The Whey Proteins of the Milk of Red Deer (*Cervus elaphus* L.)

A HOMOLOGUE OF BOVINE β -LACTOGLOBULIN

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1. The whey proteins from the milk of red deer are compared with those of cattle. Gel chromatography and electrophoresis showed a close similarity between the whey proteins of the two species in the size, mobility and relative amounts of the main constituents and in the changes in their relative amounts with time after parturition. 2. The major constituent of the deer whey was isolated. It appeared to be homologous with bovine β -lactoglobulin and had the following properties: $m = -5.2 \times 10^{-9} \text{ m}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ at 4°C and $\text{pH } 8.6$; $\text{pI} = 5.17$; $s_{20,w}^0 = 2.89 \text{ S}$; $\bar{v} = 0.748 \text{ ml/g}$; $E_{1\text{cm}}^{1\%} = 9.12$ at 278 nm ; $\Delta n/c = 1.794 \times 10^{-3} \text{ dl/g}$ at 579 nm (all at 20°C except m). Its molecular weight was that of a dimer with a subunit weight of 18000. 3. Amino acid analyses of this protein, adjusted to lysine = 15 residues showed that it contains one more residue of aspartic acid, alanine and methionine and one less glutamic acid residue and two less leucine residues than bovine β -lactoglobulin A. 4. On starch-gel electrophoresis at $\text{pH } 8.2$, this protein migrated at the same rate as bovine β -lactoglobulin B, although its isoelectric point is close to that of the bovine A variant. Milk from three out of 27 hinds examined showed a variant. This migrated in starch gel at the same rate as the bovine A variant but had a more acid $\text{pI} = 5.02$. 5. The two species whose milk whey proteins are compared represent two different families of ruminants. The similarities found support the view that the milk whey proteins of the bovids are probably typical of the suborder as a whole.

The composition of the milk of different species has been reviewed by Ben-Shaul (1962) and Jenness & Sloan (1970). Because of the scarcity of data, comparison of the protein composition is limited to the casein and whey fractions. The latter review includes a ternary diagram which shows that the milk of ruminants is characterized by a high casein/whey-protein ratio compared with other groups of mammals. Among the ruminants, all the domesticated species are bovids; their milk is easily available, but milk from other families can only be obtained from captive wild animals. Hence our knowledge of the milk proteins of ruminants mainly relates to studies on bovids. These are often taken to be typical of the suborder as a whole, but it is still desirable to extend our knowledge to the milk of other families to see whether this is true. Of these families, the cervids are the biggest and appear to have evolved earlier than the bovids (see the scheme in Young, 1962). Of the cervids, the reindeer (*Rangifer tarandus* L.) is herded but its milk proteins have not been studied in any detail. The red deer (*Cervus elaphus* L.), however, has become an experimental animal and increasing attention is being given to methods of farming it (see Blaxter *et al.*, 1974). Milk of this species has become more readily available. Arman *et al.* (1974) followed the output and gross composition of milk from several hinds at the Rowett Research Institute throughout lactation.

Their data included the casein and whey-protein contents and these showed a high ratio similar to that found in other ruminants. In the present work, the whey proteins of red-deer milk are examined in further detail and compared with those of cattle (*Bos taurus*). The major whey protein is isolated and characterized and possible variants of it are sought in milk from individual hinds.

Materials and Methods

Samples of milk at different stages of lactation and pooled milk samples were provided by frozen samples from the investigations of Arman *et al.* (1974). Further samples from individual animals were obtained from the experimental deer farm of the Hill Farming Research Organisation and Rowett Research Institute at Glensaugh, Kincardineshire. The hinds were milked under anaesthesia and after oxytocin injection as described in the above reference for captive deer at the Institute. The samples were taken in the autumn when the hinds were due to be separated from their calves.

Whey was obtained by centrifuging the milk twice for 60 min at $45000g$ at 20°C , to remove fat and casein. It was then dialysed against the buffer required for electrophoresis or gel chromatography and passed through a $0.45 \mu\text{m}$ membrane filter from

Millipore (U.K.) Ltd., London N.W.10, U.K., to remove any particles. For preparative work, pooled milk was used.

