Chemical and immunochemical characterization of caseins and the major whey proteins of rabbit milk

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Caseins were separated from whey proteins by acid precipitation of skimmed rabbit milk. Whole casein was resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis into three major bands with apparent relative molecular masses (M_r of 31 000, 29 000 and 25 000. On agarose/urea-gel electrophoresis whole casein gave three bands with electrophoretic mobilities α , β and γ . The three components were purified by DEAE-cellulose chromatography under denaturing and reducing conditions. Each was shown to have a different amino acid, hexose and phosphorus content, as well as non-identical peptide fragments after proteinase digestion. The 31 000 Da (dalton) protein, of α -electrophoretic mobility, had a high phosphorus content (4.38%, w/w); the 29 000 Da peptide, of γ -mobility, had the highest hexose content (2.2%, w/w), contained 0.8 cysteine residue per 100 amino acid residues and was susceptible to chymosin digestion corresponding thus to κ -casein; the 25 000 Da protein migrated to the β -position. The rabbit casein complex is composed of at least three caseins, two of which (α - and κ -caseins) are analogous to the caseins from ruminants. Although caseins are poor immunogens, specific antibodies were raised against total and purified polypeptides. The antiserum directed against whole casein recognized each polypeptide, each casein corresponding to a distinct precipitation line. The antisera directed against each casein polypeptide reacted exclusively with the corresponding casein and no anti-serum cross-reaction occurred between the three polypeptides. From whey, several proteins were isolated, characterized and used as antigens to raise specific antibodies. An iron-binding protein with an apparent M_r of 80 000 was shown to be immunologically and structurally identical with serum transferrin.

The mammary gland is an ideal model system for studying protein synthesis and secretion as well as the hormonal regulation of gene expression, since large amounts of caseins and whey proteins are produced during lactation in response to hormonal stimulation. The rabbit was chosen because: (1) it produces sufficient milk to isolate and characterize several milk proteins; (2) the hormonal regulation of casein and α -lactalbumin gene expression has already been investigated *in vivo* (Houdebine & Gaye, 1975; Houdebine, 1976; Devinoy & Houdebine, 1977; Devinoy *et al.*, 1978); (3) the receptor interacting with lactogenic hormones has been partially purified (Shiu & Friesen, 1974); (4) its mammary gland can be placed in organ culture

(Hurlimann & Lichaa, 1976; Houdebine & Djiane, 1980) or primary cell cultures (Kraehenbuhl *et al.*, 1975; Häuptle *et al.*, 1979); and (5) its mammary secretory immunological system has been investigated (Hurlimann & Lichaa, 1976; Kraehenbuhl *et al.*, 1975); Kühn & Kraehenbuhl, 1979a,b; Mostov *et al.*, 1980).

In the rabbit, only a few studies have been reported on caseins (Testud & Ribadeau-Dumas, 1973; Al Sarraj *et al.*, 1978a,b), in contrast with milk proteins from ruminants (Thompson & Farrell, 1974). Antisera against rabbit caseins have been prepared (Houdebine & Gaye, 1975; Al Sarraj *et al.*, 1978b) and used to analyse immunochemically the various rabbit caseins. Although the antisera were insufficiently characterized in terms of their specificity, Al Sarraj and his co-workers (Al

Abbreviation used: SDS, sodium dodecyl sulphate.

Sarraj *et al.*, 1978b) concluded that the rabbit caseins were immunologically related. Some rabbit whey proteins have been extensively studied, such as α -lactalbumin, the amino acid sequence of which has been established (Hopp & Woods, 1979) and secretory component, a membrane glycoprotein that mediates uptake and transport of polymeric immunoglobulins and is finally released into milk (Kühn & Kraehenbuhl, 1979a,b; Mostov *et al.*, 1980). Rabbit milk, unlike that of most mammals, does not contain lactoferrin, an iron-binding protein structurally different from the serum iron-binding protein transferrin (Masson & Heremans, 1971). In the present study we have isolated an 80000 Da (dalton) iron-binding protein from milk and compared its chemical and immunochemical properties with those of the serum transferrin.

