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The Characterization of the Whey Proteins of Guinea-Pig Milk

THE ISOLATION AND PROPERTIES OF α-LACTALBUMIN

BY K. BREW* AND P. N. CAMPBELL

Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W. 1

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1. The whey proteins of guinea-pig milk were examined by electrophoresis on paper, cellulose acetate, starch gel and polyacrylamide gel. 2. Two major proteins were detected, one of which was identified as blood serum albumin. 3. The major whey protein was isolated by CM-cellulose chromatography and on columns of Sephadex G-100. 4. The amino acid composition of the protein, taken in conjunction with its other properties, indicated that the major whey protein in guinea-pig milk is homologous with cow α-lactalbumin and that β-lactoglobulin is absent from guinea-pig milk. 5. Guinea-pig α-lactalbumin, which was obtained crystalline, had mol.wt. 15800, *N*-terminal lysine and *C*-terminal glutamine.

As a preliminary to a study of the biosynthesis of milk proteins by guinea-pig mammary gland, it was necessary to determine the nature of the proteins in guinea-pig whey. Turba & Hilpert (1961) isolated the major whey protein by means of paper electrophoresis. They assumed that the protein was homologous with cow β-lactoglobulin, presumably because of its high mobility on paper electrophoresis at pH 8·6.

In the present study various methods have been developed for isolating the major protein of guinea-pig whey, having in mind that the methods should be applicable to the separation of the proteins from tissue extracts. The properties of the protein have been determined and its relationship to the proteins of bovine whey established.

Some aspects of this work have been reported previously in a preliminary form (Brew & Campbell, 1965).

MATERIALS AND METHODS

Collection of milk. Lactating guinea pigs were separated from their litters overnight and the milk was gently expressed from the mammary glands by hand. Milk ejection occurred after a delay of only a few minutes and about 5ml. of milk was obtained from each animal.

Preparation of guinea-pig whey. The pH of the milk was adjusted to 4·5 (pH meter) by the addition of N-acetic acid. The precipitated casein was removed by centrifuging at 8000*g* for 5min., the layer of fat skimmed from the surface and the whey collected. The precipitated casein was washed by resuspending it in 0·15M-NaCl by using a glass rod, and the washings and whey were combined, dialysed against repeatedly changed distilled water for 48hr. and freeze-dried. The dry preparation of whey was stored at −15°.

Extraction of guinea-pig whey with ethanolic hydrochloric acid. Freeze-dried whey was suspended in 0·03N-HCl in 85% (v/v) ethanol (approx. 10mg. of dried whey/ml.) by using a small all-glass homogenizer. The suspension was centrifuged at 8000*g* for 10min. and the insoluble material re-extracted with a similar volume of 0·03N-HCl in 85% ethanol by the same method. The soluble extracts were combined, dialysed against frequently changed distilled water and freeze-dried.

Preparation of albumin fraction of guinea-pig milk. Guinea-pig milk was diluted 1:1 with distilled water to bring its protein concentration closer to that of cow's milk. The albumin fraction was prepared by a method similar to that described by Aschaffenburg & Drewry (1957) for cow's milk. The milk was warmed to 37° in a water bath. Sodium sulphate (20g./100ml. of warm milk) was added while stirring. The precipitate was removed by centrifuging at 8000*g*. The volume of the supernatant was measured and 20g. of $(NH_4)_2SO_4$/100ml. was added with stirring. The precipitate (albumin fraction) was collected by filtration, dissolved in a small volume of water, dialysed against repeatedly changed distilled water for 48hr. and freeze-dried.

Preparation of cow β-lactoglobulin. The method of Aschaffenburg & Drewry (1957) was followed. The protein precipitated from their supernatant (F_2) by $(NH_4)_2SO_4$ was dialysed against distilled water and freeze-dried.

Electrophoretic analysis. (*a*) With cellulose acetate. Electrophoresis was carried out on cellulose acetate membranes of 2·5cm. width (Oxo Ltd., Oxoid Division, London, S.E. 1) in veronal buffer, pH 8·6, *I* 0·05, as described for rat serum proteins by Campbell, Greengard & Kernot (1960).