The major whey protein was isolated by either of the following two procedures. (1) The whey proteins were fractionated by preparative-scale gel chromatography, and the component that was eluted in the same position as bovine β -lactoglobulin, when run in the column, was bulked and concentrated by ultrafiltration with a PM 10 membrane from Amicon, High Wycombe, Bucks., U.K. The fraction was then run a second time on the column, concentrated again and passed through a $0.45\mu\text{m}$ membrane filter. This preparation was further purified by isoelectric focusing or ion-exchange chromatography, before it was used for amino acid analyses or sedimentation-equilibrium experiments. (2) The whey proteins were fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation by Method IIb of McKenzie (1971a) for bovine β -lactoglobulin. The fraction obtained was concentrated by pervaporation after exhaustive dialysis against water, and then purified by ion-exchange chromatography. The resulting preparations are subsequently referred to as the deer whey protein.

Bovine β -lactoglobulin A, lots 14 and 16, or AB, lot 45 (Pentex), from Miles Laboratories, Stoke Poges, Bucks., U.K., was used as a reference protein or test substance for the experimental methods used.

Protein concentration

The concentration of protein in the whey solutions was estimated from refractive increment measurements by using an average value for the specific refractive increment ($\Delta n/c$) of 1.85×10^{-3} dl/g.

The concentration of the purified deer whey protein in experimental solutions was obtained from refractive increment or absorbance measurements by using the experimentally determined values for its specific refractive increment and specific extinction coefficient. These were based on measurements with a stock solution dialysed against 0.1 M-KCl and standardized by dry-weight determinations (constant weight over P_2O_5 at $18-20^\circ\text{C}$ and a pressure of a few mTorr).

Refractive measurements were made at 20°C in a differential refractometer based on the design of Cecil & Ogston (1951) with the mercury yellow line at 579 nm.

U.v. spectra were recorded at 20°C with a Cary 15 spectrophotometer supplied by Varian Associates Ltd., Walton-on-Thames, Surrey, U.K. Absorbance measurements at the maximum in the u.v. were made with a Beckman model DU spectrophotometer.

Chromatography

Chromatographic separations in dilute buffers were based on the gel Sephadex G-75 or the ion-exchanger DEAE-Sephadex A-50 from Pharmacia (G.B.) Ltd.,

London W.5, U.K. The proteins were dissolved in or dialysed against the eluting or starting buffer before they were applied to the columns and eluted at room temperature ($18-20^\circ\text{C}$).

Analytical gel chromatography made use of two columns, in series, calibrated with the following molecular-weight markers: bovine plasma albumin (mol.wt. 67000) and bovine γ -globulin (150000), from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; α -lactalbumin (15500) from Koch-Light, Colnbrook, Bucks., U.K.; bovine β -lactoglobulin (crystallized, Pentex) (36000) and chymotrypsinogen A ($6 \times$ crystallized) (25700) from Miles Laboratories; ovalbumin (45000) from Sigma (London) Chemical Co., Kingston, Surrey, U.K.; Blue Dextran (2×10^6) from Pharmacia (G.B.) Ltd. Further experimental details are given in the legend to Fig. 2.

Preparative gel chromatography was carried out on a column ($5\text{cm} \times 100\text{cm}$) with upwards flow in 0.15 M-NaCl/0.05 M-imidazole/HCl, pH 6.5, containing 0.02% NaN_3 , at about 30 ml/h. About 1 g of whey proteins was concentrated to 25 ml in buffer by ultrafiltration for application to the column. The void volume was 650 ml, and 15 ml fractions were collected.

Small-scale purification (10–30 mg of protein in 1 ml) of preparations, before sedimentation-equilibrium experiments, was effected by gel chromatography on a $0.9\text{cm} \times 150\text{cm}$ column.

An estimate of the subunit weight of the isolated protein was obtained by gel chromatography on a column ($1\text{cm} \times 90\text{cm}$) (Wright Scientific Ltd., Kenley, Surrey, U.K.) of Sepharose 6B [Pharmacia (G.B.) Ltd], by using upward flow in 6 M-guanidine hydrochloride. The guanidine hydrochloride was purified as described by Nozaki (1972), and the column was prepared and operated approximately as described by Mann & Fish (1972). The column was calibrated with ovalbumin, chymotrypsinogen A and bovine β -lactoglobulin (monomer 18400), referred to above, and with chymotrypsin (B chain 14000, C chain 10000) (Worthington) from Cambrian Chemical Co., Croydon, Surrey, U.K., insulin (A chain 2300, B chain 3400) from BDH, Poole, Dorset, U.K., and bacitracin A (1411) from Sigma Chemical Co. The reduced proteins were alkylated with iodoacetamide before being put on the column, and the molecular-weight values adjusted for the introduction of amidocarboxymethyl groups.