Well-defined antisera recognizing specifically caseins or whey proteins would be useful probes to identify from lactating mammary glands the translation products of cytoplasmic polyadenylated RNA and hence their corresponding mRNA species, to study the hormonal control of protein synthesis and secretion in systems *in vitro*, and by using immuno-chemical methods, to determine whether different cell types process the various milk proteins. Thus we raised antibodies against the three rabbit caseins and several whey proteins, defined their specificity and developed radioimmunoassays.

Materials and methods

Materials

Alkaline phosphatase type VII from calf intestinal mucosa (380 units/mg of protein) and chymosin (rennin) (21 units/mg) were obtained from Sigma, St. Louis, MO, U.S.A.; neuraminidase from *Vibrio cholerae* (0.1 unit/ml), chymotrypsinogen A and Coomassie Brilliant Blue from Serva, Heidelberg, Germany; chymotrypsin (62 units/ml) from Worthington Biochemical Corporation, Freehold, NJ, U.S.A.; *Staphylococcus aureus* V8 proteinase (lot no. 36-900-1), bovine serum albumin and ovalbumin from Miles Laboratories, Stoke Poges, Slough, Berks., U.K.; acrylamide, methylenebisacrylamide and 1-ethyl-2-[3-[1-ethylnaphtho(1,2-d)-thiazolin-2-ylidene]-2-methylpropenyl]naphtho(1,2-d)-thiazolium bromide ('Stains All') from Eastman Kodak Co., Rochester, N.Y., U.S.A.; DEAE-cellulose DE-22 from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; Enzymo beads and Bio-Rad AG1-X2 resin from Bio-Rad Laboratories, Richmond, CA, U.S.A.; QAE (quaternary aminoethyl)-Sephadex, Sepharose 4B, 2B and Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala, Sweden; Freund's complete adjuvant from Difco Laboratories, Detroit, MI, U.S.A. All other reagents were of analytical grade.

Methods

Purification of rabbit milk proteins. Milk obtained from outbred New Zealand rabbits during the first 7 days after parturition was centrifuged at 2500 g for 30 min at 4°C. The fat layer and the cell pellet were discarded, leaving skimmed milk.

(a) Purification of caseins. Caseins were precipitated isoelectrically from skimmed milk as described by McMeekin *et al.* (1959). The precipitate was washed four times with acidified distilled water. These caseins were then fractionated on a DEAE-cellulose column equilibrated with 10 mM-imidazole/HCl buffer, pH 7.0, containing 3.3 M-urea, 1% β -mercaptoethanol and 10 mM-EDTA, and the bound proteins were eluted from the column with a linear gradient of 0–0.6 M-NaCl (Thompson, 1966).

The breakthrough volume contained 21% of the recovered proteins and was collected in two fractions (1a and 1b). The peak eluted at 0.07 M-NaCl was 6% of the recovered material (fraction 2). The highest peak, eluted at 0.185 M-NaCl, represented 56% of the proteins (fraction 3), whereas the last peak (fraction 4), eluted at 0.285 M-NaCl, represented 17% of the recovered material. Each fraction was analysed on an SDS/10%-polyacrylamide gel. Fraction 1a was enriched in a 29000 Da casein, but contaminated by some whey proteins of high M_r (150000) identified as immunoglobulin G. Fraction 1b, obtained when the column was rinsed with the loading buffer, consisted exclusively of the 29000 Da casein. Fraction 2 contained mainly a low- M_r protein identified as α -lactalbumin, although a 29000 Da protein was also detected. Fraction 3 was enriched in a 31000 Da casein and fraction 4 in a 25000 Da casein. Complete purification of these two caseins could not be achieved by recycling the enriched fractions on DEAE-cellulose columns under the same conditions. Thus these two proteins were further purified by electrophoretic elution of each protein from SDS/polyacrylamide preparative gels followed by acetone precipitation to remove SDS. Homogeneity of each casein preparation was assessed by SDS/polyacrylamide-gel electrophoresis, as shown in Fig. 1 (below).