(*b*) With starch gel. Horizontal starch-gel electrophoresis (Smithies, 1955) was carried out by using the discontinuous buffer system of Poulik (1957) and 13% of hydrolysed starch (lot 201–1; Connaught Laboratories, Toronto,

* Present address: Department of Biochemistry, Duke University Medical Center, Durham, N.C., U.S.A.

Canada). Gels of size 0·6cm. × 5cm. × 15cm. were prepared and samples of protein (0·5–1·0mg. in 10μl. of gel buffer) applied on small pieces of Whatman 3MM paper. Electrophoresis with a current of 10mA through the gel was continued until the buffer boundary was approx. 3cm. from the anode end of the gel (about 4hr.). The gel was sliced horizontally, stained with a saturated solution of Nigrosin (George T. Gurr Ltd., London, S.W. 6) in methanol–water–acetic acid (5:5:1, by vol.) for 30min., de-stained by washing with methanol–water–acetic acid–glycerol (5:5:1:1, by vol.) and stored under glycerol.

(*c*) With polyacrylamide. Disk electrophoresis was carried out as described by Davis (1965).

Fractionation on columns of CM-cellulose. CM-cellulose (Serva from George T. Gurr Ltd.) was washed with N-NaOH, distilled water, N-acetic acid and 15mM-acetate buffer, pH5·0 (15ml. of N-acetic acid/l., adjusted to pH5·0 with N-NaOH). The resin was packed into glass columns with sintered-glass disks in a single pouring whenever possible. Acetate buffer (15mM) was passed through the column until the pH of the effluent rose to pH5·0. Before application to the column, the sample of whey protein was dissolved in 15mM-acetate buffer, pH5·0, and dialysed against this buffer for 18hr.

Protein was eluted either stepwise with 0·05M-NaCl in 15mM-acetate and 0·5M-NaCl in 15mM-acetate, pH5·0, or by gradient with 0·05M-NaCl in 15mM-acetate, pH5·0, which was added to a 250ml. fixed-volume mixing vessel fitted with a magnetic stirrer and containing 15mM-acetate buffer, pH5·0.

Fractionation on Sephadex G-100. Sephadex G-100 (Pharmacia Ltd., Uppsala, Sweden) was swollen in 0·1M-ammonium acetate–0·4M-NaCl (30ml./g.) on a magnetic stirrer for 48hr. and packed into glass columns blocked either with a sintered-glass disk or glass wool, on top of which was placed a 1cm. layer of acid-washed sand. The gel was poured continuously into the column with continual draining of buffer and frequent agitation of the gel as it packed. A filter-paper disk was placed on top of the final column and buffer passed through for 24hr. The sample of whey protein was dissolved in 1·5ml. of buffer and solid sucrose added to increase the density. The solution was layered on top of the bed with a 2ml. syringe fitted with a needle and a piece of polythene capillary tubing.

The components were eluted with 0·1M-ammonium acetate–0·4M-NaCl. Fractions were collected at timed intervals and the positions of protein peaks obtained from E_{280}. The determination of the elution volume of the components was facilitated by collecting the fractions in weighed tubes, which enabled the weight of effluent to be determined by re-weighing. Peak positions were estimated by extrapolating the ascending and descending sides of the protein peaks.

Determination of N-terminal amino acid of protein. The protein was treated with 1-fluoro-2,4-dinitrobenzene by the method of Sanger (1945). The DNP derivative of 4–5mg. of protein was hydrolysed with 0·5ml. of constant-boiling HCl (twice glass-distilled) under N_2 in a sealed glass tube at 105° for 16hr. The ether-soluble DNP-amino acids of the hydrolysate were examined by paper chromatography with the two-dimensional pentan-1-ol–phthalate and 1·5M-Na_2SO_4–phthalate system of Pairent & Williamson (1960) and on the toluene–pyridine–2-chloroethanol–0·8N-NH_3 and 1·5M-phosphate system of Levy (1954). The aqueous phase was examined on the system of Pairent & Williamson (1960) and on the 2-methylbutan-2-ol–phthalate system of Blackburn & Lowther (1951).