Ion-exchange chromatography was carried out in a column ($0.9\text{cm} \times 15\text{cm}$ or $1.6\text{cm} \times 20\text{cm}$) from Pharmacia (G.B.) Ltd., at 20 ml/h. The salt gradient was formed between 0.1 and 0.5 M-NaCl in 0.05 M-imidazole/HCl, pH 6.5.

All 0.9cm columns were eluted at about 5 ml/h. Elution was followed at 280 nm with a Uvicord 11 column monitor from LKB Instruments, South Croydon, Surrey, U.K., or to show the peaks on a salt

gradient, with a differential refractometer (Labodur; Winopal Forschung, Hanover, Germany).

Electrophoretic techniques

Paper electrophoresis was carried out on horizontally suspended Whatman 3 MM filter paper made by W. and R. Balston, Maidstone, Kent, U.K., in a buffer containing 0.05 M-sodium diethylbarbiturate/0.01 M-diethylbarbituric acid, pH 8.6, for 16 h at 0.5 mA/cm width of paper. The proteins were stained with a solution containing Lissamine Green (0.5 g/dl), acetic acid (5 ml/dl) and ethanol (25 ml/dl).

Starch-gel electrophoresis was carried out by the method of Smithies (1955), but with the discontinuous buffer system of Ferguson & Wallace (1961).

Boundary electrophoresis was carried out in the electrophoresis-diffusion apparatus, type no. 3021, made by LKB Producter AB, Bromma, Sweden. For further details see the legend to Fig. 1.

Electrofocusing experiments were performed in a sucrose-density-gradient column of capacity about 110 ml, made by Consulta, Farsta, Sweden, as described by Svensson (1962), by using Ampholine 1809-116 (LKB Instruments Ltd.) as carrier ampholytes for the pH range 4–6. The column was cooled with tap water for the first 16 h and then maintained for a further 24 h at 20°C with the applied potential difference held at 500 V. The contents were finally displaced downwards through a Uvicord II column monitor operating at 280 nm and collected in about 1 ml fractions for pH measurement.

Sedimentation analyses

These were performed in a model E analytical ultracentrifuge, from Beckman-RIIC, Glenrothes, Fife, Scotland, U.K., in an An-D rotor at 20°C unless otherwise stated. The schlieren and interference optical systems were carefully aligned and focused as described by Richards *et al.* (1971*a,b*). Photographs were recorded on Commercial Ortho film, type 4180, from Kodak, London W.1, U.K. Comparison of photographic records was carried out on a Shadomaster projection microscope, supplied by Buck and Hickman, Glasgow, Scotland, U.K. It was provided with $\times 10$ and $\times 50$ objectives and a co-ordinate table modified to hold plates and films. The calculations of the results were made with Fortran programs on an IBM 1130 computer.

Velocity experiments were performed in standard double-sector 12 and 6 mm cells and for the lowest protein concentrations in a 12 mm synthetic-boundary cell. The centrepiece of this cell had been modified by lapping away the original capillaries and re-scribing them with the outermost capillary nearer to the centre of rotation, about 8.5 mm from the cell bottom. This provided a longer path for the migration of the synthetic boundary. The sedimentation coefficients were obtained from a linear least-squares fit of the position

of the maximum ordinate on the schlieren photograph against time or a quadratic least-square fit when this was better. The values obtained with different starting concentrations were compared at the concentration obtaining at the mid-time of the observations, and used to give the concentration-dependence of the sedimentation coefficient.

Equilibrium experiments were of the long-column meniscus-depletion type of Chervenka (1970), and used a standard capillary synthetic-boundary centrepiece. Absence of precession was determined with an independently mounted cathetometer focused on one of the fringes flanking the image of the air/solution interface obtained with the schlieren optical system. Interference photographs were evaluated in the same way as those in a high-speed equilibrium experiment of Yphantis (1964).

The buffers used in the sedimentation experiments were as follows: 0.15 M-NaCl/0.05 M-imidazole/HCl, pH 6.5; 0.15 M-NaCl/0.05 M-sodium acetate/acetic acid, pH 5.2; 0.15 M-NaCl/0.05 M-sodium acetate/acetic acid, pH 4.6; 0.2 M-NaCl/0.1 M-Tris/HCl, pH 8.1. They are subsequently referred to by their pH.

