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α-Lactalbumin and lactose concentrations in rat milk during lactation

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Homogeneous rat α-lactalbumin was prepared from whey by chromatography on DEAE-Sephadex A-50 and Ultrogel AcA 44. Two biologically active forms of α-lactalbumin were apparent after ion-exchange chromatography, but on gel filtration the combined forms were eluted as a single peak with a molecular weight of approx. 33000. The molecular weight when determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was 15100. Antiserum to α-lactalbumin was prepared from rabbits, and single radial immunodiffusion was used to measure the concentration of α-lactalbumin in milk expressed from rats during lactation and for 2 days after the cessation of lactation. A significant positive correlation ($r = 0.89$) between the concentrations of α-lactalbumin and lactose was obtained for the first 20 days of lactation. This is consistent with the suggestion that α-lactalbumin may control the concentration of lactose in milk. However, a significant negative correlation ($r = -0.91$) between the concentration of α-lactalbumin and lactose was obtained for 2 days after the cessation of lactation on day 20.

Lactose synthase (EC 2.4.1.22) becomes functional in mammary tissue when the substrate specificity of galactosyltransferase is directed towards glucose by the 'specifier' protein, α-lactalbumin (Brew *et al.*, 1968). Galactosyltransferase is membrane-bound and appears to be localized in the Golgi apparatus (Keenan *et al.*, 1970), so that the synthesis of lactose occurs on the luminal side of the Golgi body (Kuhn & White, 1975*a*). Since lactose and α-lactalbumin are subsequently secreted into the alveolar lumen (Brew & Campbell, 1967; Linzell & Peaker, 1971), Brew (1969) suggested that the activity of lactose synthase is controlled by the flux of α-lactalbumin from its site of synthesis through the membranes of the Golgi body. On the basis of kinetic studies (Brodbeck & Ebner, 1966; Morrison & Ebner, 1971*a*,*b*,*c*), it has been predicted that the amount of lactose in milk should be proportional to the α-lactalbumin content (Ebner & McKenzie, 1972). This reasoning was supported by the results of Ley & Jenness (1970), showing a significant positive correlation between the concentration of these two components in the milk of six selected species.

The present paper describes the purification and assay of rat α-lactalbumin. In addition, the changes in the concentration of both α-lactalbumin and lactose were determined during the first 20 days of lactation and for 2 days after the cessation of lactation to establish the relationship between these two components in rat milk.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

α-Lactalbumin (grade II, from bovine milk), α-chymotrypsinogen A (type II, from bovine pancreas), ribonuclease A (type III-A, from bovine pancreas), serum albumin (fraction V, bovine), ovalbumin (grade V), cytochrome *c* (type II-A, from horse heart), lactase (grade II, from *Saccharomyces fragilis*), peroxidase (type I, from horseradish) and glucose oxidase (type II, from *Aspergillus niger*) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel AcA 44 was purchased from LKB, Uppsala, Sweden, and Aquacide I-A from Calbiochem Behring (Australia) Pty. Ltd., Carlingford, N.S.W., Australia. Oxytocin (Pitocin) was from Parke-Davis and Co., Sydney, N.S.W., Australia.

Milk collection

Rats were from the Wistar albino strain of *Rattus norwegicus* from our colony. The litter and dam were separated for 1.0h. The dam was then maintained under light diethyl ether anaesthesia,

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injected with 0.1 i.u. of oxytocin and the milk was expressed manually. Milk samples (0.5 ml) were obtained at 0, 1, 2, 3, 4, 5, 8, 15 and 20 days of lactation. Additional samples were collected at 1 and 2 days after the litter was removed from the dams at day 20 of lactation. A total of 44 rats were studied, and no rat was milked on more than one occasion. α-Lactalbumin was purified from milk collected from rats 1 day after they had been separated from their litters at day 20 of lactation. Milk was stored frozen at −20°C both for analysis of α-lactalbumin, serum albumin and lactose and for protein purification.