Determination of amino acid composition of protein. Samples of freeze-dried protein (6–7mg.) were hydrolysed with 3·0ml. of constant-boiling HCl (twice glass-distilled) for 24 and 72hr. in sealed tubes under vacuum at 110°. A sample of performic acid-oxidized protein (method of Hirs, 1956) was hydrolysed in a similar manner but for 24hr. only. The dry weight of protein used for the digests was calculated from their N content, the conversion factor being derived after determination of the N content of the dry protein.

The amino acid analyses were kindly carried out on duplicate or triplicate samples by using an automatic amino acid analyser (Spackman, Stein & Moore, 1958) by Mr J. M. Wilkinson of the Wright-Fleming Institute. Tryptophan was determined by spectrophotometry on solutions of the whole protein by the method of Crumpton & Wilkinson (1963).

Determination of C-terminal amino acid. Freeze-dried protein (2–3mg.), dissolved in 1·0ml. of 1% $NaHCO_3$, was digested with 1% or 5% of its weight of carboxypeptidase A-DFP (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for varying lengths of time at 25° by the method of Fraenkel-Conrat, Harris & Levy (1955). Trichloroacetic acid was added to 5% (w/v) to precipitate the protein and, after centrifuging, the supernatant was passed through a small column (1cm. × 0·5cm.) of Zeo-Karb 225 (H^+ form) to adsorb the amino acids. The amino acids were eluted with 5N-NH_3 and the solvent was removed in a desiccator over P_2O_5. The amino acids were identified by two-dimensional paper chromatography on 28cm. × 28cm. squares of Whatman no. 1 paper with butan-1-ol–acetic acid–water (12:3:5, by vol.) in the first dimension and phenol–NH_3 [phenol–water (4:1, w/v), 200ml.; aq. NH_3 (sp.gr. 0·88), 1ml.] in the second (Smith, 1958). A standard mixture of 0·075μmole of each of the protein amino acids was chromatographed under identical conditions to enable identifications to be made.

Determination of nitrogen. The micro-Kjeldahl–Nessler method of Gonzalez Cadavid & Paladini (1964) was used.

RESULTS

Characterization of the whey proteins. Three media were employed successfully for the electrophoresis of whey protein: cellulose acetate, starch gel and polyacrylamide gel. In all cases, in contrast with electrophoresis on paper, the whey proteins of guinea-pig milk were resolved effectively.

Electrophoresis with cellulose acetate membranes at pH8·6 had sufficient resolving power to separate the two common variants, A and B, of cow β-lactoglobulin (Fig. 1). With this medium two major protein bands were detected in guinea-pig whey. Both components were present in the albumin fraction (see the Materials and Methods section) of whey and were obtained, free of the minor whey proteins, by extraction of freeze-dried whey with 0·03N-hydrochloric acid in 85% ethanol

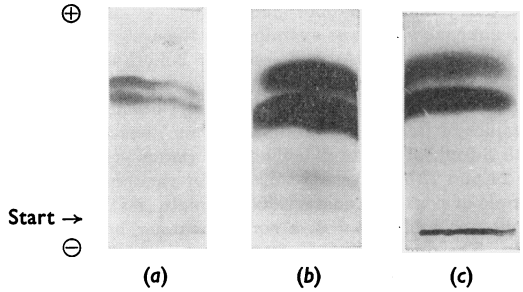

Fig. 1. Electrophoresis of whey-protein fractions on cellulose acetate membranes. Electrophoresis was in veronal buffer (pH 8·6; I 0·05). A current of 2·6 mA (150 v) was applied for 5 hr. The proteins were stained with azocarmine. (*a*) Cow β-lactoglobulin; (*b*) albumin fraction of guinea-pig whey; (*c*) protein extracted from guinea-pig whey with 0·03 N-HCl in 85% ethanol.