The partial specific volume of the deer whey protein was calculated from the densities of the stock solution referred to above and of weight dilutions made from it with the 0.2 M-KCl against which it had been dialysed.

Densities of solutions were measured at 20°C in a precision density meter designed by Stabinger *et al.* (1967), model DMA-02C, made by Anton Paar K.G., Graz, Austria. The temperature was regulated with a circulating water bath, type 01-PT-623, made by Heto, Birkerød, Denmark. It remained within $\pm 0.005^\circ\text{C}$ over a period of several hours, but showed a diurnal drift of a few hundredths of a degree depending on the ambient temperature. The density-meter constant was determined for the density range 1.00–1.14 g/dl at 20°C by measurements on KCl solutions which had been made dust- and bacteria-free by passage through a $0.45\ \mu\text{m}$ membrane filter, and whose density at 20°C was subsequently measured by the differential pycnometric method of Washburn & Smith (1934). The value for the density of pure water was taken from Kell (1967).

Viscosities of buffers were measured with suspended level Ubbelohde viscometers in a bath kept at $20 \pm 0.01^\circ\text{C}$, supplied by Townson and Mercer, Edinburgh, Scotland, U.K.

pH measurements

These were all carried out at 20°C with a Radiometer pH-meter, model 26, from V. A. Howe and Co., London S.W.6, U.K.

Amino acid analysis

Portions of solution containing 1–3 mg of protein were freeze-dried in hydrolysis tubes and then

Table 1. *Electrophoretic mobilities and composition of red-deer whey proteins by boundary electrophoresis*

Details of samples and experiments and designation of components are given in the legend to Fig. 1. Values for bovine whey proteins from the literature are included for comparison. A and D refer to data from the ascending and descending boundaries respectively. (1) Polis *et al.* (1950a), albumin from milk whey; (2) Polis *et al.* (1950b), bovine β -lactoglobulin; (3) Gordon & Semmett (1953), α -lactalbumin; (4) Smith (1948), colostrum whey immunoglobulins; (5) analysis of Ayrshire milk whey, taken from Rolleri *et al.* (1956), in which samples at the start and end of lactation were excluded.

		$10^9 \times \text{Mobility (m}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1})$					
Component ...		1	2	3	4	5	
Deer							
7-day sample	A	-6.6	-5.4	-4.3	-3.5 -2.9	-2.4 -2.0 -1.3	
	D	-6.5	-5.2	-4.1	-3.1	-2.3 -1.8 -1.4	
Pooled samples	A	-7.0		-4.3		-2.2	
	D	-6.7		-4.0		-2.0	
Bovine							
Separated proteins	D	-6.7 (1)	-5.1 (2)	-4.2 (3)		-2.2 to -1.8 (4)	
		Composition (%)					
Component ...		1	2	3	4	5	
Deer							
7-day sample	D	3	63	17	6	11	
Bovine (5)		5.5	61.3	21.7		11.5	

hydrolysed in the evacuated tube for 24, 48 and 72 h, each in duplicate, and prepared for analysis by the procedure of Moore (1963). Samples of the hydrolysates were analysed by ion-exchange chromatography on a Technicon amino acid analyser NC1 as described by Davidson *et al.* (1974). Cysteic acid and methionine sulphone were determined on the same system after performic acid oxidation of the protein as described by Moore (1963). Tryptophan and tyrosine were estimated by the method of Edelhoch (1967).

Results and Discussion

Milk whey proteins

The results of some boundary-electrophoresis experiments are shown in Table 1 and Fig. 1. The schlieren diagram Fig. 1(a) of the 7-day sample is similar to a typical diagram for the whey proteins of bovine milk given by Jenness & Patton (1959). Later on in the experiment on this sample, component 5 resolved into three components in the γ -globulin region (see Fig. 1c). Components 1, 2, 3 and 5 have mobilities close to those of bovine albumin, β -lactoglobulin, α -lactalbumin and whey immunoglobulins. Values for the bovine proteins taken from the literature are included in Table 1. Component 4 could be similar to the so called 'protease peptone' fraction of bovine whey. The relative composition obtained from