(b) Purification of the iron-binding protein. Rabbit skimmed milk was acidified to pH 4.6 by addition of acetic acid. Precipitated material was removed by centrifugation and the supernatant (whey) adjusted to pH 7.0 with 0.5 M-NaOH. The whey was then chromatographed on a column of Sephadex G-200. The proteins were recovered in four peaks and analysed by SDS/polyacrylamide-gel electrophoresis. The milk iron-binding protein was recovered in the third peak eluted from the G-200 Sephadex column. The protein was then purified by chromatography on QAE-Sephadex (elution at 0.10 M-NaCl with a linear NaCl gradient). Rabbit transferrin was prepared from serum by precipi-

tation with 60%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in water, dialysed against 10 mM-sodium phosphate buffer, pH 7.4, chromatographed on a column of DEAE-cellulose equilibrated with the same buffer, and eluted at 0.10 M-NaCl with a linear gradient of 0–0.4 M-NaCl.

Analytical methods. SDS/polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis in a vertical slab-gel (20 cm $\times$ 25 cm) apparatus was performed as described by Maizel (1971) with an acrylamide/bisacrylamide weight ratio of 75:2. Gels of 10, 13 and 5–13% (gradient) polyacrylamide were used for both analytical and preparative purposes. Some SDS/polyacrylamide gels contained 3 M-urea. Peptide analysis of partial proteolytic digests was performed in 15% gels containing 10% (w/v) sucrose. Gels were stained for glyco- and phospho-proteins with 'Stains All' as described by Green & Pastewka (1976).

Agarose-gel electrophoresis. Electrophoresis was for 1 h at 100 mV and 5 mA in horizontal slab gels (10 cm $\times$ 20 cm) containing 2% agarose in 0.025 M-Tris/sodium barbital buffer (pH 8.6)/0.2 mM-calcium citrate/3.3 M-urea. The electrode buffer consisted of 0.05 M-Tris/barbital, pH 8.6.

Electrophoretic elution of proteins from SDS/polyacrylamide gels. Proteins were eluted from SDS/polyacrylamide gels into a dialysis bag by overnight electrophoresis. SDS was removed by precipitation with 9 vol. of acetone at -20°C . The precipitate was redissolved in water containing NH_4HCO_3 (0.4 M-final concn.) and re-precipitated twice with acetone. The final precipitate was dissolved in water, dialysed against water for 5–6 h and freeze-dried.

Enzymic-digestion methods. Alkaline phosphatase. Purified caseins were dephosphorylated with alkaline phosphatase as described by Green & Pastewka (1973). The reaction was performed in 5 mM-sodium acetate buffer, pH 6.2, containing 1 mM-MgCl₂ and 1 unit ($\mu\text{mol}/\text{min}$) of enzyme for 7 μg of casein at 37°C for 4 h.

Neuraminidase. Sialic acid was removed from the purified proteins by digestion with neuraminidase (1 unit of enzyme for 1 μg of protein) in 0.5 ml of 0.05 M-sodium acetate buffer, pH 5.6, containing 100 mM-NaCl and 10 mM-CaCl₂ at 37°C for 1 h (Green & Pastewka, 1975).

Chymosin (rennin). Purified caseins (2–3 mg/ml) were digested with rennin (0.1–0.5 mg/ml) in 2 mM-phosphate buffer, pH 6.3, at 37°C for 1 h (Green & Pastewka, 1976).

Chymotrypsin and *S. aureus* V8 proteinase. Purified caseins, milk and serum transferrin were dissolved in a buffer containing 25 mM-Tris/HCl, pH 6.8, 0.5% SDS and 2.0 M-urea with heating at 100°C for 2 min. The samples were cooled and enzymes were added to the protein mixture at a ratio

of 1:5 or 1:10 (w/w) with final enzyme concentrations of 50 $\mu\text{g}/\text{ml}$ for α -chymotrypsin and 25 $\mu\text{g}/\text{ml}$ for *S. aureus* proteinase (Cleveland *et al.*, 1977). Proteolysis was performed at 37°C for 1 h and the reaction stopped by adding 10% β -mercaptoethanol and 2% SDS (final concns.) to the reaction mixture, followed by heating at 100°C for 2 min.

Amino acid analysis. A sample (1 mg) of purified proteins was hydrolysed, *in vacuo*, in 0.5 ml of 6 M constant-boiling HCl for 24 h at 110°C (Mayes *et al.*, 1973) in vacuum-hydrolysis tubes (Pierce; from Kontron, Zurich, Switzerland). The hydrolysate was freeze-dried under reduced pressure, dissolved in citrate/chloride buffer, pH 1.9, and analysed in a Biotronic (Frankfurt, Germany) LC 6000 autoanalyser, with a Resin Purum DC 6A column (Purum Chemical Corp., Sunnyvale, CA, U.S.A.). The amino acid composition was calculated as a mean $\pm$ S.D. of two measurements.