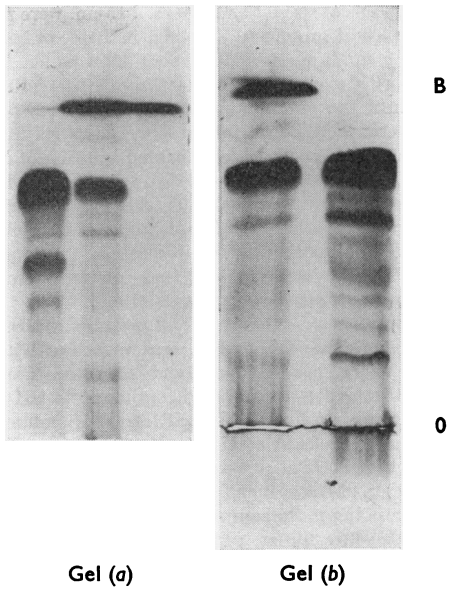

Fig. 2. Electrophoresis of guinea-pig whey and serum proteins with starch gel. The discontinuous buffer system of Poulik (1957) was used. The gel buffer was 76 mM-tris–5 mM-citric acid and the tank buffer 0·3 M-boric acid–0·05 N-NaOH. A current of 10 mA was applied for 4 hr. The proteins were stained with nigrosine. Gel (*a*): left to right, guinea-pig serum albumin, guinea-pig whey, purified major whey protein. Gel (*b*): left to right, whey, serum (O marks the point of application of the protein sample; B marks the position of the buffer boundary at the termination of electrophoresis: the major whey protein runs on this boundary).

(see Fig. 1). It has previously been shown that serum albumin and β-lactoglobulin are soluble in high concentrations of ethanol under acidic conditions (for references see Michael, 1962). Of the two proteins in the albumin fraction that with the higher electrophoretic mobility on cellulose membranes had the same mobility as guinea-pig blood serum albumin run on an adjacent strip under the same conditions. The protein also bound bromophenol blue when this dye was added before electrophoresis. These characteristics, together with its solubility in acidified ethanol and its solubility properties in solutions of ammonium sulphate and sodium sulphate, are consistent with the protein being identified as serum albumin.

The second protein in the albumin fraction of whey with the slower electrophoretic mobility on cellulose acetate is presumably the protein isolated by Turba & Hilpert (1961) by paper electrophoresis.

Horizontal starch-gel electrophoresis with the discontinuous buffer system of Poulik (1957) was also effective in resolving the whey proteins (Fig. 2). This buffer system gave sharp bands and resolution could be completed in 4 hr. The comparative mobility of the two proteins in the albumin fraction of whey protein on starch gel was reversed compared with their behaviour on cellulose acetate. The slower component had the same mobility as serum albumin. The other component ran at the salt boundary. The results with polyacrylamide gel were almost identical with those with starch gel. It may be noted in Fig. 2 that the minor components of guinea-pig whey have mobilities that coincide on starch gel closely with the components of serum. This is consistent with the passage of small amounts of serum proteins to the milk.

Isolation of the major whey protein. Ion-exchange chromatography with CM-cellulose and gel filtration with Sephadex G-100 were both effective in isolating the major whey protein. With CM-cellulose, the major protein was the only component eluted by a gradient to 0·05 M-sodium chloride in 15 mM-acetate buffer, pH 5·0, apart from an initial 'breakthrough' peak. When 0·5 M-sodium chloride was applied serum albumin and the minor whey components were eluted (Fig. 3).

With Sephadex G-100, three protein peaks were obtained (Fig. 4). The first apparently came through in the void volume of the gel and contained opalescent material. The second showed the same elution volume as guinea-pig serum albumin, and the third, most retarded, peak contained the purified major whey protein. This method had the advantage that no regeneration of the column was necessary between fractionations. The separation was so effective that high loadings of whey could be used without any adverse effect on the fractionation. Yields of protein fractions per ml. of whole milk

were: total protein, 89mg.; casein, 70mg.; major whey protein, 5mg.