Fig. 1. *Boundary electrophoresis of whey proteins from milk of red deer in 0.1M-sodium diethylbarbiturate buffer at pH8.6 and 4°C*

(a) Sample 7 days after parturition; descending boundary after 214 min at 6.6 V/cm, $c = 0.8$ g/dl. (b) Pooled samples: dialysed whey stored overnight at 4°C and centrifuged to clarify it; descending boundary after 205 min at 6.5 V/cm, $c = 0.9$ g/dl (initial). (c) Same experiment as (a) but after 259 min. Boundaries 1–5 are due to components which are compared with bovine whey proteins in Table 1. ϵ , Stationary boundary; $\leftarrow$, direction of migration.

measurements of the boundary areas together with comparable analyses of bovine whey proteins from the literature are also given in the Table. The values show good agreement.

The effect of storage at pH 8.6 and 4°C on whey from a pooled milk sample is shown in Fig. 1(b). This resulted in the loss of component 2 by precipitation. This process is presumably analogous to the slow irreversible denaturation of bovine β -lactoglobulin under alkaline conditions studied by Groves *et al.* (1951).

A series of analytical gel chromatograms of samples of milk whey obtained at different stages of lactation are shown in Fig. 2. They show that the column gave a good resolution of the main protein components and that these were eluted in positions corresponding to the bovine marker proteins. The component eluted like bovine β -lactoglobulin showed an asymmetry similar to that shown by this protein and its homologues in the milk of other bovids, when run on this column at pH 8.1. The results also indicate the changes in the relative concentrations during lactation. The change from colostrum to milk secretion appears to be similar to that which takes place in bovids. Paper electrophoresis of the whey samples

showed similar changes. In this connexion, paper electrophoresis of sera obtained from a newborn deer calf before and after suckling confirms that the young of this species depend on the ingestion of colostrum for their circulating γ -globulins.

Major whey protein

Preparative gel chromatography at pH 6.5 usually yielded about 300 mg of the major protein fraction per 100 ml of the pooled milk. From the elution diagram the fraction appeared to be homogeneous with respect to molecular weight at this pH. The neutral pH was chosen to avoid the size inhomogeneity and the instability in more alkaline buffers shown by the analytical-gel-chromatography and boundary-electrophoresis experiments. Electrofocusing experiments, described below, revealed that the fraction contained a minor component with a more acid isoelectric point. This was removed by electrofocusing or ion-exchange chromatography.

Fractionation with $(\text{NH}_4)_2\text{SO}_4$ gave a concentrated preparation containing about 2.8 g of protein from 1 litre of pooled milk. The preparation failed to crystallize when dialysed extensively at pH 5.2 against distilled water and it contained a trace of a protein comparable with bovine α -lactalbumin. This was subsequently removed by ion-exchange chromatography. Both procedures yielded a final preparation which showed a single zone on starch-gel electrophoresis.

Electrofocusing experiments on the deer whey-protein preparation, and on bovine β -lactoglobulin AB for comparison, are shown in Figs. 3(a) and 3(c). Fig. 3(a) shows the minor and major components in the fraction from pooled deer milk, to which reference is made above. Their isoelectric points at 20°C were 5.02 (4) and 5.17 (6) respectively. The values found for the bovine A and B variants were 5.18 (4) and 5.32 (4). These values had s.d. ± 0.02 and the numbers of determinations are given in parentheses. The results for the bovine proteins show good agreement with those obtained by Kaplan & Foster (1971), using electrofocusing, i.e. 5.21 and 5.34 respectively. The isoelectric point of the major component of the deer whey fraction is very close to that of bovine β -lactoglobulin A. That the isoelectric points are not quite identical was confirmed by electrofocusing experiments on mixtures of the deer and bovine proteins. They showed a reproducible difference of 0.01–0.02 in isoelectric points, illustrated by Fig. 3(d).

In contrast with these experiments, when the deer and bovine proteins were compared by starch-gel electrophoresis, the major component from the deer whey fraction migrated at the same rate as the bovine β -lactoglobulin B and not as the A variant.

Some specific properties of the deer whey protein measured at 20°C were as follows: the partial specific volume from densities of solutions in 0.2M-KCl was

Fig. 2. Analytical gel chromatography of proteins of milk whey of red deer at different stages of lactation

A 2 ml sample was put on a Sephadex G-75 column (two 100 cm $\times$ 0.9 cm columns in series) in 0.2M-NaCl/0.1M-Tris/HCl, pH 8.1, containing 0.02% NaN_3 . Elution diagrams show percentage transmission (T) plotted against elution volume (V_e). Marker proteins were: γ , bovine γ -globulin; A, bovine plasma albumin; β -L, bovine β -lactoglobulin; α -L, bovine α -lactalbumin.