Carbohydrate analysis. Purified proteins (2–4 mg) were hydrolysed in 0.5 M-HCl/methanol (1 ml) for 16 h at 65°C , and the carbohydrate analysed as described by Werner *et al.* (1978).

Phosphorus content. This was determined by the method of Fiske & SubbaRow (1925) as modified by Bamann *et al.* (1948).

Immunochemical methods. Antisera against caseins. These were produced in rats and a goat. Rats, 20 in all, were injected with 100–200 μg of acid-precipitated caseins per injection in complete Freund's adjuvant subcutaneously and intramuscularly at 15-day intervals. They were bled after the fifth injection and their sera pooled. The antiserum was then absorbed with glutaraldehyde-polymerized normal rabbit serum and whey proteins. A goat was injected at 10-day intervals with 2 mg of whole caseins (cross-linked with 0.5% glutaraldehyde) per injection in complete Freund's adjuvant and then bled after the fifth injection.

Specific antisera for α - and κ -caseins were prepared in rats by using caseins purified by DEAE-cellulose chromatography. Antigen in complete Freund's adjuvant (100 μg) was injected at 10-day intervals.

Antiserum against the milk iron-binding protein (transferrin). This was raised in a goat by intramuscular injections of 2 mg of antigen (per injection in complete Freund's adjuvant at 15-day intervals). The goat was bled after the fourth injection.

Antiserum against α -lactalbumin. This was raised in goat by using the same protocol as that described for the iron-binding protein. An antiserum against α -lactalbumin was also raised in rats by weekly intramuscular and subcutaneous injections of 100 μg of antigen (per injection) in complete Freund's adjuvant. α -Lactalbumin recovered from a Sephadex G-200 column was further purified by chro-

matography on QAE-Sephadex column equilibrated with 10mM-phosphate buffer, pH 7.4. The protein eluted at 0.07M-NaCl by a linear NaCl gradient was identified as α -lactalbumin by virtue of its lactose synthetase activity (Palmiter, 1969).

Results

Protein composition of rabbit milk

Proteins from skimmed milk were separated by isoelectric precipitation and each fraction was analysed by SDS/polyacrylamide-gel electrophoresis. About 70% of the total milk protein is acid-precipitable and operationally defined as caseins. The acid-soluble proteins (whey proteins) are resolved into about ten major bands with apparent M_r values ranging between 12 000 and 400 000. The whey proteins associated with the secretory immune system have been previously characterized, as reported elsewhere (Kühn & Kraehenbuhl, 1979a,b).

Caseins

Physicochemical properties. Size. The acid-precipitable proteins were separated into three distinct bands with apparent M_r values of 31 000, 29 000 and 25 000 on SDS/polyacrylamide-gel electrophoresis. A fourth low- M_r (~22 000) protein contaminating

the acid-precipitable material was identified as α -lactalbumin by its lactose synthetase activity. Each casein was purified by combining ion-exchange chromatography and preparative SDS/polyacrylamide-gel electrophoresis (see under 'Methods') (Fig. 1a).

Electrophoretic mobility. Agarose-gel electrophoresis in the presence of urea resolved whole caseins into three components migrating to α -, β - and γ -positions (serum proteins acting as standards). The purified components showed single bands corresponding to mobilities α , γ and β for the 31 000, 29 000 and 25 000 Da components respectively (Fig. 1b).

Chemical composition. The chemical composition of the three caseins given in Table 1 shows the three caseins to be different from each other. All three components are relatively rich in glutamic acid, proline and leucine. Half-cystine residues are present only in the 29 000 Da component. The carbohydrate and phosphorus contents determined only for the 31 000 and 29 000 Da components are also given in Table 1.

Phosphatase and neuraminidase digestion. Purified caseins (2.5–3 μ g), and acid-precipitated whole casein (6–10 μ g) were resolved on SDS/13%-polyacrylamide-gel-electrophoresis gels and stained with

Table 1. *Amino acid composition, phosphorus and carbohydrate contents of the rabbit casein components*
The experimental protocol is given under 'Methods'. Abbreviation used: N.D., not determined.