Measurement of α-lactalbumin, serum albumin and lactose

α-Lactalbumin and serum albumin were measured by the single radial-immunodiffusion technique of Mancini *et al.* (1965). Lactose concentration was determined by the method of Kuhn & Lowenstein (1967) in milk diluted 1:100 (v/v) in water. No correction was made for the presence of endogenous glucose. However, published values of the concentration of both lactose and glucose in milk during established lactation (Kuhn, 1972; Kuhn & White, 1975*b*) suggest that the overestimation of lactose would be less than 1.0%.

Purification of α-lactalbumin and serum albumin and preparation of antisera

The purification of rat serum albumin and preparation of the specific antiserum have been reported previously (Yeoh & Morgan, 1974). Rat α-lactalbumin was purified by a modification of the method described by Phillips & Jenness (1971) for the isolation of human α-lactalbumin. Fat was removed from the milk by centrifugation at 15000 ***g*** for 30 min at 4°C. The skim milk was removed by suction and the pH was adjusted to 4.6 with 1.0 M-HCl. The precipitated casein was removed by centrifugation at 15000 ***g*** for 1 h at 4°C and the whey protein was dialysed at 4°C against 20 litres of 0.5 mM-sodium phosphate buffer, pH 7.0, for at least 36 h with five buffer changes and then freeze-dried. A fraction of the whey protein was dissolved in 10 ml of 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl, and chromatographed on a column (2.5 cm × 45 cm) of DEAE-Sephadex A-50, which had been equilibrated with the same buffer. The protein was eluted with a linear gradient of 0.10–0.37 M-NaCl in 1.4 litres of the buffer. Protein absorbance of the fractions was determined at 280 nm. The galactosyltransferase activity was used to identify fractions containing α-lactalbumin (Ebner *et al.*, 1972). Both α-lactalbumin and milk serum albumin were subsequently quantified in column fractions by single radial immunodiffusion. The α-lactalbumin fractions were combined, dialysed overnight at 4°C against 100 vol. of distilled water, and freeze-dried. Alternatively the α-lactalbumin was concentrated by dialysis against Aquacide I-A for 8–10 h at 4°C. A sample of α-lactalbumin was chromatographed on a column (2.5 cm × 90 cm) of Ultrogel AcA 44 equilibrated and eluted with 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Fractions (5 ml) were eluted at a flow rate of 25 ml/h. α-Lactalbumin and serum albumin were quantified by single radial immunodiffusion. Both proteins were dialysed overnight at 4°C against distilled water and freeze-dried. To obtain

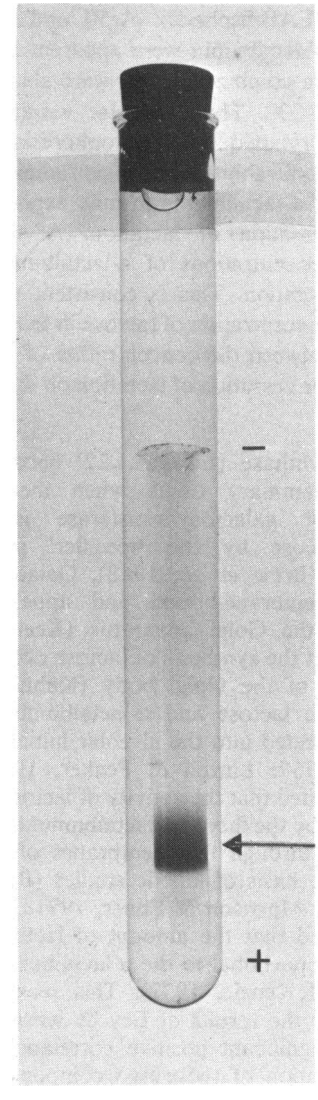

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of α-lactalbumin peak eluted from Ultrogel AcA 44*
The acrylamide monomer concentration was 10%. The arrow indicates the position of migration of α-lactalbumin.

homogeneous α-lactalbumin, the peak was re-chromatographed on the Ultrogel AcA 44 column.

Preparation of antiserum. Rabbits were injected intradermally at 15–20 sites along the back with either 1.0mg of α-lactalbumin or 6–8mg of whey protein [in 1.0ml of 0.9% (w/v) NaCl with 1.0ml of complete Freund's adjuvant]. Animals received further injections subcutaneously at 14-day intervals and satisfactory antisera were obtained within 8–10 weeks.