Fig. 3. Electrofocusing experiments comparing the protein preparation from milk whey of red deer with bovine β -lactoglobulin

About 10mg of each protein preparation was used. T = percentage transmission. The diagrams are aligned according to the pI obtained from the recorded zones from the measured pH gradients (not shown). (a) Fraction from pooled deer milk. (b) Preparation from milk of hind Rhum 5. (c) Bovine β -lactoglobulin AB. (d) Mixture of the preparation used in (b) and the bovine β -lactoglobulin AB used in (c).

$0.748 \pm 0.001(4)$ ml/g; the value calculated from the amino acid composition by the method of Cohn & Edsall (1943) was 0.746 ml/g. The specific refractive

increment ($\Delta n/c$) at 579 nm, in 0.2M-KCl, was $1.794 \times 10^{-3}(2)$ dl/g and the specific extinction coefficient at 278 nm in dilute buffer at pH 6.5 or at 279 nm in 6M-guanidine hydrochloride was $9.12 \pm 0.01(4)$ dl/g. These results compare closely with values of these properties for bovine β -lactoglobulin given by Goodrich *et al.* (1969), Charlwood (1957) and Williams (1972) respectively. The value for the specific extinction coefficient given by Townsend *et al.* (1960) is a bit higher.

The sedimentation-velocity experiments on the deer whey protein at pH 6.5 can be condensed to the following relations between the normalized sedimentation coefficient in S units and the protein concentration, in g/dl, at the mid-time of observation:

$$s_{20,w} = 2.888(\pm 0.009)[1 - 0.072(\pm 0.002)c]$$

from seven experiments in a standard double-sector cell over the concentration range 0.8–2.4 g/dl, and

$$s_{20,w} = 2.9001(\pm 0.0006)[1 - 0.0968(\pm 0.0003)c]$$

from six experiments in the modified capillary synthetic-boundary cell, over the lower concentration range 0.1–1.0 g/dl.

The effect of temperature and pH on the sedimentation coefficient was studied by using a standard double-sector cell. With concentrations of 0.7–0.9 g/dl at the mid-time of the experiment the sedimentation coefficient was determined at 20° and 5°C and at four different pH reactions. At pH 4.6, 5.2 and 6.5 the sedimentation coefficient was in the range 2.70–2.74S, in agreement with the values calculated by using the first of the expressions for $s_{20,w}$ given above. At pH 8.1, however, the sedimentation was appreciably slower, with $s_{20,w} = 2.54$ S compared with an expected value of 2.72S.

In the sedimentation-equilibrium experiments, a reproducible distribution of concentrations in the cell could only be obtained when no oil was used to form a false bottom. The results of the experiments are summarized in Table 2. This gives the weight- and z-averages of the molecular weight of the different preparations. The difference between these averages varied with the preparation and indicated that the third preparation appeared to be the most homogeneous one. This preparation had been obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation and ion-exchange chromatography and had a molecular weight ($\pm$ S.D.) $\bar{M}_w = 34100 \pm 300(6)$.

Gel chromatography in 6M-guanidine hydrochloride gave a mol.wt. of about 18000. This value is confirmed by the amino acid analyses given below, from which a subunit wt. of 18085 was calculated. The deer whey protein therefore normally exists, like its bovine homologue, mainly as a dimer of this subunit size.

The results of the amino acid analyses were first calculated on the basis of 18000 g dry wt. This gave

Table 2. *Summary of sedimentation-equilibrium experiments by the long-column meniscus-depletion method*

The preparations were examined in buffer at pH 6.5 and at speeds in the range 20000–25000 rev./min. Two equilibrium patterns obtained at different times were evaluated for each experiment. The first series of experiments was carried out in duplicate at each concentration. Further experimental details are given in the Materials and Methods section. The average molecular weights are given, $\pm$ s.d. with the numbers of determinations in parentheses.