Amino acid	Casein M_r ...	Composition (residues/100 residues)		
		31 000	29 000	25 000
Aspartic acid		6.0	5.9	6.3
Threonine		4.9	4.9	5.2
Serine		5.8	5.1	5.1
Glutamic acid		24.9	18.3	19.3
Proline		7.5	13.6	6.8
Glycine		1.3	1.9	4.6
Alanine		4.5	4.3	3.6
Valine		6.5	6.6	7.3
Half-cystine		0	0.8	0
Methionine		1.5	2.9	2.1
Isoleucine		2.8	6.0	8.7
Leucine		12.7	14.2	5.2
Tyrosine		5.4	2.1	5.5
Phenylalanine		2.7	4.6	5.3
Lysine		5.9	6.0	9.2
Histidine		1.6	2.5	2.4
Arginine		6.9	3.1	3.3
Phosphorus (% w/w)		4.38	0.57	N.D.
Total carbohydrate (% w/w)		0.38	2.2	N.D.
Mannose		0.01	0.2	
Galactose		0.09	0.7	
Glucose		0.08	0.3	
N-Acetylgalactosamine		0.03	0.4	
N-Acetylglucosamine		0.06	0.3	
N-Acetylneuraminic acid		0.11	0.3

Fig. 1. *Polyacrylamide- and agarose-gel electrophoresis* (a) 0.1% SDS/3.3 M-urea/10%-polyacrylamide-gel electrophoresis. 1, Acid-precipitated whole caseins; 2–4, purified caseins (2, 31000 Da casein; 3, 29000 Da casein; 4, 25000 Da casein). (b) 2%-Agarose-gel electrophoresis in 0.025 M-Tris/barbital buffer, pH 8.6, and 3.3 M-urea. 1, Acid-precipitated whole caseins; 2, κ -casein (29000 Da); 3, α -casein (31000 Da); 4, casein x (25000 Da).

Fig. 2. *Digestion of caseins*

(a) Rabbit caseins digested with phosphatase and neuraminidase. The detailed protocol is given under 'Methods'. 0.1% SDS/13%-polyacrylamide-gel electrophoresis staining with 'Stains All' were used. Only bands that stained blue are seen on this photograph, since a filter was used to eliminate the red-stained bands. The position of the bands stained in red are indicated by arrows (slots 5 and 7). Slots 1 and 8, acid-precipitated whole caseins; 2, 5 and 9, 31000 Da casein; 3, 6 and 10, 29000 Da casein; 4, 7 and 11, 25000 Da casein; 1–4, undigested caseins; 5–7, phosphatase-digested caseins; 8–11, neuraminidase-digested caseins. (b) Rabbit caseins digested with rennin. The detailed protocol is given under 'Methods'. 0.1% SDS/3.3 M-urea/13%-polyacrylamide-gel electrophoresis was used and staining was with Coomassie Blue. Slots 1 and 4, 25000 Da casein; 2 and 5, 29000 Da casein; 3 and 6, 31000 Da casein; 1–3, rennin digestion [rennin/casein ratio 1:25 (w/w)]; 4–6, rennin digestion [rennin/casein ratio 1:5 (w/w)]. (c) Peptide maps of rabbit caseins. The protocol for partial proteolysis is given under 'Methods'. 0.1% SDS/10% sucrose/15%-polyacrylamide-gel electrophoresis was performed. Before being stained with Coomassie Blue, the gels were fixed in 10% trichloroacetic acid/5% sulphosalicylic acid/30% methanol. Slots 1 and 4, 31000 Da casein; 2 and 5, 29000 Da casein; 3 and 6, 25000 Da casein; 1–3, *S. aureus* V8 proteinase digests; 4–6, chymotrypsin digests.

'Stains All', which stains protein red but phosphoproteins and sialoglycoprotein blue. The three caseins stained blue (Fig. 2a). Alkaline phosphatase treatment of each casein resulted in a loss of the blue colour for the 31000 and 25000 Da caseins, and a partial loss of blue stain for the 29000 Da band. Neuraminidase treatment decreased the blue staining of the 29000 Da casein without measurable change in blue staining of the two other caseins.