Properties of the major whey protein. (1) Molecular weight. This was determined by comparing the elution position of the protein on a 95cm. × 2cm. column of Sephadex G-100 with the elution positions of proteins of known molecular weight, as described by Whitaker (1963) and Andrews (1964). The buffer system used (0·1M-ammonium acetate–0·4M-sodium chloride) was originally described by Whitaker (1963) as being of sufficiently high ionic strength to eliminate ion-exchange effects with the gel. Standard proteins were chosen that have been shown by Andrews (1964) to exhibit no anomalous dissociation effects on Sephadex. The results are shown in Fig. 5.

Increasing loadings (2·3, 4·2 and 14·0mg.) of the major whey protein had no effect on the elution volume. Such a change in elution volume has been found with haemoglobin and cow β-lactoglobulin by Andrews (1964) and is probably produced by the association of monomeric units at higher loadings. From the plot of log(mol. wt.) against elution position, a value of 15800 was obtained compared with a minimum weight for cow β-lactoglobulin of 18250. These properties suggested that the protein was more similar to cow α-lactalbumin (mol. wt. 15500; Gordon & Ziegler, 1955) than β-lactoglobulin.

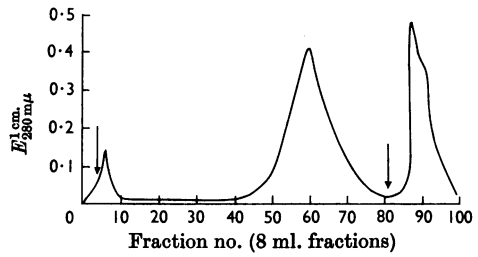

Fig. 3. Fractionation of guinea-pig whey protein by ion-exchange chromatography with CM-cellulose. Freeze-dried whey (145mg.) was applied to a 14cm. × 2·5cm. column equilibrated with 15mM-acetate buffer, pH5·0. The only protein eluted (apart from a small break-through peak) by the gradient to 0·05M-NaCl in acetate buffer (first arrow) was shown by the peak in fraction numbers 50–70. This was identified as the major whey protein. The protein eluted by stepwise elution with 0·5M-NaCl (second arrow) consisted of serum albumin and a number of minor constituents of the whey.

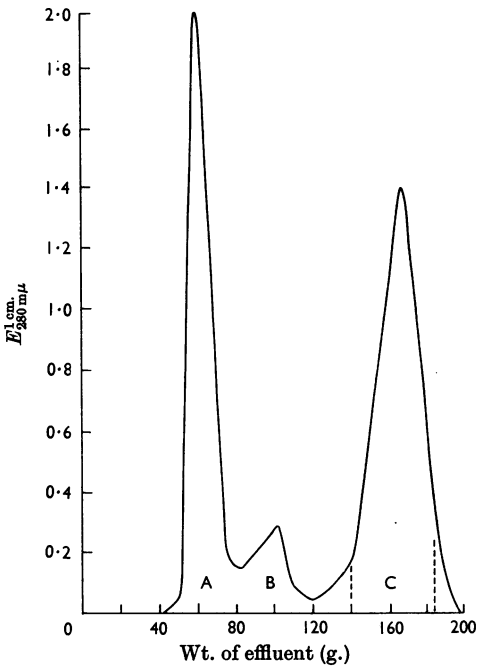

Fig. 4. Fractionation of guinea-pig whey protein by gel filtration with Sephadex G-100. Freeze-dried whey (52mg.) was applied to a 95cm. × 2cm. column equilibrated with 0·1M-ammonium acetate–0·4M-NaCl. The major whey protein was responsible for peak C. The protein in peak B had the same elution volume as guinea-pig serum albumin.