Preparation	Method of preparation	Initial concn. (g/dl)	$10^{-3} \times \bar{M}_w$	$10^{-3} \times \bar{M}_z$
Deer whey protein	Gel chromatography	0.04, 0.10, 0.25	30.7 ± 1.2	37.3 ± 2.2 (12)
	Gel chromatography and ion-exchange chromatography	0.05, 0.10	32.6 ± 0.2	35.0 ± 1.0 (4)
	(NH ₄) ₂ SO ₄ precipitation and ion-exchange chromatography	0.08, 0.21, 0.37	34.1 ± 0.3	34.4 ± 0.6 (6)
Bovine β -lactoglobulin A		0.05, 0.10, 0.20	33.9 ± 0.8	35.6 ± 0.9 (6)

Table 3. *Amino acid analyses: comparison of the deer whey protein with bovine β -lactoglobulin A*

For experimental details see the Materials and Methods section. The number of residues, $\pm$ s.e. of the mean or of the estimate, is given relative to 15 residues of lysine. On this basis the subunit weight of the deer whey protein is 18085.

		Deer whey protein			Bovine β -lactoglobulin A		
	Residues	No. of residues	$\pm$ s.e.	No. of determinations	No. of residues	$\pm$ s.e.	No. of determinations
Hydrolysis	Asp	17.01	0.16	8	15.97	0.08	5
	Thr*	8.26	0.17	8	8.26	0.22	6
	Ser*	6.89	0.18	8	6.88	0.18	6
	Glu	23.07	0.12	8	24.18	0.12	6
	Pro	8.5	0.3	8	8.5	0.4	4
	Gly	3.06	0.04	8	3.02	0.02	6
	Ala	14.91	0.08	8	13.98	0.07	6
	Val	10.04	0.03	8	9.85	0.05	6
	Cys*	5.23	0.30	4	4.89	0.24	4
	Met	4.51	0.09	8	3.66	0.04	6
	Ile†	9.66	0.16	8	9.71	0.07	6
	Leu	18.98	0.04	7	20.73	0.12	6
	Tyr	3.58	0.08	8	3.63	0.03	6
	Phe	3.88	0.03	8	3.82	0.02	6
	Lys	15			15		
	His	2.00	0.03	8	1.96	0.04	6
	Arg	2.96	0.01	8	2.96	0.02	6
Hydrolysis after oxidation with performic acid‡	Cys(O ₃ H)	5.05	0.13	3	5.00	0.15	4
	Met (O ₂)	4.88	0.19	4	4.24	0.21	4
	Ala	15			14		
U.v. absorptions§	Trp	1.97	0.03	4	1.91	0.07	4
	Tyr	3.85	0.03	4	3.75	0.07	4

* Estimate by extrapolation to zero time of digestion.

† Estimate by extrapolation to infinite time of digestion.

‡ These results are related to those on the unoxidized sample by the given values for alanine.

§ These results are calculated relative to 18000g dry wt. A sample of bovine β -lactoglobulin AB was analysed.

few values which were close to integral numbers of residues. The results for lysine were very consistent in both proteins and averaged 14.1 and 14.2 residues for the deer and bovine proteins respectively. The complete sequence of bovine β -lactoglobulin has

been determined by Braunitzer *et al.* (1973); it confirms earlier estimates that the protein contains 15 residues of lysine. Our results for this protein were therefore recalculated relative to this value. The discrepancy is probably due to errors in protein determi-

nation and/or losses by adsorption and other processes in the handling of acid hydrolysates as discussed by Robel (1973). Similar considerations probably apply to our results on the deer whey protein. These were recalculated relative to both lysine = 14 residues and lysine = 15 residues. With the latter value a greater number of almost integral numbers of residues was obtained, comparable with the results for the bovine protein. The results for both proteins are therefore given in Table 3 relative to lysine = 15 residues. On this basis the main differences in the integral numbers of residues between the two proteins are: the deer protein contained one more residue of aspartic acid, alanine and methionine and one less glutamic acid residue and two less leucine residues than bovine β -lactoglobulin A. The total number of residues found was 160 for each protein. This is two short of the total number sequenced in bovine β -lactoglobulin A by Braunitzer *et al.* (1973) (one less glutamic acid and one less leucine residue). Some later replicate analyses, unavoidably on a different lot of the bovine protein, with a single digestion time of 36h, showed good agreement with the results in Table 3 for glutamic acid and other amino acids not affected by the time of hydrolysis, but the value obtained for leucine, 21.67 ± 0.02 (4), was close to the expected 22 residues. In spite of these discrepancies in the control analyses of the bovine protein, we think, as the two proteins were hydrolysed together and analysed in alternate sequence, that the results give the probable integral differences between them.