Rennin digestion. The three casein components digested with rennin (5–6 μ g per sample) were analysed on an SDS/13% polyacrylamide slab gel and compared with the undigested caseins and rennin. The results, shown in Fig. 2(b), indicate that only the 29000 Da band was cleaved into four fragments, with apparent M_r values of 22000, 17000, 12000 and 7000.

Peptide mapping. The peptide maps of chymotryptic and *Staphylococcus aureus*-proteinase digests of the purified casein components are shown in Fig. 2(c). Each casein produced a distinct pattern of 15–20 bands, with M_r values ranging between 2500 and 25000 Da. No homology between the three caseins was observed with either enzyme.

Immunological properties. Antisera against acid-precipitable whole casein. Rat anti-(whole casein) sera revealed three antigenically distinct components when tested against whole caseins or milk (Fig. 3). These antisera also contained low-titre antibodies directed against rabbit secretory immunoglobulin A and α -lactalbumin, which were removed by absorption with rabbit serum and whey proteins.

This antiserum recognized specifically each purified casein (Fig. 3a). The three caseins were immunologically distinct and antigenically unrelated when analysed by double immunodiffusion (Fig. 3b). The goat anti-(whole casein) serum reacted with the

Fig. 3. Immunoelectrophoresis and double immunodiffusion

(a) Immunoelectrophoresis of rabbit caseins allowed to react with rat serum directed against whole casein and absorbed with rabbit serum and whey proteins. 1. Whole caseins; 2. 31 000 Da casein; 3. 29 000 Da casein; 4. 25 000 Da casein. (b) Double immunodiffusion of rabbit caseins. Rat anti-(whole casein) (α -wc) serum ($15\mu\text{l}$) was loaded in the centre well and allowed to react with the 31 000 Da casein (c31 KDa), the 29 000 Da casein (c29 KDa), and the 25 000 Da casein (c25 KDa). Note that no fusion of precipitation lines is observed between the three caseins. (c) Immunoelectrophoresis of whole rabbit casein allowed to react with rat antisera specific for each casein. 1 rat anti-(31 000 Da casein) serum; 2, rat anti-(29 000 Da casein) serum; 3, rat anti-(25 000 Da casein) serum.

31 000 Da casein and with a low- M_r protein identified as α -lactalbumin. By appropriate absorption the goat antiserum was then made specific to either the 31 000 Da casein or to α -lactalbumin. On immunoelectrophoresis no immunoprecipitation occurred with the 29 000 and 25 000 Da caseins.

Antisera against individual caseins. Rat antisera directed against DEAE-cellulose-purified 29 000 Da casein (fraction 1) or 31 000 Da casein (fraction 3)

Fig. 4. Peptide digests of rabbit milk and serum transferrin

The protocol for partial proteolysis is given under 'Methods'. 0.1% SDS/10% sucrose/15%-polyacrylamide-gel electrophoresis was performed and the gels were treated and stained as indicated in Fig. 2(c). Lanes 1, 2 and 8, rabbit serum transferrin; 3, 7 and 10, rabbit milk transferrin; 4 and 9, mixture of serum and milk transferrin; 2-4, chymotrypsin digests; 7-9, *S. aureus* V8 proteinase digests; 5, chymotrypsin alone (same amount of enzyme as in lanes 2-4); 6, V8 proteinase alone (same amount of enzyme as in lanes 7-9).

Fig. 5. Immunoelectrophoresis of rabbit transferrin with a goat anti-(milk iron-binding protein) antiserum
1, Rabbit milk; 2, the milk iron-binding protein; 3, rabbit serum.

were absorbed with rabbit serum and whey proteins. Each antiserum was further absorbed with the appropriate purified caseins to make them specific against only one casein. An anti-(25 000 Da casein) serum was obtained by absorption of anti-(whole caseins) with 31 000 and 29 000 Da caseins. Each antiserum tested by double immunodiffusion, immunoelectrophoresis or two-dimensional immunoelectrophoresis using milk, whole caseins or individual caseins as source of antigen, reacted exclusively with the corresponding casein (Fig. 3c). When the caseins were digested with rennin and then tested by immunoelectrophoresis with anti-(whole casein) antiserum only the precipitation with the 29 000 Da casein disappeared (results not shown).