Criteria of purity. α-Lactalbumin was demonstrated to be homogeneous by the presence of a single protein band (Fig. 1) after SDS/polyacrylamide-gel electrophoresis in 10% gels (Weber & Osborn, 1969). In addition, immunoelectrophoresis was performed on microscope slides with 1% (w/v) agarose in 0.02M-barbital/acetate buffer, pH8.2. Electrophoresis of the samples was carried out for 90min with a current of 10mA and voltage gradient of 9V/cm. Slides were developed with specific antisera in a humid compartment at room temperature for 24h. The gels were washed with four changes of 0.9% (w/v) NaCl and stained for protein with 0.5% (w/v) Coomassie Brilliant Blue in acetic acid/methanol (9:1, v/v).

Immunoelectrophoresis of α-lactalbumin with rabbit antiserum to rat whey gave a single precipitation line, and also rat whey protein gave a single precipitation line against rabbit antiserum to rat α-lactalbumin. In addition, rabbit antiserum to rat serum albumin gave a single precipitation line with rat whey and serum albumin purified from rat whey.

Molecular-weight estimation. The molecular weight of α-lactalbumin was estimated by SDS/polyacrylamide-gel electrophoresis in 10% gels (Weber & Osborn, 1969), with Bromophenol Blue as the tracker dye, and bovine serum albumin, ovalbumin, α-chymotrypsinogen A, ribonuclease A and cytochrome *c* as standard proteins, with mol.wts. of 68000, 45000, 25000, 13700 and 12400 respectively.

Results and discussion

Purification of α-lactalbumin

α-Lactalbumin was purified from milk expressed after the removal of litters for 24h in late lactation. This procedure provided optimal conditions for the collection of a relatively large volume of rat milk with a high concentration of α-lactalbumin (Fig. 3).

The chromatographic separation of whey protein on DEAE-Sephadex A-50 is shown in Figs. 2(*a*) and 2(*b*). The A_{280} showed three major peaks (I,II, III). Galactosyltransferase activity was localized in peak I and lactose synthase activity (α-lactalbumin) was found in both peaks II and III (Fig. 2*a*). Subsequent radial immunodiffusion showed the presence of both α-lactalbumin and serum albumin in peak II and α-lactalbumin alone in peak III (Fig. 2*b*). When peaks II and III were combined, gel filtration on Ultrogel AcA 44 resolved α-lactalbumin and serum albumin into two separate peaks (Fig. 2*c*). Electrophoresis of the α-lactalbumin peak (Fig. 2*c*) on 10% polyacrylamide gel in the presence of SDS showed a single homogeneous band of protein (Fig. 1).

Estimation of molecular weight

The molecular weight of α-lactalbumin determined by SDS/polyacrylamide-gel electrophoresis was 15100. However, the final separation of α-lactalbumin and serum albumin on the Ultrogel AcA 44 column (Fig. 2*c*) was complicated by the α-lactalbumin exhibiting an apparently higher molecular weight. The elution of α-lactalbumin relative to protein markers of known molecular weight (Fig. 2*c*) suggested that rat α-lactalbumin was eluted from Ultrogel AcA 44 with a mol.wt. just above 33000. These findings are similar to the observations of Brown *et al.* (1977), who found two protein peaks containing α-lactalbumin after passage through DEAE-cellulose 32, but only a single peak of α-lactalbumin on chromatography of whey on Bio-Gel P150. However, in this latter study the molecular weight of α-lactalbumin when determined by both molecular sieving and SDS/polyacrylamide-gel electrophoresis was 26000–28000.

Three major charged forms of α-lactalbumin each with activity in the lactose synthase reaction were demonstrated by Brown *et al.* (1977), using polyacrylamide-gel electrophoresis. Each of these forms of α-lactalbumin contained carbohydrate. Multiple glycoprotein forms of α-lactalbumin have been described by Qasba & Chakrabartty (1978) and McKenzie & Larson (1978), and Lingappa *et al.* (1978) translated and glycosylated rat α-lactalbumin *in vitro*. Andrews (1965, 1970) demonstrated that glycoproteins display anomalous behaviour on gel filtration and give molecular-weight estimations up to twice the actual value. However, in the present study the molecular weight obtained for α-lactalbumin (15100) by SDS/polyacrylamide-gel electrophoresis is in close agreement with the value obtained for α-lactalbumin from a number of other species when calculated from its amino acid composition (Brew *et al.*, 1968; Phillips & Jenness, 1971; Schmidt & Ebner, 1971). The higher-value obtained by gel filtration may be related to the glycoprotein nature of this milk protein.