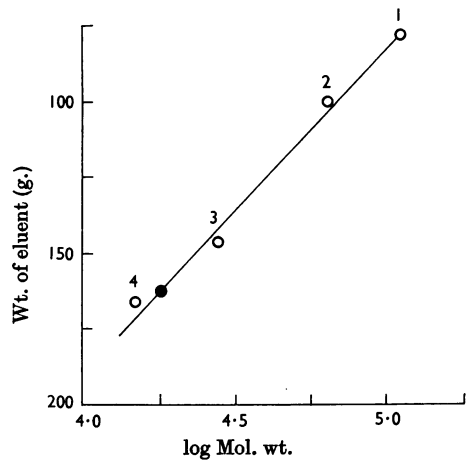

Fig. 5. Determination of the molecular weight of the major guinea-pig whey protein from its elution position on Sephadex G-100. Loadings of between 2 and 5mg. of the standard proteins were used. Molecular weights of standard proteins are taken from Whitaker (1963). Loadings of 2·3, 4·2 and 14mg. of the major whey protein showed elution positions of 168, 165 and 169g. respectively for the protein peak. The value of 168g. is used in the graph and a value of 15800 was obtained for the molecular weight of the protein. ○, Standard proteins (1, serum albumin; 2, ovalbumin; 3, trypsin; 4, cytochrome *c*); ●, major whey protein.

Table 1. *Amino acid analysis of the major guinea-pig whey protein*

Conditions for hydrolysis, determination of N and estimation of tryptophan are given in the Materials and Methods section.

Amino acid	m-moles per 100g. of protein			Residues per mole (15800g.) of protein	Bovine α-lactalbumin
	24hr.	72hr.	final		
Aspartic acid	132·2	137·8	134·9	21·3	21·8
Threonine	36·35	30·3	39·4	6·2	7·2
Serine	41·2	25·2	47·0	7·7	7·0
Glutamic acid	78·4	81·0	79·7	12·6	13·6
Proline	18·9	17·2	18·1	2·9	2·1
Glycine	28·3	29·1	28·7	4·4	6·6
Alanine	35·2	36·2	35·7	5·7	3·7
Valine	22·1	22·9	22·9	3·6	6·2
Methionine	6·1	6·9	6·5	1·0	1·0
Isoleucine	77·9	82·7	82·7	13·1	8·1
Leucine	94·3	99·1	99·1	15·6	13·7
Tyrosine	32·35	31·9	32·8	5·2	4·6
Phenylalanine	19·9	21·5	20·7	3·4	4·2
Cysteic acid	48·1		48·1	7·7	8·2
Lysine	72·2	73·2	72·7	11·4	12·2
Histidine	26·48	27·60	27·0	4·3	2·9
Arginine	14·7	14·7	14·7	2·3	1·0
Tryptophan				3·3	5

(2) Amino acid composition. The protein was analysed for amino acids after hydrolysis. The results given in Table 1 show a marked general similarity between the guinea-pig whey protein and cow α-lactalbumin. The whey protein is therefore designated guinea-pig α-lactalbumin.

(3) Crystals. As it was remarkably stable, the α-lactalbumin could be conveniently collected from column effluents by adding trichloroacetic acid to 5% to precipitate the protein and centrifuging at high speed for a few minutes to sediment. The protein precipitated in this way dissolved in distilled water, and on dialysis against distilled water for about 24hr. crystals of the protein formed. Unfortunately, probably as a result of the rapid rate of crystallization, these crystals were mainly irregular in form.

(4) *N*-terminal amino acid. By using the 1-fluoro-2,4-dinitrobenzene technique, lysine was the only *N*-terminal amino acid detected. Yields of 0·18 mole of the bis-DNP-amino acid/15800g. of α-lactalbumin were obtained. However, Thompson (1951) has reported that extensive destruction of this DNP-amino acid occurs during acid hydrolysis when tryptophan is present. The presence of three residues of tryptophan in the protein is consistent with such destruction.

DNP-arginine was absent from the aqueous phase of hydrolysates of the DNP-protein.

(5) *C*-terminal amino acid. Glutamine was the only amino acid released from α-lactalbumin by the action of carboxypeptidase A under the conditions used, even when digestion of the protein with 5% (w/w) enzyme was continued for 19hr. It is concluded that glutamine is the *C*-terminal amino acid and that this is preceded in the protein by a residue joined in a peptide linkage that is not attacked by carboxypeptidase A.

