molecular weight to the caseins. Application of this expression to the caseins (Table 3) shows that,

although there is some indication of homology between some of the proteins, it is not as strict as that indicated for the trypsins. In particular, some similarities exist between guinea-pig caseins A and B, but similarities also exist between guinea-pig casein B and the bovine α_{S1} - and κ -caseins. The results suggest that there may be a high evolutionary rate for the amino acid sequences of the caseins, a situation that has already been described for para- κ -caseins (Mercier *et al.*, 1976) by comparison of the primary structures of a number of species. However, further comparison of the caseins must await sequence analysis.

We conclude that guinea-pig milk contains four major organ-specific proteins, each markedly different on the basis of amino acid composition and tryptic peptide 'mapping'. We cannot, however, exclude the presence of some microheterogeneity within the individual casein preparations, particularly casein B. The guinea-pig caseins in common with all other caseins are phosphoproteins and contain typically high concentrations of glutamic acid and proline. It is probable that casein C is also a glycoprotein, though as the sugar content is low, it is conceivable that the glycolytic residues may be associated with the minor protein contaminant.

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