α-Lactalbumin and lactose in milk during lactation

The concentrations of α-lactalbumin and lactose throughout lactation and weaning are shown in Fig. 3. The significant positive correlation ($r = 0.89$, $n = 45$, $P < 0.001$) between the concentration of lactose and α-lactalbumin during lactation in the rat

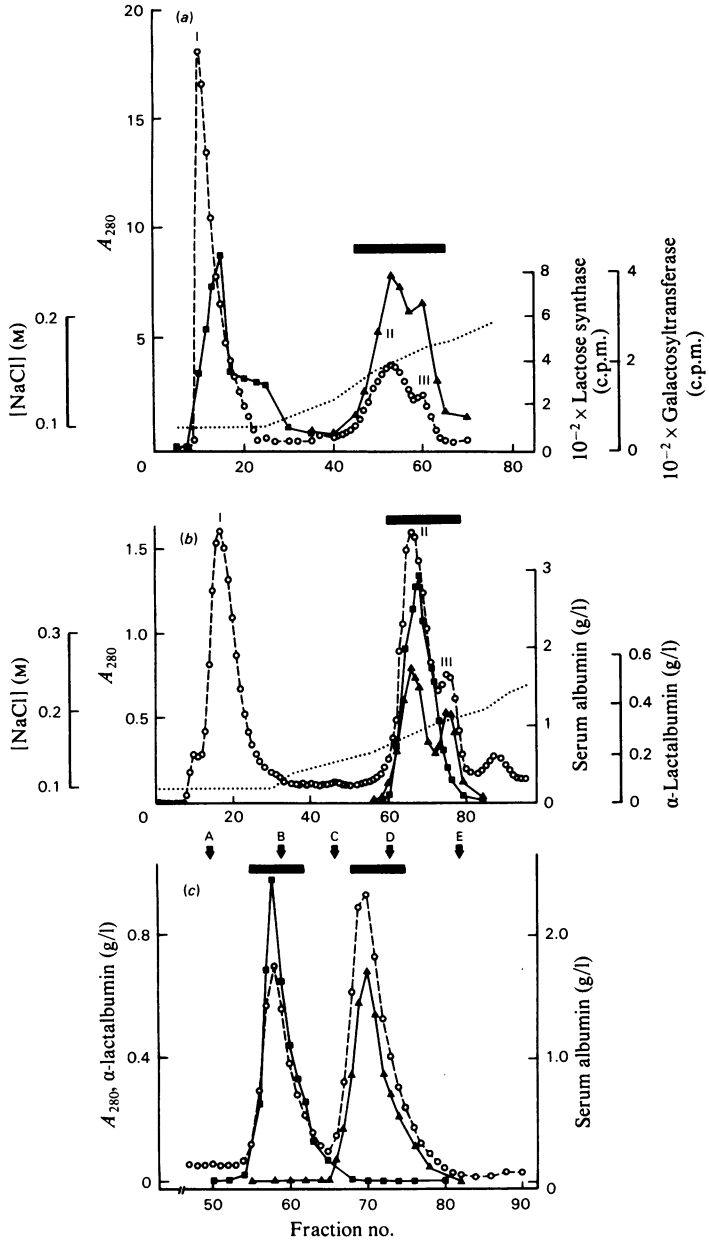

Fig. 2. *Chromatography of rat whey protein on DEAE-Sephadex A-50 and Ultrogel AcA 44*