The iron-binding protein. An 80 000 Da protein purified from rabbit milk by gel filtration and

ion-exchange chromatography was injected into rats and one goat. When tested against rabbit serum, the antiserum precipitated a protein migrating to the same position as serum transferrin (Fig. 4). Rabbit serum transferrin showed complete immunological identity with the milk iron-binding protein. Milk iron-binding protein and serum transferrin migrated to identical positions on SDS/polyacrylamide-gel electrophoresis and when subjected to partial proteolysis generated the same peptide pattern with both chymotrypsin and *S. aureus* proteinase (Fig. 5).

Discussion

Rabbit milk proteins, in common with other mammalian milk proteins, are separated by isoelectric precipitation into precipitable caseins and soluble whey proteins. The caseins were fractionated by DEAE-cellulose chromatography in the presence of urea and β -mercaptoethanol as described for bovine caseins by Thompson (1966). This procedure was also successfully used for sheep (Alais & Jollès, 1967), human (Groves & Gordon, 1970), rat (Nardacci *et al.*, 1978), mouse (Enami & Nandi, 1977), guinea pig (Craig *et al.*, 1978) and rabbit caseins (Testud & Ribadeau-Dumas, 1973; Al Sarraj *et al.*, 1978a). The elution pattern we observed for rabbit caseins was similar to that reported by Al Sarraj *et al.* (1978a). However, unlike those authors, our caseins were recovered in only three peaks, since the fourth peak (fraction 2) contained mainly a protein identified as α -lactalbumin by the lactose synthetase assay. This protein did not contain phosphorus and had a high half-cystine content. Its amino acid composition, similar to that reported by Al Sarraj *et al.* (1978a), was in agreement with that derived from the sequence of rabbit α -lactalbumin (Hopp & Woods, 1979). The discrepancies between our results and those of Al Sarraj and his co-workers (Al Sarraj *et al.*, 1978a) can be explained if whey protein contaminated their whole casein. Such a contamination has been observed in several species (Feldman, 1974). Since ion-exchange chromatography or gel filtration (Craig *et al.*, 1978) is not sufficient to achieve purification of each casein, more drastic treatments have to be used to dissociate the casein micelles. This can be achieved with an ionic detergent such as SDS used under reducing conditions, followed by preparative SDS/polyacrylamide-gel electrophoresis. Each casein purified by this protocol was homogeneous when electrophoresed in SDS/urea/polyacrylamide or alkaline-urea gels. Their apparent M_r values were 31 000, 29 000 and 25 000, with corresponding α , γ - and β -electrophoretic mobilities. The presence of three caseins in rabbit milk was previously described by Houdebine & Gaye (1975) and in other milks from several species such as the rat (Rosen *et al.*, 1975), guinea

pig (Craig *et al.*, 1978) and cow (Thompson, 1966). The three caseins we isolated were rich in glutamic acid and proline, a property they share with bovine (Jollès, 1972), guinea-pig (Craig *et al.*, 1978) and human caseins (Nagasawa *et al.*, 1967). The amino acid composition and the one-dimensional peptide patterns obtained by SDS/polyacrylamide-gel electrophoresis of α -chymotrypsin or *S. aureus* proteinase digests indicate that each of the three caseins have distinct amino acid sequences.

The 31 000 Da casein with α -mobility has a phosphorus content of ~4.5% (w/w) and does not contain cystine residues. These are the properties of bovine α_{s1} casein, except that the rabbit α -casein contains ~0.4% (w/w) hexoses, hexosamines and neuraminic acid, which are absent in the cow casein α_{s1} . The amino acid composition of rabbit α -casein is similar to that of polypeptide 3 described by Al Sarraj *et al.* (1978a) and component 2b of Testud & Ribadeau-Dumas (1973).

The 25 000 Da casein with β -mobility stains blue with 'Stains All' and red after phosphatase treatment. Thus we concluded that it is a phosphoprotein with little neuraminic acid. The amount of glutamic acid, proline and leucine is similar to that of component 4b described by Al Sarraj *et al.* (1978a), but little homology is seen with the β -casein from the cow (Groves & Gordon, 1970) or human (Ribadeau-Dumas *et al.*, 1972). For this reason we termed this casein 'casein x'.

The 29 000 Da casein with γ -mobility exhibits several properties of cow κ -casein (Mackinlay & Wake, 1971; Jollès *et al.*, 1970), such as low phosphorus content, the highest carbohydrate content of the three caseins, presence of cystine and susceptibility to rennin digestion. The amino acid composition is similar to polypeptide 1 of Al Sarraj *et al.* (1978a) and component 9 of Testud & Ribadeau-Dumas (1973).