Variants

The electrofocusing experiments described above (see Fig. 3a) showed that the fraction obtained from pooled milk by gel chromatography contained a small amount of a second component. As this component differed electrophoretically but not in size from the deer whey protein, it could be a genetic variant of it. Starch-gel electrophoresis of whey from the individual hinds that had contributed to the pooled supply showed that one, from the hind Rhum 5, contained two main components in about equal amounts and which migrated at the same rates as bovine β -lactoglobulin A and B. However, electrofocusing experiments (see Fig. 3b) showed that their isoelectric points were more acid than those of the corresponding bovine variants. The isoelectric point of the new, faster migrating, component was the same as that of the minor component seen in Fig. 3(a) at 5.02.

Additional milk samples were obtained to provide wheys from a total of 27 individual hinds. These were examined for variants of the major whey proteins by starch-gel electrophoresis. They all contained the slower-migrating variant studied here. Three contained both this protein and the faster-migrating variant, already found in the sample from the hind

Rhum 5. No other types were found. These results may be compared with some obtained by starch-gel electrophoresis of whey from two other cervids. Shubin *et al.* (1971) showed one form of a presumed β -lactoglobulin in the milk of elk (*Alces alces* L.) and one in the milk of reindeer. These migrated like bovine β -lactoglobulin A and B respectively.

The sedimentation-equilibrium experiments, together with the value for the subunit weight obtained by gel chromatography in 6M-guanidine hydrochloride and the formula weight based on the amino acid analyses, show that the deer whey protein exists as a monomer-dimer system. The results of experiments on different preparations showed a variable relation between the weight- and z-average molecular weights. As the preparations appeared homogeneous on gel chromatography, starch-gel electrophoresis and in sedimentation-velocity experiments, the differences observed are probably due to different relations between the monomer and dimer.

The bovine variant A was shown by Timasheff & Townend (1961) to form a tetramer at acid pH values and low temperatures. The deer protein, however, showed no such tendency in the sedimentation-velocity experiment at pH 4.6 and 5°C.

It has been noted that the deer whey protein resembles the bovine A variant in its isoelectric point, but migrates in starch-gel electrophoresis at the rate of the B variant. Tanford *et al.* (1959) showed that the bovine variants undergo a conformational change at pH 7.5. This is accompanied by an increase in effective volume and the exposure of hidden carboxyl groups. The low value for the sedimentation coefficient of the deer protein at pH 8.1 compared with its value at pH 6.5 can be interpreted as evidence for a conformational change in this protein too. From this it seems that both proteins have a different conformation under the conditions of starch-gel electrophoresis at pH 8.2 compared with that obtaining in the electrofocusing experiments. The amino acid analyses indicate that the two proteins contain the same number of imidazole, ϵ -amino and guanidinium groups per subunit. The electrophoretic behaviour of the deer whey protein could therefore be explained if the protein contained one less total ionizable carboxyl group than the bovine protein, and if none of these groups was hidden in the deer protein in the isoelectric state.

In the absence of sequence studies on the deer whey protein, it is speculative to say much about homology. However, reference to Grantham's (1974) table of chemical differences correlated with the relative substitution frequency suggests that, of the differences shown, aspartic acid could be a replacement for glutamic acid, and an alanine and a methionine residue could be replacements for two leucine residues. These differences in the proteins from two species belonging to different families, cervids and bovids, are no greater than those collated by

McKenzie (1971*b*) between species within the bovids, such as sheep and cattle, in agreement with the slowness of macromolecular compared with organismal evolution discussed by King & Wilson (1975).

The present results show that the close similarity of whey-protein composition among the bovids, demonstrated by gel chromatography and electrophoretic methods, extends to other families of ruminants, such as the cervids. This similarity extends in particular to the major whey protein, bovine β -lactoglobulin, and its homologues in the other species. In all of these it appears as a monomer-dimer system with a subunit wt. of about 18000 and shows small differences in amino acid composition. However, when the milk proteins of a different suborder of Artiodactyles, the suiformes, are considered, the major whey proteins, as shown for the pig (*Sus scrofa*) by Kessler & Brew (1970), is sufficiently different in its amino acid composition that it exists as a monomer.

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