(*a*) Whey protein was applied to a DEAE-Sephadex A-50 column and the protein (○) eluted with a linear gradient of NaCl (....). Fractions (10ml) were collected at a flow rate of 30ml/h. Galactosyltransferase (■) and lactose synthase (▲) activity were located and the appropriate fractions (peaks II and III) pooled as indicated by the bar. (*b*) Whey protein was applied to a DEAE-Sephadex A-50 column and the protein (○) eluted with a linear gradient of NaCl (....). Fractions (8ml) were collected at a flow rate of 25ml/h. α-Lactalbumin (▲) and albumin (■) were located by radial immunodiffusion and the appropriate fractions pooled as indicated by the bar. (*c*) A fraction from combined peaks II and III was applied to an Ultrogel AcA 44 column and 5ml fractions were eluted at a flow rate of 25ml/h. Protein absorbance (○) was measured at 280nm and the fractions which contained α-lactalbumin (▲) and albumin (■) were identified and pooled as indicated by the bars. Proteins of known molecular weight were chromatographed under the conditions described above. The protein standards indicated by the arrows are: A, Blue Dextran (V_0); B, bovine serum albumin (68000); C, ovalbumin (45000); D, α-chymotrypsinogen A (25000); E, bovine α-lactalbumin (14300).

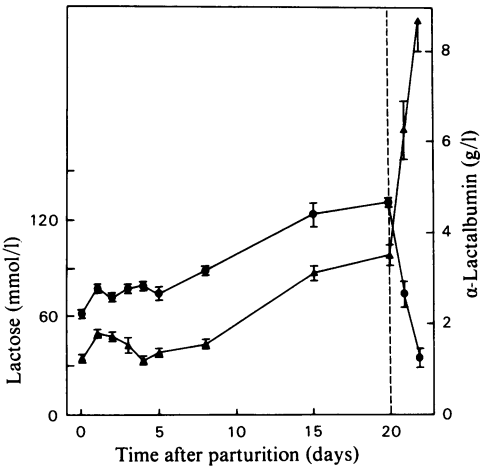

Fig. 3. *Lactose and α-lactalbumin concentration in rat milk during lactation and after the cessation of suckling*
Throughout the first 20 days of lactation, the dams were separated from their litters for 1.0h, and the milk was collected from each nipple and pooled. Additional milk samples were obtained for 2 days after the cessation of suckling at day 20 of lactation (dashed line). The concentration of lactose (●) and α-lactalbumin (▲) was determined in each milk sample. Each value represents the mean ± S.E.M. for four observations. The litter size was 10.7 ± 1.2 pups (mean ± S.D., $n = 44$).

(Fig. 3) is in agreement with the prediction of Ebner & McKenzie (1972), and lends support to the model proposed by Brew (1969) implicating α-lactalbumin in the control of lactose synthesis. In addition, these findings show that extreme caution should be exercised in interpreting the significance of the interspecies correlation for lactose and α-lactalbumin by Ley & Jenness (1970). These workers appear to have measured the concentrations of lactose and α-lactalbumin in different samples of milk. Since the changes in these components during lactation are large, it is important to make comparisons at the same stage of lactation. The ranges of α-lactalbumin and lactose values during the first 20 days of lactation in the rat encompass the concentration of α-lactalbumin reported for all species except the human, and the concentration of lactose reported for all species except the human and pig (see Ley & Jenness, 1970).

After the cessation of suckling at 20 days of lactation, the involution of the mammary gland was accompanied by a rapid decline in the concentration and presumably synthesis of lactose, but an increase in the concentration of α-lactalbumin in the mammary secretion (Fig. 3). A significant negative correlation ($r = -0.91$, $n = 9$, $P < 0.001$) was obtained between the concentration of lactose and α-lactalbumin for the 2 days after the cessation of lactation (Fig. 3). This apparently contradictory observation could be due to a fall in the concentration of intracellular glucose leading to diminished lactose synthesis and secretion. The resulting decrease in osmotic water movement would then lead to an increased concentration of α-lactalbumin after the cessation of lactation.

An observation of lactose and α-lactalbumin concentrations in human milk during the first 7 days of lactation (Kulski *et al.*, 1977) showed that there was a significant negative correlation between these two milk components during this period. This contradictory result for the relationship between lactose and α-lactalbumin during the initiation of lactation in the human, as compared with the changes throughout lactation in the rat, indicates that the mechanism suggested by Brew (1969) may not apply to all species.

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