By analogy with the bovine system, we propose that the following nomenclature be established for the rabbit: α -casein (M_r 31 000), κ -casein (M_r 29 000) and casein x (M_r 25 000).

Rat antisera raised against acid-precipitated rabbit casein revealed three antigenically distinct components in whole casein, each with different immunogenicity and each with no cross-reactivity. Thus α -casein was the most immunogenic, whereas casein x and casein κ reacted only weakly when tested with anti-(whole casein) antisera by double immunodiffusion, immunoelectrophoresis or two-dimensional immunoelectrophoresis. Previously an antiserum against rabbit casein has been described by Houdebine & Gaye (1975), but its specificity was not defined. In addition, Al Sarraj *et al.* (1978b) raised an antiserum against glutaraldehyde-cross-linked rabbit whole casein. However, this serum showed complete immunological identity among the

five polypeptides they isolated from DEAE-cellulose columns. Such cross-reactivities between the individual caseins were also reported for human (Pick *et al.*, 1976), cow (Garnier *et al.*, 1964) and mouse caseins (Feldman & Hohmann, 1971). By contrast, Feldman & Ceriani (1970) and Terry *et al.* (1975) obtained antisera against mouse and rat caseins that reacted specifically with the individual caseins. In the case of the rabbit one can explain the cross-reactivity observed by Al Sarraj *et al.* (1978b) as a reaction of the anti-casein serum with the more immunogenic casein that may contaminate the various DEAE-cellulose fractions. The antiserum of Al Sarraj *et al.* (1978b) probably recognizes the rabbit α -casein, in view of the low immunogenicity of the two other caseins. This is supported by our observation that no antibodies are produced against κ -casein and casein x when we used the same immunization protocol.

The major rabbit whey proteins were purified from the acid-soluble proteins of skimmed milk. These proteins, identified by specific antisera we have prepared consist of secretory immunoglobulin A and immunoglobulin G, an iron-binding protein, serum albumin, α -lactalbumin and low- M_r unidentified polypeptides. α -Lactalbumin, an iron-binding protein and the secretory component that binds to immunoglobulin A dimer are synthesized by the mammary epithelial cells (Hurlimann & Lichaa, 1976; Häuptle *et al.*, 1979), whereas immunoglobulin G and serum albumin are derived from the bloodstream (Hurlimann & Lichaa, 1976), and immunoglobulin A dimer is produced by local plasma cells in the mammary gland (Kraehenbuhl *et al.*, 1975).

The iron-binding protein from the whey is structurally and antigenically identical with serum transferrin (Figs. 4 and 5), a gene product of the liver (Morgan, 1969; MacKnight *et al.*, 1980). However, in the rabbit, milk transferrin is synthesized by the mammary gland (Häuptle *et al.*, 1979; Suard *et al.*, 1982). Thus, in the rabbit, transferrin is expressed by two organs, the mammary gland and the liver. In most other mammals, the mammary gland expresses an iron-binding protein that is structurally and immunologically unrelated to serum transferrin. This protein, termed lactoferrin, cannot be detected in rabbit milk (Masson & Heremans, 1971).

In conclusion, our results indicate that rabbit caseins are made up of at least three structurally and antigenically distinct proteins that share similar properties with ruminant caseins. This is further supported by the immunoprecipitation of three individual peptides from cell-free translation of polyadenylated RNA isolated from lactating mammary gland [see the following paper (Suard *et al.*, 1982)]. Antibodies have been raised against major whey proteins, including an iron-binding protein

structurally identical with serum transferrin and α -lactalbumin. Radioimmunoassays were developed for each milk protein we have characterized in the present study and can be used to investigate the hormonal regulation of gene expression in mammary primary cell cultures.

Note added in proof

Mercier & Gaye (1980) have isolated and analysed from the DEAE-cellulose fraction 2 a 29000 Da casein that exhibits sequence homology with the bovine β -casein. Since our fraction 2 was heavily contaminated with α -lactalbumin, we have not isolated and characterized this 29000 Da β -casein, which co-migrated on SDS/polyacrylamide-gel electrophoresis with κ -casein.

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