(6) Isoelectric point. Electrophoresis of α-lactalbumin on cellulose acetate at pH 7·0 and 6·0 (sodium phosphate buffers), at pH 4·5 and 4·2 (sodium lactate buffers) and at pH 3·4 and 2·4 (glycine–hydrochloric acid buffers), as a result of which the mobility of the protein was determined relative to glucose, gave on extrapolation a value of 4·8 for the isoelectric pH. It is realized that glucose is not entirely suitable as a solute for determination of the correction owing to electro-endosmosis (Legatt-Bailey, 1962).

DISCUSSION

The major protein components in whey from the ruminants buffalo, sheep and goat, are homologous with cow β-lactoglobulin (Mawal, Barnabas & Barnabas, 1965; Bell & McKenzie, 1964; Maubois, Pion & Dumas, 1965; Phillips & Jenness, 1965). Unlike these milks, that of guinea pig contains as its main whey protein, a component that is homologous with cow α-lactalbumin. Although the *N*- and *C*-terminal amino acids differ from those of cow α-lactalbumin, they are both consistent with

single base changes in the controlling genes (glutamic acid/lysine, messenger RNA triplet sequences ApApA, GpApA, leucine/glutamine, triplet sequences CpUpA, CpApA; see Nirenberg *et al.* 1965), and the amino acid compositions show marked similarities. 'Fingerprints' of guinea-pig α-lactalbumin, reduced and carboxymethylated and digested with trypsin under controlled conditions, showed 13 peptides, only two of which contained arginine, as judged by the Sakaguchi reaction of Jepson & Smith (1953), a result entirely consistent with the amino acid composition and molecular weight reported in the present work.

Guinea-pig milk is not unique in the difference of its major whey protein from that of the cow. The major protein of human whey shows a sedimentation coefficient 1·7–1·73 s (Maeno & Kiyosawa, 1962; Johansson, 1958). This protein may also be homologous with cow α-lactalbumin, which has a sedimentation coefficient 1·75 s (Gordon & Semmett, 1953) and is certainly very different from β-lactoglobulin (2·83 s) (Cecil & Ogston, 1949).

The whey proteins of a number of individual guinea pigs were examined by cellulose acetate electrophoresis, but no evidence for genetic variation in the α-lactalbumin was obtained. Moreover, the product obtained by fractionation on Sephadex was homogeneous on starch and polyacrylamide gel. These results do not, of course, exclude the possible existence of genetic variants, since the amino acid substitutions may not produce changes that show up on electrophoresis of the resultant proteins.

The only milks hitherto reported to contain β-lactoglobulin are from ruminants. From the results on human and guinea-pig milks, it seems possible that β-lactoglobulin is confined to the milk of ruminants, or possibly a group of mammals including the ruminants, whereas α-lactalbumin has a much wider distribution. The investigation of this point might be of taxonomic interest.

Both in the guinea pig and the cow the α-lactalbumin represents less than 6% of the total milk protein, so that it is probably of little nutritional importance, but the physiological role of this protein is unknown. In this respect the markedly similar molecular weight, amino acid composition and *N*- and *C*-terminal amino acids for α-lactalbumin and those lysozymes so far investigated (e.g. see Jollés, 1960) may be noted. To the extent that the properties mentioned reflect similar primary structures the α-lactalbumins may have evolved by gradual modification from lysozyme, which is found in the milk of many species. Guinea-pig α-lactalbumin had, however, no detectable lysozyme activity.

The presence of high concentrations of blood serum albumin relative to the major whey protein in guinea-pig milk was observed by Turba & Hilpert (1961). It seems probable that the milk contains small amounts of some of the other blood serum proteins. This is consistent with the suggestion of Laurell & Morgan (1965) that the mammary gland acts partially as a molecular sieve in relation to the transfer of blood serum proteins to milk.

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